The 9th International Congress of Comparative Physiology and Biochemistry

FROM MOLECULES TO MACROPHYSIOLOGY

23-28 August 2015

ICCPB 2015

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FROM MOLECULES TO MACROPHYSIOLOGY

23-28 August 2015

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WELCOME

It is our pleasure to welcome you to Kraków, Poland for the 2015 International Congress of Comparative Physiology and Biochemistry (ICCPB2015). Congress meetings have been successfully organized every 4 years since 1983. ICCPB2015 in Kraków is already our 9th meeting. We are happy to inform you that you are amongst over 350 fellow biologists who have contributed to building the program of the Congress under the headline 'From Molecules to Macrophysiology'.

We hope that the atmosphere of the Congress in Kraków will be especially stimulating. Kraków with its medieval architecture offers endless opportunities for inspiration and unconventional thinking. It is exactly here, at the Jagiellonian University in Kraków, that Nicolaus Copernicus started his scientific education which finally led him to his revolutionary ideas that challenged old models of the universe.

We wish you an inspiring and pleasant stay in Kraków,

Paweł Koteja, Ph.D.
Institute of Environmental Sciences, Jagiellonian University, Kraków, Poland
Chair of the ICCPB2015 Local Organizing Committee

Marek Konarzewski, Ph.D.
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INVITED PLENARY SPEAKERS

Dr. Kevin Gaston

Professor of Biodiversity & Conservation and the Founding Director of the Environment & Sustainability Institute at the University of Exeter.

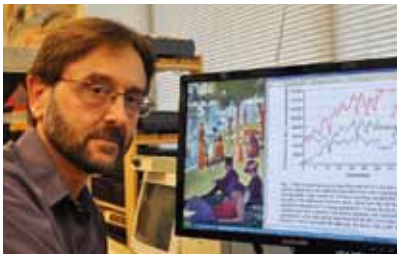
Kevin Gaston is an ardent proponent of studying the implications of physiological variation over large spatial and temporal scales and one of the founders of both macroecology and macrophysiology. He has authored over 450 papers and is an ISI Web of Knowledge Highly Cited Researcher. He has been a Leverhulme Research Fellow, recipient of a Royal Society-Wolfson Research Merit Award (2006-11), and in 2011 was elected to Academia Europaea.

As an invited speaker, Kevin will talk about macrophysiology, which investigates variation in physiological traits over large geographical and temporal scales, and it studies ecological implications of this variation. Macrophysiology strives for identification of patterns and understanding their origin. In doing so it attempts to address pressing global issues such as climate change, habitat destruction, invasive species, overexploitation and pollution.

Dr. John R. Speakman

Professor of Zoology in the School of Biological Sciences, University of Aberdeen and a '1000 Talents' professor in the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences in Beijing.

John Speakman did his PhD at the University of Stirling, Scotland. He was given the special expert status by the Chinese government, the highest honorary status for a foreign national in China. He was awarded several international prizes including the Zoological Society of London scientific medal, the Royal Society of Edinburgh/Saltire Society Scottish Science Medal, the Kwarazmi International festival Guest of Honor, the Chinese Academy of Sciences Bing Zhi Forum International Professorship, the CAS-Novo-Nordisk Foundation 'Great Wall' professorship. John Speakman was elected the Fellow of the Royal Society of Edinburgh and the Fellow of the Academy of Medical Sciences. He was also elected to the European Academy of Sciences (Academia europaea). John Speakman studies the causes and consequences of inter-individual variation in energy expenditure, especially the consequences of variation in energy demands for fat storage and the linkage between energy demands and ageing. His work provided a background to understanding the physiological basis of animal and human responses to energy imbalance including obesity and diabetes. He is a recognised international authority in the use of stable isotope methodologies to study energy expenditure (in particular the doubly-labelled water method). He has published over 400 peer reviewed scientific papers, including twice on the cover of Nature. He is on the editorial boards of Science and Biology Letters and several other journals and was a director on the board of the Company of biologists between 2005 and 2011.



Dr. Theodore Garland, Jr.

Professor of Biology at the Department of Biology, University of California, Riverside, Editor in Chief of the journal Physiological and Biochemical Zoology.

Ted Garland did his PhD at the University of California, Irvine. His main research interests concern the evolution of complex behavioral and physiological adaptations, especially related to locomotor performance of terrestrial vertebrates. He has become a renowned proponent of two major approaches in modern evolutionary physiology: quantitative genetics combined with experimental evolution, and phylogenetically informed comparative analyses. The former approach has led him to develop a unique study system – mice selectively bred for increased voluntary exercise on wheels. The experiment started in 1993 and has so far resulted in more than 120 research papers. His co-edited book, Experimental Evolution (2009), is a benchmark in the field. Methods and statistical software developed by Ted to consider phylogenetic perspectives in data analysis are now popular tools in a wide range of research areas. He has published almost 260 papers that have been cited a total of nearly 23,000 times. As an invited speaker, Ted will talk about his two approaches as complementary tools for resolving questions in comparative, ecological, and evolutionary physiology.

Dr. Michael Angilletta, Jr.

Professor and the Associate Director of Undergraduate Programs in the School of Life Sciences at Arizona State University.

Mike Angilletta studies the impacts of temperature on organisms and their consequences for ecological and evolutionary processes. His research on thermal biology has generated more than 70 scientific papers, but he is best known for his award-winning book, Thermal Adaptation. Mike has a passion for teaching biology at the introductory level, combining a love for thinking broadly with a flair for telling funny stories. He was recently voted ASU's Funniest Teacher, an honor that he cherishes more than any academic achievement.

As an invited speaker, Mike will talk about that ways that physiologists can contribute to the emerging discipline of macrosystems ecology, a computationally intensive approach to scaling from organismal processes to regional patterns. His talk will focus on theory and experiments that address the impacts of climate change on the distributions of species.

Dr. Elżbieta Pyza

Professor of Cell Biology and Neurobiology, Head of the Department of Cell Biology and Imaging, Institute of Zoology, and Scanning Electron Microscopy of the Faculty of Biology and Earth Sciences, Jagiellonian University, Kraków, Poland.

Elżbieta Pyza studies circadian neuroplasticity - a new type plasticity in the nervous system. She received the Canadian NSERC and Polish Academy of Sciences Awards and a scholarship of the Howard Hughes Medical Institute. She was also awarded with the President of Poland Silver Cross for scientific achievements. She is a corresponding member of the Polish Academy of Arts and Sciences and the Chairwoman of the Commission on Embryology and Morphology of the Academy.

Elżbieta Pyza was first, in collaboration with Dr. Ian Meinertzhagen from Dalhousie University, Halifax, Canada, to detect cyclic oscillations in synaptic contacts and morphology of neurons in the visual system of Drosophila melanogaster and other fly species. This phenomenon is called circadian neuroplasticity and recently it was also observed in mice. Now she and her collaborators are studying the regulation of circadian neuroplasticity by circadian clocks and mechanisms of this process.

As an invited speaker, Elżbieta will talk about circadian rhythms in the visual system of Drosophila and in the somatosensory cortex of mice.

Dr. Marek Konarzewski

Professor of Biology at the Institute of Biology, University of Białystok, Poland and the Corresponding Member of Polish Academy of Sciences.

Marek Konarzewski pursued his PhD and D.Sc. degrees at the Polish Academy of Sciences Institute of Ecology and then returned to the University of Białystok, after postdoctoral work in Dr. Jared Diamond's laboratory at the University of California, Los Angeles.

Marek's research interests interface evolutionary ecology, eco-physiology and quantitative genetics of vertebrates. He uses both laboratory and natural settings to study physiological traits (mostly related to metabolism) from molecular to whole-animal levels. He is a member of Editorial Board of Physiological and Biochemical Zoology and serves as an Associate Editor of Functional Ecology.

As an invited speaker, Marek will address implications of the cell size for the key organismal traits - body size and metabolic rates and their links to major molecular traits - genome size and pathways such as mTOR. His presentation will be co-authored by Dr. Jan Kozłowski, Professor of Biology at the Institute of Environmental Sciences, Jagiellonian University.

Student Fee-Waiver Awards

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The PBZ Awards were given to:

Emily Cornelius – *University of Wisconsin-Madison, USA:* oral presentation O.20.9 (page 239)

Olga Fedoruk – *Ternopil National Pedagogical University, Ukraine:* poster P.5.1 (page 86)

Olga Kushniryk – *Yuriy Fedkovych Chernivtsi National University, Ukraine:* poster P.27.1 (page 339)

Nastashya Wall – *McMaster University, Canada:* poster P.20.2 (page 242)

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Plenary lectures

KEYNOTE LECTURE

Macrophysiology - progress and prospects

Kevin Gaston

Environment and Sustainability Institute, University of Exeter, UK; K.J.Gaston@exeter.ac.uk

Macrophysiology is the investigation of variation in physiological traits over large geographic and temporal scales and the ecological implications of this variation. As an explicitly defined field of investigation it is a decade old. It thus seems timely to re-examine its conceptual foundations and methodological approaches, the insights it has provided and the challenges the field is presently facing.

From Thermal Physiology to Macrosystems Ecology: Building a predictive theory for real world problems

Michael J. Angilletta Jr.[1], Ofir Levy[1], Lauren Buckley[2], Tim Keitt[3], Michael W. Sears[4]

[1]Arizona State University, USA; [2]University of Washington, USA; [3]University of Texas at Austin, USA; [4]Clemson University, USA; angilletta@asu.edu

The growing interest in economic and environmental impacts of climate change has thrust physiologists into the spotlight. As if explaining physiological adaptation to past climates wasn't hard enough, policy makers, lay people, and other scientists would like us to tell them whether physiological adaptation will help species to persist decades from now. My talk addresses three major challenges of using physiological research to forecast ecological impacts of climate change. First, we must scale from the macroclimates derived from global circulation models to the microclimates experienced by organisms. This challenge requires us to downscale climatic data from a resolution of 1000 km to a resolution of 1 m or less. Then, we must use these microclimates to infer acute and chronic effects on the physiological performance of organisms. This second challenge requires us to design experiments in which microclimatic conditions correspond to likely scenarios for past and future environments. Finally, we must aggregate performances of organisms to predict the dynamics of populations. This third challenge requires individual-based models parameterized with physiological data, as well and behavioral and life-historical data. To meet these challenges, I unwittingly journeyed between two points in my career: from a researcher who studies the thermal physiology of lizards to a member of a team that integrates climate modeling, organismal biology, and landscape ecology. I will tell the story of that journey, highlighting recent efforts to understand the potential impacts of climate change on North American lizards.

Born to Run: replicated selective breeding for high levels of voluntary exercise in house mice

Theodore Garland, Jr.

Department of Biology, University of California, Riverside, USA;
theodore.garland@ucr.edu

Selection experiments are an important tool in comparative, ecological, and evolutionary physiology (1). Since 1993, we have bred 4 replicate lines of mice for high levels of voluntary wheel running on days 5 & 6 of a 6-day period of wheel access when they are young adults. These High Runner (HR) lines are compared with 4 non-selected Control (C) lines to test for correlated responses to selection at various levels of biological organization. Wheel running responded rapidly to selection before reaching apparent limits at generation 16-28, depending on line and sex. Wheel running has not increased appreciably in the HR lines for an additional 40+ generations of selective breeding. Causes of the selection limits are not yet understood, but they are not simply due to an exhaustion of additive genetic variance for daily running (2). An additional hybrid HR line is now under selection in an attempt to break the limit, possibly in a sex-specific manner (3). At various generations, HR lines have been shown to have elevated maximal oxygen consumption and treadmill endurance, reduced body size and body fat, larger hearts, increased baseline circulating corticosterone concentrations, larger brains, altered motivational systems and responses to dopaminergic drugs, increased hindlimb symmetry, larger femoral heads, altered semicircular canal shape. Current research is focused in several complementary areas, including genetic analyses, the evolution of brain size (4), effects of early-life environmental conditions (including maternal effects) on adult phenotypes (5), and integrating of research materials (e.g., digital images of skeletal elements) into educational activities (6).

References: (1) Storz *et al.* 2015. Am. J. Physiol. In press. (2) Careau et al. 2013. Evolution 67:3102-3119. (3) Hannon *et al.* 2011. Behav. Genet. 41:615-624. (4) Kolb et al. 2013. J. Exp. Biol. 216:515-523. (5) Acosta *et al.* 2015. Physiol. Behav. In press. (6) Radojcic *et al.* 2014. Science Scope 37:51-60.

Circadian plasticity in the brain of insects and mammals

Elżbieta Pyza

Department of Cell Biology and Imaging, Institute of Zoology, Jagiellonian University, Kraków, Poland; elzbieta.pyza@uj.edu.pl

Plasticity of the brain enable vertebrates and invertebrates to process sensory information but also to learn and to form memory. It has been reported, as changes in the properties and in the number of contacts between neurons – synaptic plasticity and in the structure of neurons – neuronal plasticity, in response to external stimuli. We have found a new type of plasticity, circadian plasticity, that occurs in the brain of flies and mice. This is also a new type of circadian rhythms detected at the level of nerve cells in the visual system of flies and in the barrel cortex, a part of somatosensory cortex of mice.

In the visual system of the fruit fly *Drosophila melanogaster* synapses and the presynaptic protein BRP cycle during the day and night and these rhythms are maintained in constant darkness (DD) indicating their circadian origin. The rhythms in synapses between the retina photoreceptors and the first order interneurons (tetrad synapses) and in BRP in those synapses are correlated with cyclic changes of size of interneuron axons and dendrites [1].

In the barrel cortex of mice, where cortical representation of whiskers are located, inhibitory synapses show the circadian rhythm in the number, with more synapses during the night when animals are highly active in running wheels. In turn the number of excitatory synapses increases during the day but this rhythm is not circadian. Moreover, the number of double synapse dendritic spines increases during the night but single ones during the day [2]. These results suggest that inhibitory synapses may be added to the existing single synapse dendritic spines, with one excitatory synapse, to decrease excitation during the period of high flow of sensory information.

Both examples of circadian plasticity which have been detected in flies and mice indicate spontaneous circadian remodelling of the brain that is important for different brain functions and behaviour during the day and night.

[1] Górska-Andrzejak J, Damulewicz M, Pyza E 2015 Circadian changes in neuronal networks. *Current Opinion in Insect Science* 7:1-6.

[2] Jasińska M, Grzegorczyk A, Jasek E, Litwin JA Kossut M, Barbacka-Surowiak G, Pyza E 2014 Daily rhythm of synapse turnover in mouse somatosensory cortex. *Acta Neurobiol Exp* 74:104-110.

The physiological basis of life history trade-offs: is there a role for oxidative stress?

John R. Speakman[1, 2]

[1]*Institute of Biological and Environmental Sciences, University of Aberdeen, UK;*
[2]*State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, People's Republic of China;*
j.speakman@abdn.ac.uk

Life history theory concerns the trade-offs that determine the patterns of investment by animals between reproduction, growth and survival. It is widely recognised that physiology and energetics play a role in the mediation of life history trade-offs, but the details remain obscure. As life history theory concerns aspects of investment in the soma that have consequences for survival, understanding the physiological basis of life histories is also related to understanding the fundamental process of ageing. One idea from the field of ageing that has gained considerable traction in the area of life histories is that life history trade-offs may be mediated by free-radical production and oxidative stress. In this lecture I will explore the basis of this idea and present recent published and unpublished data which provide tests of its predictions.

Macrophysiology from the cell size perspective

Marek Konarzewski[1], Jan Kozlowski[2]

[1]*Institute of Biology, University of Białystok, Poland; marekk@uwb.edu.pl*
[2]*Institute of Environmental Sciences, Jagiellonian University, Poland; jan.kozlowski@uj.edu.pl*

Albert Einstein once famously pointed to a peculiar phenomenon of a relative uniformity of the cell size across an enormous range of body size variation found in animal kingdom. Why and how then the cells of relatively low range of sizes can be building blocks of a mouse and a whale? Or was Einstein, after all wrong, and even a small cell size variation observed among different taxa translates into a variety of life histories and macrophysiological patterns? We argue that many of those patterns (such as the Peto's paradox, temperature-size rule and ultimately, scaling of metabolic rates) cannot be fully explained without studies on mechanisms differentiating the coupling between cellular size, metabolism, and body mass. Mounting evidence suggest that whole-body metabolic rates are simultaneously affected by the interplay between the primary physical factors (surface-to volume ratios of the nuclei and cells) and biological factors (chromatin density related to genome size directly affecting the intensity of transcription and translation) and molecular crowding affecting major metabolic pathways in cell matrix, such as mTOR. As a result, equally large organisms still differ many-fold in their metabolic rates. We therefore call for a revival of studies linking the cell size with the key organismal traits, which may greatly contribute to better understanding of the mechanisms that give rise to macrophysiological patterns.

Symposia

Ion Homeostasis and Functional Regulation in Aquatic Animals: Molecular and Integrative Studies

Organizers (*Canadian Society of Zoologists* and *Japanese Society for CPB*):

Greg Goss, *University of Alberta, Canada*
Toyoji Kaneko, *University of Tokyo, Japan*
Pung-Pung Hwang, *Academia Sinica, Taiwan*

Prolactin and cortisol mediate the maintenance of the ionocytes expressing Na^+, Cl^- cotransporter in Mozambique tilapia: Exploring with a newly-developed gill filament incubation system

Soichi Watanabe, Toyoji Kaneko

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Endocrine control of osmoregulation is essential for teleosts to adapt to various aquatic environments. Prolactin (PRL) is known as a fundamental endocrine factor for hyperosmoregulation in teleost fishes, and acts on ionocytes in the gills to maintain body fluid ion concentrations at constant levels in freshwater conditions. Cortisol is also known as an osmoregulation-related steroid in teleosts; however, its precise function is still controversial. Here, we investigated more detailed effects of PRL and roles of cortisol on ionocytes of Mozambique tilapia (*Oreochromis mossambicus*) in freshwater, using a newly-developed gill filament incubation system. In the gill filament incubation, cell viability was highly improved by an incision made along the filament. PRL was showed to maintain the density of freshwater-type ionocytes in isolated gill filaments; this effect of PRL is not achieved by the activation of cell proliferation, but by the maintenance of existing ionocytes. Cortisol did not show any distinct effect on ionocyte density in isolated gill filaments. We also assessed effects of PRL and cortisol on gene expressions of Na^+, Cl^- cotransporter (NCC), Na^+/H^+ exchanger-3 (NHE3), Na^+/K^+-ATPase α-subunit (NKAa) 1a and NKAa1b. PRL maintained NCC and NKAa1a expressions, and cortisol showed a synergistic effect with PRL on NCC and NKAa1a expressions, though cortisol alone exerts no effect on these genes. PRL treatment also maintained normal NCC localization at the apical membrane of the ionocytes. These results indicate that PRL maintains freshwater-type ionocytes, and that cortisol stimulates the function of ionocytes maintained by PRL.

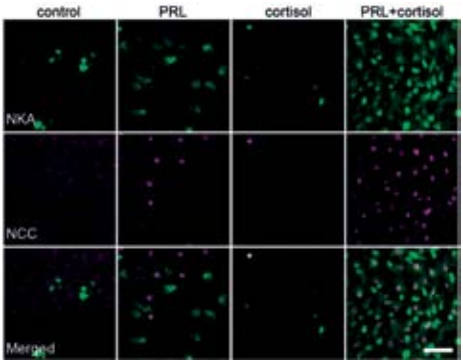

Figure: Immunohistochemical observation of the ionocytes on the 72 h-incubated gill filament from freshwater-adapted fish.

A convergent model representing proton and ammonia transport through epithelium in cephalopods

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Cephalopods have evolved complex sensory systems and an active lifestyle to compete with fish for similar resources in the marine environment. Their highly active lifestyle and their extensive protein metabolism has led to substantial acid-base regulatory abilities enabling these organisms to cope with CO_2 induced acid-base disturbances. Despite strong acid-base regulatory abilities described for cephalopods, the mechanistic basis for extracellular pH homeostasis is still poorly understood in these non-model organisms. According to our present knowledge we propose that extracellular pH stabilization during environmental acid-base disturbances is achieved via active HCO_3^- buffering and H^+ secretion in cephalopods. In convergence to teleost, cephalopods possess an ontogeny-dependent shift in ion-regulatory epithelia with epidermal ionocytes being the major site of embryonic acid-base regulation, while gill epithelia are the predominant site for pH regulation and ammonia excretion in adults. And a higher rate of proton removal from the blood under acidified conditions was accompanied by increased NH_4^+ excretion rates. These findings indicate conserved mechanisms for acid-base regulation and nitrogen excretion in marine species, which represents an exciting research direction addressing the evolution of pH regulatory systems.

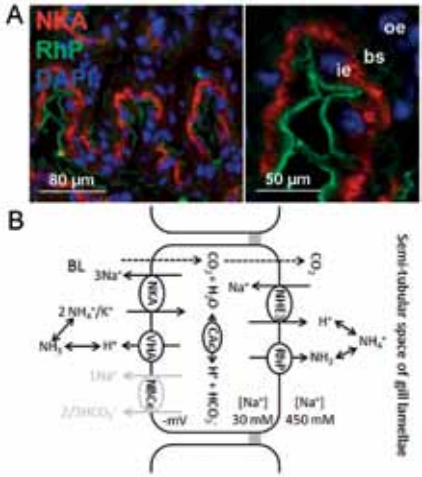

Figure Identification of NH_4^+ transport pathways in cephalopod branchial epithelia.

Immunohistochemical analyses demonstrate the presence of RhP in apical membranes of the inner epithelium of the third order gill lamellae (A). Hypothetical model for the transport of NH_4^+ across the branchial epithelium (B). NH_4^+ formation is supported by basolateral VHA and is imported into the cell by the NKA. At the apical membrane NH_4^+ is deprotonated and NH_3 and H^+ are separately transported into the semi-tubular space where a trapping of NH_4^+ is proposed.

The osmorespiratory compromise: regulation of paracellular and transcellular permeabilities of the gills

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In recent years, the focus of ionoregulatory research has been on the various mechanisms responsible for the active uptake of ions (in freshwater) or their active excretion (in sea water). However, an equally important, and often overlooked topic is the mechanisms governing the passive diffusive fluxes of ions in the opposite direction, as well as water movements. The gills have multiple functions, of which iono/osmo-regulation is only one. Respiratory gas exchange is at least equally important, and the gills must balance the homeostatic needs of these two functions – the "osmorespiratory compromise[2]" During exercise or environmental hypoxia, the requirements for O2 uptake increase, met by increases in the effective permeability of the gills to respiratory gases achieved by elevations in exchange area, reduced blood-to-water diffusion distance, and altered blood perfusion patterns. Traditionally, the "cost" of these adjustments was unfavorable elevation in passive ion and water fluxes, and these were thought to occur through mainly paracellular pathways. However the discovery of at least one species, the Amazonian oscar (*Astronotus ocellatus*), which during acute hypoxia actually reduces branchial ion and water permeabilities[5], does not change gill paracellular permeability[5], but exhibits profound changes in gill cellular morphology[1] has led to a re-assessment of this interpretation. I will discuss recent published and unpublished findings on methods for assessing gill paracellular versus transcellular permeability[3], on species surveys of the osmorespiratory compromise[4], and on responses to exercise versus hypoxia, with particular focus on the oscar, the rainbow trout (*Oncorhynchus mykiss*), and the common killifish (*Fundulus heteroclitus*) (NSERC Discovery, CNPq).

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Acid-base regulation is linked to salt excretion of ionocytes in seawater-acclimated medaka (*Oxyzias latipes*)

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Branchial ionocyte of seawater (SW) teleosts is responsible for salt secretion and acid-base regulation. The apical Na^+/H^+ exchanger (NHE), cytosolic carbonic anhydrase (CA) and basolateral HCO_3^-/Cl^- exchanger (AE) in ionocytes were suggested as the major transporters for acid-base regulation. We hypothesized that this function (acid-base regulation) is also linked to the salt (Cl^-) secretion of ionocytes because of the basolateral AE. In this study, SW-acclimated medaka was used to test this hypothesis. A scanning ion-selective electrode technique (SIET) was applied to measure H^+ and Cl^- secretion by ionocytes in the skin of larvae. Treatments with inhibitors of NHE, CA and AE effectively suppressed both H^+ and Cl^- secretion by ionocytes. In situ hybridization and immunocytochemistry colocalized NHE3 (*slc9a3*), CA2 (*ca2*) and AE1 (*slc4a1*) in the skin ionocytes. A real-time qPCR analysis showed that branchial mRNA expressions of *slc9a3*, *ca2* and *slc4a1* were induced by SW; *slc9a3* and *slc4a1* were induced by acidified SW. This study showed that acid-base regulation is linked to salt secretion of ionocytes and modified the mechanism of salt secretion in SW teleost.

A study of calcium homeostasis regulation in the euryhaline flounder

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The concentration of calcium in extracellular fluid is precisely regulated throughout vertebrates from fish to mammals. The ability to sense changes in plasma-ionized calcium is mediated by the calcium-sensing receptor (CaSR), which is an integral component in regulating PTH secretion from the parathyroid glands of mammals. Parathyroid-hormone Related Protein (PTHrP) is a peptide found in many different organisms, both in higher and lower vertebrates and is one of the hypercalcaemic factors in mammals. Its role in lower vertebrates has been recently established. Parathyroid hormone related peptide (PTHrP) has been implicated in calcium and osmoregulatory physiology of fish as circulating levels change in accordance with osmotic state. Flounder (*Platichthysflesus*) is a teleost fish and in the evolutionary scale is considered a lower vertebrate. To investigate Parathyroid-hormone Related Protein (PTHrP) and parathyroid hormone receptors (PTHRs) and their functions in teleost fishes, we have cloned and identified sites of mRNA expression of the PTHrP1,PTHrP2,PTH1R and PTH3R in the flounder. Using quantitative PCR (QPCR), PTHrP1,PTHrP2,PTH1R and PTH3R mRNA expression has been measured in gill, kidney, bladder and gut of chronically adapted seawater (SW) and freshwater (FW) flounder. QPCR measures of PTHrP1 andPTH1R mRNA expression indicated similar levels for FW and SW flounder. PTHrP2 and PTH3R mRNA expression levels in FW adapted flounder gills were approximately 2-fold higher than those in SW adapted fish. The differential expression in PTH3R in SW and FW gill implies an underlying role in osmoregulation and perhaps a calcium regulatory function in fish as the gill is the major site of ion regulation. The *in vivo* responses of this system to the ip administration of EGTA rapidly induced hypocalcemia, and a concomitant increasing PTH_{1-34} and lowering of plasma STC-1.Calcimimetic administration(1 mg/kg R-568) rapidly increased plasma STC-1 levels, and reduced plasma concentrations of calcium when compared with S-568-treated controls. Together, these findings support an evolutionary conserved role for the CaSR in the endocrine regulation of calcium before the appearance of parathyroid glands in tetrapods.

Distributional changes in gill ionocytes in Japanese sea bass after transfer from seawater to freshwater

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We examined the distributional change in branchial ionocytes of Japanese sea bass. In several teleost species, including Japanese sea bass, immuno-histochemical observation revealed a clear salinity-induced change in the distributional pattern of ionocytes. In freshwater (FW), ionocytes were distributed on both filaments and lamellae, whereas lamellar ionocytes disappeared in seawater (SW). To observe the detailed distributional change in ionocytes, we first examined the ion-transporting mechanism of ionocytes in this species. Immunohistochemical observations showed that SW-acclimated fish had one predominant ionocyte type, which possessed cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel and Na^+/H^+ exchanger-3 (NHE3) in the apical membrane and $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and Na^+/K^+-ATPase (NKA) in the basolateral membrane. On the other hand, ionocytes of FW-acclimated fish were largely classified into two types: 1) cells with apical NHE3 and basolateral NKCC and NKA; and 2) cells with apical Na^+/Cl^- cotransporter (NCC) and basolateral NKA. Next, we examined the distributional change in ionocytes after transfer from SW to FW, applying immunohistochemical staining with anti-NHE3, anti-NKA and anti-NKCC/NCC (T4) antibodies. In fish reared in SW as controls, ionocytes expressing NHE3 and NKCC were localized exclusively in the filaments and absent from the lamellae. The density of NHE3/NKCC-immunoreactive ionocytes in lamellae increased with time after transfer to FW. NCC-immunoreactive ionocytes appeared in the filaments at 3 days after transfer. Together with the functional plasticity of ionocytes reported previously in other fish, our findings suggested that, on transfer from SW to FW, pre-existing NHE3/NKCC-immuno-reactive ionocytes transformed into FW type by losing apical CFTR and spread their distribution over lamellae. On the other hand, NCC-immunoreactive ionocytes were specific for FW and emerged from filaments.

ASIC4.2 is involved in Na^+ uptake in zebrafish (*Danio rerio*) larvae

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The molecular identity of the mechanism of Na^+ uptake across the gills of freshwater fish remains the subject of lively debate. Despite the extensive evidence for NHE mediated Na^+ uptake, the thermodynamic constraints on NHE function suggest that alternative mechanisms may need to exist. Recently, it has been demonstrated that acid sensing ion channels (ASICs) are present in the ionocytes of freshwater rainbow trout and zebrafish gill, and are involved in uptake of Na^+ in these species. To further investigate the role that ASIC4 may play in freshwater fish Na^+ acquisition, we used morpholino knockdown to inhibit translation of ASIC4.2 in zebrafish larvae. While knockdown of ASIC4.2 had no effect on zebrafish larvae reared in normal medium (Na^+=600 µM), it resulted in reduced Na^+ uptake in the morphants reared in low-Na^+ (Na^+=50 µM) medium. Moreover, staining with anti-ASIC4.2 antibody and ConconavalinA revealed that in zebrafish larvae, ASIC4.2 localizes to the apical membrane of HR MRCs. Findings from this study suggest that ASICs play a role in Na^+ uptake in freshwater fish reared in very soft water.

Plasma membrane Ca^{2+} ATPase in stereocilia is required for the functioning of mechanotransducer channels in lateral-line hair cells of zebrafish

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The sensory hair cells in mammalian inner ear are responsible for sound transduction. Sound waves deflect hair bundles and open the mechanotransducer (MET) channels, which initiate mechanotransduction. The MET channels expressed in stereocilia are cation channels with high Ca^{2+} permeability. In mammalian, mutation of Plasma membrane Ca^{2+} ATPase (PMCA) caused hearing loss however the mechanism is not well understood. In this study, zebrafish lateral line is used as an animal model to investigate the role of PMCA in mechanotransduction of hair cell. The protein expression of PMCA was showed in stereocilia of lateral line hair cell in 72 hpf zebrafish larvae. We used the scanning ion-electrode technique (SIET) for functionally assaying MET channel in hair cell. The MET channel mediated Ca^{2+} influx detected by SIET was suppressed by treatment of PMCA inhibitors (o-vanadate, eosin Y). Morpholino knockdown of PMCA1 also showed reduced MET channel mediated Ca^{2+} influx and abnormal swimming behaviors. These results indicated that PMCA was critical for maintaining the function of MET channel.

Hormone control of fish ion regulation: Zebrafish as a model

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Fish have sophistcicated mechanims of ionic and acid-base regulaiton for mainting body fluid homeostasis through gill ionocytes. Many hormones have been proposed to control the ionic and acid-base regulation mechanisms in fish but lack convincing evidences to support. Zerbafish, an emerging model with several research advantages, was used to explore the related issues. In zebrafish gill and embryonic skin, different types of ionocytes were identified to carry out ion transport functions through respective sets of ion transporters, and the molecular mechanisms of ionocytes proliferation and differentiation were also dissected. These provide a competent platform to precisely study the ion transport pathways and ionocytes that a hormone targets. The expression or the synthesis of isotocin, cortisol, vitamin D, stanniocalcin (STC-1) and/or their receptors were found to be affected by different ion levels or pH. Loss- and gain-of-function experiments showed that isotocin stimulates the ion transport functions of ionocytyes through regulating the proliferation of p63-labeled stem cells and the differentiation of Foxi3a-labled ionocytes progenitors. Cortisol and vitamin D also exert their positive actions on zebrafish ion transport through stimulating the Foxi3a-mediated ionocytes differentiation. Contrarily, STC-1 reveals the negative actions by suppressing the Foxi3a expression and thus the ionocytes differentiation. The similar approaches were used to demonstrate the involvement of endothelin (EDN1) signaling in the activation of acid secretion function in a specific group of acid-secreting ionocytes upon acute acidification in aquatic environment. EDN1 expression was increased at 4 h after low pH treatment. Gain- and loss-of-function of EDN1 (or the receptor) respectively resulted in stimulation and suppression of the acid secretion function without effects on the H^{+}-ATPase translation, implying a regulation at the post-translational level. This suggests the role of END1 in the acute regulation of acid secretion mechanism. As such, to cope with changing environment these hormones control fish ionic and acid-base homeostasis through regulations of the activities/functions of target ion transporters or ionocytes at the level(s) of transcription, translation or post-translation, and/or different developmental stages of the cells.

Phosphoenolpyruvate carboxykinase (PEPCK)-mediated gluconeogenesis accelerates acid secretion by H^+-ATPase-expressing ionocytes during acidosis

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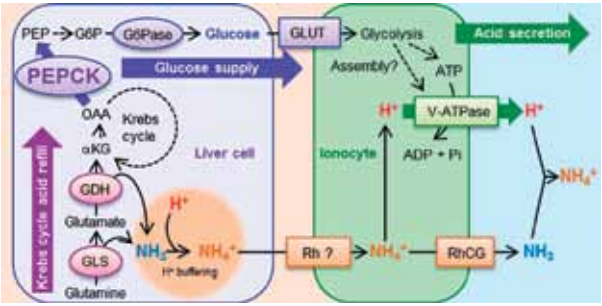
^{V}acuolar-Type H^+-ATPase (V-ATPase) takes central roles in pumping H+ through cell membranes of diverse organisms, which is essential for surviving acid-base fluctuating lifestyles or environments. Although glucose is believed to be an important energy source to drive V-ATPase, the link between acid secretion and glucose supply remains unclear. In the present study, we used zebrafish larva as an *in vivo* model to show a role of the PEPCK, a key enzyme for gluconeogenesis, in H+ secretion by V-ATPase-expressing ionocytes. When zebrafish larvae were exposed to low-pH stress, H^+ secretion by H^+-ATPase-rich (HR) cells and Pck1 (zebrafish PEPCK) expression in liver was increased within 6 h. Although knockdown of *pck1* abolished acid-induced H^+ secretion by HR cells, glucose supplementation to yolk could rescue this effect. Analysis of body amino acid contents showed drastic decreases in glutamine and glutamate levels in the low-pH exposed larvae, and a gene coding for mitochondrial glutaminase was highly expressed in the liver of these larvae. Through a series of the experiments, we found that PEPCK-mediated gluconeogenesis provides additional glucose to drive V-ATPase during acidosis, and glutamine and glutamate are probably the major source for such acute glucose production.

Figure: A schematic view showing the acid-induced metabolic shift and gluconeogenesis in the liver supporting enhanced H^+ secretion by HR cells.



Effect of dissolved organic carbon from different sources in the Amazon basin (Brazil) on the osmoregulatory compromise of tambaqui (*Colossoma macropomum*) challenged by acid water

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Acid waters have been recognized to inhibit the active uptake of Na^+ and Cl^-, while stimulating massive diffusive ion losses in freshwater fishes. Dissolved organic carbon (DOC) seems to ameliorate the effects of low pH exposure, modulating branchial Na^+ transport systems and the electrical properties of fish gill membrane. These physiological effects mediated by DOC on gill functions have been directly associated with DOC physicochemical properties. Therefore, we analyzed the responses of osmoregulatory parameters in the Amazonian fish species *Colossoma macropomum* challenged by acid water in presence of three different sources of natural DOC from Amazon Basin (São Gabriel da Cachoeira, SGC; Novo Airão, NA and Iranduba, IRD), which, in turn, displayed important differences in their physicochemical properties. Our findings demonstrate, so far, that SGC DOC directly affects gill permeability in tambaqui challenged by acid water, as seen as by the more negative TEP, which seems to be related with the high aromaticity of the DOC molecules from SGC. However, in contrast to our initial hypothesis, Na^+ and Cl^- net losses were significantly stimulated in tambaqui exposed to low pH in the presence of SGC DOC, resulting in lowered levels of both Na^+ and Cl^- in plasma of fish. The initial results indicate that the SGC DOC clearly affected the ionic homeostasis of tambaqui, but exerted a “non-protective” effect against the deleterious effects of low pH exposure. Thus, the comparative study of the physiological responses mediated by DOC from the lower Rio Negro and from the Solimões River in tambaqui will highlight the nature and magnitude of the direct effects of DOC molecules on physiological functions of gills of native Amazon fish.

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Hematological changes during elimination of copper in Prussian carp after short-term exposure to sublethal concentration of this metal in water.

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The aim of this study was to investigate hematological changes occurring in the peripheral blood of Prussian carp during the purification after the short exposure (2.5 or 5 hours) to sublethal concentration of copper in the water (2 mg Cu/L). Hematological parameters (RBC, WBC, hematocrit) were specified and additionally Cu, Fe and Zn concentration was determinate in collected tissues (gills, muscles, liver, kidney). Heavy metals level analysis was performed by AAS method using an atomic absorption spectrometer ATI UNICAM 929. Exposure of Prussian carp to Cu caused a decrease of RBC and WBC, as well as fluctuating changes in hematocrit index, which indicates the toxic influence of Cu on the basic hematological parameters. Statistically significant correlations of RBC and WBC on the exposure duration (2.5 or 5 hours) were observed during 5 hours after exposure cessation. The highest concentration and rate of elimination of Cu was observed in the gills of the examined fish. Periods of exposure used in the experiment did not affect either the maximum observed concentration levels of Cu, Fe, Zn and on the duration of the purification, but statistically significant correlations of studied parameters to the exposure duration were noted. After exposure to Cu the decrease of Fe and increase of Zn levels were observed in tissues.

Comparison of strategies in calcium balance for hypothermic acclimation between tilapia (*Oreochromis mossambicus*) and goldfish (*Carassius auratus*)

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Most freshwater teleosts live and grow in a much lower Ca^{2+} concentration environment compared to their body fluids. Therefore, they must uptake Ca^{2+} from the water by the active transportation mainly through the gill. The transportation is depended on several Ca^{2+} transporters include ECaC (Epithelial Calcium Channel), Ca^{2+}-ATPase and Na^{+}/Ca^{2+} exchanger (NCX). In addition, the teleosts are poikilothermic animals and therefore the body temperature will be fluctuated following the change of the seasons. When the environmental temperature decreased, freshwater fishes need to change the parameters such as Ca^{2+} -dependent ATPase kinetics (Mcardle and Johnston, 1982) and activity. However, the studies on the Ca^{2+} uptake acclimation under cold challenge are mainly focusing on the sarcocplasm reticulum but are relatively scarce on the whole body Ca^{2+} regulation.

We have used eurythermic goldfish and steothermic tilapia as the comparisons for the cold acclimation study. Upon the cold challenge, goldfish can adjust the Ca^{2+} influx and netflux rates back to the normal status but not the tilapia. In addition, by the measurement of the Km and Vmax for Ca^{2+} influx rate we also detected that binding affinity and maximum transportation velocity are both increased in the goldfish but not in the tilapia. Finally, we can detect the decrease of the whole body Ca^{2+} content is more obvious in tilapia than in goldfish.

Kinetics and pH optima of gill Na/K-ATPase from white sturgeon (*Acipenser transmontanus*) following exposure to elevated salinity and aquatic hypercarbia

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The white sturgeon (*Acipenser transmontanus*) is a euryhaline fish with a tremendous capacity to tolerate aquatic hypercarbia. This study examined juvenile white sturgeon during a 10-day exposure to either elevated salinity (20ppt) or aquatic hypercarbia (elevated $PwCO_2$: ~6 kPa) or a combination of both stressors. Aquatic hypercarbia induced an immediate extracellular acidosis (from pH 7.65 to pH 7.2) while seawater exposure following aquatic hypercarbia caused an ~30% increase in plasma osmolality (from ~250 to ~ 330 mOsmol/kg). During this acclimation any proteins exposed to the extracellular environment must be able to maintain proper function despite large changes in plasma pH, $PwCO_2$, total osmolality and ion concentrations. Gill Na/K-ATPase is a primary active transporter involved in powering both acid-base balance and ion regulation in fish. Since this transporter is localized to the basolateral membrane with extracellular domains exposed to the plasma we chose to study the kinetics and pH optima of this protein in sturgeon during their acclimation to both elevated salinity and aquatic hypercarbia. Under these conditions, gill Na/K-ATPase activity increased six-fold while protein levels increased 2.5 fold. The pH optima for gill Na/K-ATPase was found to be ~7.6 in control fish as well as those exposed to both elevated salinity and hypercarbia, coinciding with control plasma pH levels. Na/K-ATPase activity was maintained over a broad pH range suggesting this enzyme can continue to function despite the wide fluctuations in pH experienced during an acid-base disturbance.

The effect of copper intoxication moment for embryonic development, hatching and early life stages of Prussian carp (*Carassius gibelio* Bloch) larvae

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The aim of presented study was to estimate the effect of cooper exposition during the embryonic development and juvenile stages of Prussian carp (*Carassius gibelio* Bloch). Copper is an essential trace element required for homeostasis of organisms, but in polluted environment together with other heavy metals copper exerts adverse effect on aquatic organisms, even at the early stages of their development. The experiment was divided into two parts, first tested the impact of Cu on embryonic development and hatching success, while the second one investigated the reaction of hatched larvae to this metal during 94 days.

First the eggs, then larvae were kept in the laboratory condition, in water containing copper ($1 mg \cdot L^{-1}$). The sample of eggs obtained from 5 sexually matured females of Prussian carp were divided into two dishes and incubated for 4 days after activation with common carp (*Cyprinus carpio* L.) sperm. The mortality, hatching rate, number of hatched larvae and number of deformed larvae was observed.

In the first part of experiment tested concentration of Cu accelerated hatching and decreases the percent of larvae survival. It was also observed that the percentage of deformed larvae was highly significantly higher than in the control group. In the second part of experiment it was shown that cooper ($1 mg \cdot L^{-1}$) did not significantly affect the growth and weight gain of Prussian carp (*Carassius gibelio*, Bloch) larvae during the time of observation.

Summing up the obtained results shown that copper is a heavy metal that has highly teratogenic effects on Prussian carp larvae and significantly interferes the most important developmental stage - embryogenesis.

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Freshwater chloride levels dramatically influences post feeding acid-base recovery and SDA in rainbow trout

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Acid secretion into the stomach after food consumption is accompained by base secretion into the blood. This lead to a rapid rise in blood pH and [HCO_3^-] (the "alkaline tide"), recovery from which involves regulatory processes at the gill, intestine and kidney. In freshwater most HCO_3^- excretion or uptake occurs at fish gills, in exchange for Cl^-, facilitated by Cl^-/HCO_3^- transport. Freshwater rainbow trout has the widest global distribution of any aquacultured species. However, "freshwater" is not a single homogeneous environment and water chemistry relevant to ion transport physiology in fish (including chloride) naturally vary by over 100-fold geographically (Hartmann et al., 2014). To date, however, the effects freshwater Cl^- levels on post-prandial physiology and on the cost of food digestion have not been explored. Since water Cl^- levels would directly influence branchial Cl^-/HCO_3^- exchanges, we hyphothesised that Cl^- levels in freshwater would affect recovery from the alkaline tide and potentially also the cost of digestion (i.e. Specific Dynamic Action or SDA). In adult rainbow trout at 15 °C and voluntarily feeding on a 3 % ration, experimentally varying environmental [Cl^-] (0.05, 0.5 and 5 mM) lead to dramatic changes in bicarbonate, ammonia, and net acid-base fluxes. High Cl^- increased the peak rate of net base excretion by 13-fold (from 1.5-3h), post-prandial recovery was ~2.5 times earlier, and the cumulative net HCO_3^- flux was 2-fold higher. SDA was also affected by external Cl^- levels, lasting 26% longer under low Cl^- levels. The net SDA (total O_2 consumption associated with digestion), however, was lowest at the middle freshwater chloride level (0.5 mM). These data have implications for the energetics of fish growth both in aquaculture and in natural settings, based on geographical variations in water chemistry.

Hartmann J, et al. 2014. A brief overview of the GLObal RIver CHemistry Database, GLORICH. *Geochem Earth Surf Ges-10* 10: 23-7.

Insect Molecular Biology - the Postgenomic Era

Organizer (*Deutsche Zoologische Gesellschaft*):

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RNA-interference- a tool for evaluating hormonal functions in insects

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RNA interference (RNAi) is a natural cellular process by which mRNA is targeted for degradation by a small interfering RNA that contains a strand complementary to a fragment of the target mRNA. This results in a sequence-specific inhibition of gene expression. The discovery of RNAi enabled the use of loss-of-function analyses in many non-model insects with still unknown genome. The dsRNA application can be performed by injection, feeding, or even by spraying. Ecdysteroids and juvenile hormones (JH) are known as key hormonal regulators in insect larval development and metamorphosis. Moreover, both types of hormones are involved in the regulation of reproduction in adult insects. Besides the classical developmental hormones, numerous neuropeptides are integrated in these processes, showing highly species-specific functions.

The present review deals with recent contributions from our laboratory describing the role of juvenile hormone and neuropeptides in insect life phase transition and egg production, using the RNA-interference technique for a specific knockdown of enzymes from the JH pathway and of neuropeptide preprohormones controlling JH biosynthesis:

(1) Enzymes involved in the JH pathway of medflies (*Ceratitis capitata*; Diptera) during reproduction; (2) The role of allatotropin on the titer of juvenile hormones during larval development in *Spodoptera frugiperda* (Lepidoptera); (3) The role of o allatotropin and allatostatin type C on the transfer of juvenile hormones from males to females during mating in *S. frugiperda* (Lepidoptera); (4) The role of allatotropin and allatostatins type B and C in the pupal stage of *Tribolium castaneum* (Coleoptera); (5) The role of allatostatin type A on the synthesis of juvenile hormone III in termites (*Mastotermes darwiniensis*; Isoptera).

Neuropeptides are highly pleiotropic in function and their particular tasks have to be ascertained in each species. Besides its use in exploring gene functions, RNA-interference has a huge potential to be used in insect pest management.

Molecular dissection of insect seasonality using RNAi: Roles of the circadian clock genes

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The bean bug *(Riptortus pedestris)*

Photoperiodism is a seasonal timing mechanism that enables organisms to coordinate their development and physiology to annual changes in the environment in response to day length (photoperiod) as an upcoming seasonal cue. Many insects adopt photoperiodism to overcome harsh seasons unfavourable for continuous growth and reproduction by entering diapause, a hormonally-regulated developmental/reproductive arrest. Our focal species, the bean bug *Riptortus pedestris*, also shows clear photoperiodism; adults reproduce under long days, whereas enter reproductive diapause and accumulate much lipid under short days for overwintering.

One of the important physiological systems in photoperiodism is the photoperiodic time measurement that measure and assess the length of day or night. It has been widely accepted in many organisms that a circadian clock is deeply involved in the photoperiodic time measurement system (Bünning's hypothesis). However, there has been little conclusive evidence in insects. We have approached this issue by using RNA interference (RNAi) of several circadian clock genes. We found that RNAi of clock genes clearly disrupts (1) the circadian rhythm, (2) photoperiodic regulation of ovarian development in females, (3) photoperiodic regulation of secretory fluid accumulation in the ectodermal accessory gland reservoir in males, and (4) photoperiodic regulation of lipid accumulation, which is regulated by a distinct cascade from the reproductive organ development. These results indicate that the circadian clock resides as a core system in photoperiodism, possibly in the photoperiodic time measurement system.

Frontiers in cold hardiness: An "omics" world

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Molecular studies of cold and frozen systems – be they naturally cold-hardy animals, or artificially cryopreserved cells/tissues/organs – all have one thing in common. This is a need to identify and quantify biochemical markers of stress, injury and survival. Assessing complex cell functions has traditionally been difficult and time consuming – absorbing many grad student careers for energy-intensive methods like gene screening, PCR, immunoblotting and enzyme assay. However, new technologies have emerged recently that are re-focusing research efforts and allowing huge amounts of data to be gathered from very small cell/tissue samples. Among these, accelerated rates of genome sequencing are giving us access to genomic tools to find inherent differences in genes between species, and much easier, faster and cheaper tools are now available for metabolic analysis: e.g. RNA seq or RT^2 profiler arrays for screening stress-responsive transcriptomes, proteome analysis via mass spectrometry, and Luminex technology that allows multiple protein analytes to be assessed simultaneously in a multiplex format. Crucial data on metabolic status also comes from analyzing the patterns of posttranslational modification of macromolecules including the phosphoproteome and the epigenome. This presentation will assess various options for the use of new metabolic and genomic tools in the investigation of insect cold hardiness with a particular focus on recent work from my lab that has analyzed cold/freeze responsive microRNA expression and posttranslational control of enzymes in goldenrod gall-forming insects, *Eurosta solidaginis* and *Epiblema scudderiana*. For comparative- and cryo-biochemistry, the future is bright as we now have the capacity to apply many new molecular technologies to explore stress response and adaptation. Studies in my lab are supported by NSERC Canada. For more information go to: www.carleton.ca/~kbstorey.

Silks from insects – From natural diversities to technical applications

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Since all known silks which have evolved multiple times are heterogeneous in both molecular structure and biological function, it is difficult to postulate a distinct definition of silk: Silk belongs to the family of structural extracorporeal proteins with highly repetitive sequences, which are processed starting with a concentrated silk solution. This definition excludes e.g. hair which is fabricated out of low concentrations of keratin. In particular, silks are fibers, which distinguish them from other secreted structural proteins such as glues produced by marine organisms. On a molecular level, one specific characteristic of silk is the semicrystalline structure based on a distinct amino acid sequence. Crystallites are embedded in an amorphous matrix and contribute to the unique mechanical properties of silk. Throughout all known silk types, the fraction of crystalline structures varies from low to high amounts with direct impact on the mechanics of the fibers.

Silks are only produced by arthropods; the silkworm (larva of *Bombyx mori*) and spiders (*Araneae*) are probably the most prominent producers of silk. Only few examples of aquatic silks are known, e.g. the marine silk of amphipods, or the insect silk produced under water by the larvae of midges and caddisflies. The majority of silks is produced in terrestrial environments, and during evolution an impressive variety of different silks has emerged especially within the class of insects.

Here, an overview of insect silk will be given, focusing on the relation between molecular structure and biological function as well as the processing of silk. Additionally, examples of possible biomedical and technical applications of insect silk will be illustrated.

Neuenfeldt M, Scheibel T 2014 Silks From Insects: From Natural Diversity to Applications. *Insect Molecular Biology and Ecology* 376-400.

Molecular mechanisms behind the evolution of a plant-derived chemical defense in the model system *Chrysomelina* leaf beetles

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Leaf beetles (Chrysomelidae) of the taxon Chrysomelina are caught in the trophic sandwich between plant defenses and predators and parasitoids. The immature chrysomeline beetles developed defensive glands in which sequestered plant secondary metabolites are converted, stored and released in case of attack by their natural enemies. To achieve exploitation of phytochemicals, the larvae evolved transport and metabolic mechanisms already present in the iridoid (cyclopentanoid monoterpenoids) de novo producing Chrysomelina species. The underlying mechanisms of sequestration were selected being adaptive to host pant chemistry which allowed a Chrysomelina lineage to escape the plant constraints. This host plant switch is accompanied by phenotypic plasticity in the chemical defense contributing to create an enemy-free space as a refuge against carnivores but may also have consequences for the mutualistic interactions in an ecosystem. The knowledge of transport preferences, enzyme kinetics, and gene architecture in the chemical defense of the taxon Chrysomelina donates to our holistic understanding of evolutionary processes leading to adaptive radiation of herbivores and plants. From our recent studies it became clear that not only enzymatic reactions, e.g. catalyzed by non-selective glucosidases or selective oxidoreductases, found in the secretions of sequestering species, but also transport processes essential for sequestration are already developed in the ancestral species. Comparative studies on iridoid de novo producing and sequestering larvae have indicated the complex influx-efflux transport network guiding deterrent precursors through barriers of different selectivity as a general principle found in Chrysomelina. This network includes particularly broad-spectrum ATP binding cassette transporters acting in excretion mechanisms by Malpighian tubules and defensive glands which provide a flexible base to build on a plant dependent chemical defense. Hence, although the use of phytochemicals for anti-predator defenses appears to be a spectacular evolutionary innovation, it requires only a few modifications from ancestral processes.

3-Nitropropionic esters of isoxazoline glycosides protect leaf beetle larvae: A new defensive concept

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Leaf beetles larvae have developed an impressive diversity of chemical deterrents to defend themselves against predators, which they can discharge from specialized pair wise glandular reservoirs on their back upon attack. The reservoirs represent "bioreactors" performing all late reactions of the deterrent-production, starting from plant-derived (sequestered) or *de novo* synthesized glucosides of non-toxic, early precursors of their genuine defenses [1]. Recently we discovered in the hemolymph of all studied chrysomelina species 3-nitropropionic esters of isoxazoline glycosides. Generally the 3-nitro ester is attached to the primary hydroxyl group of the glucoside moiety, but esters with the hydroxyl group at the hexose moiety are also known. Previously these esters were described as adult defenses [2]. Although glucose esters of 3-nitropropionic acid occur in host plants of the leaf beetles, the compounds are biosynthesized *de novo* in the fat body of the larvae from ß-alanine sequestered from their food plants. Labelled b-alanine (13C and 15N) is incorporated into the isoxazoline moiety and the b-nitropropionate moiety. 3-Nitropropionate is known as a potent mitochondrial inhibitor inactivating the succinate dehydrogenase and, hence, ATP-generation. We assume, that the deterrents, presented as droplets in the presence of a predator, act as aposematic volatile signals. Most likely, the effect of the deterrents on the predators is strongly enhanced and supported by their negative experiences after consumption of toxic larvae [3].

1) A. Burse *et al.*, Always being well prepared for defense: The production of deterrents by juvenile Chrysomelina beetles (Chrysomelidae). 2009 *Phytochemistry*, 70, 1899-1909; 2) J. Pasteels, The value of defensive compounds as taxonomic characters in the classification of leaf beetles. 1993, *Biochemical Systematics and Ecology* 21, 135-142; 3) W. Boland *et al.*, Two lines of defense in juvenile leaf beetles; esters of 3-nitropropionic acid in the hemolymph and aposematic warning by volatiles. 2015 Submitted.

Identification, transcriptional analysis and functional assays of Pheromone Biosynthesis Activating Neuropeptide (PBAN) in the European corn borer, *Ostrinia nubilalis*

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The European corn borer (ECB), *Ostrinia nubilalis* Hbn. (Lepidoptera; Crambidae, Pyraustinae) has two pheromone strains (Z and E) that produce and respond to opposite ratios (97:3; 1:99) of (*Z)*-11- and (*E)*-11-tetradecenyl acetate. Sex pheromone synthesis in female moths is regulated by Pheromone Biosynthesis Activating Neuropeptide (PBAN). ECB is broadly studied, however the nucleotide sequence and regulatory role of its PBAN was unknown.

We cloned the full-length cDNA of Diapause Hormone-PBAN (*Ostnu*-DH-PBAN). The ORF of *Ostnu*-DH-PBAN is 603 bp in length encoding five putative neuropeptides: DH (24 aa), alpha-Subesophageal Ganglion Neuropeptide (SGNP) (7 aa), beta-SGNP (20 aa), PBAN (37 aa) and gamma-SGNP (8 aa). Interestingly, other Crambidae species (*Maruca vitrata*, *Omphisa fuscidentalis*) also have 37-aa-long PBAN. The putative peptide sequences were found to be identical in *Z* (Hungarian) and *E* (Slovenian) pheromone strains; while some allelic variations in cDNA sequence were observed within both strains.

The major component of the pheromone blend was detected with GC-MS. *In vivo* administration of full-length synthetic *Ostnu*-PBAN (5.8 pmol) resulted in two- to threefold pheromone production in three-day-old virgin or mated females. *Ostnu*-PBAN injection restored the pheromone titer to almost the normal level one day after decapitation in both strains. A significantly higher pheromone titer was demonstrated as an effect of *Ostnu*-PBAN even in mid-photophase, when normally it is not detectable.

The RT-qPCR assays have shown that *Ostnu*-DH-PBAN mRNA expression was highly brain-specific in both sexes. The expression profile of genes involved in pheromonogenesis and their regulation by *Ostnu*-PBAN will be also presented.

This work was supported by Hungarian Research Funds OTKA K100421 and OTKA K104011, in parallel with TÉT_12_FR-2-2014-0009 (France and Hungary) international collaborative agreement.

The path of the toxin: investigating transport mechanisms of secondary host plant compounds in a leaf beetle

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The chemical arms race between plants and herbivorous insects is characteristic of the evolutionary seesaw between selective pressure and adaptation. Chrysomelids, a large family of beetles feeding on numerous different plants, harbour many species which are not only able to tolerate toxic plant compounds, but also to sequester them and use them in their own defensive secretions. This sequestration necessitates mechanisms to transport the corresponding substances from the intestines to defensive glands.

Cardiac glycosides form one prominent class of compounds that defend plants against herbivores. Their ability to inhibit the ubiquitous Na,K-ATPase leads to a high potential toxicity throughout the animal kingdom. Nevertheless, specialized insects from different orders feed on cardenolide-containing plants. Several of them, e.g. the dogbane beetle *Chrysochus auratus,* possess an insensitive Na,K-ATPase. However, in the beetle's nervous system, *C. auratus* expresses a sensitive form of the enzyme, raising the question how cardenolides are prevented from accessing the nervous tissue.

We here investigate how the beetle protects its nervous system at its "blood brain barrier" and how it transports cardenolides from intestine to defensive glands. Both processes require transport against concentration gradients. Two families of trans-membrane proteins have so far been reported to transport cardenolides: ABC-transporters and Oatps (organic anion transporting polypeptides). Starting from RNAseq data of *C. auratus*, we cloned candidates from each gene family and expressed them in different cell systems to test for their functional capability of transporting cardenolides *in vitro*.

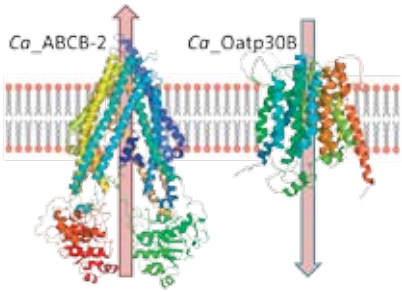

Figure: Homology models of a putative cardenolide efflux transporter (ABC, left) and a putative cardenolide influx transporter (Oatp, right) from *C. auratus*.

Dominant suppressors of p53-induced apoptosis in *Drosophila melanogaster*

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Cells seriously damaged or unwanted in development are removed by the process of programmed cell death, apoptosis. The central mediator of the apoptotic cascade is the transcription factor p53. By removing abnormal, cancerous cells, *p53* is one of the most important tumor suppressor genes.

While the genetic cascade leading to caspase activation is well characterized, less is known about the upstream regulation of *p53* activity, especially its negative regulators, which can inhibit p53 activation and apoptosis. The single *p53* gene, *Dmp53* in *Drosophila melanogaster* is a useful model to study *p53* function and regulation. As the molecular structure, function and the interaction network of the mammalian and fruitfly *p53* orthologs are largely similar, the results gained in *D. melanogaster* can be interpreted in the mammalian system.

To recover dominant suppressors of p53-induced apoptosis, we made an over-expression screen in the fruitfly by making use of the Gal4-UAS gene activation system. We identified eight genes: organic cation transporter 2 (*orct2*), the unknown *CG3773*, cAMP-dependent protein kinase-R2 (*Pka-R2*), *Regena* (*Rga*), the CDC25 mitotic regulator phosphatase *string* (*stg*), the Zink finger-containing *crooked legs* (*crol*), the MAP KKK protein kinase (*Mekk1*) and the transcription elongation factor *Spt5* (*Spt5*). Overexpression of these genes suppresses the apoptotic effect induced in the eye („rough eye" phenotype) by the elevated level of Dmp53. For the majority of them, the interaction with *p53* was not known previously. While all these genes suppress the p53-induced apoptosis, only the *CDC25* ortholog *string* can suppress the ionizing radiation-induced cell death.

The study was sponsored by the Hungarian Research Fund, OTKA K75774.

Vitellogenin expression in *Spodoptera exigua* from the line selected towards cadmium resistance

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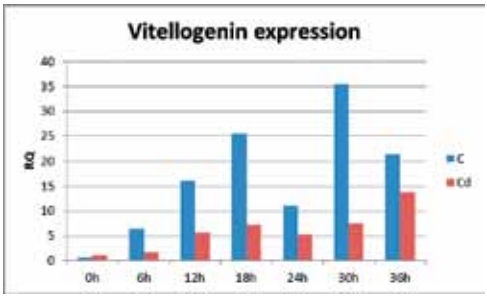
In organisms existing under permanent stress a range of various protective proteins are synthesized. An enhanced and long lasting synthesis of such proteins can be connected with the decrease or even cease of the synthesis of other proteins, e.g. vitellogenin (Vg; precursor of egg yolk protein). This may result in diminishing egg production and disturbance of reproduction. The allocation of considerable part of energy toward the synthesis of protective proteins might be disadvantageous for the organism. Thus, in this study we decided to estimate how prolonged contact of *S. exigua* with cadmium (for over 120 generations) influenced the level of vitellogenin (Vg) expression in female bodies. The assessment of Vg expression in females was measured in the fat body in consecutive time points (0, 6, 12, 18, 24, 30 and 36 hrs) after adult eclosion.

The most common method for analysing qRT-PCR data is to normalize mRNA levels of a target gene to internal reference genes. However, in certain insect species the reference genes may not be stable. This is the case in *S. exigua*. The differences in the levels of functional gene expression result in the changes of signal transduction, protein synthesis, and metabolism among different development time points. Therefore, in this study, internal and external (from plasmid) Actin gene was used as a reference. Research shows that expression of Vg in female fat body from the cadmium line is lower in time points 6, 12, 18, 24 and 30 hrs in comparison to insects from the control. Moreover, in both lines a decrease of gene expression at 24 hrs was observed. Most likely, allocation of energy in individuals from cadmium line is shifted toward removal of effects of heavy metals instead of vitellogenin production.



This project was supported by National Science Centre, Poland (decision DEC-2013/09/B/NZ9/01754)

Early Life Experience and its Consequences for Adult Life and Future Generations

Organizers:

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Early life effects on later life performance: mechanisms and time scales

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The environmental conditions experienced during development can have significant effects on later life performance, potentially shaping the future phenotype at every stage from shortly after conception (e.g. by influencing maternal provisioning of the embryo) until the late stages of juvenile growth. This mix of environmental and maternal effects can influence a diverse range of morphological, physiological and behavioural traits, and can have long term consequences for reproductive performance, the rate of senescence, and longevity.

We will illustrate these effects with examples drawn from our research on a range of vertebrate species (zebra finch, shag, starling, brown trout, three-spined stickleback), studied both in the laboratory and in the field. We will show, for instance, that the position of the egg in the ovary influences both the behaviour and metabolic rate of the resulting offspring, that juvenile growth trajectory influences later reproductive performance, and that exposure to environmental or social stressors in early life influence both cognitive performance and stress responsiveness in adulthood. Multiple mechanistic processes underlie these phenotypic effects and their consequences, including changes to hormonal systems, oxidative stress and rates of telomere erosion, which are linked to faster senescence and shorter lifespan. Taken together, these studies suggest that early-life circumstances can program animal development in ways that shape its life history. We will also discuss whether or not these influences are an adaptive response to a given set of circumstances or represent a disruption of development with adverse consequences.

Maternal photoperiodic programming of reproductive development

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Seasonal physiology has evolved as an adaptive strategy to predictably changing environments, with photoperiod used as the main environmental timing cue. The seasonal physiological state is also influenced by the animal's history, allowing changes in physiology in anticipation to the seasons. The importance of history–dependent photoperiodic programming applies to the developmental strategies of the young, timed by the photoperiod received by the mother during gestation. In mammals, photoperiod is internally transduced by the pineal hormone melatonin, which during pregnancy, reaches the fetus via the placenta affecting fetal photoperiodic responses. Melatonin acts through a hypothalamic network involving thyroid stimulating hormone subunit ß (TSHß) in the pars tuberalis, type 2 (Dio2) and 3 (Dio3) thyroid hormone deiodinases in the tanycytes.

We investigated the programming effect of maternal melatonin on the hypothalamic mechanisms regulating seasonal physiology using pregnant female Siberian hamsters (*Phodopus sungorus*) placed under long (LP: 16h light (L):8h dark (D)) or short (SP: 8L:16D) photoperiod until pups' weaning. At weaning, half of the offspring from both groups were transferred to intermediate photoperiod (IP: 14L:10D) and their testicular development and hypothalamic gene expression was studied during the juvenile period. Offspring gestated in SP showed increased testicular development when exposed to IP as compared to the IP exposed animals gestated in LP. In IP exposed animals, TSH expression in the pars tuberalis was identical but there was a strong change in the expression of Dio2 and Dio3 only in the SP gestated animals suggesting a profound alteration of the hypothalamic thyroid system by the melatonin signal received during gestation. Our results show that the photoperiod experienced during gestation is able to program the adult offspring hypothalamic gene expression in response to the same melatonin input.

Nutrition during pregnancy: Consequences for the next generation

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Over twenty years ago epidemiological studies revealed an association between birth weight and risk of type 2 diabetes and cardiovascular disease. This led to the developmental origins of health and disease hypothesis – the concept that the environment experienced during critical periods of development has a permanent impact on our long-term health. Nutrition is thought to be one key early environmental factor that is thought to be an important early environmental factor that can have a major impact on offspring metabolic and cardiovascular health. Animal models have been invaluable in identifying the mechanisms underlying these programming effects. Three mechanisms have emerged: (i) permanent structural changes as a consequence of an organ being exposed to a suboptimal level of nutrient or hormone required for its development (ii) accelerated cellular ageing as a consequence of increased oxidative stress and (iii) epigenetic programming through changes in DNA methylation, histone modifications and miRNAs. Further understanding of these mechanisms will give the potential to develop markers of disease risk and help design of rational intervention strategies.

Prenatal glucocorticoids and the programming of adult disease

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Epidemiological and experimental evidence suggests that an adverse fetal environment permanently 'programmes' cell-specific gene expression and hence physiology leading to increased risks of cardiometabolic and neuropsychiatric disorders in later life. We hypothesised that fetal glucocorticoid overexposure might underpin this link.

In genetically identical rodents, prenatal stress, glucocorticoid over-exposure or inhibition/knockout of 11ß-hydroxysteroid dehydrogenase type 2 (11β-HSD2), the feto-placental 'barrier' to maternal glucocorticoids, reduces birth weight and causes permanent hypertension, hyperglycaemia, increased hypothalamic-pituitary-adrenal (HPA) axis activity and anxiety-related behaviours in adult offspring. The phenotype persists into a second generation, transmitting via male or female lines. However, whilst first and second generation phenotypes are similar, the molecular mechanisms and epigenetic markers differ in each generation, implying strong selection for phenotype but rather militating against neo-Lamarckian notions of 'epigenetic inheritance'.

In humans, placental 11β-HSD2 activity correlates directly with birth weight and inversely with infant blood pressure. Moreover, low birth weight babies have higher plasma cortisol levels throughout adult life, indicating HPA programming. Indeed, maternal glucocorticoid therapy or ingestion of liquorice (which inhibits 11β-HSD) alters offspring cognition, behaviour and HPA axis function. Furthermore, exposure to the Nazi Holocaust exerted permanent effects upon glucocorticoid levels and steroid metabolism, effects dependent upon the age at exposure. The second (un-exposed) generation also shows altered cortisol levels and metabolism, but again with discordance in detail between generations.

Effects of early life exposure to high fat diet on brain development

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The hypothalamus plays a key role in regulating energy balance. During early life, nutritional alterations may have structural and functional consequences on the programming of hypothalamic feeding systems. However, the mechanisms programming the development of hypothalamic circuits are not well understood. To address this question, female C57BL/6 mice were fed normal chow or HFD during the lactation. We sacrificed the offspring at some important time windows for hypothalamic development to examine the changes of the hypothalamic architecture and function. We found that maternal body mass and energy intake were higher when fed the HFD. HFD also increased pup mass and the fat mass of the offspring. Furthermore, maternal HFD suppressed offspring's hypothalamic neurogenesis on day 15 and day 21 during lactation (no effect on day 9), and increased two inflammation markers (IgG1 and IL-6). Using lipidomics to analyse composition of the milk indicated triglycerides in the HFD is unlikely to be the factor causing the high fat diet effects. These data provide new insights into the mechanisms by which alteration of postnatal nutrition may have long-term consequences on body weight regulation in the offspring.

Differential expression of milk miRNA during the lactation cycle of the marsupial tammar wallaby (*Macropus eugenii*)

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Lactation is a key aspect of mammalian evolution for adaptation of various reproductive strategies along different mammalian lineages. Marsupials, such as tammar wallaby, adopted a short gestation and a relatively long lactation cycle; the newborn is immature at birth and significant development occurs postnatally during lactation. Continuous changes of tammar milk composition may contribute to development and immune protection of pouch young. To address the putative contribution of newly identified secretory milk miRNA in these processes, high throughput sequencing of miRNAs collected from tammar milk at different time points of lactation was conducted. A comparative analysis was performed to find distribution of miRNA in milk and blood serum of lactating wallaby.

The results confirmed that tammar milk contains significant quantities of miRNA and revealed differentially expressed miRNAs during the lactation cycle. Majority of these miRNAs are identified as putative markers of mammary gland activity and functional candidate signals to assist growth and timed development of the young. Comparative analysis of miRNA distribution in milk and blood serum suggests that milk miRNAs are primarily expressed from mammary gland rather than transferred from maternal circulating blood, likely through a new putative exosomal secretory pathway. In contrast, highly expressed milk miRNAs could be detected at significantly higher levels in neonate blood serum in comparison to adult blood, suggesting that milk miRNAs may be absorbed through the gut of the young. The function of miRNA in mammary gland development and secretory activity has been proposed, but results from the current study also support a differential role of milk miRNA in regulation of development in the pouch young, revealing a new potential molecular communication between mother and young during mammalian lactation.

Changes in luteal DICER1 mRNA and protein expression during pregnancy in pigs: Effect of antiandrogen treatment

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DICER1 is an RNase III enzyme required for microRNA processing. MicroRNAs are non-coding small RNAs that serve as posttranscriptional gene regulators and control a wide range of biological functions. The recent findings indicated that microRNA-mediated gene regulation is an important mechanism in controlling of mammalian reproduction, including luteal tissue functions. In pigs, corpus luteum (CL) is the main source of progesterone through the entire gestation. Our prior study revealed that experimentally induced androgen deficiency during late pregnancy in pigs leads to diminished luteal progesterone synthesis. Therefore, the present study aimed to assess whether antiandrogen flutamide influences DICER1 expression in the porcine CL of pregnancy. Pregnant sows were allotted into three flutamide-treated and respective control groups. Flutamide was administered subcutaneously (50 mg/kg bw, seven times every day) between days 43-49, 83-89 or 101-107 of gestation. CLs (n=8-11/each group) were obtained on day 50 (GD50), 90 (GD90) or 108 (GD108) of gestation. The expression of DICER1 was conducted at mRNA and protein level using real-time PCR and Western-blot analyses, respectively. Moreover, immunolocalization of this enzyme was performed. Results were analyzed using Mann-Whitney U-test. Flutamide treatment led to upregulation of DICER1 mRNA and protein expression only on GD90 and GD108 when compared to respective controls. Interestingly, DICER1 mRNA and protein levels did not change among controls, while increased gradually among flutamide-exposed groups with pregnancy progression. Positive DICER1 immunolocalization was found in large luteal cells in all control and flutamide-treated groups. In summary, the study provides evidence that antiandrogen flutamide upregulates DICER1 luteal expression during late pregnancy in pigs, suggesting that microRNAs might be involved in the regulation of androgen-dependent genes expression in the porcine CLs.

Supported by NSC (grant No. DEC-2011/03/D/NZ4/00303 dedicated to MG).

Augmentation of salt chemotaxis learning due to chronic nicotine exposure is modulated by dopamine signaling in the nematode *Caenorhabditis elegans*

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The chemotactic response of wild-type NaCl-conditioned nematodes, which have been pre-exposed to 100 mM NaCl, is markedly smaller than that of mock-conditioned nematodes. This phenomenon is known as salt chemotaxis learning (Tomioka et al., 2006). We have previously shown that acute nicotine exposure impairs salt chemotaxis learning in the nematode *Caenorhabditis elegans* and found that serotonin signaling plays an essential role in modulating the acute effects of nicotine (Matsuura et al., 2013).

In the present study, we investigated the effects of chronic nicotine exposure on salt chemotaxis learning in *C. elegans* to determine its underlying mechanisms. The chemotactic response of wild-type NaCl-conditioned nematodes, which were maintained on a growth medium containing 0.3 mM nicotine throughout the larval stage, was markedly smaller than that of NaCl-conditioned nematodes, which were maintained on the same medium but without nicotine. This indicates that chronic nicotine exposure causes augmentation of salt chemotaxis learning.

Several mutants were analyzed to clarify the molecular mechanism underlying this augmentation. Salt chemotaxis learning was augmented when *tph-1* mutants with a defect in serotonin synthesis were pre-exposed to nicotine throughout the larval stage. In contrast, salt chemotaxis learning was not augmented when *bas-1* and *cat-2* mutants with a defect in either serotonin and dopamine production or dopamine synthesis alone were pre-exposed to nicotine throughout the larval stage. However, the augmentation of salt chemotaxis learning due to chronic nicotine exposure was observed when *bas-1* and *cat-2* mutants were maintained on growth plates that included 20 mM dopamine. These results indicate that dopamine signaling plays an essential role in augmentation (Matsuura and Urushihata, 2015).

1) Matsuura T, Miura H, Nishino A. 2013 *Neurosci Res*. 77:155–161; 2) Matsuura T, Urushihata T. 2015 *Biosci Biotechnol Biochem*. 79:462–469; 3) Tomioka M, Adachi T, Suzuki H, *et al*. 2006 *Neuron*. 51:613–625.

Effects of nicotine exposure period during larval stages on salt chemotaxis learning in the nematode *Caenorhabditis elegans*

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It is known that the chemotactic response of wild-type NaCl-conditioned nematodes, which are pre-exposed to 100 mM NaCl, is significantly smaller than that of mock-conditioned nematodes. This phenomenon is referred to as salt chemotaxis learning. We previously showed that salt chemotaxis learning is inhibited by acute nicotine exposure and that serotonin signaling plays an essential role in this inhibition (Matsuura et al., 2013). We also showed that the learning is augmented by chronic nicotine exposure and that dopamine signaling is involved in the augmentation (Matsuura and Urushihata, 2015).

The present study investigated the effect of nicotine exposure period during larval stages on salt chemotaxis learning in the nematode *Caenorhabditis elegans*. Salt chemotaxis learning in the young adult stage was inhibited when the nematodes were pre-exposed to 0.3 mM nicotine during the first, second, third, or fourth larval stages. The inhibition of salt chemotaxis learning decreased with increase in the period of nicotine exposure, and chronic nicotine exposure augmented the learning.

We subsequently focused on the inhibition of salt chemotaxis learning due to nicotine exposure during the first larval stage to clarify the mechanism underlying the learning inhibition. Salt chemotaxis learning was inhibited when *cat-2* mutants, which show a defect in dopamine synthesis, were pre-exposed to nicotine during the first larval stage. In contrast, inhibition of salt chemotaxis learning was not observed when *bas-1* (defective serotonin and dopamine production) and *tph-1* (defective serotonin synthesis) mutants were pre-exposed to nicotine during the first larval stage. However, the inhibition of salt chemotaxis learning was observed when *bas-1* and *tph-1* mutants were maintained on a growth plate that included 200 mM serotonin. These results indicate that serotonin is involved in the inhibition of salt chemotaxis learning due to nicotine exposure during the first larval stage.

1) Matsuura T, Miura H, Nishino A. 2013 *Neurosci Res*. 77:155-161; 2) Matsuura T, Urushihata T. 2015 *Biosci Biotechnol Biochem*. 79:462-469

Maternal testosterone influences the acute-phase immune response in Japanese quail offspring

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Maternal testosterone (T) can shape behavioural and physiological phenotype of offspring, including the immune system. Effects on the immune system are of evolutionary and practical importance but obtained results are often contradictory, reflecting different experimental models. In our group we have selected Japanese quail for high (HET) and low (LET) egg yolk T content and in this model we study effects of maternal T on the immune system of offspring.

We analysed effects of experimentally increased yolk T content in eggs of both lines on immune response of quail chicks. We collected eggs from both lines and injected half of them with 50 ng of testosterone (eT). After hatching we assessed the acute-phase immune response on the basis of non-specific antibodies, heterophile/lymphocyte (He/Ly) ratio and gene expression of interleukine-6 and chemokine K203 after lipopolysaccharide (LPS) injection.

Basal levels of plasma non-specific antibodies were higher in HET than LET line. Antibody response to LPS did not differ between lines, but was stimulated by eT 2 and 8 days after initial LPS injection. Three hours after second LPS injection, the acute decrease of plasma antibodies was more pronounced in quail treated with eT without any line differences. In response to LPS, higher gene expression of interleukine-6 and chemokine K203 in the spleen was found in the HET as compared to LET line. Treatment with T stimulated gene expression of both immunological mediators in LET line, whereas a numerical decrease was found in HET line. A similar trend was seen in He/Ly ratio.

Our data imply that various parts of the immune system can have different sensitivity to maternal T in the yolk. We demonstrated that increased maternal T can enhance non-specific response to immune challenge in quail chicks and these effects may depend on the genetically determined responsiveness of embryos to yolk hormones.

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Incubation in hypoxia affects lung morphology in chicks

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Hypoxia during incubation is known to induce many morphophysiological changes in hatchlings, including alterations in metabolic and ventilatory responses to hypoxia. It is also demonstrated that hypoxia effects depend on the phase of embryonic development, the called critical windows of development. Pulmonary structure maturity occurs at the last third of incubation and no data exist about hypoxia effects at this incubation phase regarding lung post-hatching structure in precocious birds. Thus, we aimed at investigating the effect of reducing O_2 (15% O2) in the air from day 12 to 19 of incubation on the lung structure of 1 and 10 days old chicks. Lungs from chicks from hypoxic and normoxic incubation were fixed, extracted from the body and prepared using stereology technique for measuring lung volume, the density that each structure represents in lung volume(%), and absolute volume that each structure represents(cm^3). The structures quantifying were air cappilaries, blood cappilaries, atria, infundibula, parabronchi, interparabronchial tissue and blood vessels. One-day-old chicks incubated in hypoxia presented higherdensity of air cappilaries (16,7% for hypoxia and 14,1% for normoxia incubation), blood cappilaries (26% for hypoxia and 22,4% for normoxia incubation) and less density of atria (6,7% for hypoxia and 11,5% for normoxia incubation) compared with chicks incubated in normoxia. Ten days old chicks from hypoxia incubation showed higher density of air cappilaries (17,4% for hypoxia and 13,4% for normoxia incubation) and less density of atria (5,81% for hypoxia and 8,05% for normoxia incubation) compared with chicks incubated in normoxia. Our results suggest, for the first time, that reducing concentration of oxygen in the air during the late phase of embrionic development seems to increase the area for gas exchanges in lungs of chicks, at least up to 10 days post hatching.

Female reproduction and resource investment into major life history in Indian mealmoth (Lepidoptera: Pyralidae)

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Quantifying how insects maximise overall fitness at different body sizes is key to understanding how they allocate resources into different life history functions. Here, we appraise female reproductive life history and resource allocation between survival and reproduction at different body size in the Indian mealmoth, *Plodia interpunctella* Hubner (Lepidoptera: Pyralidae). We predicted that female body size should influence life-history trade-offs between reproduction and survival, in other words current and future reproduction. We measured individual reproductive life history using the ovigeny index, lifetime potential fecundity, initial egg load and initial oocyte load. We also used an established insect nutrient assay protocol through which resource investment was determined from macronutrient allocations (e.g. protein, carbohydrate, glycogen and lipid) in different newly emerged female tissues related to reproduction and survival. Ovigeny index declined significantly with body size, but due only to changes in potential lifetime fecundity. Increasing body size in female *Plodia* increased initial oocyte load, but not initial egg load. Smaller females invested more resources into protein, carbohydrate, glycogen and triglyceride in reproductive and somatic tissue than larger females, but not lipids. This implies that early reproduction requires major resource investment into both of these tissue types. Overall, these data illustrate how resource investment in smaller female insects can favour early reproduction. However, early reproduction is apparently costly for female *Plodia* as investment into initial egg load reduces female lifespan and future reproduction.

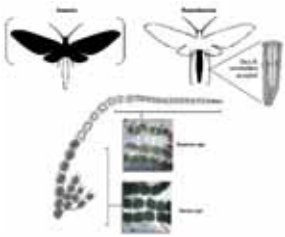

Figure showing *Plodia* body parts link to somatic and reproductive components and image of immature and mature eggs.

Brood size manipulation effects on nestling growth, ageing markers (telomeres) and oxidative stress parameters in a wild passerine

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Several studies have suggested that nestling developmental state might be especially constrained by environmental conditions, such as resource availability or sibling competition among others, with strong fitness consequences. Telomeres are good predictors of lifespan or life expectancy because they get shorter with age, they are very sensitive to oxidative stress and there are large levels of individual variation in age-related attrition levels. We predicted that a manipulation of early development conditions in nestlings through a brood size manipulation would result in changes in telomere length, likely mediated by differential levels of oxidative stress.

We experimentally manipulated 90 spotless starling (*Sturnus unicolor*) nests, with a total of 250 nestlings, creating reduced, control and enlarged broods. Our treatment significantly affected chick body mass and telomere length at 14 days of age in both first and second broods. Using linear mixed-effect models we found a general quadratic effect of brood manipulation on telomere length, but also a significant interaction between treatment and brood order. After separating the analysis by broods (first and second), we found a significant quadratic effect of the manipulation in telomere length within first broods, whereas in second broods there was a negative linear effect.

In addition, we checked several blood and plasma markers previously used to estimate the imbalance between the production of reactive species and the antioxidant metabolism status, that is, oxidative stress. Such parameters include glutathione levels in erythrocytes, plasma levels of malondialdehyde (lipid peroxidation product) and plasma antioxidant capacity based on lipid mobilization (triglycerides) and acid uric amount. We will consider the relationships between these markers, glucocorticoid levels in plasma and feathers and telomere length.

Maternal and offspring responses to increased variability in ambient temperature experienced by gravid lizards (*Eremias multiocellata*)

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Temperature variability, which is predicted to increase in the following decades, might have substantial impacts on the fitness of organisms. To understand how temperature variability would affect maternal thermoregulation and offspring traits, we experimentally manipulated maternal thermal environments by exposing gravid lizards (*Eremias multiocellata*) to low, mediate and high temperature variability treatments with the same average temperature, respectively. Our results showed that (1) the body temperatures of gravid lizards decreased as temperature variability increased, (2) high temperature variability impaired the cognitive ability of offspring, but did not affect the morphology and locomotor performance of offspring, (3) offspring from mothers experienced high temperature variability increased their selected body temperatures and metabolic rate, and therefore showed relatively high growth rate. These results suggest that lizards may not be able to maintain optimal body temperatures simply by behavioral thermoregulation under thermal conditions with increased temperature variability, and maternal thermal environments may shape the physiological responses of offspring, which partly buffer the effect of high temperature variability.

Transgenerational effects of mean of temperature and thermal variability on performance curve in *Drosophila melanogaster*

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Environmental signals during development and early life can have long-term consequences for morphology, physiology, behavior and life history of organisms and can potentially impact on future generations. We studied the impact of mean ambient temperature and thermal variability on locomotion performance in *Drosophila melanogaster* during development (egg→adult) and in the future generation. Adults were capture from the wild in Central Chile and maintained under controlled conditions (24°C and LD = 12:12). Flies from third laboratory generation were randomly assigned to three acclimation treatments (28 ± 0°C (28C), 28 ± 4°C (28V) and 30 ± 0°C (30C)) based in the upper limit to viability in *Drosophila melanogaster* in order to obtain experimental parents. Parent individuals were placed in bottles to reproduction at each of three acclimation temperatures (28C, 28V and 30C) to obtain the offspring (F1). We quantified the effect of temperature on walking speed on adult flies at a sequence of temperatures from 16 to 40°C. The results revealed that temperature at which locomotion performance is maximized (*To*) varied significantly among acclimation treatments in parental individuals; in flies reared at 28C *To* diminished by 2 and 1°C relative to 28V and 30C, respectively. *To* of F1 was associated with acclimation temperature of parents, the maximum performance (υ_{max}) increased significantly in offspring reared in the same treatment that parental population. The results revealed the existence of transgenerational effect of mean of temperature and thermal variability on performance curve parameters in *Drosophila melanogaster.*

Telomeres and Life Histories

Organizers:

Pat Monaghan, *University of Glasgow, UK*
Francois Criscuolo, *CNRS Strasbourg, France*

Telomeres and life history, an introduction

François Criscuolo[1], Pat Monaghan[2]

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Telomeres, well studied in a biomedical context, have only been studied from an evolutionary and ecological perspective in the past 10-15 years, and there is still much that we do not know. Evolutionary biologists are interested in how telomere dynamics influence longevity and life history variation. These studies have yielded key insights into their potential role in mediating life history trade-offs involving longevity, for example in relation to growth, reproduction and immune function. The pattern of inheritance of telomere length and relationships with age, physical performance and health status has been highlighted in a broad range of animal species. However, some more recent studies have produced contradictory results, suggesting that short telomeres do not always carry a lifespan penalty, and that there may be key differences among taxa in how telomere are regulated. We need to broaden our understanding of how telomere dynamics are influenced by environmental circumstances at different life stages and how this influences fitness outcomes. We also need more comparative studies to further our understanding of how telomere dynamics have evolved in relation to specific biological or physiological traits. The different talks in this session will shed light on telomere regulation in reptiles, birds and mammals, in relation to morphs, parasitism, sociality and hibernation.

Telomere dynamics in hibernating mammals: effects of prolonged hypometabolism and interbout arousals

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Torpor and hibernation are elements of a life history strategy that are common across a wide diversity of mammalian taxa. The benefits of this tactic are thought to include reduced energy expenditure, lower rates of predation and increased longevity/reproductive lifespan.

We have investigated the effects of torpor and hibernation on telomere dynamics in a range of small rodent species. Our results suggest a complicated pattern of telomere length variation strongly influenced by hypometabolism and the high energy demands of arousals from torpor bouts. The suppressed metabolic rate associated with torpor appears to decrease the extent of telomere loss and thus slow the rate of aging. Individuals using more torpor during hibernation maintain relative telomere length (RTL) compared to those animals spending longer times in a euthermic state. However we have also found a strong influence from the number of interbout arousals during hibernation on the rate of telomere loss. More arousal bouts lead to an increased loss of RTL across the entire hibernation season. Therefore hibernation may represent a trade-off between the benefits of increased survival probability and the costs of somatic maintenance.

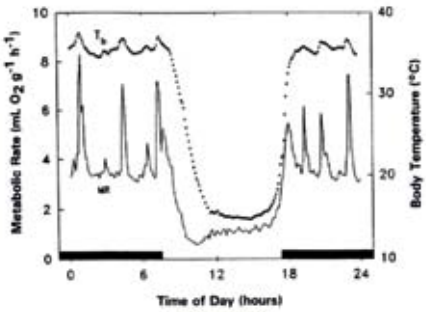

Figure: Example of a typical torpor bout with reduced metabolic rate and decreased body temperature[1].

Ruf, T., & Heldmaier, G. (1992). The impact of daily torpor on energy requirements in the Djungarian hamster, *Phodopus sungorus*. *Physiological zoology*, 994-1010.

Telomere dynamics and life-history allocation in a wild mammal

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A key challenge in evolutionary biology is to explain the extensive variation in life-history traits, particularly that seen within species. This variation in traits such as lifespan and timing of reproduction may arise as consequence of trade-offs, whereby no single strategy consistently outperforms others. As limited energy availability precludes simultaneous investment in reproduction, growth and maintenance, and the payoffs of these investments at different life-stages vary between individuals depending on their quality, the optimal pattern of allocating resources across the lifespan also varies. Therefore, strategic allocation of resources is expected to evolve, leading to differences in lifespan and timing of reproduction depending on individual traits and the conditions faced during development and growth.

Telomere length of an individual reflects the genetic makeup of an individual, as well as telomere attrition during fetal development and with advancing age. As telomeres erode with chronic stress and oxidative and immunological challenges, telomere length can be thought of as a measure that integrates genetic, environmental and even social information. Telomere length has therefore the potential to act as a cue for life-history allocation patterns, and so may mediate life-history decisions an individual makes, such as investing into reproduction versus maintenance or survival at a given age.

We use a long-term study system of a wild population of banded mongooses (*Mungos mungo*) to investigate the potential proximate role telomere length plays in mediating life-history allocation. Banded mongooses are an ideal model system, as there is extensive variation in both lifespan and timing of reproduction between the sexes and among individuals. We use qPCR to investigate the relative explanatory power of TL length and TL attrition rates in predicting lifespan and life-history trait variation in the wild.

Hidden cost of infection: Chronic malaria erodes telomeres and lifespan

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Recovery from infection is not always complete, and mild chronic infections may persist. These chronic infections are assumed to cause no damage to host; the potential for any long-term effect on Darwinian fitness is poorly understood. Great reed warblers pick up various species of malaria parasites while wintering in the tropics and after initial acute malaria, are asymptomatically infected for life. Although these birds are chronically infected with malaria parasites, their behavior seems unaffected and they show no short-term cost of infection. However, over the long-term we found that these asymptomatic low-level chronic infection reduced lifespan, as well as lifetime number and quality of offspring. Furthermore, the delayed fitness effects were mediated through faster degradation of telomeres (the protective caps of the ends of chromosomes), a result supported by controlled infection experiments on birds in captivity. The results of this study imply that chronic infection may be causing a series of small adverse effects that accumulate and eventually impair phenotypic quality and Darwinian fitness.

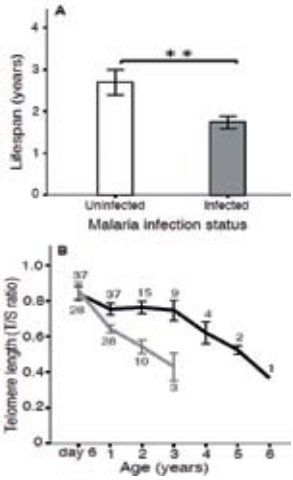

Figure: (A) Wild birds infected (gray bar) with chronic malaria infections showed lower lifespan ($p = 0.004$), (B) this effect was manifested through faster telomere shortening (gray line; Age × malaria status, $p < 0.0001$).

Asghar M 2015. Hidden cost of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. *Science* 347: 436-438.

Do infected wild passerines get shorter telomeres?

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Telomeres play vital role in ageing and longevity. They can serve as a reliable biomarker of ageing and organism condition. Moreover, telomeres are thought to provide a key to predict lifespan and survival. Avian malaria, a vector-borne disease, is also known to reduce lifespan. Detrimental effects of infections can drive various life history trade-offs and telomere dynamics may be as well altered while facing costly disease. Here we aim at linking telomere loss and avian malaria caused by parasites from genus *Plasmodium* and *Haemoproteus*. Malaria lineages and telomere length were evaluated using PCR-based techniques. We employ long term data from the blue tit (*Cyanistes caeruleus*), a free-ranging passerine inhabiting island of Gotland. In this population infection prevalence is 65%, so constitutes a serious selective pressure. We examine the connection between infection status, its intensity and telomere length in birds of the same age. We hypothesize that telomeres may be reduced in animals experiencing malaria. We look into sex-specific effects as higher prevalence of infection is observed in males, however sex-specific telomere dynamics differences are rare.

Early life nutrition, telomere dynamics and life history plasticity in birds

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Loss and restoration of telomeres, the protective ends of eukaryotic chromosomes, play a key role in cellular death and replacement, and thereby in ageing. Most telomere loss occurs during early development, a life stage where organisms are exposed to a number of factors that can induce oxidative stress, an important factor contributing to telomere loss. It has recently been proposed that dietary antioxidants might play an important role in telomere protection due to their antioxidant properties. In birds, the protective effect of dietary antioxidants on telomeres might take place either during embryo development (i.e. via maternal allocation of antioxidants into the egg yolk) or during post-natal growth period (i.e. via parental feeding). In a series of experiments with zebra finches, we investigated 1) intra-clutch variation in maternally-derived dietary antioxidants and embryo telomere length, 2) whether within-clutch differences in embryo telomere length persist during post-natal development and 3) whether dietary antioxidants may help to prevent the loss of telomere length after hatching.

We found that the level of maternally-derived dietary antioxidants in the egg-yolk were markedly lower in the last compared to earlier laid eggs, resulting in shorter telomere length in the embryos from these last eggs. Interestingly, these differences in telomere length among siblings persisted until adulthood. In addition, by experimentally manipulating the intake of dietary antioxidants during post-natal development, we also found that females fed with a high antioxidant diet during sexual maturation showed reduced telomere loss. Together, these results indicate that dietary antioxidants can influence rates of cellular senescence during different stages of the development. However, differences in antioxidant requirement and allocation strategies, probably linked to the development of sexual maturation, may lead to sex-specific differences in ageing rates.

Eat, celibate and elongate your telomeres: Influence of supplemental feeding on telomere dynamics in a small arboreal rodent

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Pulsed resource consumers have to cope with a high variation in food availability, and extra costs of reproduction are difficult to afford in years of low food abundance. Edible dormice (*Glis glis*) are highly specialized seed predators which only reproduce in mast years of beech. In mast failure years entire populations can skip reproduction. Especially juveniles need high caloric food in late summer to gain enough body fat reserves to survive the upcoming hibernation season. Due to this peculiar life history strategy, dormice show a low number of reproductive bouts throughout their life, in combination with a high longevity (up to 12 years). Although previous studies have shown that dormice are able to reverse prior cellular damage in terms of telomere shortening, it is unclear which endogenous or environmental factors govern somatic maintenance and repair.

To investigate the effects of food availability on telomere dynamics we conducted a feeding experiment in a free-living population in the Vienna Woods (Austria) during a mast-failure year. Our results indicate that changes in telomere length during the active season (between May and September) were affected by food availability. Animals that experienced an artificial mast situation (i.e., sunflower seeds provided into nest-boxes) elongated their telomeres more than animals without supplemental food (i.e., experiencing a mast failure year). These effects were independent of age, sex and body weight of the individuals. We therefore conclude that dormice use the availability of high-caloric food (seeds) to invest energy into somatic maintenance.

Morph-specific telomere dynamics and responses to TA65 treatment in an Australian Lizard

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Australian painted dragon lizards occur in different morphs with respect to head colour (red, orange, yellow, blue) and the presence or absence of a 'bib'. In a series of studies, we have analysed to what extent head colours identifies different reproductive tactics (red are aggressive and territorial, whereas e.g., yellow males are sperm competitive sneakers). Bibbed males have an investment strategy that seems more heavily biased towards reproduction than to self-maintenance. For example, experimental data in wild animals showed that bibbed males in three workload groups (carrying a 'load' of silicone coated lead, a Styrofoam control, or being sham operated), consistently lost more body condition through the experiment. In a lab experiment, we exposed males to TA65 (a herbal drug with synthetic telomerase effects in some taxa) in order to assess whether males produced longer or shorter telomeres in response to TA65 treatment. We could not verify an immediate, factorial effect of treatment (TA65-males did not have longer telomeres than controls). However, morphs differed in telomere length with yellow males having longer telomeres than red, aggressive males, and non-bibbed males with higher self-maintenance having longer telomeres than bibbed ones.

Thyroid Hormones as Master Regulators: Comparative and Developmental Perspectives

Organizer:

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Sponsored by the Journal of Neuroendocrinology

Thyroid hormones regulate thermal acclimation in fish

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Thermal acclimation is often cited as the most important defense ecotherms have against climate change, but the overarching mechanisms that coordinate this process are not known. Thyroid hormone (TH) mediates thermal responses in mammals, and its physiological effects appear similar across vertebrates. We therefore hypothesized that TH regulates thermal acclimation in ectotherms so that metabolism, muscle function and cardiac performance are optimized to the prevailing ambient temperature. We pharmacologically induced hypothyroidism in zebrafish (*Danio rerio*) during acclimation to cold (18˚C) and warm (28˚C) conditions. We measured an array of whole animal and tissue-specific response parameters to assess metabolic, cardiac and skeletal muscle performance. We found that TH enhanced swimming performance during cold acclimation, but had little to no effect in the warm acclimated fish (Little *et al*. 2013). This general trend, where fish were more sensitive to TH during cold acclimation, was mirrored in response measures for metabolism, cardiac function (Little and Seebacher 2014) and skeletal muscle performance (Little and Seebacher 2013). We suggest that the properties that underlie the role of TH in thermal acclimation (temperature sensitivity and metabolic control) may have predisposed this hormone for a regulatory role in the evolution of endothermy.

1) Little AG, Kunisure T, Kannan K and Seebacher F 2013 *BMC Biology* 11:26; 2) Little AG and Seebacher F 2013 *The Journal of Experimental Biology 216*:3514-3521; 3) Little AG and Seebacher F 2014 *The Journal of Experimental Biology* 217:718-725.

Effects of thyroid hormone manipulations on growth and metabolism of american alligator hatchlings

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Thyroid hormones play an important role in vertebrate growth, development and metabolism. However, few details are known about their influence on postnatal development of reptilian archosaurs, such as the American alligator. To explore the role of thyroid hormones in the neonate American alligator we orally administered triiodothyronine (T_3), saline solution 0.9% and a thyroperoxidase inhibitor, Methimazole (MMI), for 4 weeks to induce hyperthyroidism and hypothyroidism, respectively. We measured systemic O_2 consumption (VO_2) by flow-through respirometry (at 30 °C), followed by permeabilized-muscle mitochondrial respiration of cardiac and thigh muscle using Oroboros O2K high-resolution respirometry. We measured hematocrit and morphometrics. Plasma $T_3$was measured using Accubind® ELISA kit.

VO_2 analysis of T_3, control and MMI groups showed that the hyperthyroid group (T_3) had significantly higher systemic VO_2 compared with control. The hypothyroid group (MMI) had significantly lower VO_2, suggesting that T_3 acts as a metabolic regulator at the systemic level. Cardiac ventricle and liver mass were decreased in T_3 group but SVL and femur length were decreased in MMI groups, suggesting T_3 promotes structural bone growth.

Similar to responses at whole body and tissue level, T_3 treatment increased mitochondrial respiration of cardiac ventricle and skeletal muscle fibers at OXP-CI+II, OXP-CII, and cytochrome oxidase (COX) when compared with control and MMI groups. When compared to an endothermic archosaur such as the Pekin duck, the T_3 group of alligators have similar OXP-CI and OXP-CI+II values. We concluded that thyroid hormone directly effects metabolic rate at the organelle, tissue, and whole animal levels. The results suggest thyroid hormones play a pivotal role in neonate alligators, not only in regulating growth and systemic metabolism, but also mitochondrial respiration and associated energy production capabilities therein.

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Thyroid modulation of oxidative balance in birds

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By concomitantly up-regulating anabolic and catabolic enzymes, thyroid hormones (THs) stimulate mitochondrial oxidations and basal metabolism. Given that mitochondria are a major source of intracellular reactive oxygen species (ROS), TH-induced mitochondrial hypermetabolism was repeatedly associated with increased oxidative stress in mammalian tissues (1). In rodents for instance, THs stimulate ROS generation and impair antioxidant defenses, thus generating an oxidative imbalance favoring oxidative injuries.

This link between TH and oxidative stress cannot however be generalized to all endotherms, as studies in birds led to different conclusions. Pharmacological manipulation of thyroid status to produce hypo-and hyperthyroid ducklings (*Cairina moschata*) revealed that the ROS production per oxygen consumed (free radical leak) of isolated muscle mitochondria was inversely related to plasma T_3 levels. Physiological cold-induced hyperthyroidism also reduced mitochondrial ROS production in ducklings and penguins (*Aptenodytes patagonicus*). Conversely, heat-stress that reduces TH levels induces ROS overproduction in chicken (*Gallus gallus*) skeletal muscle mitochondria (2). In all models, ROS production was inversely related to the expression of avian mitochondrial uncoupling protein. In accordance with changes in ROS production, hypothyroid ducklings had lower oxidized lipids and DNA while mitochondrial membranes of hyperthyroid ducklings or cold-acclimatized penguins exhibited higher unsaturation and peroxidation indexes than in controls indicating a greater susceptibility to free radicals. Changes in antioxidant enzymes were not consistently related to THs in birds but activities tended to be positively related with thyroid status. Reviewed data indicate that birds and mammals present fundamental differences in their capacity to handle TH-induced alteration of oxidative balance. Avian capacity to mitigate TH-related oxidative imbalance might contribute to the high longevity of birds despite high energy expenditure.

1) Venditti P & Di Meo S (2006) Cell Mol Life Sci 63: 414-434. 2) Kikusato M & Toyomizu M (2013) PLOS One 8(5): e64412.

How to build a furnace: The role of t_3 in development of endothermy in altricial birds, including the red-winged blackbird (*Agelaius phoeniceus*)

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Avian development occurs across a spectrum of functional maturity at hatching ranging from precocial to altricial. Altricial red-winged blackbirds (RWBB) rapidly mature in the nest before fledging around 11 days posthatch. During this period, neonates undergo physiological and metabolic changes associated with development of endothermic phenotype.

Thyroid hormones (TH), key regulators of avian metabolism, are thought to influence development of endothermy. Altricial species, which remain non endothermic for days after hatch, show similarly delayed maturation of the thyroidal axis. This delayed timing suggests a relationship between peak circulating TH and obtainment of endothermy.

To better understand the role of TH in RWBB endothermic developmental trajectory, we characterized whole animal O_2 consumption (VO_2) and ventilation, and mitochondrial respiration of permeabilized fibers from breast, thigh, and heart after plasma TH levels were altered via administration of the thryoperoxidase inhibitor, Methimazole (MMI), during the first 5 days of neonatal life.

5dph MMI treated animals exhibited lower plasma T_3 than control. Body mass of hypothyroid animals did not differ from control, but fractional heart mass of 5 and 7dph was lower. Hypothyroid animals showed delayed maturation in $VO2_2$ response when faced with decreasing temperature, but acheived an endothermic phenotype by 9dph. Wing chord and femur length of MMI hatchlings was shorter than control. Hypothyroid neonates had lower mitochondrial respiration when compared with control animals.

Our data suggest TH plays an active role in systemic development of endothermic capacity, especially in the first days after hatching for fast growing species like the RWBB. In the neonate avian multiple systems develop in concert to produce an endothermic phenotype, but reduced TH can delay maturation of endothermic capacity in altricial species.

The evolution of endothermy is explained by thyroid hormone mediated responses to cold in early vertebrates

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The evolution of endothermy is one of the most intriguing and consistently debated topics in vertebrate biology, but the proximate mechanisms that mediated its evolution are unknown. Here, we suggest that the function of thyroid hormone in regulating physiological processes in response to cold is key to understanding the evolution of endothermy. We argue that the capacity of early chordates to produce thyroid hormone internally was the first step in this evolutionary process. Selection could then act on the capacity of thyroid hormone to regulate metabolism, muscle force production and cardiac performance to maintain their function against the negative thermodynamic effects of decreasing temperature. Thyroid-mediated cold acclimation would have been the principal selective advantage. The actions of thyroid hormone during cold acclimation in zebrafish are very similar to its role during endothermic thermogenesis. The thyroid-mediated increases in metabolism and locomotor performance in ectotherms eventually resulted in sufficient heat production to affect body temperature. From this point onwards, increased body temperature per se could be of selective advantage and reinforce thyroid-induced increases in physiological rates. Selection for increased body temperature would promote those mechanisms that maximise heat production, such as increased Na^+/K^+-ATPase activity, futile cycling by SERCA, and mitochondrial uncoupling, all of which are regulated by thyroid hormone. The specific end point of this broader evolutionary process would be endothermic thermoregulation. However, considering the evolution of endothermy in isolation is misleading because the selective advantages that drove the evolutionary process were independent from endothermy. In other words, without the selective advantages of thyroid-mediated cold acclimation in fish, there would be no endotherms.

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Endocrine disruption in the marsh frogs exposed to organic nanoscale complexes of cobalt and zinc

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Engineered metal-containing nanoscale materials (Me-NCs) represent a novel environmental challenge, and the toxic impacts of Me-NC are not well understood. In this study, we compared the effects of Co^{2+} and Zn^{2+} and their nanoscale complexes (NC) derived from N-vinylpyrrolidone in a model amphibian *Pelophylax ridibundus*. Males were exposed for 14 days to waterborne Co^{2+} (50 µg/L), Zn^{2+} (100 µg/L), or the corresponding concentrations of Co-NC, Zn-NC or the polymeric substance (PS) to assess whether the toxic mechanisms are shared between Me-NC and their corresponding metals. Levels of cortisol, vitellogenin, thyrotropin (TSH) in the blood and deiodinase activity in the liver were measured as biomarkers of the endocrine disruption. CYP450 and GST activities, indices of cytotoxicity and metal levels were also evaluated. Exposure to Co^{2+} upregulated TSH and deiodinase; Co^{2+} and Co-NC exposures caused elevation of CYP450, whereas Zn^{2+}, Zn-NC and PS downregulated CYP450, decreased cortisol levels and activated vitellogenesis. The endocrine disrupting and cytotoxic effects were more strongly and differently induced by Me-NCs and PS compared to free metals, and were more severe in frogs than in bivalves and fish studied previously (Falfushynska *et al.*, 2012, 2014) indicating greater sensitivity of amphibians to these pollutants.

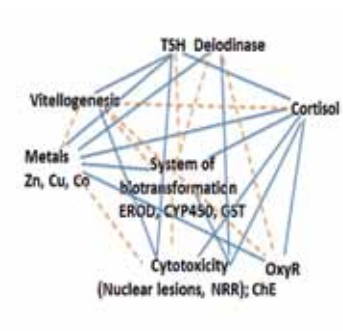

Figure: A schematic representation of the relationships between the studied biological traits in the frog from treatment groups: blue solid and red dotted connector lines represent relationships with positive and negative correlations, respectively.

1) Falfushynska H *et al.* 2012 *Chemosphere* 88:925936; 2) Falfushynska H *et al.* 2014 *Comp. Biochem. Physiol* 160C: 66–74.

Effect of TRH factor on prolactin secretion in lambs – in vitro study

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In seasonally breeding animals process of initiation and maintenance of lactation requires the presence of various hormones and growth factors. Prolactin (PRL) plays a major role in lactopoesis process. In recent years, attention has been paid on the role of thyroid hormones in the process of initiating and maintaining lactation in small ruminants. The objective of the conducted studies was to determine the effect of TRH factor on prolactin secretion process in vitro. The studies were carried out on 40 days old pituitary glands, collected from lambs. Collected pituitary glands were dissected and cut in half along the longitudinal fissure so that each half contained both glandular and nervous parts. The in vitro incubation of the glands was performed for 3 h in Parker's medium at 37°C. The control pituitary glands were incubated in Parker's medium, while the experimental pituitary glands were incubated in medium containing TRH factor (30 µg/ml). Each times intervals of 15 min. administered and collected 1ml of medium from each sample. The concentration of prolactin in the medium were determined by RIA method. During first 15 min. of incubation, prolactin concentration in control group was (76,29±17,8 µg/ml) and was significantly ($P \leq 0,01$) lower for a group of TRH (82,84±18,1 µg/ml). After 45 min. of incubation, prolactin concentration (control group) was (70,79±15,2 µg/ml) and (72,34±16,1 µg/ml) (TRH group) and was significantly ($P \leq 0,05$) lower. In second hour of incubation, prolactin concentration was still significantly ($P \leq 0,01$) lower in control group (16,35±8,1 µg/ml), than in TRH group (26,13±11,2 µg/ml. In third hour of incubation, differences in prolactin concentration between control group (12,8±3,4 µg/ml) and TRH group (14,6±4,1 µg/ml) were still statistically significant ($P \leq 0,05$). The study results indicate that TRH is a stimulatory factor of prolactin secretion in young lambs.

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Neural Basis of Olfaction: Insight into How the Olfactory System Can Evolve

Organizers (*Japanese Society for CPB*):

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Olfactory representation from the periphery to higher brain centers in *Drosophila melanogaster*

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Odor information is first detected by olfactory receptors and processed through multiple receptor-glomerular channels in the first order olfactory center, then reformatted into higher brain centers and eventually perceived by animals. To reveal the logic of olfactory processing, it is fundamental to comprehensively map odor representations from the glomerular channels and follow the flow of information into higher brain centers. We used *in vivo* whole-cell patch-clamp recordings in *Drosophila melanogaster* and characterized odor response profiles of antennal lobe (AL) projection neurons (PNs) originating from 31 glomeruli (> 60 % of total ~50 glomeruli). First, comparison of odor responses of PNs with olfactory sensory neurons at the same glomeruli revealed that combinatorial odor representations activating multiple glomerular channels are generally conserved. Next, functional odor coding map was reconstructed at the AL and higher brain centers. We found attractive and aversive odors are separately represented by different clusters of glomeruli in the AL, and these representations are further distinguished by the two separate regions in both the mushroom body and the lateral horn. Furthermore, we found evidence that odor information carried by a labelled line and combinatorial patterns converge to the same region in the lateral horn, suggesting functional convergence for aversive odors. These results provide a global picture of the olfactory circuit design underlying innate olfactory behaviors.

Olfactory coding in the dual olfactory pathway of the honeybee

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Insects constitute a well-established model for the study of the neural processes underlying olfactory perception. In insects, odors are detected by sensory neurons located in the antennae, whose axons project to a primary processing center, the antennal lobe (AL). There, the olfactory message is reshaped and further conveyed to higher-order centers, the mushroom bodies (MB) and the lateral horn (LH). Among insects, Hymenoptera present a striking olfactory system with a clear neural dichotomy from the periphery to higher-order centers, based on two main tracts of projection neurons: the medial and lateral antennal lobe tracts (m-ALT and l-ALT). To unravel the functional role of these two pathways, we performed in vivo calcium imaging on the brain of the honeybee Apis mellifera, a social insect that relies on both floral odors for efficient foraging and pheromones for social communication. Our recordings at the level of the AL reveal global redundancy of floral odors coding in both pathways but unravel some specificities for encoding chemical group and carbon chain length of odor molecules. We also compared the responses of honey bee pheromonal compounds, and found that queen and brood pheromones are differentially encoded by these pathways, whereas worker pheromones induce redundant activity. At the level of the LH, we showed odor-specific maps and a conservation of odor-similarity relationships, with a clear segregation of odorants based on pheromone type. The LH thus contains an odor-specific map with distinct representations for the different bee pheromones, a prerequisite for eliciting specific behaviors.

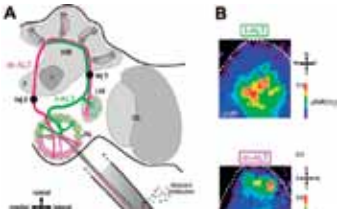

Figure: A) Dual olfactory pathway of the honey bee brain. AN, antennal nerve; MB, mushroom bodies; LH, lateral horn; m-ALT and l-ALT, median and lateral antennal lobe tract; OL, optical lobe. B) Odor-induced calcium signals in the AL for l-ALT PNs (top) and for m-ALT PNs (bottom). Relative fluorescence changes ($\Delta R/R\%$) are presented in a false-color code, from dark blue to red. From Carcaud *et al.*, 2015

The antennal lobe organization and function in the related species of heliothine moths

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In many male moth species, the antennal lobe is the primary olfactory center in the brain for processing odor information of sex pheromones and host volatiles. Correspondingly, the antennal lobe is divided into at least two compartments, marcoglomerular complex and ordinary glomeruli. The closely related heliothine moths possess such antennal lobes and show consistency in the organization. However, corresponding to the species-specific pheromone system, the macroglomerular complex shows species-specific organization. In this study, we revisit the antennal lobe and the macroglomerular complex of the male *Helicoverpa armigera*, *Helicoverpa assulta* and *Heliothis virescens* with emphasis on the organization of glomerulus. The glomerular organization of macroglomerular complex as well as other regions is compared in the number, shape, size and position and the function.

Glomerular size matters in pheromone preference of the European corn borer central nervous system

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The European corn borer (*Ostrinia nubilalis*) is a textbook example of pheromone polymorphism and is a model of evolution of sexual communication in insects. Two strains exist, which produce and prefer opposite ratios of their two-component pheromone blend. These strains do not freely interbreed in sympatry. Genetic studies have identified the gene, which is responsible for the dimorphism in female pheromone production. Although it is known that male preference is determined by a single sex-linked locus, the gene is as of yet unknown. Using activity dependent anterograde staining and intracellular recordings combined with immunohistochemistry, we found that the male preference is mediated by an antennal factor, which reverses the functional topology in the antennal lobe. Extensive intracellular recordings and neuronal fills of the hybrids, male of which prefer intermediate ratios, demonstrate that EZ and ZE hybrids have an E-type functional topology. However, the size of E and Z glomeruli and the antennal response are intermediate in the hybrids. Paternal backcrosses show a pattern predicted under sex-linkage. No differences were found in frequency and types of projection neurons encountered between the parental strains, their hybrid, and backcrosses.

Pheromones mediating copulation and attractionin *Drosophila*

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Intraspecific olfactory signals known as pheromones play important roles in insect mating systems. In the model *Drosophila melanogaster*, a key part of the pheromone-detecting system has remained enigmatic through many years of research in terms of both its behavioral significance and its activating ligands. Here we show that Or47b-and Or88a-expressing olfactory sensory neurons (OSNs) detect the fly-produced odorants methyl laurate (ML), methyl myristate, and methyl palmitate. Fruitless (fru^M)-positive Or47b-expressing OSNs detect ML exclusively, and Or47b- and Or47b-expressing OSNs are required for optimal male copulation behavior. In addition, activation of Or47b-expressing OSNs in the male is sufficient to provide a competitive mating advantage. We further find that the vigorous male courtship displayed toward oenocyte-less flies is attributed to an oenocyte-independent sustained production of the Or47b ligand, ML. In addition, we reveal that Or88a-expressing OSNs respond to all three compounds, and that these neurons are necessary and sufficient for attraction behavior in both males and females. Beyond the OSN level, information regarding the three fly odorants is transferred from the antennal lobe to higher brain centers in two dedicated neural lines. Finally,we find that both Or47b- and Or88a-based systems and their ligands are remarkably conserved over a number of drosophilid species. Taken together, our results close a significant gap in the understanding of the olfactory background to Drosophila mating and attraction behavior; while reproductive isolation barriers between species are created mainly by species-specific signals, the mating enhancing signal in several Drosophila species is conserved.

The ecological and physiological significance of olfactory sensory neuron co-localization

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Insect olfactory sensory neurons (OSN) are stereotypically grouped into sensilla on the chemosensory organs. The functional significance of OSN co-localization is poorly understood, but two hypotheses have been proposed. Firstly, co-localization might allow for signal modulation in the periphery via cross talk between OSNs sharing the same extracellular environment. Secondly, co-localization allows for coincidence detection of odor filaments, which should improve the discrimination of closely separated odor sources. We performed electrophysiology and field experiments on the European spruce bark beetle (*Ips typographus*), and found support for both hypotheses. In this species, the aggregation pheromone component *cis*-verbenol is detected by an OSN that is co-localized with an OSN detecting the host-derived behavioral antagonist 1,8-cineole. To test if co-localization allows for signal modulation in the periphery, we studied the responses of these OSNs to binary mixtures of the compounds. In support of the hypothesis, the response of the *cis*-verbenol OSN was strongly inhibited by the simultaneous response of the 1,8-cineole OSN. To test whether co-localization also improves odor source discrimination, we designed a field trapping experiment to investigate the beetles' response to odor source spacing. We separated the aggregation pheromone source from the 1,8-cineole source (0-48 cm), and compared the catches with those obtained in response to the same spacing between the pheromone and verbenone – a behavioral antagonist detected by OSNs that are not co-localized with pheromone OSNs. Consistent with the hypothesis, trap catch increased with distance between odor sources more for 1,8-cineole than for verbenone. In summary, our results suggest that (I) co-localization of OSNs allows for peripheral signal modulation, and (II) it also improves the discrimination of closely separated odor sources. Thus, selection for peripheral mixture processing and improved odor source discrimination ability could both underlie the strict co-localization of OSNs that is seen across the Insecta.

The distribution of octopamine and tyramine and their receptors in the honey bee brain

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Since Erspamer and Boretti first described the biogenic amine octopamine (OA) in the salivary gland of octopus as a molecule with "adrenaline-like" action, decades of extensive studies have demonstrated the important role of OA and its precursor tyramine (TA) play in invertebrate physiology and behavior. OA is synthesized from tyrosine. First it is converted to TA by the enzyme tyrosine decarboxylase then to OA by beta-hydroxylase. Until recently, thr role of TA was considered to be only the precursor of OA. However, the recent discovery of the action of TA and its localization indicate that TA has its own functions with its own source independently of that of OA. Here we describe the source of the OA and TA in the antenna, antennal lobe and mushroom body of the honey bee using specific antibodies against each biogenic amine. We show that ventral unpaired median (VUM) neurons are both OArgic and TArgic. We found that varicose-like fibers from VUM neurons are mostly located in the rind/cortex area of antennal lobe glomeruli. In addition, we found that mushroom bodies receive intensive innervation from the paired large TArgic mushroom body neurons located in the ventro-rostral protocerebrum. Both amines trigger intracellular signaling pathways by binding with different affinities to G-protein coupled OA/TA receptors. Activation of OA1 receptors increases the intracellular Ca^{2+} concentration (Sinakevitch et al., 2011, 2013). Activation OA-2 beta increases cAMP, whereas activation of AmTYR1 receptors inhibits adenosine 3',5'-cyclic monophosphate (cAMP). Here we used newly developed antibodies against AmTyr1 receptors to characterize its distribution in the antenna, antennal lobe and mushroom body in combination of the neurobiotin tracing of neurons that are important components of these networks. We found that AmTyr1 is expressed in the presynaptic sites of the olfactory receptor neuron axons in the antennal lobe as well in the projection neuron endings in the calyx. We present these data in the contact of a growing understanding of modulation by OA and TA in these networks in the honey bee brain.

1) Sinakevitch, I., *et al.* 2013 *Front System Neurosci doi:10.3389; 2)* Sinakevitch, I., *et al.* 2011 *PlosOne* 6(1): e14536. doi:10.1371

Expression levels of N-methyl-D-aspartate receptors in the chum salmon *Oncorhynchus keta*: Effect of seawater acclimation during the parr-smolt transformation

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This study investigated the adaptive capacity, olfactory memory, and imprinting ability of chum salmon *Oncorhynchus keta* during parr-smolt transformation, in response to salinity changes, when discharged into the sea. We examined changes on N-methyl-D-aspartate receptors (NMDARs; NRs) in different growth stages (early parr, parr, and early smolt) from freshwater (FW) to seawater (SW). Expression levels of NR genes mRNA and concentration of cortisol, T_3, T_4, dopamine and Na^+/K^+-ATPase activity significantly increased at salinity change condition. Moreover, in cultured brain cells, NRs were significantly lower in all groups treated with MK-801 (an antagonist of NRs) than in the early parr stage group in the FW treatment. We confirmed that the reduction in mRNA expression levels of NRs increased from the early parr to the early smolt stage. The information reported here should be taken into account in future studies on the relationship between memory factors of natal streams and homing mechanisms in Salmonidae.

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (2014R1A2A2A01002747).

Chemical World Sensing: A Joining Scope of Chemical Ecology and Neuroscience

Organizers (*Japanese Society for CPB*):

Ryohei Kanzaki, *University of Tokyo, Japan*
Mamiko Ozaki, *Kobe University, Japan*

Dietary preference by food flavor: Cross-modal pathway of the gustatory and olfactory inputs

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Flowers of different plant species have different scents having different chemical compositions. Hence, every floral scent would not uniformly affect the feeding preferences of insects. The blowfly, *Phormia regina*, is a nectar feeder insect. When the fly feeds on the nectar in a flower, its olfactory organs, antennae and maxillary palps, are exposed to the floral scent. In this situation, the fly perceives sweet taste of the nectar and at the same time particular scent of the flower, olfactory information of which affects the feeding preference. Here we show that the floral scents of 50 plant species brought various effects on the sucrose feeding response measured by the proboscis extension reflex (PER) test: Those floral scents were first categorized into three groups, based on their increasing/decreasing effects on the PER threshold concentration of sucrose, by which we knew whether the fly innately likes, ignores or dislikes them. Moreover, memory of feeding experiences with the floral scents variously influenced the PER threshold. After dietary experiences of the sucrose solutions scented with flowers, the tested scents did not always show the same effects as before. Considering their effects on the PER threshold of sucrose both with and without the dietary experiences, we could categorized the effects of 50 tested scents into 16 of the 27 theoretically predicted types. When we repeated the similar PER tests in the flies whose antennae or maxillary palps were ablated prior to the PER test or the dietary experience, the results indicated that olfactory inputs through two types of olfactory organs take different roles in forming or modifying feeding preference. Furthermore, we found a neuronal rout suggesting gustatory receptor neuron from the labellum and olfactory receptor neuron from the maxillary palp directly interacts with the processing gustatory information within the primary gustatory center of the fly brain.

Dietary rescue of altered metabolism gene reveals unexpected *Drosophila* mating cues

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To develop and reproduce, animals need long-chain mono- and polyunsaturated fatty acids (MUFAs; PUFAs). Although some UFAs can be synthesized by the organism, others must be provided by the diet. The gene *desat1,* involved in *Drosophila melanogaster* UFA metabolism, is necessary for both larval development and for adult sex pheromone communication. After characterizing *desat1* expression in larval tissues, we found that larvae in which *desat1* expression was knocked down throughout development died during the larval stages when raised on standard food. By contrast pure MUFAs or PUFAs— but not saturated FAs—added to the larval diet rescued animals to adulthood with the best effect being obtained with oleic acid (C18:1). Male and female mating behavior and fertility were very differently affected by preimaginal UFA-rich diet. Adult diet also strongly influenced several aspects of reproduction: flies raised on C18:1-rich diet showed increased mating performance compared to flies raised on standard adult diet. Whereas cuticular pheromones transferred from wild-type flies caused a significant rescue of mating performance in male and female flies in which *desat1* function had been knocked down, cues produced by C18:1-adult fed transgenic knockdown males had no such effect. Therefore, both larval and adult *desat1* expression control sex-specific mating signals. This work shows that supplementing the diet with specific fatty acids rescues pre-adulthood lethality and reproduction in a severe metabolic mutant and also reveals novel mating cues. A similar nutrigenetics approach may be useful, in other metabolic mutants, to uncover cryptic effects otherwise masked by severe developmental defects.

Naturalist-inspired chemical ecology: Targeting decision making in nature

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A century ago, our understanding of behavior was dominated by naturalists. Today, we can assess and manipulate nervous systems at genetic, molecular and physiological levels. Despite our tremendous focus on understanding the brain, we still know remarkably little about how even simple brains generate complex behaviors. By studying natural behavior and the processing of natural stimuli across animals, we can unite these modern scientific techniques with natural observations to truly understand how brains make decisions in our complex world.

We employ a comparative approach to understand how insect brains parse the complex natural chemical environment to generate decisions. Our current research focuses on two major questions: By what mechanisms can nervous systems evolve to generate novel decisions? And second, how do nervous systems adapt to make the same decisions in diverse environments? For the first question, we examine the chemosensory basis for sympatric speciation events in *Rhagoletis* flies. Our current studies indicate that genetic changes in *Rhagoletis* host preference mainly target the brain. The stereotyped, innate, and heritable nature of host search also implies a dedicated sensory pathway consistent across populations. For the second question, we study how similar transcontinental species of hoverflies pollinate in subarctic Swedish meadows, the Himalayan mountains, and even South Indian rainforests. We endeavor that our comparisons will allow us to generate overarching principles for decision making constrained by fundamental concepts, rather than specific physiologies.

Mapping behaviourally relevant odour signals from the periphery to higher-order centres in the helithine moth brain

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Among the moth species most extensively studied within chemosensory research are the heliothine moths, a monophyletic subfamily consisting of numerous noctuid species distributed in all five continents. This group is particularly fascinating because sympatric species use female-produced compounds to communicate not only within but also across the species. Thus, the male-specific olfactory system of these moths typically includes two sub-arrangements, one carrying pheromone information underlying attraction and sexual behavior and the other signal information originating from females of sympatric species, underlying rejection. By performing intracellular recording/staining of antennal-lobe projection neurons combined with confocal microscopy and computational modelling we have investigated how the two categories of behaviorally relevant odors are represented in the higher brain centers. The stimuli included two species-specific pheromone compounds and one interspecific signal. The results are discussed in relation to previous findings on the arrangement in the periphery, including a pattern of sensory neuron categories that are compartmentalized according to distinct rules.

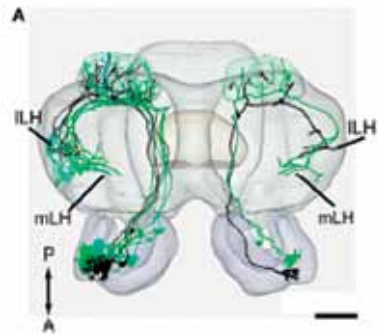

Figure: Individually reconstructed projection neurons registered into a standard brain atlas. As shown, the two types of pheromone neurons (green and turquoise) target an overlapping region partly segregated from that innervated by neurons carrying signals originating from a heterospecific female (black). Adapted from Zhao *et al.* 2014.

Pheromone and non-pheromone odor cleanup from an insect antenna: the role of grooming

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Fast changes in concentration of intermittent odor signal should be resolved by insect olfactory system to navigate in natural environment as well as agricultural and urban settings. Surface of olfactory appendages is covered by waxes and liquid hydrocarbons which prevent cuticle from water loss. Hydrophobic odor molecules get easily dissolved in this lipid layer and should be removed to maintain high temporal resolution of olfactory signals. Insects evolved specific enzymatic systems destroying highly species-specific signals, sex pheromones, found inside sensilar lumen as well as on the body surfaces (Vogt et al, 1985; Vogt, Riddiford, 1986). General odorants are not species-specific and very diverse chemical substances, they were not shown to be enzymatically cleared from the surface of antennal cuticle. Our data show that grooming behavior in American cockroach, *Periplaneta americana*, is altered under general odorant presence, but do not change significantly upon low dose pheromone stimulation. Thus, grooming provides nonspecific odor elimination from antennal surface for the wide range of olfactory stimuli.

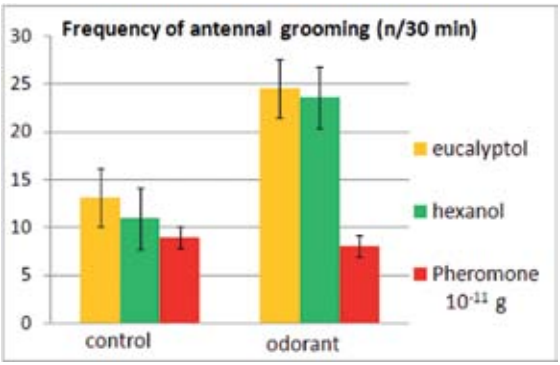

Figure: The number of antennal groomings performed during 30 min in either clean air (control) or in odorant supplied to the test chamber. Each animal was presented first with control air and later with odorant. Means±SE.

Vogt R.G. Riddiford L.M., Prestwich, G.D. *PNAS*. 1985. V. 82(24), 8827-8831; Vogt R.G. and Riddiford L.M. *J. Chem. Ecol*. 1986. V.12, No. 2. P. 469-482.

This work is supported by RFBR grant #13-04-00610a.

Neural basis of pheromone orientation in the silkmoth: From genes, neural networks, and behavior to robots

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To elucidate the dynamic information processing in a brain underlying adaptive behavior, it is necessary to understand the behavior and corresponding neural activities. This requires animals which have clear relationships between behavior and corresponding neural activities. Insects are precisely such animals and one of the adaptive behaviors of insects is high-accuracy odor source orientation. In order to understand the neural basis of odor source orientation in the silkmoth, we have worked at multiple levels, from genes, single cells of the neural system to the actual behavior. Relatively small size of the moth brain and a large body of identified neuron data are being used to attempt rebuilding behaviorally relevant circuits of the moth brain by means of realistic biophysical simulations. Moreover, in order to know the relationships between neural activity and behavior, we have developed a novel 'insect-robot hybrid system'. This experimental system has a mobile robot as its body. The robot is controlled by the insect through its behavior (i.e., an insect-controlled robot) or the neural activities recorded from the brain (i.e., a brain-controlled robot) (Figure). As we can arbitrarily control the motor output of the robot, we can intervene at the relationship between the insect and the environmental conditions. This system will contribute to better understanding of the neural basis of adaptive behavior. At first in this lecture, odor-source orientation in the male silkmoth and its neural basis will be introduced. Second, the extent of adaptation in the behavioral strategy, as governed by the neural system and investigated via a robotic implementation, will be shown.

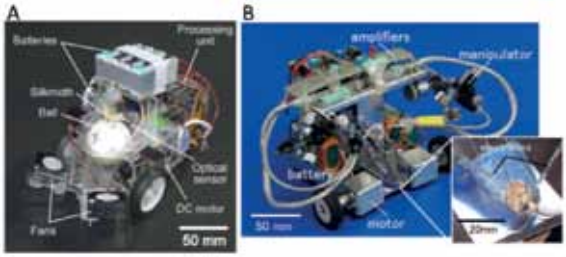

Figure: Insect-robot hybrid system. (A) Insect-controlled robot. (B) Brain-controlled robot.

Carbon dioxide sensitivity and its role in multifunctional neurons in the mollusk *Onchidium*

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Intrinsically photoresponsive neurons in the abdominal ganglion of the amphibious mollusk *Onchidium* named Ip-1 and Ip-2 (Ip-1/2) react to several different stimuli. These neurons respond to light with slow hyperpolarization and to CO_2 stimulation with slow depolarization. In this study, increasing the concentration of CO_2 in the air caused hyperventilation and enlargement of the pneumostome in the intact animal. In a semi-intact preparation, pouring artificial seawater (ASW) with dissolved CO_2 onto the central ganglia caused the previously closed pneumostome to open. In an ASW environment, Ip-1/2 neurons depolarized even under conditions of constant pH (alkaline ASW) and after dissolution of CO_2. This depolarization prolonged the firing of action potentials in Ip-1/2 neurons. Adding protons H^+ to ASW caused Ip-1/2 depolarization only when the neurons' membranes were depolarized to a potential above the resting potential. Furthermore, in the presence of the carbonic anhydrase inhibitor acetazolamide (AZ), CO_2-induced excitation in Ip-1/2 neurons was increased in both normal and alkaline ASW. These results suggest that when dissolved in ASW, CO_2 directly induced the depolarizing response in Ip-1/2 neurons. Since Ip-1/2 neurons participate in pneumostome opening, these results suggest that increased CO_2 levels in ASW directly stimulate CO_2-sensitive central neurons, promoting ventilation.

Figure: *Onchidium verruculatum Cuvier* (synonym; *Peronia verruculatum*)

Arrow shows a pneumostome; an increase of CO_2 in air (5%) caused a larger pneumostome opening than that in normal conditions, as observed in the intact animal preparation.

The structural change of the perireceptor proteins, OBP and CSP, upon ligand binding

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The much amount of soluble proteins were found in the hydrophilic lymph or mucus layers of olfactory or chemosensory organ. They can bind the hydrophobic compounds (odorant and/or pheromone), transport the odorant or pheromone to its receptor and modulate the receptor functions. We tentatively named such proteins perireceptor proteins (PRPs). So far, two classes of perireceptor protein were found, odorant-binding protein (OBPs) and chemosensory protein (CSPs). [1)] The Cp-Lip1 is an OBP found from Japanese newt (*Cynops pyrrhogaster*) [2)], and CjapCSP7 is a CSP found from the Japanese carpenter ant (*Camponotus japonicus*).

In this research, we expressed and purified functional forms of two perireceptor proteins, and analysed the structural and functional changes of PRPs upon ligand binding using fluorescence binding assay and CD (circular dichroism) measurements.

1) Pelosi, *et al.*, *Chemical senses* 30.suppl 1, i291-i292. (2005); 2) Iwasa T, *et al.*, *The Japanese journal of taste and smell research* 15(2), 211-220, (2008)

Photoreception Regulating Animal Physiology and Behavior

Organizers (*Japanese Society for CPB*):

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Retinal photoreceptors for body color change in zebrafish

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In response to ambient light levels, many cold-blood vertebrates darken or lighten their body colors. The light-induced body color change, also called "background adaptation" (Fig. 1), is mediated by ocular photoreception in teleosts, as shown in the classical study by von Frisch (1911). This study aims to identify the photoreceptive molecule(s) responsible for the light-induced body color change by using zebrafish as an animal model. To this end, we first generated a transgenic zebrafish line, in which rods and cones in the retina can be conditionally and selectively ablated. The rod-less cone-less zebrafish larvae exhibited no significant difference from the intact animals in the body color change under a saturated light condition. This light-induced behavior is thus mediated by non-rod non-cone retinal photoreceptor neurons (Fig. 2), possibly expressing non-visual opsins such as VAL-opsins and melanopsins. To investigate the light signaling process, we determined the spectral sensitivity for the light-induced body color change in the wild-type zebrafish. The estimated action spectrum suggested involvement of multiple opsin-type molecules in this photic regulation. Experimental data using the rod-less cone-less larvae as well as pharmacological inhibition will be presented to discuss this hypothesis.

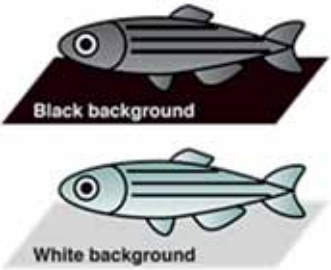

Fig. 1. Background adaptation in teleosts.

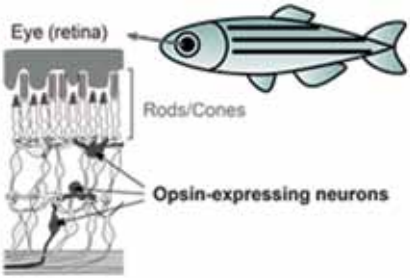

Fig. 2. Photoreceptor cells in teleost retina.

Colour regulates mammalian biological timing

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Dawn and dusk are key portions of the day for synchronizing the mammalian circadian system to the solar cycle and involve changes in both the quantity ('irradiance') and quality ('colour') of light. It is well known that animals use the variation in irradiance to align their behaviour and physiology with environmental light:dark cycles. By contrast, whether changes in colour also contribute to mammalian entrainment has, until recently, remained unexplored. Here I highlight our new data that addresses this question. First, using environmental measurements, we find that blue-yellow colour discrimination within the mammalian retina can provide a highly reliable method of estimating sun-position across twilight. Moreover, using electrophysiological recordings, we find that a subset of neurons in the mouse suprachiasmatic circadian clock display the cone-dependent spectral opponency required to make use of this information. Thus, our data show that some clock neurons are highly sensitive to changes in spectral composition occurring over twilight and that this input dictates their response to changes in irradiance. Finally, we confirm that spectral changes occurring during twilight are required for appropriate circadian alignment under natural conditions, using physiological recordings from mice housed under photoperiods with simulated dawn/dusk transitions. Together, our data reveal a new sensory mechanism for estimating time of day that would be available to any mammalian species capable of chromatic vision.

Wavelength discrimination in the pineal photoreception of lower vertebrates

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In the pineal organs of lampreys and teleosts, as well as in the pineal-related organs of frogs and lizards, the ratio of UV to visible lights in environmental light is detected, which suggests that lower vertebrates can discriminate different wavelengths of light with the pineal and related organs independently of eyes. We have already found that parapinopsin, which was first identified from the catfish pineal and parapineal organs, is the UV sensitive opsin underlying pineal UV reception in the lamprey by spectroscopic, immunohistochemical and electrophysiological analyses (1). We also isolated the parapinopsin from rainbow trout, clawed frog and iguana, of which the pineal and related organs were reported to detect ratio of UV and visible lights (1, 2). In addition, parapinopsin has some molecular properties different from those of vertebrate visual UV opsins. The findings suggest that parapinopsin provides a common molecular basis for pineal UV reception in the wavelength discrimination. However, the molecular basis for the visible light reception in the pineal wavelength discrimination remains unclear although it is suggested that green-sensitive parietopsin underlies the wavelength discrimination by UV and green light receptions as a partner of parapinopsin in the iguana parietal eye (2). In this study, we explored opsins underlying the visible light-sensitivity in the pineal wavelength discrimination by analyzing them spectroscopically, histochemically and electrophysiologically.

1) M. Koyanagi, E. Kawano, Y. Kinugawa, T. Oishi, Y. Shichida, S. Tamotsu and A. Terakita: *Proc. Natl. Acad. Sci. USA* 101, 6687-6691 (2004); 2) S. Wada, E. Kawano-Yamashita, M. Koyanagi and A. Terakita: *PLoS One*, e39003 (2012)

A deep brain photoreceptor involvement in innate perceptual discriminatory behaviour in medaka fish larvae

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Evidence has accumulated for several light-sensitive structures outside the eyes which influence behaviour through non-image-forming photoreception. Extra-retinal photoreception has been best characterized in non-mammalian vertebrates and occurs at several sites, including the pineal complex and by so-called "deep brain" photoreceptors. Recently we were able to identify specific cholinergic inter- and motorneurons located in delineated brain domains of adult medaka fish which were proven to be light-sensitive due to the expression of two groups of non-visual photoreceptors, VAL- and TMT-opsins (Fischer *et al.*, 2013). We speculate that environmental light can modulate the information processing in the vertebrate brain by changing the physiological characteristics of opsin-expressing neurons, and thus shaping relevant perceptual behaviour outputs. Using the highly efficient transcription activator-like effector nucleases (TALENs) method for targeted genome editing, we successfully generated homozygous mutant lines for *tmt1b*. These tmt1b-mutant fishes, when assessed in an ethological prey/predator size discrimination assay, revealed significant differences ($p<0.05$) in their stimulus-driven responses when compared with wild-type siblings. Interesting, by changing ambient light intensity, it was possible to manipulate the perceptual ability of the tmt1b-mutant fishes, which suggests a light-mediated change of the intrinsic valences of relevant stimuli. To start to mechanistically understand the different behaviour responses seen in the perception-mediated assay, a quantitative mRNA sequence transcriptome was made, comparing different anatomical parts of the adult medaka brain among age-matched wild-type and *tmt1b* homozygous sibling fishes. With this strategy, we obtained a limited but strong set of candidate genes that, due to their different levels of expression between genotypes, might explain how a light cue can in fact shape vertebrate behaviour.

Dissecting the nature of non-cephalic putative photoreceptors in the marine bristleworm *Platynereis dumerilii*

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[1]Max F. Perutz Laboratories and Research Platform "Marine Rhythms of Life", [2]CIBIV - Center for Integrative Bioinformatics Vienna, University of Vienna, Vienna; CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna; florian.raible@mfpl.ac.at

There is increasing evidence for the presence of opsin-positive neurons outside the brains of animals, but the function of these putative light receptors, as well as their evolutionary relationship with cephalic photoreceptors remain enigmatic. We have recently uncovered non-cephalic opsin-positive cells in the marine bristle worm *Platynereis dumerilii*, a model system for chronobiology and evolutionary developmental biology. These cells share expression of a distinct *opsin* gene (*r-opsin1*) as well as a Gq-type G protein alpha subunit (*1*) with adult eye photoreceptors, consistent with the notion that they are light sensitive.

In order to further investigate the nature of these non-cephalic cells, we have taken advantage of a stable transgenic strain that expresses an EGFP cassette under control of *r-opsin1* regulatory sequence, and highlights both cephalic and non-cephalic *r-opsin1* cells *in vivo* (*1*). We have developed a robust protocol for the dissociation of dissected worms and selective enrichment of EGFP-positive cells using FACS. By qPCR analysis, we have validated that RNA derived from sorted cells is highly enriched for *r-opsin1* transcript compared with unsorted cells. Optimization of an RNA amplification protocol has further allowed us to generate transcriptome profiles of small pools of cells. Consistent with our qPCR analyses, *r-opsin1* transcripts are highly enriched in EGFP-positive transcriptomes. Further bioinformatic analyses reveal additional co-enriched transcripts. These comprise genes commonly expressed in EGFP-positive cells from both heads and trunks, but also transcripts specific for each tissue. Together, our data provide a strong molecular entry point into the characterization of non-cephalic opsin cells, as well as their comparison with cephalic photoreceptors of worms and other species.

(1) Backfisch *et al.*, *Proc. Natl. Acad. Sci. U.S.A*. 110, 193–198 (2013).

Effects of LED spectra on the expression of vertebrate ancient long opsin and maturation-related hormones in goldfish *Carassius auratus*: Profiles following exogenous gonadotropin hormone

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We investigated the effect of light spectra on vertebrate ancient long opsin (VAL-opsin) and sex hormones in the goldfish *Carassius auratus* using red and green light-emitting diodes (LEDs). In addition, we investigated the effect of in vivo injection of exogenous gonadotropin hormone (GTH; 5 μg/g body mass) on VAL-opsin levels. We measured changes in the expression levels of gonadotropin-releasing hormone (GnRH: sGnRH and cGnRH-II), GTH (GTHα, LHβ, and FSHβ), and estrogen receptor (ER: ERα, ERβ1, and ERβ2) mRNAs, and GnRH and GTH proteins. Furthermore, we measured changes in plasma 17β-estradiol (E2) levels. In fish exposed to green light spectra and 5 μg/g GTH injection, VAL-opsin levels was significantly higher than the control (white) and red light-exposed groups. In fish exposed to green light spectra, the expression levels of GnRHs, ERs, and plasma E2 were significantly higher than those of the control and red light-exposed groups. These results indicate that GTH injection activates VAL-opsin and sex hormones and that green light spectra influence VAL-opsin in goldfish. In addition, red light is rapidly absorbed by water molecules and cannot be detected by fish. Thus, green light appears to trigger VAL-opsin to promote the expression of sexual maturation-related genes in fish. These results provide insight into the environmental, physiological, and molecular processes underlying fish development.

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (2014R1A2A2A01002747).

Identification and localization of opsin transcripts in the Antarctic krill *Euphausia superba*

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[1]Department of Life Sciences and Biotechnology, University of Ferrara, Italy; [2]Department of Biology, University of Padova, Italy; [3]Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Bremerhaven, Germany; [4]Australian Antarctic Division, Kingston, Australia; bru@unife.it

Opsin photopigments have been identified in most animal species and their functions are related to vision and phototactic behaviour. In the last thirty years, hundreds of opsins have been identified and sequenced. The traditional view divided opsins in three classes: "*r-type*" found in rhabdomeric photoreceptors of protostomes, the "*c-type*" found in ciliary photoreceptors of vertebrate rods and cones and the "*Group 4*" (e.g. peropsin). Recently, c-opsins were also detected in protostomes. We investigated the opsin repertoire of the Antarctic krill (*Euphausia superba*). *E. superba* is an interesting model to study the evolution of opsins. It inhabits almost all circumpolar marine environments, including abyssal depths and the under-ice habitat, and shows a characteristic diel vertical migration during which passes across different photic environments. Using a gene candidate approach, we identified 4 different "r-type" opsins in the krill transcriptome. Phylogenetic analysis showed that 3 of them (named Rh1, Rh2, Rh3) belong in the Middle Wavelength Sensitive ($\lambda_{max} \approx$ 450-500 nm) group and one falls in the Long Wavelength Sensitive ($\lambda_{max} \approx$ 500-550 nm) group. Hydropathy analysis (Kyte-Doolittle method) of the sequence predicted seven transmembrane domains, an extracellular amino terminus and an intracellular carboxyl tail. All key amino acids including the lysine in the VII transmembrane domain are present. Comparison of the *E. superba* opsin sequences with invertebrate orthologs showed a sequence similarity >60%. qPCR analysis showed that Antarctic krill opsins are expressed in different organs, such as compound eyes, brain and abdomen. Interestingly, we confirmed previous findings concerning daily fluctuations in abundance for opsin mRNA in the head, with a peak during the photoperiod.

Comparative Physiology of Gastrointestinal Tract and Enteric Nervous System

Organizers (*Japanese Society for CPB*):

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Hiroshi Shimizu, *King Abdullah University of Science and Technology, Kingdom of Saudi Arabia*

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Anatomical distribution of ghrelin peptide and preproghrelin and ghrelin receptor *ghs-r1a* in forebrain and gastrointestinal tract of goldfish (*Carassius auratus*)

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Ghrelin is an orexigenic peptide mainly synthesized in the gastrointestinal tract of vertebrates that acts through specific membrane receptors coupled to G proteins. In goldfish ghrelin and ghrelin receptors (GHS-R1a1, GHS-R1a2, GHS-R2a1 and GHS-R2a2) have been detected in brain and peripheral locations by PCR. However, there is scarce information about the anatomical distribution of the ghrelinergic system in teleosts. The aim of the present study was to investigate the anatomical location of both ghrelin and ghrelin receptor, GHS-R1a, in goldfish (*Carassius auratus*). For this purpose, fish were maintained under a 12L:12D photoperiod and fed daily (2 h after the lights on, ZT 2). Immunohistochemistry with the antibody against ghrelin peptide and *in situ* hybridization for *preproghrelin* and *ghs-r1a* were performed in brain, esophagus, intestinal bulb, J-loop and anterior intestine. *Preproghrelin* and *ghs-r1a* ghrelin receptor genes as well as ghrelin peptide were located in the mucosal epithelium of all the studied sections from the esophagus to the anterior intestine, mainly in the mucosal epithelium and also in the submucosal layer, as previously reported for other teleosts and vertebrates. Moreover, *ghs-r1a* ghrelin receptor gene expression showed a widespread distribution in brain, particularly in some hypothalamic nuclei (preoptic nucleus, nucleus of lateral recess, anterior periventricular nucleus and preoptic periventricular nucleus), and certain extrahypothalamic areas (valvula of the cerebellum and *torus longitudinalis*). The presence of the ghrelin in these encephalic areas that has been previously linked with other orexigenic and anorexigenic systems as NPYergic and orexinergic systems in teleosts, including goldfish, support the role of ghrelin as a modulator of food intake and energy balance.

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Comparative physiology of rhythmic movements of the gastrointestinal tract regulated by the enteric nervous system in gastropods (*Mollusca*)

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The digestive system of gastropods is highly diverse, as this taxonomic class includes carnivores, herbivores, scavengers, suspension feeders, and parasites. The structure and function of gastrointestinal (GI) tract varies depending on the feeding habits even among herbivorous gastropods, which share a similar ingestive system that uses a buccal mass and radular apparatus.

Three gastropod species were used in this study: *Aplysia* sp. and *Bursatella leachii* from Aplysiidae, and *Lymnaea stagnalis* from Pulmonata. *Aplysia* feeds on large seaweeds, *Bursatella* on epilithic algae, and *Lymnaea* on epilithic algae and water plants. *Lymnaea* has the longest esophagus among the three species, and *Aplysia* has a much larger crop than *Bursatella* and *Lymnaea*. In all three species, peripheral neurons in the enteric nervous system (ENS) exhibited autonomous synchronous periodic-burst activity. Simultaneous recording of the neuronal activities and movements of the gastrointestinal tract revealed that the periodic bursts were followed by contractions of the gizzard and peristaltic movements in the crop in *Aplysia.* In *Bursatella* and *Lymnaea,* these events were followed by contractions of the crop and peristaltic movements in the esophagus. These observations show that the ENS contains pacemaker neurons responsible for the neurogenic rhythmicity of these movements. The pacemaker region was localized on the posterior gizzard in *Aplysia* and on the crop in *Bursatella* and *Lymnaea*. Therefore, in all cases, rhythmic neuronal activity originates distal to regions that undergo peristalsis.

In summary, GI tract motility and the site of the pacemaker region of *Bursatella* were more similar to those of *Lymnaea* than those of *Aplysia*, even though the latter species is more closely related to *Bursatella.* We will discuss how ENS functions in the GI tract evolved to suit specific diets.

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Neural control of the hindgut in a penaeid shrimp, *Marsupenaeus japonicus*

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The digestive tract in decapods crustacean is composed of three functional regions, the foregut, midgut, and hindgut. While control mechanisms of the foregut by the stomatogastric nervous system had been well known, neural control of the midgut and hindgut had been less investigated (cf. 1). We studied on neural control mechanisms of hindgut movement, using a penaeid shrimp, Marsupenaeus japonicus.

The musculature of the digestive tract in the shrimp is composed of two types of striated muscles, the outer circular muscle and the inner longitudinal muscle, as in other decapods (cf. 2). After the posterior intestinal nerve (PIN) arose from the dorsal root of the 6th abdominal ganglion (AG6), PIN run anteriorly on the ventral surface of the hindgut. When repetitive stimuli were applied to PIN, the stimulation induced rhythmic contraction with increase of tonus in the hindgut. The excitatory effects of the stimulation were blocked by a cholinergic antagonist, atropine.

Several types of efferent impulse units were extracellularly recorded from PIN. Spontaneous rhythmic burst activities were observed when the ventral nerve cord was intact. After the anterior connectives of AG6 were cut, the rhythmic burst activities ceased. Perfusion of AG6 with the Ca^{2+}-deficient saline depressed spontaneous activities in PIN. The repetitive stimuli applied to the anterior connectives of AG6 evoked excitatory effects on impulse activities in PIN. Some neuronal somata were observed in AG6 by retrograde Co^{2+}-labeling through PIN (cf. 3). These neuronal somata may be candidates for excitatory neurons of the hindgut.

From these results, it was suggested that PIN included excitatory fibers on the hindgut muscle and one of them might be cholinergic. While somata of the hindgut excitatory neurons would be located in AG6, rhythmic activities of these neurons might be due to neurons in other central ganglia.

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Neuromodulation for behavior in the insect stomatogastric nervous system

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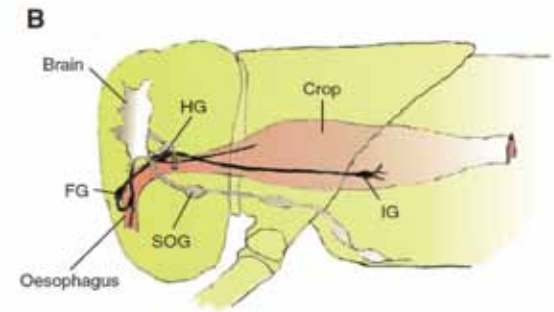
A fundamental question in neurobiology concerns the way by which the function of the nervous system is modified to allow an animal the behavioral plasticity needed to adapt to the changing demands of its environment. Modulation of neural circuits for behavior has been the focus of considerable work over the last decades. Neuromodulators, primarily biogenic amines and peptides, induce, coordinate and shape behavior, by acting simultaneously at many target points in the nervous systems. Substantial progress has been made by studying rhythmic behaviors and the central pattern generating (CPG) circuits that generate them. The ability of the network to produce a variety of different motor patterns, and thus a number of related behaviors, is a fundamental feature of CPG networks. Insect neural networks have been in the forefront of research on CPGs and their modulation.

For over a decade we have been studying the neuronal networks in the locust stomatogastric nervous system (STNS). This system, which is closely associated with the brain and subesophageal ganglion (SOG), is comprised of a set of inter-connected ganglia; the frontal ganglion (FG), which innervates the esophagus, and the hypocerebral ganglion (HG) and paired ingluvial ganglia, innervating the anterior and posterior portions of the crop, respectively (Figure below). Chemical modulation, as well as sensory inputs from the gut and inputs from other neural centers enables the STNS networks to induce multiple and complex foregut rhythmic patterns, depending on the animal's physiological and behavioral state.



This presentation will summarize many years of investigation into the neuromodulation and circuit interaction in the locust STNS, and their role in the generation and control of the system's motor output.

Peptides and peptide receptors controlling food intake and digestion in locusts

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Despite their huge biodiversity, some fundamental characteristics are shared by all animals. Metazoans are heterotrophs, implicating the basic need for the intake and digestion of food, as well as for the intestinal absorption of nutrients. Therefore, it is crucial that animals can rely on physiological mechanisms for the control of these essential processes. Nutrient-sensing, hormonal and neuronal signaling systems are playing an important role in this complex regulation.

In this lecture, we will consider a few neuropeptide-mediated pathways that are implicated in the physiological regulation of feeding and digestion in insects. In particular, we will discuss recent data obtained in the locusts, *Schistocerca gregaria* and *Locusta migratoria*, which are swarm-forming pest species that irregularly devastate the agricultural production in large areas of the world. In a physiological and neurobiological research context, they have proven to be interesting experimental model organisms. Moreover, RNA interference constitutes a highly efficient and robust method to silence the expression of peptide precursors and/or receptors in several insect species, including locusts. We have identified several insect neuropeptide precursors and receptors and will report on our recent physiological and molecular biological studies that further illustrate the role of neuropeptides, such as CRF/DH, NPF, sNPF and SK, in the regulation of food intake, digestive enzyme secretion and gut motility in locusts. The general aim of our work is to contribute to a better understanding of the regulation of these complex processes, as well as of the possible functional interactions between different regulatory pathways in an integrative physiological context.

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Digestive movements in Hydra: Regional distribution of the capacity of esophageal reflex and peristaltic reflex movements

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Hydra is a freshwater organism that belongs to class Hydrozoa of phylum Cnidaria. Since the digestive cavity of Hydra is apparently a hollow space, diffusion of digestive enzymes and digested materials was considered to be characteristic of the digestive process. We provided evidence against this common understanding by demonstrating that three digestive movements (esophageal, peristaltic, and defecation) occur depending upon the time after the ingestion of prey (Shimizu *et al*., 2004). Unlike mammals, however, regional functional difference was not observed suggesting that the specification of digestive tract occurred later in evolution.

In order to examine the regional difference in the capacity of digestive movements, we made Hydra tissue of homogeneous positional origins P1, P2 and P3 correspondent to upper, middle and lower position of the body column respectively by grafting tissue pieces in a ring in tandem using fish lines. We then examined the pattern of the movements by means of digital time-lapse techniques. The result showed that there is a definite difference in the pattern of movements depending upon the positional origin of the tissue suggesting that regional specification of the digestive tract does occur even in a very short digestive tract and even in primitive invertebrates.

Regional specification of the digestive tract has been studied in relation to the pattern of expression of *Hox* orthologues. The present result is the first demonstration that the regional specification occurs independently of *Hox* patterning. This is because it is now generally accepted that duplication of *Hox* orthologues took place after Cnidaria diverged from common ancestor. Possible molecular mechanism for the specification will be discussed.

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The Role of Monoamines in Modulating Behavior

Organizers:

John Swallow, *University of Colorado, USA*
Jaime Grace, *University of Colorado, USA*
Andrew Bubak, *University of Colorado, USA*

Serotonergic mediation of aggression and opponent assessment in stalk-eyed flies

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The stalk-eyed fly, *Teleopsis dalmanni,* is a sexually dimorphic species characterized as having elongated eyestalks containing eye bulbs protruding laterally from the head. This trait is exaggerated in males and is used during aggressive displays and ritualized combat for access to feeding and roosting sites, typically resulting in the individual with longer eye span winning the contest. However, non-morphological features, such as central serotonergic (5-HT) activity, can also influence individual aggression and have profound effects on fighting intensity and outcome. In order to understand the effects of 5-HT and gain further insight into potential mechanisms mediating stalk-eyed fly aggression, we manipulated central 5-HT concentrations by administering the metabolic precursor, 5-hydroxytryptophan (5-HTP), and staged two different fighting paradigms. The first involved size-matched pairs with one opponent treated for elevated neural 5-HT levels. This resulted in elevated aggression in individuals with higher 5-HT as well as an increased probability of winning the fight against opponents with lower levels of 5-HT. The second experiment aimed at understanding assessment strategies between opponents. Size-mismatched pairs were pitted against each other with half the smaller opponents treated for heightened 5-HT. We discovered that 5-HT increased aggression in the smaller opponents despite the perception of a larger opponent leading to increased fight initiations, engagement in more high-intensity behaviors, and fewer retreats compared to untreated smaller opponents. Furthermore, larger males that faced a treated, hyper-aggressive smaller opponent altered their fighting strategy by preemptively escalating fights to physical, high-intensity attacks. Collectively, these results indicate a significant role for 5-HT in male stalk-eyed fly aggression as well as a modulatory role in rival assessment.

The self-organization of pavement ant wars: Individual decision making and brain monoamines

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Social insect colonies are regulated as non-hierarchical, distributed systems in which ants must make individual decisions that are informed by local information cues. Changes in colony behavior occur collectively because of the many decisions of individuals. Here, we report how brain monoamines affect the decisions of individual ants after detecting chemical cues during interactions with nestmate ants, fighting with non-nestmate ants, or exposure to nestmate and non-nestmate chemical cues during the self-organization of warfare by pavement ant (*Tetramorium caespitum*) colonies. Ant colonies are closed societies from which intruders are excluded and colony membership for pavement ants is coded in cuticular hydrocarbon based cues. Wars are hostile conflicts in which members of one society coordinate their efforts to kill or injure members or disrupt the normal functioning of another. Animal wars seem to arise from changes to social context or circumstance instead of primarily from the inherent aggressive tendencies of individuals and are examples of how cooperation within a society can lead to aggression between members of opposing societies. The pavement ant is a tramp species well known for its ant wars in which thousands of workers from two colonies fight. Fighting is ritualized; ants fight by grabbing another ant's mandibles with its own and pairs undergo what can be described as a "push-of-war" while other ants recruit more workers to the battle and few, if any, ants die during the battle. What are the rules that influence the collective-organization of these "wars" and how are cooperative and agonistic behaviors modulated in individuals by brain monoamines? We report that serotonin (5-HT) levels are higher in ant brains after interactions with nestmate ants or their chemical cues. In contrast, dopamine levels are elevated in ant brains after fighting with non-nestmate ants and after exposure to their chemical cues. Brain octopamine levels were also elevated in ants that fought non-nestmates.

Controlling the decision to fight or flee – the roles of octopamine and nitric oxide in cricket aggression

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Fighting is dangerous, which is why all animals choose to flee once the costs outweigh the benefits, but the mechanisms underlying the decision to fight or flee are largely unknown. Our work on crickets provides rare insights into how this can be achieved simply by exploiting the powers of neuromodulation. As in many animals, aggression in crickets is promoted by physical exertion, winning a conflict, and possession of resources. Experiments with aminergic receptor agonists and antagonists revealed that the effect of these diverse experiences is each mediated by octopamine, the invertebrate analogue of noradrenalin (review: Stevenson & Rillich, Front. Neurosci 6:116, 2012). By interfering with information exchanged by agonistic signals during fighting we found that crickets flee as soon as the sum of their opponent's aversive actions exceeds a critical amount. This impact of the opponent's actions is mediated by the NO/cGMP signalling pathway, which increases susceptibility to aversive stimuli and with it the likelihood to flee (Stevenson & Rillich, Science Adv. 1:e1500060, 2015). Activation of the NO/cGMP pathway in subordinates also leads to a prolonged period of reduced aggressiveness, known in many animals as the loser effect. While octopamine and dopamine can both induce early recovery from the loser effect, dopamine is necessary (Rillich & Stevenson, Hormones Behav. 66:915-922, 2014). We conclude that the decision to persist in fighting in crickets is promoted by octopamine, which sets the threshold at which activation of the NO/cGMP pathway invokes the decision to flee (Rillich & Stevenson, Front. Behav. Neurosci 9:95, 2015 in response to aversive experiences accumulated during fighting (Figure).

Monoamine receptors and swarming behaviour in locusts

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The desert locust, *Schistocerca gregaria*, can undergo a density-dependent phase transition between a cryptic solitarious and a swarming gregarious phase that differ in many aspects of behaviour, physiology and morphology. The first characteristic that changes during phase transition is behaviour. The gregarious behaviour is triggered within a couple of hours of forced crowding. This reflects conditions in nature where they come together because sources have become rare, and thus the gregarious phase is necessary to migrate to more favourable places.

During this period in which the behavioural changes occur, the levels of multiple monoamines changed rapidly in the nervous system of the desert locust, mainly caused by tactile stimulation (Rogers *et al.*2003, 2004). The rise of serotonin levels was shown to be necessary as well as sufficient to induce the behavioural change (Anstey *et al.* 2009). By using RNA interference and pharmacological blockers, we demonstrated that protein kinase A (PKA) is absolutely crucial for this initial behavioural change in the desert locust and that this process thus resembles classical forms of learning.

Also in other locust species PKA was shown to be crucial for the initial behavioural change (Ma *et al.* 2011), however other biogenic amines seem to be more important than serotonin upstream.

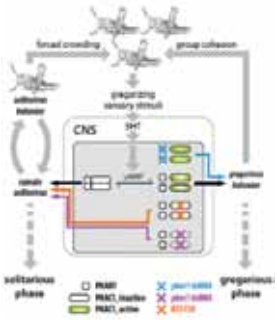

Figure: Molecular processes underlying the behavioural phase transition in *Schistocerca gregaria*

Research Foundation of Flanders and the KU Leuven Research Foundation.

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Biparental behavior in the burying beetle, *Nicrophorus orbicollis*: a role for dopamine?

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Burying beetles, Nicrophorus orbicollis, have facultative biparental care and depend on locating a rare reproductive resource, a small vertebrate carcass that they bury and prepare as food for the young. After eggs are laid in the soil nearby, both male and female remain in the reproductive chamber to guard and feed the newly-hatched, altricial larvae. During a breeding cycle, burying beetles typically switch from aggression towards conspecifics to pair bonding and parental care. Importantly, previous studies of reproductive physiology indicate that in N. orbicollis hormonal and social factors appear to interact to mediate a vertebrate-like plasticity in the modulation of parental behavior. For example, the levels of juvenile hormone (JH), the main insect gonadotropin, are known to rise and fall at key time points during breeding, and JH levels appear responsive to stimuli from a mate or young. However, JH reproductive plasticity does not seem sufficient to explain the elaborate control of parental care. To further elucidate the neurophysiological substrate of this complex behavior, we have established the reproductive profiles of several key neuromodulators of insect social behavior, the biogenic amines octopamine (OA), dopamine (DA) and serotonin (5-HT). Intriguingly, quantification of monoamine levels in individual beetles revealed that after 24 hr of care, when parental feedings approach peak rates, brain levels (pg/µg protein) of DA (mean ± SEM) are significantly elevated in parental beetles (19.8 ± 2.6) compared to nonbreeding, unmated males and females (16.5 ± 2.1) matched for age and colony background ($P<0.05$). In contrast, OA and 5-HT levels did not significantly differ between breeding and nonbreeding beetles. These results thus provide the first support for a functional link between DA and the neuromodulation of parental behavior in the burying beetles. Additional functional work will characterize the OA, DA and 5-HT receptors and their subtypes, and use pharmacological treatments to further assess the link between central monoamine function and N. orbicollis parental behavior.

The salivary glands of *Calliphora vicina* as a model system for analysing the cellular actions of serotonin and crosstalk between intracellular signalling pathways

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The salivary glands of the blowfly *Calliphora vicina* are an established model system for studying cellular signal transduction processes. In this non-innervated gland, the biogenic amine serotonin (5-hydroxytryptamine; 5-HT) acts as a neurohormone and stimulates fluid secretion by activating both inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} and cyclic adenosine 3´,5´-monophosphate (cAMP) signaling pathways in the secretory cells. More recent studies indicate a complex functional crosstalk between the two signaling pathways and we are only just beginning to understand the molecular basis and physiological significance of such crosstalk. In order to characterize the signal-inducing 5-HT receptors, we cloned two cDNAs (*Cv5-ht2*, *Cv5-ht7*) that share high similarity with mammalian 5-HT_2 and 5-HT_7 receptor genes, respectively. RT-PCR demonstrated that both receptors are expressed in the salivary glands and brain. Stimulation of *Cv5-ht2*-transfected mammalian cells with 5-HT elevates cytosolic [Ca^{2+}] in a dose-dependent manner (EC50 = 24 nM). In *Cv5ht7*-transfected cells, 5-HT produces a dose-dependent increase in [cAMP]i (EC_{50} = 4 nM). We studied the pharmacological profile for both receptors. Substances that appear to act as specific ligands of either Cv5-HT_2 or Cv5-HT_7 in the heterologous expression system were also tested in intact blowfly salivary gland preparations. We observed that 5-methoxytryptamine (100 nM) activates only the Cv5-HT_2 receptor, 5-carboxamidotryptamine (300 nM) activates only the Cv5HT_7 receptor, and clozapine (1 µM) antagonizes the effects of 5-HT *via* Cv5HT_7 in blowfly salivary glands, providing means for the selective activation of each of the two 5-HT receptor subtypes. This study represents the first comprehensive molecular and pharmacological characterization of two 5-HT receptors in the blowfly and permits the analysis of the physiological role of these receptors, even when co-expressed in cells, and of the modes of interaction between the Ca^{2+}- and cAMP-signaling cascades.

Biogenic amines modulate opposite forms of learning and synaptic plasticity in the honey bee (*Apis mellifera*)

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Biogenic amines mediate the probabilities of antagonistic behavioral states in invertebrates(*1*). In honey bees, octopamine has been implicated as a key part of the reinforcement for appetitive associative conditioning, such as, when a honey bee associates the odor of a flower with nectar. This kind of conditioning is critical for a honey bee forager as it learns about flowers that present resources that the colony needs for survival. However, foragers must also learn about the other types of flowers that do not present nectar. In a natural setting, for example, some flowers that have already been pollinated no longer present nectar, and the odor bouquet they give off changes slightly from unpollinated flowers in the same patch. Honey bees learn to avoid unrewarding flowers via non-associative 'odor habituation', also called Latent Inhibition. We have shown that a second biogenic amine – tyramine – is associated with this form of learning. Interestingly, The same Ventral Unpaired Medial (VUM) neurons in the insect CNS produce, and may synaptically release, both biogenic amines(*1*). This raises the possibility that two opposing forms of conditioning are driven by co-release of these neuromodulators in the CNS. We have studied both forms of learning behaviorally as well as in the Antennal Lobes (AL) and Mushroom Bodies (MB) of the honey bee brain. Both of these neuropils are important for implementing synaptic plasticity. We show how the outputs of these areas of the brain are shaped by both kinds of experience, and via computational models we propose the specific synapses in the neural networks that must be modified in order to reproduce empirical observations. Finally, we have developed a computational model for decision making that integrates associative and non-associative mechanisms, and it replicates well the patterns of behavior observed in individual honey bees during conditioning studies(*2*).

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Monamine receptors and honey bee behavior

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Honeybees exhibit a fascinating division of labor among their sterile female workers. They perform a wide range of different tasks as they age. The most distinct tasks are nursing (providing food for the brood) and foraging (collecting pollen and nectar). These differences in task performance correlate with different sensory response thresholds, for example for visual and gustatory stimuli. These physiological and behavioral processes are known to be under the control or modulation of biogenic amines such as serotonin (5-hydroxytryptamine, 5-HT) and tyramine. Knowing the mechanisms how these substances act on the cellular level is fundamental to understand their role in behavior. Very important parts of the signaling cascades are represented by respective receptors, which transfer an extracellular signal into an intracellular one.

Here we present the results of the characterization of the respective receptors. This includes a wide range of different types of analyses, for instance genomic organization, expression analysis, and functional analysis of the intracellular pathways.

Octopamine underlies the honeybee's physiological response to hunger stress

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The biogenic amine octopamine (OA) is the invertebrate structural homolog of the invertebrate stress hormone norepinephrine. OA seems to play multiple roles regulating an insect's behaviour and has long been thought to be the transmitter of the reward pathway in insects thereby playing a crucial role in appetitive learning and memory formation. Moreover, OA is also known to be involved in processing hunger and stress thereby regulating an animal's approach towards food.

In this study we aimed towards understanding and integrating these multiple roles of OA in the honeybee, *Apis mellifera*. We demonstrate an involvement of OA in the sugar metabolism of honeybees. In line, OA impacts the bees' sucrose sensitivity, sucrose uptake, and survival.

Taken together, our results indicate a role of OA in regulating a bee's physiological and behavioural response to their feeding status. Thus we conclude that OA in honeybees acts similar as the stress hormone norepinephrine in vertebrates regulating an animal's metabolisms and its stimulus responsiveness in stressful situations.

Role of monoamines in modulating fish behaviour

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The organisation of the brain monoaminergic systems appears to have been highly conserved across the vertebrate subphylum. In fish as well as in other vertebrates brain monoamines, dopamine (DA), norepinephrine (NE) and serotonin (5-HT), mainly act as neuromodulators with complex effects on multiple functions. These systems are activated in response to stress, and to some degree catecholamines and 5-HT appear to have opposing effects, DA and NE promoting active responses whereas 5-HT seems to induces withdrawal. The 5-HT system acts stimulatory on the hypothalamic-pituitary-interrenal axis but it is also important for stress coping and long-term stimulation of the 5-HT system counteracts stress-induced elevation of plasma cortisol. Fish displaying divergent stress coping styles (proactive vs. reactive) differ in 5-HT and DA functions. During agonistic interactions brain monoaminergic systems are activated and the 5-HT system appear to inhibit aggressive behaviour. However, this inhibitory effect is only observed following long-term activation of the 5-HT system. In teleost fish, brain monoaminergic function is also related to life history traits.

Monoamines are critical for the ability of exercise to modulate brain function and behaviour

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The myriad beneficial effects of exercise include improved learning and memory and reduced incidence of stress-related psychiatric disorders such as anxiety. Research using animal models has revealed a critical role for monoamines in mediating the behavioral effects of exercise. Repeated voluntary exercise activates locus coeruleus norepinephrine neurons [1], which in turn leads to enhanced brain-derived neurotrophic factor (BDNF) expression in the hippocampus [2, 3] and improved hippocampal-dependent learning and memory [4]. Exercise also produces neuroadaptations in brain serotonergic (5-HT) systems [5] including a reduction in striatal and amygdala 5-HT2C receptors [6]. These adaptations contribute to protection against maladaptive behavioral consequences of acute stressor exposure including interference with goal-directed learning and anxiety [7]. Finally, acute exercise can augment fear extinction learning [8] and prevent the relapse of anxiety following extinction [9]. Recent data are consistent with a role for enhanced dopamine 1 receptor signaling in the dorsal striatum in mediating the ability of acute exercise to prevent the relapse of fear following extinction. Together, these data suggest that monoamines and adaptations within monoaminergic systems are important for the cognitive, stress-protective, and anxiolytic effects of exercise.

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The effect of dopamine and noradrenaline reuptake inhibitor on swiminduced aerobic metabolism in bank voles from a selection experiment

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The actual physical performance is determined not only by biophysical and physiological limitations, but also by a behavioral characteristic – motivation. We applied an experimental evolution approach combined with pharmacological manipulation to test a hypothesis that evolution of increased aerobic exercise performance can be triggered by evolution of motivation to undertake physical activity. The study was based on a unique model system: bank voles (*Myodes glareolus*) selected towards high swim-induced aerobic metabolism (VO_2 swim). In generation 19, voles from the four selected A-lines achieved 55% higher VO_2swim than those from unselected C-lines. Because the voles could float on the water surface with only a minimum activity, the maximum rate of aerobic metabolism achieved in that test depended not only on their aerobic capacity, but also on motivation to undertake intensive activity. We measured the VO2swim after intraperitoneal injections of saline and 20mg/kg or 30mg/kg bupropion, a reuptake inhibitor that modulates activity of neurotransmitters putatively involved in regulating physical performance: dopamine (DA) and noradrenaline (NA). It has been shown that bupropion injections resulted in increased physical performance in mice (1) and rats (2). We expected that the drug would generally increase the activity and if the difference between A and C lines is associated with an altered DA or NA transmission, the effect should be weaker in the A-lines. However, the drug had no effect on the achieved level of VO_2swim. The outcome can be due to the fact that "positive stress" induced by contact with water resulted in motivation sufficient to compensate the effect of the drug. Thus, the results did not allow firm conclusions concerning involvement of the motivation mechanisms dependent on these neurotransmitters in evolution of increased aerobic exercise performance in the experimental evolution model system. Funding: NCN UMO-2014/13/N/NZ4/04824; DS/WBINOZ/INOS/757; DS/MND/WBINOZ/INOS/20/2014; DS/MND/WBINOZ/INOS/27/2013

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Central administration of cannabinoid ligands modulates passive avoidance learning in olfactory bulbectomized rats

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The present study investigated the sub-chronic effects of cannabinoid CB1 receptor agonist HU-210 and CB1 receptor antagonist SR 141716A on learning and memory of rats with a model of depression (bilateral olfactory bulbectomy, OBX). HU-210 (5 µg) and SR 141716A (3 µg) were microinjected intracerebroventricularly (icv) in OBX rats and a passive avoidance task (step through) was used as a test for learning and memory. The seven day icv administration of HU-210 in OBX rats prolonged the latency time on the retention test (3 hours after the training sessions) and increased the percentage of rats that have reached the learning criterion by (14 %) on the 3rd hour and by (29 %) on the 24th hour as compared to the saline-treated OBX controls (0%), while the cannabinoid CB1 receptor antagonist SR 141716A did not affect significantly the performance of OBX rats. However, despite the observed learning and memory enhancing effect, HU-210 failed to abolish completely the bulbectomy-related deteriorated performance of OBX rats in the step through test. These findings suggest a modulatory effect of CB1 receptors in learning and memory processes of rats with an OBX model of depression.

Maintaining the *Drosophila* larval heart in situ: Modulators and stretch activated channels

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The *Drosophila* heart is of interest as a genetic and physiologic model for developmental studies, pharmacological screening, investigating the ionic bases for pacemaker activity as well as understanding the modulation of pacemaker activity. To study cardiac physiology in vivo a suitable saline is necessary to maintain heart activity. Recently a modified HL3 saline has shown promise in maintaining the heart rate (HR) (de Castro *et al.*, 2014). However, this minimal saline, which is pH stable, does not maintain the HR for long periods of time for electrophysiological or imaging studies. In the current study, a cocktail of octopamine (OA), dopamine (DA), acetylcholine (Ach) and serotonin (5−HT) maintains a stable HR for longer periods of time. Each of the modulators separately increases HR frequency. Even without the common hemolymph sugars (trehalose and sucrose), the HR is maintained. With the cocktail of modulators, the HR stays stable for 2 hours. Given that TRPA receptors (stretch-activated) are expressed in the heart, I examined their role in maintaining HR. We developed an apparatus to simulate body wall contraction and relaxation on the heart tube as well as with a saline profusion system. Profusion was less invasive than stretching the body, and promoted larval HR maintenance. Currently, we are investigating the effect of pharmacologically inhibiting stretch activated ion channels as well as using RNAi knockdown in the intact larvae.

de Castro, C. *et al.*, (2014). Examining the usefulness of various salines by physiological assays designed for larval Drosophila melanogaster: heart rate, synaptic transmission at neuromuscular junction and central circuits in the CNS. *Journal of Comparative Physiology* A 200:83–92

Using genomics to address the role of serotonin in modulating aggression

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The role of serotonin in modulating aggression in animals is complex. In the stalk-eyed fly, *Teleopsis dalmanni*, serotonin appears to exert sexually dimorphic effects on the expression of aggressive behaviors. In previous studies, treatment of males and females through dietary supplement with 5-hydroxytryptophan (5-HTP) causes a significant elevation in brain levels of serotonin (5-HT). Males experiencing artificially elevated 5-HT levels exhibit increased aggression while females show reduced aggression compared to untreated control individuals in staged contests. We performed an RNA-seq study to determine which genes may be differentially regulated due to elevated 5-HT levels. Our analysis revealed a number of genes in both males and females whose expression differed across treatments. Several candidate genes were identified that were differentially regulated in either males or females or both. These genes will be discussed in the context of their potential to influence aggression, and in particular, their potential to contribute to the sexually dimorphic effects of serotonin observed through behavioral analyses.

Sex-specific effects of serotonin on aggression in a sexually dimorphic stalk-eyed fly

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Stalk-eyed flies, *Teleopsis dalmanni*, experience intense sexual selection, both intersexual and intrasexual, as males compete over access to territories and females. Both male and female *T. dalmanni* have eyes at the end of stalks, but male eyestalks are significantly longer, accounting for as much as 9% of body mass. Males with longer eyestalks are both more attractive to females and more likely to win aggressive contests with other males. Serotonin (5-HT) plays a critical role in modulating aggressive behavior: males with artificially elevated 5-HT levels are more likely to win competitions with size-matched opponents over a food source. However, the increase in aggression in response to elevated 5-HT may be sexually dimorphic. In this study, we examined whether females treated with 5-HTP (5-hydroxytryptophan, the serotonin precursor that is converted to serotonin in the brain) are more likely to exhibit aggressive behaviors towards other females in size-matched contests. Intriguingly, we found that females treated with 5-HTP exhibited fewer aggressive behaviors and were less likely to win aggressive competitions. These studies illuminate the sexually dimorphic role of serotonin in modulating aggression.

Biological Timing System Using Circadian Clocks in Invertebrates

Organizers (*Japanese Society for CPB*):

Elzbieta Pyza, *Jagiellonian University, Poland*
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Daily and seasonal patterns in serotonin levels in haemolymph and tissues of the freshwater mussels *Unio tumidus*

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Light is regarded as the strongest Zeitgeber for biological rhythms in organisms. Body of bivalves is usually light protected by their shell; furthermore, semi burrowing unionids live buried within the deposits, nevertheless they have the photoreceptors within their incurrent aperture. Photoreceptors of organisms drive the light information to the nervous system, e.g., by the serotoninergic nerves. Serotonin (5-HT) levels in haemolymph of the marine gastropod *Aplysia* are regulated by light-dark conditions. Little is known how unionoids transfer the light signals and have a light or clock-depending rhythmicity in serotonin levels.

Unio tumidus were kept *in situ* in lake littoral in natural photoperiod (LD) and constant darkness (DD) conditions in spring, summer, autumn and winter (12:12h, 16:8h, 12:12h and 8:16h light:darkness); every 4 hours haemolymph was isolated from 5 adults (or 10 in summer), tissues were immediately preserved in a fixative. The level of serotonin was assessed by the ELISA method; the distribution of the 5-HT in tissues - by immunohistochemistry. We observed an endogenously driven circadian rhythm of serotonin level in haemolymph in *U. tumidus;* nevertheless the influence of light on haemolymph 5-HT levels was visible. We observed lower levels of 5-HT during the light phase of the day, and higher, doubled levels in the darkness in autumn and summer. During summer we observed that the daily changes of serotonin level in haemolymph are sustained in DD, however the results from LD conditions show a strong influence of the long day light on *U. tumidus* physiology. During winter's long night we observed a continuous, generally higher level of the haemolymph serotonin in LD conditions and continuous low levels in DD. At spring we observed constant medium values of 5-HT in haemolymph in both LD and DD. No daily and seasonal changes in 5-HT levels in bivalve tissues were observed.

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Glia-related circadian modulation of neuronal circuits in the visual system of Diptera

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Glial cells that express the two core genes of the circadian clock, *period* (*per*) and *timeless* (*tim*), belong to peripheral circadian oscillators and decidedly contribute to circadian plasticity of Diptera visual system (Fig.). The epithelial glial cells (eGl) of the first optic neuropil (lamina) have been shown to display robust rhythmic changes in their volume and in the expression level of the catalytic subunit of sodium pump (Na^+/K^+-ATPase α subunit). Their modulatory input affects the circadian rhythm of abundance of the presynaptic protein Bruchpilot in photoreceptor terminals. At the same time they influence the rhythm of morphological changes of the most conspicuous postsynaptic partners of the photoreceptors, the L1 and L2 monopolar cells.

Our most recent studies revealed that glial cells of the second optic neuropil (medulla) of *Drosophila melanogaster* (dMnGl) (Fig.) that lie in the vicinity of the terminals of circadian clock ventral lateral neurons (LNvs) may play a particular role among glial oscillators. They possess receptors for the main circadian neurotransmitter, the neuropeptide Pigment-Dispersing Factor (PDF) released by LNvs, and they express PER at significantly higher level than other types of glia. Interestingly, expression of PER in dMnGl is still stronger by 50% in *Pdf*0 mutants. Such results indicate that when the pacemaker LNvs are silent (in PDF-free environment) the glial damped oscillators can increase the amplitude of PER oscillations to strengthen their circadian activity. Consequently, some form of PDF-based communication between the LNvs and dMnGl must take place.

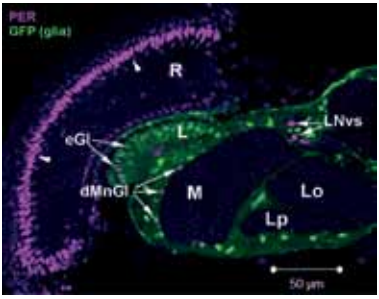

Figure: The optic lobe of *Drosophila melanogaster* showing targeted expression of Green Fluorescent Protein (GFP) to glial cells and immunolabeled with anti-PER serum. PER-positive nuclei (magenta) belong to the ventral lateral clock neurons (LNvs), the compound eye photoreceptors (arrow heads) and the glial oscillators, such as dMnGl and eGL. R-retina, L-lamina, M-medulla, Lo and Lp-parts of lobula.

Timing of eclosion behaviour in *Drosophila*

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Eclosion in the fruit fly *Drosophila melanogaster* is a classic example for a circadian-timed behaviour [1]. While eclosion motor behaviour itself is orchestrated via a peptidergic signalling cascade [2], pigment-dispersing factor (PDF)-positive clock neurons as well as a functional peripheral clock in the prothoracic gland are essential for the timing of eclosion to the early morning [3]. It is thus likely that some communication between circadian clock neurons, eclosion-related peptidergic cells and the prothoracic gland exists.

To identify underlying signaling pathways, we are combining neurogenetics, anatomy and live cell imaging. Our focus is on peptidergic neurons as these neurons can signal neuronally as well as hormonally. In this talk, we will report on the functional significance of the various so-far tested peptidergic neurons for the circadian control of eclosion.

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Circabidian rhythm in the large black chafer *Holotrichia parallela*

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Many organisms synchronize their own activities with an environmental cycle, such as a day, tide, month, and year, appearing on the earth. However, it has been reported that adults of the large black chafer *Holotrichia parallela* (Insecta, Coleoptera: Scarabaeidae) show 2-days periodicity in their occurrence on the ground and pheromone production[1, 2]. However, chronobiological study on this species has not been done yet. In this study, we examined their activity rhythm in the field and laboratory. In the riverbed of Yamatogawa River in Osaka (34° 35′ 14″ N, 135° 30′ 16″ E), collection by a pheromone trap from June to October (a season) revealed that the male beetles flew to the Chinese elm *Ulmus parvifolia* at sunset and away from it at sunrise. Their activity was strictly associated with sun light through the season. A mark and recapture experiment was made for 2 seasons at 2 trees of *U. parvifolia*. The total number of the marked beetles was 598, and the rates of recaptured beetles which were repeatedly captured on the tree more than 3 times in a season were 26.1% in the 1st and 20.2% in the 2nd season. Among these beetles, 68% appeared on even days counted from the marked day, that is, 2, 4, 6 days after the marking or later even days. On the tree they were eating leaves. Copulation was also observed sometimes (picture). In the laboratory, the beetles appeared on the ground during darkness every 2 LD (12 h-light and 12-h darkness) cycles, and under constant conditions the rhythm freeran with a period of 47.6±0.6 h (n=13). Phase responses of this rhythm to light pulses seemed to show that 2 repetitions of a phase response curve during a 'circabidian' period of about 48 h. These results suggest that *H. parallela* visit the trees every 2-nights for eating or sometimes copulation, and this periodicity is caused by an endogenous circabidian rhythm. It is probable that the circabidian rhythm is constituted by a circadian clock.

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Morph-specific daily rhythms in global gene expression and the juvenile hormone titer in the wing-polymorphic cricket, *Gryllus firmus*

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The existence and importance of circadian rhythms in organismal adaptation are poorly understood issues in evolutionary biology. We previously identified a dramatic morph-specific circadian rhythm for the blood concentration of the important insect hormone, juvenile hormone (JH) during adulthood, in both laboratory and field populations of the wing polymorphic cricket, *Gryllus firmus*. The flight-capable morph (LW, long-winged) has fully developed wings and flight muscles, and exhibits a dramatic JH titer circadian rhythm during adulthood. By contrast, the flightless morph (SW, short-winged) has reduced and non-functional wings and flight muscles and a minimally-cyclic JH titer. The robust JH rhythm in the LW morph likely regulates aspects of nocturnal flight. In the present study we investigated the degree to which morphs differ in daily rhythms of global gene expression in the fat body that parallel the morph-specific rhythm in the blood JH titer. A significantly greater number of genes differed in expression (RNA-Seq; $P < 0.005$) in the LW morph than in the SW morph between the early and late portions of the photophase, which paralleled the greater change in the JH titer in the LW morph during that time. A subset (9) of these genes exhibited significantly greater daily rhythms in the LW morph, compared with the SW morph, over a two day period of adulthood as quantified by qPCR. The degree to which morph-specific change in gene expression is regulated by JH has yet to be determined. The number of genes exhibiting diel change in gene expression in the LW morph was significantly greater than the number differing in gene expression between the LW and SW morphs at the same time of day. Morph-specific diel change in global gene expression is a prominent but previously unidentified feature of morph adaption in *G. firmus* and possibly other ecologically-important polymorphisms.

An approach to the photoperiodic time-measurement mechanism in the cricket, *Modicogryllus siamensis*

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Photoperiodic regulation of development is widely observed in insects occurring in temperate zones. Nymphs of the cricket *Modicogryllus siamensis* show clear photoperiodic responses in their post-embryonic development. They become adult in 50~60 days after hatching in long-day conditions, while they need more than 100 days until half of them become adult in short-day conditions. Results of night interruption suggest that photo-inducible phase locates during the late night. The circadian clock is apparently involved in the photoperiodic time-measurement mechanism because disruption of the circadian clock by RNA interference of *period* gene prevented the responses to both long-days and short-days. We are thus approaching to the photoperiodic time-measurement mechanism by investigating the circadian clock and the photoreceptors. Our results have shown that opsin genes expressed in the compound eye are involved since their RNAi prevents the long-day responses. We recently found that *cryptochrome* genes (*cry1*, *cry2*) play an important role in the photoperiodic response. Their mRNA expression profiles are different between long-day and short-day conditions, and treatments with their dsRNA disrupted the long-day responses and changed the expression profile of clock genes. We then examined the effects of light given at the putative photo-inducible phase on the photoperiodic responses in crickets treated with *cry1* or *cry2* dsRNAs. The effects varied depending on the wavelengths of light and on the treated gene. Based on the results, we will discuss the role of photoreceptors and *cry*s in the cricket's photoperiodic time-measurement mechanism.

Characterization and distribution of allatostatic factors in the brown-winged green bug *Plautia stali*

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In the temperate regions, many insects anticipate seasonal changes by photoperiod and enter diapause during unfavourable seasons. Gonadal development is inhibited in insects which enter diapause at the adult stage. Juvenile hormones (JH) biosynthesized by the corpus allatum (CA) is known as a key hormone controlling gonadal development in many insects. However, the neuronal mechanisms underlying photoperiodic regulation of JH biosynthesis have not been revealed. In the brown-winged green bug *Plautia stali*, JH biosynthetic activities are clearly suppressed by short-day conditions to inhibit ovarian development, and JH biosynthetic activities are inhibited by the mid brain (mBr) which contains the pars lateralis (PL) and pars intercerebralis (PI) neurons innervating the corpus cardiacum (CC)-CA complex (Figure) *in vitro* (1). In the present study, as the first step to investigate the neuronal mechanism underlying photoperiodic control of JH biosynthesis, we characterized allatostatic molecules in the mBr of *P. stali*. Allatostatins (Dippu-AST2, 8 and 9), octopamine, dopamine and serotonin showed no significant effects on JH biosynthesis. The methanol extracts of the mBr (2 brain equivalents) showed clear inhibitory effects on JH biosynthetic activities. The inhibitory effects by the mBr were heat resistible but significantly reduced by trypsin-treatment. Next, the cephalic ganglia containing the brain and subesophageal ganglion (SEG) were divided into small regions and their methanol extracts were examined. Inhibitory effects were found in any part of the cephalic ganglia, and particularly brain regions containing the PL showed strong inhibitory effects. These results suggest that allatostatic molecules in *P. stali* are peptides containing lysine- or arginine-residues, and have also other functions than inhibition of the CA in the nervous system.

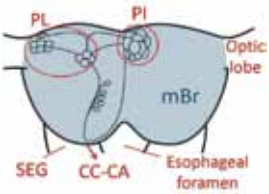

Figure: Schematic illustration of the mBr which include PI and PL neurons projecting to the CC-CA of *P. stali*. The shaded area indicates the mBr.

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Effects of the pars intercerebralis-removal on the circatidal rhythm in the mangrove cricket *Apteronemobius asahinai*

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The neuronal mechanisms underlying the circadian clock have been studied well, whereas less attention has been paid to the mechanisms of other biological clocks. The mangrove cricket *Apteronemobius asahinai* possesses circatidal and circadian clocks, and simultaneously shows a circatidal rhythm, alternation of active and inactive phases, and a circadian rhythm, modification of activity levels by suppressing activities during subjective days. In our previous study, surgical removal of the optic lobe, the principal circadian clock locus in crickets, disrupted only the circadian rhythm but not the circatidal rhythm[1]. This indicates that the principal circatidal clock is located in a region(s) different from the optic lobe. In the present study, we focused on the pars intercerebralis (PI), a neurosecretory center of the insect brain, because it was shown to be another circadian clock locus in the house cricket *Acheta domestica*[2]. We performed surgical removal of the PI and observed its effects on the locomotor activity rhythms. After the surgery, 3 of 12 crickets, which showed a circatidal rhythm before surgery, maintained a circatidal rhythm, and 7 became arrhythmic. The other 2 crickets showed a rhythm with a single active phase in a day, which seems to be a circadian rhythm. In addition, removal of the most dorsal perikarya excluding the PI, medio-dorsal perikarya including the PI, or bilateral neuropil regions lateral to the PI, also caused similar effects on the activity rhythms like removal of the PI: activity patterns varied among the crickets after the surgery. These results suggest that the PI and regions around the PI are important for the circatidal rhythm. The circatidal clock itself and/or its output pathway may reside in these regions in *A. asahinai*.

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How clock and environment influence eclosion rhythms of *Drosophila* under natural conditions

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The circadian timing of behaviour allows animals to adapt to environmental conditions and is thought to increase their fitness. A classical model for a circadian-timed behaviour is the eclosion (adult emergence) of the fruit fly. *Drosophila* eclosion is gated to the morning hours, which is commonly assumed to be an adaptation to prevent water loss and thus optimising fitness.

Laboratory studies have shown that the *Drosophila* eclosion rhythm can be entrained to different Zeitgebers like light or temperature and depends on a functional molecular clock and PDF signalling. Very little, however, is known about eclosion rhythms in nature. A major question here is whether and to what extent the circadian clock and abiotic Zeitgebers contribute to ensure that flies eclose at the right time of the day.

We started to monitor the eclosion of wildtype and mutant *Drosophila* under natural conditions using a newly developed camera-based eclosion monitoring system called WEclMon (Würzburg eclosion monitor). A statistical model allows to dissect the role of the circadian clock and abiotic factors on eclosion rhythm and a possible hierarchy of the different Zeitgebers. We present data from the first field seasons which already suggest that the relative importance of the clock and the various Zeitgebers differs between genotypes. Moreover, and unlike in the laboratory, eclosion timing seems to be influenced by multiple interdependent factors.

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Is the biological clock engaged in fat body growth and metabolism regulation during larval development of the Egyptian cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae)?

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Numerous biological processes display daily rhythmic fluctuations that are regulated by circadian clocks. Circadian rhythms are entrained by environmental stimuli, mainly light/dark and temperature cycles, but also free-run in the absence of such stimuli with a 24h period. Circadian clocks coordinate the timing of physiological and developmental processes and synchronize their schedule to the appropriate time of the day.

Most research on circadian rhythms of physiological processes in Lepidoptera is carried out on adults. In this study we performed a detailed investigation of fat body ontogenesis during larval development of the cotton leafworm *Spodoptera littoralis*. We focused on the role of the biological clock in the regulation of growth rhythm and development of the fat body. We analysed rhythm of cells proliferation in the larval fat body *in vivo*. To confirm independence of this process from the central clock located in the insect brain we examined the fat body cell proliferation rhythm *in vitro*.

We found that larvae of the last instar show daily fluctuations in body weight, with a significant increase during the night. Our data show that these changes are strongly correlated with daily fluctuations of the fat body cells' proliferation and rate of metabolism, characterized by labeling with 5-bromo-2'deoxyuridine and measurement of oxygen consumption, respectively. Statistically significant increase of proliferating cells number in ZT 12 as well as circadian changes of *period* mRNA level in this tissue cultured *in vitro* indicate the presence of independent functional peripheral clock in the larval fat body.

Obtained results indicate that the biological clock is engaged in the fat body growth and metabolism in the last instar larvae of *S. littoralis*.

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Molecular Level Approaches to Circadian Rhythm and Photoperiodism in Vertebrates

Organizers (*Japanese Society for CPB*):

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The zebrafish circadian clock: Its regulation of the cell cycle in cell culture and during embryo development

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The zebrafish circadian system is highly decentralized, with cells and tissues in the body not only containing an endogenous circadian pacemaker, but also being directly light responsive. This fact is also true in zebrafish cell lines, and also from the earliest stages of embryo development. Consequently, the circadian clock in the embryo and in vitro can be directly set simply by changing the lighting conditions in the incubator. A such zebrafish cell lines represent a value tool for the study of clock-regulated downstream or output processes at the cellular level, as no pharmacological manipulations are required to establish pacemaker synchrony in the population.

We have taken advantage of the fact that zebrafish cells contain a complete circadian system to study the regulation of cell cycle timing in vitro. In this presentation, we will describe the role played by *p21* rhythmicity in regulating the timing of S-phase entry across the circadian cycle. In addition, we will explore the idea of how S-phase is regulated to occur at different times of the day in specific tissues in zebrafish larvae, by the use of novel cell cycle regulators and specific promoter changes.

The clock mechanism begins to “work” in zebrafish larvae from the end of the first day of development, with embryos being light responsive from the moment that zygotic transcription begins. We will explore some of the downstream biology that is both light and clock regulated in early embryonic stages, including a discussion of cell cycle regulation in development. Approaches to monitor cell cycle progression dynamically in live cells and embryos will be discussed. We have employed the fluorescence ubiquitination cell cycle indicator (FUCCI) imaging of zebrafish cell lines over several cycles to explore entrainment of the cell cycle to differing day lengths (T-cycles) and cell densities. The results reveal interesting differences between clock-cell regulation at the single cell versus population level.

Cellular differentiation and circadian clock development in mammals

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Circadian clock is an intrinsic time-keeping system that regulates essential physiological functional rhythms. In the mammals, the central circadian pacemaker resides in the suprachiasmatic nucleus (SCN) of the hypothalamus, coordinating cell-autonomous molecular oscillators throughout the body to perform tissue-specific functions. The basis of molecular oscillator consists of transcriptional/translational feedback loops (TTFL) of clock genes. However, the circadian molecular oscillator does not cycle in early embryos. We have revealed that pluripotent ES cells do not display discernible circadian molecular oscillations, and the emergence of mammalian circadian molecular rhythms occurs during cellular differentiation process. The mechanisms emerging molecular oscillator as well as suppressing the circadian clock in pluripotent stem cells should promote our understanding of the cellular circadian clock system in mammals.

TRPA1 transcription is altered in the absence of TRPV1: Clock molecular machinery regulation in *Mus musculus* liver

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Daily cycles of light and of temperature are major environmental signals for organisms. Recently the interaction of opsins with TRP channels was demonstrated in photo- as well as thermo-responses (Shen, WL Science 331, 1333, 2011). We hypothesized that temperature cycles signal mammalian peripheral clocks via TRP channel activation, and that this signal synchronizes peripheral clocks. We investigated the function of TRPV1 channels by assessing clock genes expression in the liver of wild type (WT) and *TrpV1* knockout (*TrpV1*$^{-/-}$) mice in constant darkness (DD) or light-dark cycles (LD). *TrpV1*$^{-/-}$ mice displayed normal locomotor activity and body temperature, suggesting that light information from the retina to the central clock (SCN), and its processing in the suprachiasmatic nuclei are not altered in the absence of TRPV1 channel. In the liver, *Per1* and *Clock* transcripts showed temporal variation in DD, *Per2* oscillated in LD, and *Bmal1* oscillated in both conditions. No differences were observed in clock gene expression in the liver of *TrpV1*$^{-/-}$ as compared to WT animals in both LD and DD. In WT animals, liver *TrpV1* oscillated along 24 hours, peaking in the scotophase, and *TrpA1* showed constant temporal expression; in *TrpV1*$^{-/-}$ animals, *TrpA1* oscillated with a peak in the photo-phase. The differential expression of *TrpA1* in *TrpV1*$^{-/-}$ and WT mice suggests a compensatory expression of TRPA1 channel in the absence of TRPV1, and a putative role for these channels in the regulation of liver clock machinery.

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Circadian clock-regulated physiologies in mouse SCN/hippocampus and chicken pineal gland

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The periods of the circadian oscillators are generally diverged from 24 hours, while they can be adjusted (entrained) to exact 24-hr period by receiving the ambient signals such as day-night variation of light condition on earth. For the light entrainment, the circadian clock is phase-delayed or -advanced by a light pulse given at early or late night, respectively. The time-of-day-dependent phase-response to light is an important property common to all the circadian clocks, but the underlying molecular mechanism is still poorly understood. This is because the light signals are often transduced through multiple layers of the retinal signal transduction process including the synaptic transmission before being sent to the oscillatory machinery, making the molecular analysis of the output pathway difficult.

Among the vertebrate clock tissues, the chicken pineal gland is unique in that it retains intrinsic phototransduction pathways for entrainment of the intracellular clockwork. Therefore, the chicken pinealocytes appeared to provide a prominent platform for studies on the light-entrainment mechanism. Our challenge to comprehensive analysis of the chick pineal light-inducible genes revealed phase-dependent activation of transcription factors HSF1, HSF2, XBP1 and E4BP4, a series of regulators essential for clock gene expression. Roles of E4BP4 in the clockwork were further investigated in the mammalian circadian clock system. The molecular studies on the photic and non-photic input pathways to the central and peripheral clocks should reveal an important input of E4BP4-mediated signaling to the entrainment.

Genetic and epigenetic mechanisms of circadian plasticity

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The mechanism of the human circadian clock is present in nearly all cells of the body and highly conserved with other metazoans. Nevertheless, daily timing in humans is highly variable, ranging from “larks” with early phases of activity to “owls” with much later habits. Our laboratory has applied a variety of molecular methods to understanding these differences, ranging from genomic quantitative trait mapping in primary human cells, to metabolomics using human serum, and brain epigenomic studies in murine models. Overall, we have uncovered a wide variety of mechanisms, each of which contributes either statically or dynamically to “circadian plasticity”, an ability to modify biological clock mechanisms to suit environmental needs. From mice to humans, we show that the same mechanisms are operative in setting clock time: genes in which human polymorphisms affect circadian phase are also important to circadian mechanism in mice, and genes identified by global RNAi screens as “clock genes” in mice are also enriched in human genetic studies of clock function.

Understanding the mechanism of vertebrate photoperiodism by comparative approach

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Animals living in temperate zone use changes in day length to adapt to seasonal changes in environment, but mechanisms underlying seasonal (photoperiodic) time measurement are not fully understood. Japanese quail is an excellent model for the study of these mechanisms because of its rapid and dramatic response to changes in photoperiod. We have demonstrated that local thyroid hormone catabolism within the mediobasal hypothalamus (MBH) by thyroid hormone-activating enzyme (type 2 deiodinase: DIO2) regulates photoperiodism. Functional genomics analysis in quail demonstrated that long day stimulus induces thyrotropin (thyroid stimulating hormone: TSH) production in the pars tuberalis (PT) of the pituitary gland, which triggers *DIO2* expression in the ependymal cells of the MBH. In mammals, nocturnal melatonin secretion provides an endocrine signal of the photoperiod to the PT that contains melatonin receptors in high density. We have also demonstrated the involvement of TSH signaling pathway in mammals by using the TSH receptor null mice. Well known function of TSH derived from pars distalis (PD) of the pituitary gland is stimulation of thyroid gland. However, the mechanisms by which PT- and PD-TSH exert distinct functions within the body remained mystery. We found TSHs from two anatomical sources undergo different glycosylation and this tissue-specific glycosylation imparts different functions on a single hormone.

Effects of orally melatonin and long term exposure to light on serum indices and gene expression of insulin and glucagon in male Wistar rats

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Despite efforts to determine effects of melatonin on blood glucose, no clear and conclusive answer has been represented so far. To clarify this query, we investigated influence of endogenous and exogenous melatonin on genetic and serologic aspects of secretory function of pancreas in rats. Thirty adult Wistar rats were divided into 6 groups: Control, Melatonin, Light, Light and melatonin, Dark, Dark and melatonin. To achieve variable levels of endogenous melatonin, 10-day long-term exposure to light and darkness was implemented. Exogenous, melatonin was administered orally after a period of 10 days to groups 2, 4 and 6 (10 mg/kg of body weight). Blood glucose and serum levels of insulin, glucagon, and melatonin were measured by ELISA. Gene expression levels of insulin and glucagon was determined by using the Real Time PCR. Results showed an increase of blood glucose and also a decrease in serum levels of insulin after administration of melatonin without any significant difference in serum levels of glucagon. Gene expression levels of insulin in melatonin group were significantly lower than control group while their glucagon was more. We concluded that oral administration of melatonin leads to increasing blood glucose, due to inhibition of insulin and stimulation of glucagon synthesis.

Table 1: Blood glucose (mg/dl) and serum levels of insulin (IU/ml) and glucagon (ng/l) in different groups (Mean ± SEM)

Groups	Blood glucose	Insulin	Glucagon
Control	165.80 ± 14.85	4.32 ± 0.13	0.90 ± 0.13
Melatonin	213.20 ± 15.70*	2.32 ± 0.23*	0.88 ± 0.04
Light	160.20 ± 7.72	2.07 ± 0.35	0.90 ± 0.06
Light + Melatonin	196.40 ± 9.98*	2.03 ± 0.23*	0.90 ± 0.04
Dark	173.60 ± 13.80	2.02 ± 0.18	1.28 ± 0.38
Dark + Melatonin	214.00 ± 11.00*	1.72 ± 0.13*	0.82 ± 0.02

*vs Control $p<0.05$

Phase shifting the seasonal rhythmicity via melatonin in mammals originating from different geographic regions

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Photoperiodic response, varied with latitude of animal's origin or habitat, is a central component of fitness in temperate and polar environments. Seasonally breeding mammals use the annual change in the photoperiod cycle to drive rhythmic nocturnal melatonin signals from the pineal gland, providing a critical cue to time seasonal reproduction. One of the ways of phase shifting the rhythmicity is the treatment mammals with exogenous melatonin. The aim of this study was to investigate the effects of exogenous melatonin (daily supplied with water or subcutaneous implanted) on the tissue antioxidant capacity (TAC), and morphofunctional parameters of leucocytes in mammals. We used rodents (Wistar rats), that are acutely responsive to circadian phase, and some farmed carnivorous species (American mink, raccoon dog, red fox, blue (arctic) fox), that are seasonal mammals with different geographic origin.

We observed several responses to phase shifting the seasonal rhythmicity in long-lived seasonal mammals. In addition to differences in basal TAC and in morphofunctional parameters of leucocytes, various susceptibilities of mammals to exogenous melatonin can be explained by differences in their ecology and physiology. Raccoon dog, originating from East Asia, and experiencing the winter dormancy, is more responsive to melatonin than other mammals. In rats melatonin had no effect on TAC but caused the retardation of decrease in the number of lymphocytes with advancing age. Pineal melatonin acts as hormone encoding photoperiod information and orchestrating the seasonal changes in body functions. So photoperiodism cannot be disregarded when evaluating the mechanisms underlying life-historical events in any animal living at temperate and polar latitudes.

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Effects of interaction between leptin and prolactin on SOCS-3 mRNA expression in ovine pituitary

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Leptin and prolactin (PRL) represent a wide range of functions. Both hormones are involved in the processes of reproduction and regulation of the body's energy stores and their secretion change seasonally. It is believed that SOCS-3 protein, known as a suppressor of cytokine signaling, is a pivotal negative regulator of signal transduction of the mentioned hormones. The aim of this study was to investigate the effect of the interaction between leptin and PRL on the expression of SOCS-3 mRNA in the pituitary gland of sheep. We used pituitaries isolated from 10 ewes decapitated in May (LD; n=5) and November (SD; n=5). Tissues were cut into approx. 50 mg explants, which were treated with: 1) control medium or medium containing: 2) leptin (0.1 µg/ml), 3) PRL (0.1 µg/ml) or 4) leptin and PRL (0.1 µg/ml each). Incubation was maintained for different time intervals: 0, 30, 60 or 120 minutes. The level of expression of SOCS-3 mRNA was analyzed by Real-Time PCR. Results of this experiment confirmed that leptin and PRL are involved in regulation of SOCS-3 mRNA expression in ovine pituitary. During LD, leptin highly significantly ($P < 0.001$) reduced the transcription of SOCS-3, while during SD its showed a trend to enhancing the expression of this suppressors. PRL during both periods significantly reduced level of SOCS-3 mRNA in the pituitary, and this effect was highly significant in LD ($P < 0.001$) and significant in SD ($P < 0.05$). In explants subjected to simultaneous action of both hormones during LD, it was found that the expression of SOCS-3 was significantly lower ($P < 0.001$) than in control explants, significantly lower ($P < 0.05$) than in explants treated with leptin alone and comparable to the expression observed in the tissues incubated only with PRL. In SD, there were no significant differences between the level of transcripts in explants treated with the combination of leptin and PRL compared to other experimental groups. It can be presumed that the full understanding of the mechanisms of interactions between cytokines and SOCS-3 will allow in the future to use this knowledge in the study of physiological and pathological conditions both in humans and animals.

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Dopamine produces multiple effects on phototransduction cascade in frog rod photoreceptors

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In vertebrate retina, dopamine and melatonin forms a reciprocal pair of neuromediators which controls many key processes including conductivity of gap junctions and retinomotor effect. According to traditional scheme, an effect of dopamine on various photoreceptor functions is mediated by activation of dopamine D2-receptors and the following decreases of $[cAMP]_{in}$ in photoreceptor cell. Earlier, we have demonstrated that increase of $[cAMP]_{in}$ evoked by forskolin, caused multiple effect on phototransduction cascade and leads to elevation of the photoreceptor sensitivity (Astakhova *et al.*, 2012). Here we show that the effect of dopamine and quinpirole on the rod photoreceptors of frog *Rana ridibunda retina*, might only partly be explained by variation of $[cAMP]_{in}$. Thus, dopamine (but not quinpirole) decreases the sensitivity of phototransduction cascade. On the other hand, both dopamine and quinpirole slows down the activation of the cascade. The most significant difference between forskolin and dopamine action is an effect on so-called critical time constant of the cascade (τ_D). While forskolin produces a moderate increase of τ_D, both dopamine and quinpirole sufficiently slows down τ_D by factor 2.5. Thus, we conclude that the effect of dopamine on phototransduction cascade cannot be explained solely by $[cAMP]_{in}$ variation, and some other signaling pathways might be involved as well. The difference in reaction of the phototransduction cascade to dopamine and quinpirole also suggests, that dopamine might bind some others than D2-receptors.

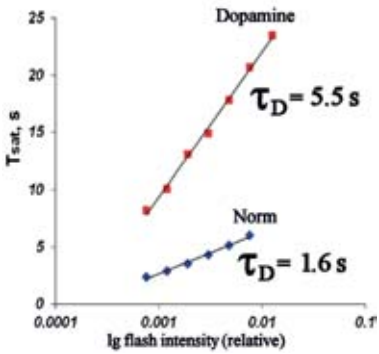

Figure: A length of stay in saturation (T_{sat}) *vs.* relative intensity of stimulation flash (s.c. Pepperberg plot). A linear approximation of curves in semilogarithmic plot gives a critical time constant, τ_D.

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Seasonal changes in liver weight and size of hepatocytes in bank voles are accompanied by changes in glycogen and fat content

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Bank voles are small, winter active rodents, which undergo regression of body and organ weight before winter, and in spring they resume the growth. Mechanism of seasonal changes in mass of internal organs still remains unclear. The present research focuses on examination of the seasonal plasticity of liver weight. We analysed livers from wild bank voles caught in different seasons of the year. The laboratory experiment was also performed to determine the role of photoperiod in the seasonal adjustment of liver property. The bank voles were held from birth under long photoperiod (LP) for 12 weeks and next half of them was transferred to short photoperiod (SP). In both parts of experiment we analysed mainly changes in size and morphological structure of hepatocytes. Using PAS-staining method we also examined glycogen deposits in the liver. We found significant differences in the liver weight between seasons, the livers of animals from summer were on average 54% bigger compared with those from winter. We also found a strong positive correlation between liver weight and hepatocyte size. Hepatocytes in livers of animals from summer were on average 64% bigger and showed significantly increased deposition of glycogen compared with those from winter. On the contrary, in vole hepatocytes from winter, deposits of glycogen were very rare but numerous lipid droplets were visible. The laboratory experiment revealed that both size of liver cells and the amount of deposition of glycogen is determined mainly by photoperiod. Switching animals to SP induced both depletion of liver mass, decrease in hepatocyte size and decrease of glycogen content in hepatocytes. The data indicate that seasonal changes in hepatocyte size and liver weight in the bank vole may be associated with changes in properties of liver, adjusting metabolism of this organ to environmental alteration. Simultaneously, decrease in food intake of animals under short photoperiod suggests that nutrient availability may be an important factor determining seasonal changes in size of hepatocytes and liver weight.

Biological Rhythms in Non-mammalian Vertebrates

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The circadian system in reptilian species - what do we know?

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Majority of biochemical physiological, and behavioral functions of animals exhibit rhythmical fluctuations, being a result of specific adaptations to the cyclic variations of the environment. The variations of photoperiod and temperature play the major role in the synchronization of daily and annual rhythms, driven by an endogenous oscillator. In reptilian species the central oscillator of the circadian clock regulating behavior and physiology is located in multiple tissues, such as the retina, pineal gland, parietal eye and in the suprachiasmatic nuclei (SCN) located in hypothalamus. The pineal body develops as an evagination of the roof of the diencephalon, whereas the parietal eye arises either as an evagination of the pineal body or as a separate diverticulum of the diencephalon. All these structures are anatomically and functionally associated with the photoreception. In reptilian species, the pineal gland plays the central role in the regulation of circadian rhythmicity and is involved in the generation and control of different circadian rhythms, such as locomotor activity, body temperature, behavioral thermoregulation, and electroretinographic responses. Some findings have indicated the that the SCN of lizards are homologous to the SCN of mammals, because are topographically similar to the SCN of mammalian species, and receive a direct retinal projection. Moreover, as in some mammals the SCN of lizard lies just dorsal to the optic chiasm and adjacent to the third ventricle, in the region of transition from the preoptic area to the hypothalamus and show to bind antibodies raised against neuropeptide Y.

Biological clock evolution in the darkness: The cavefish model

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Circadian rhythms of behavioral and physiological processes are driven by endogenous oscillators synchronized to environmental cycles. They represent an adaptive advantage that allows organisms to predict and anticipate cyclic environmental changes. The daily cycles of light-dark and the food availability are the most prevailing signals for the entrainment of circadian oscillators. However, the physiological mechanisms whereby light and food regulate the clock remain incompletely understood. To shed light on the evolution of the biological clock in vertebrates we have investigated at molecular and behavioural levels the circadian clock of cavefish. Cavefish have evolved in extreme hypogean environments characterized to constant darkness, stable temperature, and low nutrient levels. They show a convergent evolution, sharing a range of striking troglomorphic phenotypes such as anophthalmia, depigmentation, and reduced metabolic rate. We have performed a deep analysis of the circadian clock of cavefish investigating the effect of different feeding and lighting conditions on the behavioural rhythmicity and the clock gene expression. To do this we studied the Somalian cavefish *Phreatichthys andruzzii* in comparison with the zebrafish *Danio rerio*, that is normally exposed a cyclic environment. Previous investigation showed a strong feeding entrainment of locomotor activity for both species. Here, using different paradigms of periodic food availability, we show that food-entrainable oscillators are more strong in cavefish respect to zebrafish. Furthermore, in parallel, we investigated photic entrainment of locomotor activity in adult and larvae of fish exposed to different monochromatic light. Interestingly, while zebrafish showed a normal entrainment under all light-dark cycles tested, *P.andruzzii* changed the behavioral pattern (e.g. arrhythmicity, negative phototaxis) in response to the light stimulus. In order to explain at the molecular level these results, we examined the expression pattern of a set of opsin and clock gene homologs. Both behavioral and molecular characterizations can be the basis to understand these complex biological processes and also demonstrate the great utility of cavefish to study the evolution of the circadian clock.

Non-photic entrainment in fish

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The organisms that live in the Earth are subjected to geophysical variables that display cyclic variations. Most of these cyclic changes in environmental variables such as light, temperature and tides are generated by the movements of the Earth, Moon and Sun relative to each other. As these cyclic changes in the environment are constant and predictable, they have affected the biological evolution, selecting the occurrence of biological rhythms in the physiology of all living organisms, from prokaryotes to mammals. These biological rhythms confer the organisms with an adaptive advantage, as they can synchronize their physiological processes to occur in a moment of time when the effectiveness and the success would be higher and/or the cost and risk for the organisms would be lower. Among the environmental factors, or synchronisers, the most studied so far has been the light, which is the main synchroniser in mammals. However, other environmental variables seem to play also an important role on biological rhythms, especially in aquatic animals such as fish. In this presentation, we will focus on the current knowledge of the role and effects of non-photic synchronisers (temperature, food and tidal cycles) on the circadian system and biological rhythms in fish.

Crosstalk between the pineal circadian oscillator, melatonin and inflammation in the chicken exposed to the different photoperiodic conditions

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Majority of physiological processes, including immune defense, undergoes the diurnal and seasonal changes synchronized by the circadian clock, therefore the reciprocal interrelationships between the clock and clock-controlled processes are examined extensively. Avian master clock is composed of the three autonomous components comprising avian equivalent of mammalian SCN, photosensitive pineal gland and a retina. We examined the role of the pineal clock in the chicken immunity on the model non-specific immune function, i.e. experimental peritonitis, and demonstrated its season-related development. Diurnal profiles of the pineal clock gene transcription appeared to be independent of the light phase duration, being also not abolished by continuous illumination (LL conditions), whereas the influence of exogenous melatonin was dependent on the duration of its availability. Rhythmic expression of *Bmal1* appeared to provide a direct link between the circadian clock and the melatonin output pathway, while melatonin availability was involved in the canonical transcription pattern of *Per3* in the chicken pineal gland. Melatonin appeared not a sole factor responsible for the time of day- and season-related development of inflammation as there were some more (like pro-inflammatory cytokine *IL-6* and *IL-18* gene transcription) and less (immune cell circulation or acute phase protein, represented by the serum lysozyme concentration) related with the presence of melatonin. Circulating mediators of peritonitis are most probably recognized by the pineal oscillator where the core clock genes transcription was affected by inflammation. Additionally, the effect of the seasonally elicited inflammation on selected clock gene transcription evaluated in the thymus, a primary immune gland, demonstrated that transcription factor E4bp4 should be considered as a molecular link between biological clock and chicken immune system in the synchronization of inflammation with the season-related lighting conditions.

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Ectotherm and endotherm peripheral clocks: a machinery for light and temperature reception based on opsins and TRP channels?

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The interaction of opsins with transient receptor potential (TRP) channels has been demonstrated in the photo-responses of the mammalian intrinsically photosensitive retinal ganglion cells (ipRGC) and human melanocytes. Both light and UVA radiation activate melanopsin in ipRGC and rhodopsin in melanocytes, respectively, leading to the activation of phospholipase C (PLC), and the ultimate opening of TRPC6/C7 in ipRGC and TRPA1 in human melanocytes. Recently, the involvement of TRPA1 and rhodopsin was demonstrated in thermo-responses of *Drosophila*, which can be rescued by mouse melanopsin in rhodopsin mutated larvae. In *Danio rerio* and mammalian cell lines and organs, temperature has been reported to entrain clock gene expression. Nevertheless, the role of opsins and TRP channels as thermosensors, and their participation in clock gene modulation in *Danio rerio* embryonic ZEM-2S cells and murine melanocytes Melan-A, is still unknown. Based on that, we investigated the presence of TRP channels mRNA and of melanopsin and rhodopsin mRNAs and proteins in ZEM-2S and Melan-A cells. mRNA of *trpA1a, trpA1b, trpC6, trpM2, trpM4a, trpM4b/c, trpM5, trpV1*, and *trpV4* was detected in ZEM-2S cells, and of *TrpV1, TrpC7* and *TrpM8*, but not *TrpA1* and *TrpC6*, in Melan-A cells. mRNA and protein levels of melanopsins and rhodopsin were found in both cell lines. It has been established that ZEM-2S cells respond to blue light pulse with increased clock gene expression but the effect of temperature is still poorly understood; on the contrary, white light pulse does not affect clock gene expression in Melan-A cells. Bearing in mind that ZEM-2S and Melan-A cells possess the machinery capable of transducing light- and thermo-responses, our next step will be to investigate the effect of temperature on clock gene expression in both cell lines.

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Note: L.V.M. de Assis and R Jerônimo have the same contribution

Timezyme, arylalkylamine N-acetyltransferase: activity and coding genes expression in the three-spined stickleback

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Arylalkylamine N-acetyltransferase (AA-NAT) that catalyzes the conversion of serotonin to N-acetylserotonin is considered as the "rate-limiting enzyme" in biosynthesis of melatonin (Mel), hormone which plays an essential role in timekeeping in vertebrates. Rhythmic *aanat* expression, AA-NAT activity, and consequently Mel production, are driven by an internal circadian clock and external light signals. In fishes, Mel is rhythmically produced in photoreceptive pineal gland and retina. A single AA-NAT coding gene has been found in mammalian, avian, and anuran genomes. Teleost fish have two *aanats*, *aanat1* and *aanat2*, the former is expressed only in the retina and the latter in both the retina and the pineal gland. In the three-spined stickleback (*Gasterosteus aculeatus*), there are three AA-NAT coding genes, *aanat1a*, *aanat1b* and *aanat2*, which sequences are available in Ensembl genome browser. In this study, we examined genes expression in sticklebacks' brain, retina, skin, stomach, gut and kidney. Real-time PCR analysis revealed that *aanat1a*, *aanat1b* are almost exclusively expressed in retina where *aanat1a* predominates over *aanat1b*. Expression of *aanat 2* is about three times higher in brain than that in retina. In parallel, AA-NAT activity was tested in the same tissues. This study demonstrated the enzyme activity in all tissues that was consistent with an expression of the corresponding genes. Kinetic analysis suggested the different affinity of AA-NAT for serotonin in various tissues.

Daily rhythms of expression of genes involved in sex differentiation in zebrafish

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In fish sex differentiation is driven by sexual steroids, which production depends on environmental cycles (such as light and temperature) and a biological clock. Surprisingly, rhythms in the endocrine mechanisms responsible for sex ratio control remains unexplored. Here we investigated the daily rhythmic expression of genes involved in the production of sex steroids and ultimately male/female ratio in zebrafish maintained under a 12:12 h light-dark cycle at constant 27°C. We analyse the expression level in gonads and brain of female and male individuals. In gonads, the expression of aromatase (*cyp19a*, ovary) and antimullerian hormone (*amh,* testis) was rhythmic with opposite acrophases: ZT 5:13h (in the day) for cyp19a and ZT 15:39 (at night) for *amh*. In brain, the expression of *dmrt1* was also rhythmic in both sexes, in female the acrophase being ZT 6:43, in phase with ovarian aromatase expression. *Foxl2* presented a peak of expression in both sexes during the last hours of the night. Finally cyp19 b (neuronal aromatase) and cyp11b presented daily differences, especially in male where the gene expression peaked during the night. These results provide the first evidence for marked time-dependent differences in the expression of genes involved in the production of sex steroids and sex differentiation in fish.

The Evolution of Endothermy

Organizers:

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Roberto Nespolo, *Universidad Austral de Chile, Chile*
Paweł Koteja, *Jagiellonian University, Poland*

Testing the aerobic capacity model using quantitative genetics in present-day species

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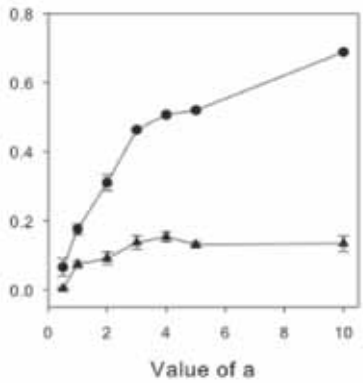
The evolution of endothermy is one of the most puzzling events in vertebrate evolution, for which several hypotheses have been proposed. The most accepted is the aerobic model, which assumes the existence of a genetic correlation between resting metabolic rate (RMR) and maximum aerobic capacity (whose standard measure is maximum metabolic rate, MMR). This model posits that directional selection acted on maximum aerobic capacity and resting metabolic rate increased as a correlated response, in turn increasing body temperature. To test this hypothesis we implemented a simple two-trait quantitative genetic model in which RMR and MMR are initially independent of each other and subject to stabilizing selection to two separate optima. We show that mutations that arise which affect both traits can lead to the evolution of a genetic correlation between the traits without any significant shifting of the two trait means. Thus the presence of a genetic correlation between RMR and MMR in living animals provides no support in and of itself for the past elevation of metabolic rate via selection on aerobic capacity. This result calls into question the testability of the hypothesis that RMR increased as a correlated response to directional selection on MMR, in turn increasing body temperature, using quantitative genetics. We think other strategies can be followed to test the aerobic model in the phylogeny of amniotes.

Figure: genetic correlation (circles; triangles represent phenotypic correlations) as a function of allelic value (a), after 100.000 generations of simulated evolution of two traits. A large (about 0.8) genetic correlation appears just as response to mutation rate (there was no functional association between the traits).



Relationship between selection for high aerobic metabolism and reproductive traits in bank voles *Myodes glareolus*

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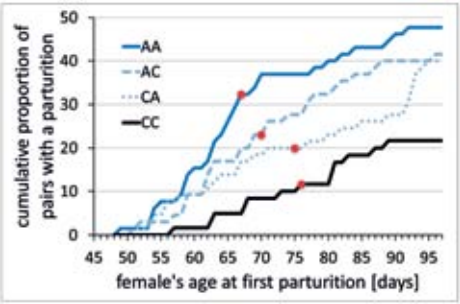
According to current concepts, selection forces leading to the evolution of endothermy acted simultaneously on several characteristics such as the aerobic exercise capacity or reproductive traits, rather than on a single performance trait. Thus, a crucial question is whether these traits are positively correlated or functional trade-offs impose limits on their correlated evolution. To answer the question we used a unique model: lines of bank voles selected for high swim-induced aerobic metabolism (A) and unselected, control lines (C). We have already shown that under regular breeding conditions the voles from A lines were giving birth sooner after the mating, and had a larger litter size and mass at birth, than those from the C lines. However, when all animals experienced the change from winter to summer photoperiod or were mated at an older age the differences faded. Therefore we hypothesized that the selection for high aerobic performance resulted primarily in accelerated readiness to reproduce. We tested this hypothesis by estimating the ability to become pregnant and deliver offspring in four types of pairs mated constantly from the age of about 24 to 80 days (±3days): both mates from A lines (AA, n=65), A-female mated with C-male (AC, n=65), C-female mated with A-male (CA, n=65), and both mates from C lines (CC, n=60). Mean age at first parturition (marked with the red dots on the Figure), calculated for successful pairs only, was lower in AA than in CC groups but the difference was not significant (p=0.23). However, the proportion of parous females was higher in AA (48%) than in CC group (22%, p=0.02), and intermediate in AC (42%) and CA (40%) groups. The result is consistent with the concept of triggering the evolution of endothermy by the correlated evolution of locomotor and reproductive performance traits.



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Basoendothermy: Schrëwdinger's legacy

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Recent evidence suggests that only a single species of eutherian mammal, survived the asteroid impact at the Cretaceous-Palaeogene boundary ~ 66 million years ago (mya, O'Leary *et al.* 2013 Science 339, 662-667). The hypothetical eutherian ancestor (dubbed Schrëwdinger by a popular contest) is postulated to have been small bodied, nocturnal and insectivorous, and likely possessed the capacity for prolonged periods of metabolic down-regulation, either in the form of daily torpor, or hibernation. The capacity for prolonged metabolic suppression would have increased the chances of surviving the harsh environments at the K-Pg boundary. Remnants of these characteristics are prevalent in extant descendants of Schrëwdinger, but especially visible in basoendotherms. These mammals are predominantly nocturnal, inhabit the lower latitudes, have highly variable body temperatures and lower than average metabolic rates. For example, free-ranging body temperatures we collected from greater hedgehog tenrecs, a basal placental mammal and ideal model basoendotherm, show how small increases in homeothermy can come at relatively low energetic costs. Thermoregulatory costs are greatly reduced in this species as a result of a high degree of variability in body temperature (thermolability). Here we review the degree of body temperature variability in modern mammalian lineages and explore the energetic consequences of thermolability in relation to climate and evolutionary history. A review of the literature shows that moderate to high levels of thermolability are much more common throughout the mammalian phylogeny than previously believed. Schrëwdinger's legacy can therefore be found not only in the basal mammalian orders (Xenarthrans and Afrotherians) but in the Eulipotyphla, Primates, Rodentia and potentially Carnivora.

Nocturnalism in mammals and the evolution of endothermy

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A nocturnal bottleneck has been postulated to be a major development in the evolution of mammals. Avoidance of predation, the development of endothermy and the resulting energy balance have been suggested to play an important role. Accumulating evidence has been obtained based on gene losses in the visual system and in the photolyase DNA repair system. This holds specifically for placental mammals (Gerkema *et al.* Proc R Soc B 2013). A recent analysis of these gene losses in monotremes, marsupial and placental mammal suggests a twofold nocturnal bottleneck during the Mesozoicum (Van der Loosdrecht *et al.* in prep). The manifold temporal niche switching after that (Gerkema *et al.* Proc R Soc B 2013) resulted in many cases of diurnality. A circadian thermo-energetics hypothesis (Hut *et al.* Prog Brain Res 2012) gives an explanation for the balance between nocturnality and diurnality. In support, at least in small mammals a metabolic induction has been demonstrated (Van der Vinne *et al.* PNAS 2014). At this moment the development of visual systems and DNA-repair systems may give the best insight in the historical time frame of nocturnality. The development of endothermy, in combination with torpor mechanisms, remains, however, crucial for an understanding of the evolution of nocturnality.

Hotter really is better: body temperature and the evolution of endothermy

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Over the past century many hypotheses have been advanced for the evolution of endothermy. In recent years considerable attention has been directed at the possible role of parental care, but the most widely accepted idea has been the *aerobic scope hypothesis* (ASH), proposed by Al Bennett and John Ruben in 1989. They suggested that endothermy arose through selection for an enhanced aerobic capacity, and that both body temperature and resting (basal) metabolic rate increased as a side-effect. The precise mechanism for the coupling between aerobic scope, resting metabolic rate and body temperature was not, however, specified.

In this talk I will examine the role of body temperature in aerobic performance, explore some of the assumptions underpinning the aerobic scope hypothesis, and suggest a modified version. In the modified aerobic scope hypothesis, a higher body temperature is not so much a consequence of the evolution of an enhanced aerobic scope, but rather the mechanism by which this was achieved.

The antiquity of endothermy in archosaurs and the metabolic consequences of abandoning endothermy in the crocodilian lineage

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Mammals and birds share characteristics that are associated with the term 'endothermy', including a high density of mitochondria, high resting and active aerobic metabolic rates, high pulmonary diffusing capacity, high cardiac outputs, and four-chambered hearts that separate high systemic and low pulmonary blood pressures. There are functional relationships between these features. Both lineages of archosaurs (crocodyloform and dinosaur-avian) are characterised by high blood pressure, four-chambered hearts and complex pulmonary design, indicating that endothermy originated among, or even earlier than, the basal archosaurs of the Late Permian, some 100 million years before the first birds. It is therefore likely that all dinosaurs were endotherms.

Five lines of evidence indicate that modern crocodilians evolved ectothermy from endothermic ancestors some time during the Mesozoic Era. These include development of the embryonic heart, flow-through lung ventilation, breathing during locomotion, bone histology and the pace of the molecular clock. The shift to ectothermy in crocodilians was associated with sit-and-wait predation in water. In this environment, endothermy and aerobic capacity are selected against, and predation involves primarily anaerobic metabolism in explosive feeding events. Aerobic metabolic rate during maximal activity accounts for less than 13% of total power generation in estuarine crocodiles, while it is about 50% in mammals. Because of the loss of aerobic capacity in crocodiles, total power generation during the first 10% of a bout of intense exercise is approximately half that of a mammal at 1 kg body mass, decreasing to only 8% in 200 kg animals. Therefore the loss of endothermy in the crocodilian lineage greatly reduced total power output during exercise.

1) Schachner, E.R. *et al*. (2013). Pulmonary anatomy in the Nile crocodile and the evolution of unidirectional airflow in Archosauria. *PeerJ* 1:e60; DOI 10.7717/peerj.60; 2) Seymour R.S. (2013). Maximal aerobic and anaerobic power generation in large crocodiles versus mammals: Implications for dinosaur gigantothermy. *PLoS ONE* 8, e69361; 3) Seymour, R.S. *et al*. (2004). Evidence for endothermic ancestors of crocodiles at the stem of archosaur evolution. *Physiological and Biochemical Zoology* 77, 1051–1067.

The Cenozoic Endotherm Pulses: The macrophysiology of mammalian endothermy

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The diversity of endothermic states increased dramatically in Cenozoic mammals coincident with global cooling and the attendant changes in vegetation. The highest probability is that the ancestral monotreme, marsupial, and placental mammal that survived the K/Pg extinction event had a low metabolic rate, low body temperature (< 36°C), and was capable of long-term hibernation. Hibernation is plesiomorphic in mammals and evolved during the Mesozoic in the tropics. The conservative endothermic features of the earliest mammals, termed basoendothermy, were probably sustained throughout the Mesozoic through stabilising selection that maintained an optimal body temperature for spermatogenesis. However, with the demise of the dinosaurs and the replacement of extensive rainforests with open woodlands, savannahs, and grasslands in the Late Cenozoic, elevated endothermy, that is, meso- and supraendothermy, evolved in tandem with cursoriality in larger mammals, in the Carnivora, Artiodactyla and Perissodactyla. The increases in metabolic rates and body temperatures evolved in tandem also with the evolution of the scrotum and externalised testes to maintain the optimal temperature for spermatogenesis. Endothermic diversity in small mammals was influenced most extensively by climate, especially cold ambient temperatures and unpredictable rainfall. These models plus new paleontological data now allow the development of more holistic models for the evolution of endothermy in birds and mammals.

Macrophysiology of endotherms: The global variation in thermal traits of birds and mammals

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The relationships among species' thermal capacities and the geographical variation of ambient climatic conditions are of key importance to understand the distribution of Life on Earth and the responses of species to climate change. Here, we assembled data on thermal tolerances from physiological experiments of more than 500 species of birds and mammals from all over the word and analyzed them, along with data on ambient climate and species' geographical distributions in a phylogenetically and spatially explicit context. We found that thermal tolerance was a poor predictor of geographical range size, and that, overall, the ambient temperature conditions that species experience across their distributions do not match well with their thermal tolerances. Macrophysiological patterns were partly incongruent for birds and mammals – a difference which may result from different adaptive capacities via behavior, morphology or physiology (Khaliq *et al.* 2014). Current environmental conditions explained a larger amount of variation in thermal traits among species than their evolutionary history. However, phylogeny was much more important than current environment for explaining the variation in physiological traits in the tropics, whereas environment was more important than phylogeny in temperate species. Using species' thermal tolerances, we show that their vulnerability to climate change increases from polar to tropical regions, underlining the high sensitivity of tropical diversity to even small changes in ambient climatic conditions. However, we also show that many species may be able to tolerate projected temperature increases across significant proportions of their ranges. Our results suggest that the relationship between thermal physiology and ambient climate varies across space and between taxa. Overall, our findings underline the need for cross-disciplinary research among physiologists, ecologists, and biogeographers, to improve future projections of biodiversity in a changing world.

Khaliq, I., Hof, C., Prinzinger, R., Böhning-Gaese, K. & Pfenninger, M. (2014) Global variation in thermal tolerances and vulnerability of endotherms to climate change. *Proceedings of The Royal Society Biological Sciences B*, 281, 20141097.

Interspecific Scaling of Metabolic Rate: Time for Synthesis?

Organizers:

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Scaling of activity metabolic rate in endothermic mammals, birds and dinosaurs

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The maximum aerobic metabolic rate of an organism is matched to the anatomy and physiology of the oxygen supply system. The structure of the cardiovascular system is governed by the requirements for blood flow rates that are adequate to supply oxygen demanded by the tissues. Thus, the radius and wall thickness of major arteries are dynamically adjusted to accommodate given flow rates and blood pressures, and arterial size can be used as a gauge for maximum metabolic rate of the organs they supply. In particular, the size of the nutrient foramen of the femur bones of mammals and birds provides an index of blood flow to the interior of the bone, which is necessary for bone remodelling in response to loading and the repair of microfractures due to locomotory activity. The scaling of blood flow index has virtually the same exponent in mammals (0.86) and birds (0.89) as maximum aerobic metabolic rate during terrestrial locomotion, but is significantly higher than the exponent for basal metabolic rate. Foramen size in mammals is a better predictor of maximum metabolic rate than is body mass. Bipedal birds perfuse their femora at approximately twice the rate as do quadrupedal mammals, reflecting differences in locomotory stress on two legs rather than four. A 1 kg cursorial bird has a femur mass and a blood flow index about two times higher than a volant bird of the same mass, again reflecting relative loading. Blood flows to the internal femoral shaft of birds and mammals are 1 – 2 orders of magnitude higher than in reptiles, as most reptiles do not remodel their long bones in response to exercise. Blood flow indices for extinct giant birds of New Zealand (moa) lie on the avian scaling regression. However, data from ten species of dinosaur are higher than both mammals and birds, indicating a highly active and aerobic lifestyle consistent with endothermy.

1) Allen, G.H., *et al*. (2014). Blood flow for bone remodelling correlates with locomotion in living and extinct birds. *Journal of Experimental Biology* 217, 2956-2962; 2) Seymour, R.S., *et al*. (2012). Blood flow to long bones indicates activity metabolism in mammals, reptiles and dinosaurs. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 279, 451-456.

What is the role of temperature in the scaling of metabolic rate?

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It has been known for over a century that metabolic rate increases with temperature, but there is no consensus as to why this should be so. The key arguments have been whether metabolic rate is determined primarily by the supply of nutrients to the cells or by demand for ATP, and whether temperature drives higher rates of metabolism, or allows it.

A problem in resolving these issues is that the synthesis of ATP comprises a complex series of events, each with a different temperature sensitivity. There is also a lack of clarity over what is meant by 'metabolism', an apparently simple term that means different things to different people. In this talk I will examine the various processes involved in ATP synthesis (intermediary metabolism) and their different temperature sensitivities, and then look at the range of ecological uses to which this ATP is put. I will use this as a background to explore how temperature might affect metabolism in theory, and compare this with data from a range of organisms.

Allometric scaling of active metabolic rate: Biomechanical causes and cellular consequences in flying insects

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Flying insects offer an attractive model to investigate metabolic rate scaling. Their mode of locomotion imposes high energy demand for muscle function, the dominating contributor to this metabolic state. This allows to uncover close associations between the scaling of morphological and biomechanical traits, dictating flight metabolic rate scaling. Furthermore, I will show how intraspecific variation found in insects can recapitulate interspecific scaling and its variation. The consequences of flight metabolic rate scaling on muscle tissue supporting flight are observed on multiple metabolic phenotypes involved in energy production or associated with cell metabolic activity. Interspecific scaling of flight muscle metabolic enzymes appears to be driven directly by flight metabolic rate scaling, although only for some components of the metabolic machinery, and according to known principles of metabolic regulation. Studies conducted at the intraspecific level also show that the same functional steps are linked to metabolic rate variation, thereby bridging the gap between intra and interspecific scaling. Finally, flight muscle membrane phospholipid composition scales with body mass and according to the proposed membrane pacemaker hypothesis, indicating how cellular properties change with tissue metabolic intensity. This work will be presented within a phylogenetic framework. Overall, this presentation will summarize multiple empirical studies and demonstrate how our understanding of metabolic rate scaling can be framed within known mechanistic physiology.

The scaling of metabolism in the perspective of DEB theory

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Kleibers law, i.e. respiration is proportional to body weight to the power 3/4, interspecifically, still generates discussion since it is generally seen as explanation for many biological scaling phenomena. Since 1986, Dynamic Energy Budget (DEB) theory offers a sound explanation for this law.

DEB theory treats Kleibers law as a special case of wider problem of co-variation of parameters across species. The rules for co-variation follow from physical dimension analysis and do not involve empirical or optimization arguments. Both maximum body weight and maximum respiration are just two functions of some parameters. They do not contain all parameters, and many interesting phenomena involve other parameters as well. Respiration, with contributions from several underlying processes, cannot serve as explanation for scaling phenomena generally. DEB theory offers a simple quantifier for where species are on the supply-to-demand spectrum, with a direct link to the ratio between peak and standard metabolism. I will also discuss the waste-to-hurry phenomenon to illustrate some points.

Ecological mechanisms affecting the body-mass scaling of metabolic rate: Toward a synthesis

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Contrary to popular belief, the body-mass scaling of metabolic rate may be not only constrained by intrinsic factors (e.g., surface area, resource-transport networks, cell size and/or other aspects of body design and composition), but also affected by various extrinsic (ecological) factors, both biotic and abiotic. I will describe and evaluate various hypothetical mechanisms by which ecological factors may affect metabolic scaling both within and among species. These mechanisms will be classified according to their effects on resource supply (as mediated by surface area or resource-transport networks) and resource demand (as mediated by body composition and various resource-using processes), in accordance with the recently proposed, synthetic "contextual multimodal theory" of metabolic scaling (Glazier, 2014). A major focus will be on how both the slope and elevation of metabolic scaling relationships can be significantly affected by adaptive and phenotypically plastic changes in behavioral activity and life-history tactics that act via their influence on resource acquisition and allocation to various biological processes and structures requiring various amounts of metabolic support. As a result, I will attempt to show that metabolic rate does not merely drive the rates of various biological and ecological processes, as proposed by the "metabolic theory of ecology", but also responds to the resource demand associated with these processes (Glazier 2015). Reciprocal causation between the rates of metabolism and other biological processes, as mediated by various regulatory systems, will be emphasized.

1) Glazier, D.S. (2014) Metabolic scaling of complex living systems. *Systems* 2(4): 451-540; 2) Glazier, D.S. (2015) Is metabolic rate a universal 'pacemaker' for biological processes? *Biological Reviews* 90(2): 377-407.

Metabolic scaling from ecophysiological and life-history perspective

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There is more and more evidence that a universal scaling exponent for metabolic rate does not exist. Even worse, non-linearity of log metabolic rate with respect to log body mass has been noticed. It seems, therefore, that allometric equations do not represent a law, but approximation, not always perfect, of non-linear relationship. With such perspective, the proper question is not on the value of slope but on the causes of non-linearity and concave downward shape. Metabolic rate is a sum of metabolic processes on organ, tissue and cellular levels. Some organs, such as brain, liver or heart have high metabolic rate even at rest; muscles are metabolically almost inert at rest but use most of ATP during intense exercises; bones, chitin and fat are metabolically almost inert all time. Thus metabolic rate must strongly depend on relative size of organs, and scaling exponents of metabolic rate must depend on slopes of allometries of particular organs. Metabolic rate may also depend, but only partly, on the size of cells. Small cells having high surface-to-volume ratio are expensive because of cost of maintaining gradients on cellular membranes. On the other hand, they may synthesize at higher speed, because large surface allows faster delivery of substrates and oxygen. The advantage or disadvantage of having small or large cells depends on ecological context, such as temperature and food availability. Furthermore, natural selection does not act directly on metabolic rate, but selects traits that maximize fitness, which is measured by reproductive value at birth converging often to lifetime offspring production. Thus mortality must impact optimal life history traits, as well as optimal physiological solutions, including relative size of organs and musculature. Scaling of metabolic rate is usually performed on interspecific level, whereas explanations invoke intraspecific level, with implicit assumption that slopes are the same. According to the theory of life history evolution based on optimal resource allocation, within-species and between-species slopes usually differ because body mass should not be treated as independent variable for metabolic rate.

Cell size changes contributed to the evolution of body size in *Carabidae* beetles: An optimal cell size theory perspective

Bartosz W. Schramm, Agnieszka Gudowska, Marcin Czarnoleski, Anna Maria Labecka, Ulf Bauchinger, Jan Kozlowski

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According to the optimal cell size theory, changes in cell size may have important consequences for physiological performance of organisms. On one hand, small cells provide larger area of plasma membranes for resource assimilation, while on the other hand, large cells spend less energy on maintaining ion gradients across membranes. The aim of this comparative study was to assess the role of cell size changes in the evolution of body size of *Carabidae* beetles. We analysed body size, body mass and ommatidia size (our proxy of cell size) in 6 species of beetles (*Carabus nemoralis, Carabus linnei, Carabus arvensis, Pterostichus burmeisteri, Abax ovalis, Carabus violaceus*) caught in the wild in two consecutive seasons. Within species, a positive correlation between the size of ommatidia and body mass was observed, and when statisticaly corrected for body mass, males possessed larger ommatidia than females in autumn and conversely smaller ommatidia in spring. We found that the size of ommatidia differed significantly among species, and to a large extent this variation was linked to the evolution of body mass: species-specific size of ommatidia scaled positively with body mass in females ($p<0.001$) and in males ($p=0.001$). We conclude that body mass and cell size may have coevolved in beetles. Ultimately, such coevolution can help understanding the origin of mass-scaling of metabolic rates.

Mass-scaling of standard metabolic rate in *Carabidae* beetles: Sex, season and breathing patterns

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Metabolism is a fundamental property of all organisms because it limits and/or facilitates almost all biological processes at different levels of organization. The rate of the biochemical processes can be estimated by the rate of respiration: oxygen consumption or carbon dioxide release. The relationship between metabolic rate (MR) and body mass (M) is expressed as a power function, $MR=aM^b$. Understanding the scaling exponent b is the key interest for several fields from ecology, and evolutionary physiology to life history evolution. This comparative study examines whether scaling exponent (b) and intercept (a) of the metabolic rates measured for carabid beetles vary between sexes, as well as spring and autumn and between utilized breathing patterns (discontinuous, cyclic and continuous gas exchange pattern). We used flow-through respirometry to measure standard metabolic rates for 8 species (*C. coriacues, C. violaceus, C. nemoralis, C. arvensis, C. auronitens, C. linnei, A. ovalis, P. burmeisteri*) of beetles caught in wild in spring (n=366) and in autumn (n=211). Interspecific metabolic rate increased allometrically with body mass. For the entire data set, relationship between log body mass (x) and log metabolic rate (y) was equal to y=-4.90+0.78x. Sex and season affect neither slope nor elevation ($p>0.05$). Whether carabid beetles employed discontinuous, cyclic and continuous gas exchange pattern significantly influenced both metabolic rate (ml CO_2*min^{-1}) and intercept ($p<0.05$). General level of the metabolic rate was higher for continuous and cyclic and the lower for discontinuous gas exchange. Our results reveal that from the three variables investigated in this study, namely sex, season and breathing mode, the latter had the largest influence on the metabolic rate scaling. We conclude that breathing pattern employed by insects should be measured to properly interpret estimations of metabolic rate in general, but more specifically in studies investigating allometric scaling of metabolic rate.

From Molecular Background to Phenotypic Variation in Metabolic Rate

Organizers:

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Aneta Książek, *University of Białystok, Poland*
Paulina Szafrańska, *Mammal Research Institute PAS, Poland*

Uncoupling protein(s) and uncoupling as determinants of metabolic rates

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The realization that uncoupling proteins exist - i.e. that there are protein(s) that has as their apparently only purpose to allow for the re-entering of proton (equivalents) from the cytosol to the mitochondrial matrix - opens for the question of the significance of these proteins for the (basal) metabolic rate in different animals. By name, there are three "uncoupling proteins - UCP1, UCP2 and UCP3 - but there is presently no reason to consider UCP2 or UCP3 as being functional uncoupling proteins - they have simply been named in this way due to their evolutionary closeness to UCP1. Even concerning UCP1, its evolutionary predecessor protoUCP1 is likely not an uncoupling protein. It is found e.g. in fish and other poikilothermic creatures. First with the evolution of true mammals did the UCP1 we know today develop. Although UCP1 is innately active (thermogenic) in isolated brown-fat mitochondria, it is inactive in unstimulated brown-fat cells, due to the inhibition of UCP1 activity caused by the purine nucleotides (ATP, ADP, GTP, GDP) in the cytosol. Only when the brown-fat cell is adrenergically stimulated, is UCP1 activated. The mechanism for this is in reality still not know, despite many decades of investigation of UCP1-containing mitochondria and cells. Likely fatty acids released from lipid droplets in the brown-fat cells activate UCP1, directly or indirectly.

In addition to UCP1 - that thus only influence metabolic rate when it is activated - metabolic rate may be determined by other proteins in the mitochondrial membrane. Especially the adenosine nucleotide transporter (ANT) has been discussed to allow for the "leak" current observed in the mitochondria from all cells; the ANT1 isomer is probably the isomer responsible for this function. How important this function is physiologically we do not know.

The possibility to alter the basal metabolic rate of humans has large interest presently, in attempts to counteract the increasing obesity problem in the world today. Indeed, increasing the metabolic rate (everything else being unaffected) would hinder the development of obesity, and mechanism to increase the basal metabolic rate are therefore widely sought presently.

Lipid signaling in the control of metabolic homeostasis

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Living organisms and individual cells continuously adapt to changes in their environment. Those changes are particularly sensitive to fluctuations in the activity of metabolic enzymes and the availability of energy substrates. Stearoyl-CoA desaturase (SCD) catalyzes the synthesis of monounsaturated fatty acids, mainly oleate and palmitoleate, which are a major component of tissue lipids. SCD1 deficient mice (SCD1-/-) reveal increased energy expenditure and decreased body adiposity due to the upregulation of genes of fatty acid oxidation and the downregulation of genes of lipid synthesis. SCD is an important component in the regulation of skeletal muscle metabolism, which affects insulin sensitivity, mitochondrial fatty acid oxidation and ceramide de novo synthesis. The lack of SCD1 gene increases the rate of fatty acid beta-oxidation through activation of the AMP-activated protein kinase pathway and by upregulating genes of fatty acid oxidation in soleus and red gastrocnemius muscles. Consistent with increased beta-oxidation, the contents of free fatty acids and long-chain acyl-CoAs are significantly decreased, which together with reduced mRNA level and activity of serine palmitoyltransferase led to reduced ceramide synthesis in oxidative muscles of SCD1-/- mice. Thus, reduced contents of free fatty acids, acyl-CoAs and ceramides as well as increased AMPK phosphorylation, might contribute to increased insulin sensitivity observed in muscle of SCD1-/- mice. Furthermore, SCD1 expression correlates with global DNA methylation level and the rate of histone acetylation in skeletal muscle. These findings raise the possibility that the mechanism by which SCD1 regulates gene expression may involve epigenetic modifications.

The influence of cell size on the variation in metabolic rate

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The rate at which a multicellular organism utilizes energy obtained from food, called the rate of metabolism, is a sum of energy expenditure of all cells that build its body. The main determinant of dramatic differences in metabolic rates between organisms is undoubtedly body mass, which depends on both: cell number and cell size. Therefore, evolution of metabolic rates of multicellular organisms is hypothesized to reflect the evolution of their cell architecture. This is likely to stem from a tight link between the sizes of cells and nuclei, which are expected to be inversely related to cell metabolism. The cell size, apart from genetic background, is mainly regulated through the target of rapamycin (mTOR) activity. mTOR integrates the input from upstream pathways responding to nutrient, oxygen, and energy availability. Activation of mTOR protein appears to increase cellular metabolism, the size of cell, and protein synthesis rate. To check for the link between cell size and the variation in metabolic rates we analyzed mTOR activity and cells sizes in mice artificially selected for high and low basal metabolic rate (BMR) as a model system. We found that selection for BMR enforced changes in cellular structure of metabolically active organs. The hepatocytes, kidney proximal tubule cell, and duodenum enterocytes were considerably smaller in mice originated from low BMR line, while their erythrocytes remained bigger in compare to their cousins from high BMR line. Moreover, individuals characterized by high basal metabolic rate (and larger metabolically active organs and bigger cells), have higher expression of mTOR gene in liver than mice from low BMR line. However, the differences in the amount of mRNA coding this protein in small intestines were not significant between selected lines. We predict that changes in mTOR activity in some tissues may follow increase in consumption rate being compensated for higher energy demands in high BMR mice. Our results suggest that changes in size of cells and its molecular background constitute a critical, albeit largely underappreciated, factor in explanation of metabolic rates variation.

Tissue-specific differences in mitochondrial properties underlie inter-individual variation in metabolic rate

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Standard metabolic rate (SMR) and maximal metabolic rate (MMR) typically vary 2-3 fold amongst individual animals of the same species, size and life history stage, despite these traits being presumed to have a significant impact on fitness. Yet, the underlying physiological differences that determine such intraspecific variation in metabolism are largely unknown.

Here we tested the hypotheses that variability among individuals in SMR and MMR is influenced by mitochondrial functioning, and that if SMR support the cost of maintaining the machinery needed for MMR, their underlying mitochondrial capacities should be shared. We examine the role of variation in mitochondrial respiratory capacities (leak and phosphorylative respiration rates) of key metabolic tissues (liver and white muscle) in brown trout *Salmo trutta* from the same life stage, environment and nutritional state.

Mass-independent MMR and SMR were found to be uncorrelated, indicating that they are under the control of separate physiological processes. Moreover, the leak respiration in liver was significantly greater in high SMR individuals and the difference in MMR between fish was positively related to the leak respiration in the white muscle.

Trout with high SMR or high MMR, far from signaling a higher phosphorylative capacity of their mitochondria, can be those individuals whose tissue-specific mitochondria have a greater leakage of proton across the mitochondrial inner membrane. These results open up a range of avenues for future research on the consistent variability among individuals in the aerobic metabolism, including the fitness consequence of such variation in mitochondrial function, given the importance of mitochondria in the conversion of resources into ATP.

Lactation-induced changes in mitochondrial metabolism and fatty acid composition

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Raising offspring represents the most energy demanding process for female mammals during which they produce a lot of heat through the exothermic process of milk production and very high food intake rates. Simultaneously, non- shivering thermogenesis is largely shut down to limit hyperthermia during lactation. We studied, if these fundamental changes in metabolism affect mitochondrial respiration, and fatty acid composition in laboratory mice. We exposed lactating C57BL/6 females to two ambient temperatures (15 °C and 21 °C) and analyzed respiration rates in isolated liver and heart mitochondria on day 18 of lactation when the offspring was weaned. We predicted that differences in ambient temperature should affect how lactation modulates mitochondrial respiration: while lower ambient temperature can induce higher uncoupled respiration in non-reproductive controls, the exothermic process of lactation might cancel out the need for heat production.

We found that liver state II and state IV respiration rates were significantly higher in the lactating animals than in non-reproducing controls. Corresponding to that we found that respiration control ratios were lower in lactating females than in non- lactating controls. Surprisingly, we did not identify any effect of lower ambient temperature on mitochondrial respiration rates. In heart mitochondria we observed the same patterns with lactating females showing lower respiration control ratios but these differences did not reach significance potentially due to the low protein content of the prepared mouse hearts. We conclude that lactation significantly increases mitochondrial uncoupling in the liver with the same trend being observed in the heart but this process does not depend on ambient temperature. Further studies are needed to identify the long-term consequences of these changes and how they can affect thermosensitivity of the females and their milk production.

Molecular-level response to selection in a mammalian model of behavioural and physiological adaptation

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Experimental evolution combined with genome or transcriptome resequencing is a promising approach for advancing our understanding of the genetic basis of adaptive evolution. We applied this strategy to investigate the molecular changes underlying response to selection for aerobic metabolism (A) and for predatory behavior (P) in the bank vole (*Myodes [=Clethrionomys] glareolus*) selection experiment. After 13 generations of selection four replicated selected lines differed from four control lines (C) in the selected trait by more than 2 standard deviations. The analyzes of the liver, heart and hippocampus transcriptomes demonstrated that genes differentiated between P and C lines are likely associated with hunger, aggression, circadian rhythms and general physiology of the nervous system, whereas genes differentiated between A and C lines can be related to mobilizing fats and sugars from body reserves, stress response and mating success.

The number of genes differentially expressed between P and C lines (149) was around two times smaller than for A vs. C comparison. On the other hand, comparison of allele frequencies with expectations from drift simulations showed overrepresentation of SNPs repeatably differentiated between P and C lines but not between A and C lines. These findings suggest that genetic architecture of the traits differ: variation in aerobic performance seems to be encoded by more variants with averaged smaller effects, whereas fewer variants of larger effects underlie predatory behavior. It shows that pattern of molecular-level response to selection can be determined by selected traits and their genetic architecture. Therefore to learn about general patterns of molecular-level response to selection we need to investigate not only multiple replicates, but also multiple quantitative traits.

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A comparative transcriptome approach to the hypoxia tolerance of the brain of the hooded seal (*Cystophora cristata*)

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During the dive, the brains of diving mammals (seals and whales) experience a reduced supply of oxygen. It has been demonstrated that the neurons of the hooded seal (*Cystophora cristata*) are more tolerant to hypoxia than those of mice. Seal neurons also better survive a reduction in the supply of glucose and high concentrations of lactate. This tolerance may be accompanied by a shift of the oxidative energy metabolism to the astrocytes while in terrestrial mammals the aerobic energy production mainly takes place in neurons.

We employed RNA-seq to study the molecular basis of the unusual hypoxia tolerance of the seal brain. Transcriptomes were generated by Illumina sequencing of the cerebral cortex of the hooded seal and the ferret (*Mustela putorius furo*), which is a terrestrial relative. Selected differentially expressed genes were verified by quantitative real-time PCR. Gene ontology (GO) and KEGG pathway analyses showed a higher expression of genes involved in metabolic processes in the brain of the hooded seal, while lower expression was found for genes involved in neuronal signaling and protein synthesis. These differences may be interpreted in terms of an energy saving strategy in the seal's brain.

We further used RNA-seq to compare the effect of hypoxia and reoxygenation on brain slices from layer V of the visual cortex of hooded seals. Compared to the normoxia control, hypoxia caused the preferential upregulation of genes related to various signaling pathways, and a down-regulation of genes involved in ion transport and other neuronal processes, indicative for a neuronal shutdown.

Environmental metabolomics reveal geographic variation in aerobic metabolism and metabolic substrates in Mongolian gerbils (*Meriones unguiculatus*)

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Mongolian gerbils (*Meriones unguiculatus*) have a large-scale distribution in northern China. Geographic physiological variations which related to energy and water metabolism are critical to animals' local adaptation and distribution. However, the underlying biochemical mechanism of such variation and its role in adaptation remains largely unknown. We used GC-MS metabolomics approach to investigate the biochemical adaptation of Mongolian gerbils from xeric (desert), transition (desert steppe) and mesic (typical steppe) environments. Gerbils in desert population had lower resting metabolic rate (RMR) and total evaporative water loss (TEWL) than mesic population. Serum metabolomics revealed that concentrations of five tricarboxylic acid cycle intermediates (citrate, cis-aconitate, α-ketoglutarate, fumarate and malate) were lower in desert population than mesic population. Gastrocnemius metabolomics and citrate synthase activity analysis showed a lower concentration of citrate and lower citrate synthase activity in desert population. These findings suggest that desert dwelling gerbils decrease RMR and TEWL via down-regulation of aerobic respiration. Gastrocneumius metabolomics also revealed that there were higher concentrations of glucose and glycolytic intermediates, but lower concentrations of lipids, amino acids and urea in desert population than mesic population. This geographic variation in metabolic substrates may enhance metabolic water production per oxygen molecule for desert population while constraining aerobic respiration to reduce RMR and TEWL.

Mitochondrial efficiency as a regulator of energy trade-offs in birds fasting in the cold

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During food shortage, energy conservation is a key priority for organisms. We recently provided evidences that flexibility in the mitochondrial oxidative phosphorylation (OxPhos) efficiency may represent a key factor responsible for the energy conservation and the remarkable fasting endurance of king penguin chicks during winter (Monternier 2014). We investigated mitochondrial OxPhos adjustments of a mature (gastrocnemius) and an immature skeletal muscle (pectoralis) in fasting Muscovy ducklings either chronically exposed to cold (CA) or reared at thermoneutrality (TN). This protocol was designed to study the energetic constraints (cold and starvation) independently. In CA fasted ducklings, OxPhos capacity decreased in both skeletal muscles, but only pectoral muscle mitochondria were more efficient at producing ATP. In TN fasted ducklings, the decreased of OxPhos capacity was concomitant with an increased OxPhos efficiency in both skeletal muscles. Interestingly, these adjustments were also observed when fasted TN ducklings were acutely exposed to cold. On the whole, these results highlight the fact that cold acclimation impairs adjustment of OxPhos efficiency in gastrocnemius, an early functional muscle which is involved in avian thermogenesis. We suggest that starvation-induced energy sparing in gastrocnemius is counterbalanced by cold acclimation and the need for a more « thermogenic », loose-coupled mitochondria in cold-acclimated ducklings. Furthermore, in pectoral muscle OxPhos efficiency improvement was found in both ducklings and king penguin chicks. However, fasting was not found to induce mitochondrial hypo-metabolism in penguin chicks. These results suggest that i) increased OxPhos efficiency could be a general feature in birds facing food deprivation to save energy; and ii) hypo-metabolism discrepancy would be due to the necessity for wild species to maintain muscular activity against predators, which requires higher mitochondrial activity level.

Monternier P.A. *et al* Mitochondrial phenotypic flexibility enhances energy savings during winter fast in king penguin chicks – *J. Exp. Biol.* 2014 217:2691-2697.

Gene profiling of glycogen storage in the Pacific oyster using next-generation sequencing

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Glycogen is an essential nutrient storage form of glucose. The Pacific oyster *Crassostrea gigas* accumulates reserves of glycogen in winter for gametogenesis and spawning in summer. After spawning during high-temperature summers, oysters become highly nutrient depleted; therefore, rapid glycogen synthesis and storage is important for recovery from these stresses. Despite the importance of glycogen storage, there is little detailed information on the underlying molecular mechanisms. Given this deficiency, we analyzed the gene expression profiles of oyster glycogen storage organs using next-generation sequencing. In October, samples of stored and non-stored glycogen were collected from oysters. The digestive gland and gonad with surrounding mantle were dissected out immediately, and complementary DNA libraries derived from extracted mRNA were sequenced using next-generation sequencing on the Illumina platform. RNA-seq reads were analyzed to estimate gene expression levels using whole-genome sequence data (1). In the digestive gland and gonad with surrounding mantle, 15,570 and 23,131 genes were expressed, respectively. The number of genes showing greater than 2-fold changes between stored and non-stored glycogen were 2,416 (15% of genes in the digestive gland) and 3,111 (13% of genes in the gonad with surrounding mantle), respectively. Gene ontology analysis showed that the largest group of genes altered between groups were from the categories “enzyme in molecular function” and “metabolism in biological process” (Figure). Pathway analysis suggested that glycogen storage is linked to the insulin signalling pathway.

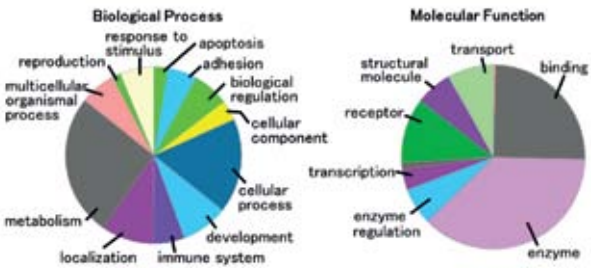

Figure: Gene ontology analysis of the digestive gland genes differ between groups.

1) Zhang G et al. 2014 *Nature* 490:49-54.

Lactate dehydrogenase distribution and localization in the brain of the hooded seal (*Cystophora cristata*) as biochemical adaptations to dive

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The brain of the hooded seal may have a higher anaerobic capacity than the brain of non-diving mammals. This hypothesis is based on the finding that the neurons of the hooded seal tolerate hypoxia and lactate better than the neurons of the mouse. Here we have compared the cerebral levels of lactate dehydrogenase (Ldh) and the buffering capacity of the hooded seal to those of ferret (*Mustela putorius furo*) and of mouse (*Mus musculus*). Our results demonstrate that the brain of the hooded seal has no higher anaerobic capacity as measured by Ldh mRNA and protein expression levels, Ldh enzyme activity, and buffering capacity. The Ldha and Ldhb amino acid sequences of the hooded seal show no significant differences compared to those of non-diving mammals. However, we observed that – compared to the brains of the mouse and of the ferret – the brain of the hooded seal has higher levels of $Ldha_1b_3$ and $Ldhb_4$ isozymes (figure: Panel A), and preferential localization of Ldhb in astrocytes (figure: Panel B). These results suggest that the brain of the hooded seal has an enhanced potential to aerobic metabolism, and that the contribution of astrocytes to aerobic metabolism is higher in the brain of the hooded seal than in the brains of the non-diving mammals. The different Ldh isozymes distribution and localization in the brain of the hooded seal may, therefore, contribute to the hypoxia and lactate tolerance of this tissue.

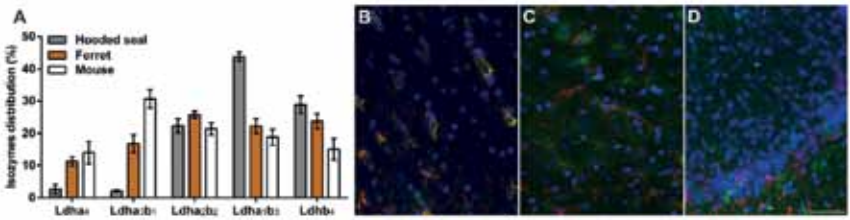

Figure: Ldh isozymes distribution (A), and Ldhb localization in the cortex of hooded seal (B), ferret (C) and mouse (D). Ldhb, green; Gfap (astrocytes), red; Ldhb-Gfap co-localization, orange/yellow; nuclei, blue. Bar represents 50 µm.

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The effect of artificial selection towards high aerobic metabolism on erythrocyte parameters in bank vole

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Achieving high aerobic exercise capacity requires efficient oxygen uptake, transport and distribution in working muscles. We applied experimental evolution approach to test the hypothesis that evolution of high aerobic metabolism results in increased blood parameters related to transport of oxygen. The research was performed on 165 blood samples from bank voles (*Myodes glareolus*) representing generation 18 or 19 of four lines selected for high aerobic metabolism (A) and four unselected control lines (C). We measured erythrocyte parameters (count/µl, cell volume, haematocrit, haemoglobin concentration, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration). Contrary to the expectation, males from the A lines had significantly less haemoglobin (p=0.01) and erythrocytes (p=0.03) per unit of blood than those from C lines (Fig. 1). The difference was not significant for females. The other blood parameters did not differ significantly between the groups. Considering that voles from the A lines achieved about 50% higher maximum rate of oxygen consumption in the swimming trials than those from C lines, the decrease in blood parameters most directly related to oxygen carrying efficiency is surprising. Apparently, oxygen carrying capacity of blood is not a crucial parameter limiting the aerobic capacity in bank voles.

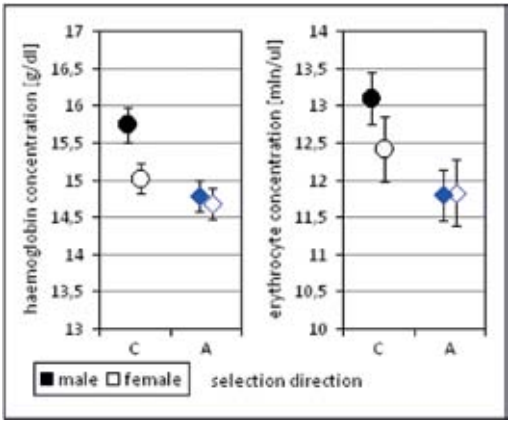

Fig. 1: Haemoglobin and erythrocyte concentration in voles from the A and C lines (data from generation 18 and 19 combined; Least Square Means±SE).

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Metabolic risk factors in mice divergently selected for BMR fed high fat high carb diets

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In recent years obesity and its concomitant health complications have become an epidemic all over the world, but the evolutionary sources of its origin still remain unknown. One of the most popular concepts in literature is the thrifty gene hypothesis (TGH) promoting the adaptive point of view of high fat gain. In accordance with TGH, individuals carrying the thrifty gens were able of enhanced fat storage making them less susceptible to periods of food scarcity. Possibly adaptive at one point, in modern day western societies consuming energy dense highly palatable foods such thrifty genotype produces morbidly obese individuals. One idea is that genes promoting the prone to obesity thrifty phenotype may be linked to low basal metabolic rates (BMR). Lower BMR equals lower basic maintenance costs, which means that all excess calories get stored as fat, especially when physical activity is limited. To verify this assumption we used mice artificially selected for divergent levels of BMR (high H-BMR, low L-BMR type mice). Animals were exposed to a 4 month long unlimited access to diets emulating the so called western diet (high fat and high carbohydrate diets) with no access to running wheels or any other form of physical training. Our preliminary results show that although H-BMR type animals consumed more food throughout the experiment they also showed higher levels of voluntary activity, gained less weight, had lower body fat content and lower levels of blood triglycerides, cholesterol and glucose than the L-BMR individuals at the end of the experimental period. However, contrary to our expectations oxidative stress damage was higher in the high BMR mice. These basic indicators suggest that genetically determined low BMR may be a factor predisposing individuals to increase adiposity in response to a western-type diet and developing a cluster of metabolic abnormalities.

Selection for high aerobic capacity during swimming has no protective effect against obesity

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The fact that physically active individuals are at lower risk of developing obesity and associated health disorders is well known. Large-scale epidemiological studies have even found low aerobic capacity in humans to be an excellent predictor for some of the components of metabolic syndrome. Recently however lots of attention has been focused on the intrinsic aerobic capacity (i.e. untrained aerobic capacity) of an organism as a possible factor affecting individual susceptibility to obesity linked diseases. Studies on animal models suggest that even in the absence of physical training the genetically determined high intrinsic aerobic capacity should have somewhat of a protective effect against weight gain and increasing adiposity. To test this assumption we fed a high fat and a high carb diet, both emulating the so called western diet plus a control diet, for four months to mice selected for high aerobic capacity during swimming in mid cold water (25°C) and unselected (randomly bred) control animals. At the end of the experimental period we found however, that contrary to the recently popular concept, there was no difference in susceptibility to the western type diets between selected and control animals. There were no significant between-line type differences in body mass, blood cholesterol, triglycerides and glucose levels as well as body fat content, only significant effects of the energy dense diets. The selected and control animals also showed no differences in voluntary activity and oxidative damage in the internal organs. Our results contradict the recent studies on animals from similar selection experiments and contradict the protective role of intrinsic aerobic capacity in obesity development, while simultaneously raising further questions about the role of physical training.

Does heat dissipation capacity limit energy budgets of lactating bank vole?

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Understanding factors limiting sustained metabolic rate (SusMR) is a central issue in ecological and evolutionary physiology. The "heat dissipation limit" (HDL) theory suggests SusMR at peak lactation is constrained by female's ability to dissipate the excess heat. To test the HDL theory, we shaved lactating bank voles (*Myodes glareolus*) to elevate their capacity to dissipate heat. The voles came from selection experiment in which lines selected for high aerobic metabolism (A) had higher metabolic rate than unselected control lines (C). We expected that shaving should increase reproductive performance (measured as milk production and litter growth at the peak of lactation), but the effect should be different in A than C voles. The number of weaned pups did not differ between the experimental groups (mean±SD: 5.0±1.7). At peak of lactation, shaving significantly increased food intake (LSM±SE; A-shaved: 14.8±0.4; A-unshaved: 12.6±0.4, C-shaved: 14.6±0.4 and C-unshaved: 12.9±0.3 g day-1; p = 0.004), daily energy expenditure (A-shaved: 108.5±3.0; A-unshaved: 97.0±3.0, C-shaved: 106.2±3.0 and C-unshaved: 95.8±2.7 kJ day-1; p = 0.008) and metabolizable energy intake (A-shaved: 220.2±8.6; A-unshaved: 199.8±8.7, C-shaved: 217.4±8.7 and C-unshaved: 196.1±8.2 kJ day-1; p=0.011) in both A and C voles. Digestive efficiency was lower in shaved voles (A :76.1±0.5, C: 75.5±0.5%) than in unshaved ones (A:77.6±0.5, C: 76.1±0.5%; p=0.012). However, shaving had no effect on milk production (mean±SE, 94.3±42.6 kJ day-1) or on litter growth (1.5±0.8 g day-1). Our data demonstrated that the shaving altered the energy budget of lactating bank voles, but the lack of simultaneous changes in reproductive performance undermines the HDL hypothesis. Furthermore, lower digestibility in the shaved than in the unshaved group provides an indirect support to the classical concept of energy budgets limited centrally by the capacity of alimentary system to digest food.

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Turn down gene expression for WAT: Anti-apoptotic signaling protects white adipose tissue in hibernating 13-lined ground squirrels

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During hibernation, the metabolic rate of 13-lined ground squirrels (*Ictidomys tridecemlineatus*) can drop to just 1-5% of normal resting rate at 37°C; body temperature can plummet as low as 5°C and heart/breathing rates fall to low values. Energy saved by using hibernation allows squirrels to survive the winter without eating, living off lipid reserves in white adipose tissue (WAT). During hibernation some energy must be used to defend against conditions that would normally be damaging for mammals (e.g. low temperatures, hypoxia, ischemia) and that can trigger cell death via apoptosis. Life or death for cells is often determined by the relative amounts and activities of the pro- and anti-apoptotic Bcl-2 family of proteins that respond to stress signals. The objective of this study was to determine how anti-apoptotic proteins respond to protect WAT cells during hibernation. Relative levels of several pro-survival anti-apoptotic proteins were quantified in WAT via immunoblotting over six time points of the torpor-arousal cycle. These included anti-apoptotic Bcl-2 family members functioning at the mitochondrial level: Bcl-2, p-Bcl-2 (S70), p-Bcl-2 (T56), Bcl-xL, p-Bcl-xL (S62), Mcl-1, p-Mcl-1 (S159) as well as caspase inhibitors downstream of the mitochondria: x-IAP and c-IAP. Bcl-xL and c-IAP exhibited no changes over torpor-arousal, but significant increases in Mcl-1 and x-IAP proteins occurred during torpor compared to euthermic levels, and significant decreases in p-Mcl-1 (S159), Bcl-2, p-Bcl-2 (T56), p-Bcl-2 (S70), and p-Bcl-xL (S62) levels were noted. Generally, these data suggest an overall increase in white adipose tissue survival efforts during hibernation.

From Molecular Machines to Performance and Life History of Ectotherms: Temperature, Oxygen, Cell and Body Size

Organizers:

Marcin Czarnoleski, *Jagiellonian University, Poland*

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Physiological performance and life history of ectotherms – a cell size perspective

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Environmental temperatures have pervasive effects on the performance and life history of ectotherms. With an increasing body temperatures, organismal functions improve until they reach a peak level, followed by a dramatic drop towards lethal temperatures. Typically, a warm ectotherm grows faster as a result of improved physiological performance but for unknown reasons it ceases growth earlier, maturing at a smaller size (temperature-size rule). Among populations, body size of ectotherms often diverges genetically such that larger individuals occur in colder environments (Bergmann's rule). A change in the size and number of cells are the proximate mechanisms behind these two ecological rules, but it is unclear whether the two cellular mechanisms have equal fitness consequences. Here, I review the current state of the theory of optimal cell size to integrate it with knowledge on the thermal-sensitivity of performance and life history. Briefly, the concept of optimal cell size predicts that a tradeoff between meeting metabolic demands and conserving energy decides about a thermal dependence of cell size. Collectively, small cells have a large surface area relative to volume, which requires more ATP to turnover phospholipids and generate electrochemical gradients. Nevertheless, organs with small cells should metabolize faster because of greater surface area for transport, shorter distances for diffusion, and more nuclei for transcription. Therefore, warmer and hypoxic environments should select ectotherms to develop smaller cells, whereas cold and normoxic environments should favor larger cells. The physiological effects of small cells reduce lags in metabolic activity and enhance performance during warming, which should play a role in shaping thermal-sensitivity during resource demanding activities (e.g. physical activity or offspring production) or during exposition to brief periods at elevated temperatures (e.g. in thermally fluctuating environments). The link between cell size and capacity to produce new tissue should decide about adaptive patterns of resource allocation to growth and reproduction. Ultimately, these patterns should determine the thermal-sensitivity of development and growth of ectotherms and lead to the origin of temperature-size rule and Bergmann's rule.

Oxygen and capacity limited thermal tolerance in animals: Connecting to ecology and evolution

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Understanding thermal ranges and limitations of organisms becomes important in light of climate change and its effects on ecosystems. The integrative concept of oxygen and capacity limited thermal tolerance (OCLTT) has successfully characterized the first thermal limits to whole animal performance and the consequences of such limits at ecosystem level. Various indicators of oxygen supply vs. demand were being analysed in species across animal phyla, representative of mostly aquatic environments. Analyses of anaerobic mitochondrial pathways provided evidence for oxygen deficiency at mitochondrial levels at thermal extremes. Oxygen recordings in (mostly venous) body fluids provided evidence for hypoxemia leading towards anaerobiosis. Steady state temperature dependent performance profiles traced the resulting thermal window and indicate the key role of aerobic metabolism in supporting steady state performances, from growth to exercise and reproduction. An underlying set of molecular to whole organism mechanisms defines oxygen demand and the capacity of oxygen supply and whole organism functional scope. At thermal extremes further mechanisms support passive tolerance. From a wider perspective, upper thermal limits across organism domains are defined by the highest levels of complexity or the largest number of compartments affected, in line with a key role for the capacity of oxygen supply systems and of demand in defining animal limits. Further studies began to explore how OCLTT principles were modulated by evolutionary adaptation, when changes to oxygen supply or demand occurred, e.g. during evolution in permanently cold polar waters or transition to breathing air. The paper will conclude with an assessment of the limits that become effective at ecosystem level (e.g. Deutsch et al., 2015) and also with how temperature induced changes in species interactions can be understood through shifts in species coexistance ranges and in relative capacities for performance.

Deutsch, C., Ferrel, A., Seibel, B., Pörtner, H.O., Huey, R.B. (2015) A metabolic constraint on marine habitat and its climatic changes. *Science* 348, 1132-1135.

Struggling to balance oxygen uptake and consumption when breathing under water: Implications for thermal tolerance and growth

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Breathing under water is challenging: the diffusion of oxygen is orders of magnitude lower in water than in air, while the higher density and viscosity of water greatly enhance the cost of breathing. Oxygen is essential for burning food and generate energy, but may become limiting for aquatic organisms that rely on gas exchange under water. However, while a shortage of oxygen quickly leads to asphyxiation, too much oxygen is toxic. Therefore, the ability to regulate oxygen consumption rates is at a premium; good respiratory control will enable ectotherms to balance oxygen toxicity against the risk of asphyxiation across a wide range of temperatures[1].

Temperature is intimately tied to oxygen budgets and hence energy budgets through thermal controls on metabolism and temperature related changes in the availability of dissolved oxygen (notably diffusivity, viscosity and solubility). I will present work showing that differences in temperatures act more strongly on ectotherms that rely on aquatic rather than on aerial gas exchange[2].

Body size is also intimately tied to oxygen budgets and hence energy budgets through size related changes in oxygen requirements and respiratory surfaces. In recent work we demonstrate that growth rates and final size attained are governed by the interactive effects of temperature and oxygen. The opposing effects of temperature on growth and adult size (giving rise to the Temperature-size Rule) were found to be manifested only under conditions where oxygen is limiting. Thus, it seems that an oxygen-based mechanism may underly the Temperature-size Rule in aquatic ectotherms[3].

An oxygen perspective focusing on the balance between oxygen supply and oxygen demand can help to explain thermal responses, and the capacity of an animal to regulate gas exchange is likely a key attribute to take into account.

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Mitochondrial function and membrane composition during temperature acclimation in alligators

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Although they do not thermoregulate by producing significant amounts of internally generated heat, ectotherms can acclimate to cold temperatures by adjusting their biochemistry. For example, some crocodilians are known to acclimate to holding temperature by changing the density of muscle mitochondria, resulting in compensation during aerobic performance[1, 2.]

We examined the biochemical and functional correlates of temperature acclimation in yearling American alligators (*Alligator mississippiensis*) that were kept, from 6 months of age, at 30°C, 20°C, or were cycled daily between 20 and 30°C. Metabolic rate, measured using flow-through respirometry, was similar among groups when tested at 20°C. However, when tested at 30°C, the alligators that had been held at 30°C and those that were cycled had higher mass-specific metabolic rates than those held at 20°C. These whole-animal metabolic rates were mirrored by oxygen consumption in mitochondria, which were measured using permeabilized thigh muscle fibers. When assayed at 20°C, oxygen consumption of mitochondria were similar, on a per gram tissue basis. However, when assayed at 30°C, mitochondrial oxygen consumption was higher in the alligators that had been held at 30°C or temperature-cycled daily.

We also investigated the lipid composition of muscle and mitochondrial membranes, which we hypothesized would be altered due to homeostatic regulation of membrane fluidity or to adjust metabolic rates. There were few differences among groups in membrane lipid composition, although the alligators held at 20°C had higher arachidonic acid in their phospholipids. Overall, our data suggest mitochondrial proliferation in thigh muscles of the two groups that reached 30°C, and we did not see evidence of thermal compensation in the animals held at 20°C.

1) Guderley, H. & Seebacher, F. 2011 *J Comp Physiol B* 181:53-64; 2) Glanville, E. J. & Seebacher, F. 2006 *J Exp Biol* 209:4869-4877

Energy expenditure and thermal adaptation in a terrestrial ectotherm: Natural selection and reciprocal transplants

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One of the central questions in evolutionary ecology is how different functional capacities impact fitness, and how it varies across populations. In a semi-natural experiment, we analyzed introduced populations of an invasive species, the garden snail (*Cornu aspersum*) in Chile. A common garden/reciprocal transplant experiment was performed in three populations that span a 1300 km latitudinal gradient and differ markedly in climate (semiarid North to humid South). We then released ca. 450 individuals per population (two generations after field-captured snails) in replicated enclosures at the range extremes. Morphological (size and shell darkness), physiological (standard metabolic rate and digestive efficiency) and life history (growth rate) traits were measured in all snails before the release. We only detected significant selection on body size (M_B), residual standard metabolic rate (SMR) and growth rate. Survival was significantly higher in snails from north than in snails from center and south, when raised at the north. However, at south survival was not different among source populations. We found negative correlational selection in MB and SMR at north (Figure) whereas at south, we only found directional selection on growth rate and M_B, and stabilizing selection on SMRR. These results suggest that selection on physiological traits related with energy allocation is pervasive, irrespective of climate and distance.

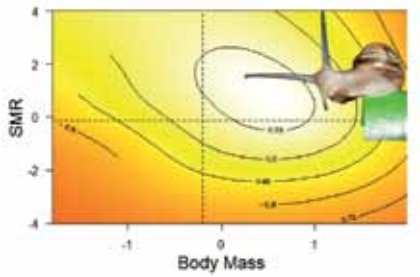

Figure: fitness contour showing negative correlational selection on (residual) standard metabolic rate (SMR) and body mass: small snails with high SMR and large snails with low SMR were promoted by selection.

High temperature tolerance in *Drosophila subobscura*: geographic variation, phenotypic plasticity and global warming

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Adaptive responses to global warming can be achieved through increasing tolerance to high temperatures via evolution or plasticity. The study of latitudinal variation of heat tolerance has been a useful approach to evaluate the evolution and adaptation to local thermal conditions. However, methodological artifacts can significantly affect our estimations of heat tolerance and then we can overlook adaptive potential and/or clinal patterns for upper thermal limits. Recently, we have proposed a framework for the study of heat tolerance taking into account the intensity and duration of heat stress, which creates a thermal tolerance landscape using the thermal death time curves (TDT). One of the most interesting outcomes of this framework was that we uncovered latitudinal patterns of upper lethal temperatures in insects that remained unnoticed in previous broad-scale comparative analyses. Here, we empirically assessed latitudinal variation in adult heat tolerance in *Drosophila subobscura* reared at two temperatures. We used two ramping assays (fast and slow heating rates) and four static stressful temperatures to estimate the TDT curves, which allow knowing the critical thermal maximum (CTmax) and thermal sensitivity to increasing stressful temperatures. We found a positive latitudinal cline for CTmax, but no clinal pattern for knockdown temperatures estimated from the ramping assays. Although high-latitude populations were more tolerant to an acute heat stress, they were also more sensitive to prolonged exposure to less stressful temperatures, supporting a trade-off between acute and chronic heat tolerance. Conversely, developmental plasticity did not affect CTmax but increased the tolerance to chronic heat exposition. The patterns observed from the TDT curves help to understand why the relationship between heat tolerance and latitude is contingent on the methodology used. Therefore, TDT framework provides a more complete and reliable measurement of heat tolerance, which is extremely important to understand and predict how species might respond to global warming.

Local thermal adaptation or countergradient variation across geographic ranges: Is there a latitudinal compensation in ectotherms?

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Environmental variation across geographical gradients is a key driver in the evolution of biological diversity, giving rise to genetic and phenotypic variation within species, and affecting survival and reproduction of organisms. Such variation has been well documented across latitudinal gradients, in which climatic conditions, season lengths, ambient temperatures and resource availability are paramount factors affecting the rate of most biological processes. Lower temperatures, extreme seasonality and shorter growing seasons at higher latitudes are expected to cause a decline in metabolic rates and annual growth rates of ectotherms. Nevertheless, if a reduction in the rates of these biological processes involves a reduction in fitness, then organisms may evolve compensatory responses for the constraints imposed by high-latitude habitats. To test the existence of a latitudinal compensation in ectotherms, we explore some alternative models of thermal evolution (e.g., local thermal adaptation and countergradient variation) using common-garden experiments to investigate the extent to which the level of energy turnover and the energy budget are affected by climatic constraints in terrestrial and marine ectotherms. Our results indicate that natural populations along broad latitudinal gradients exhibit combination of different models of thermal evolution depending on the existence of thermodynamic constraints, evolutionary trade-offs and gene flow.

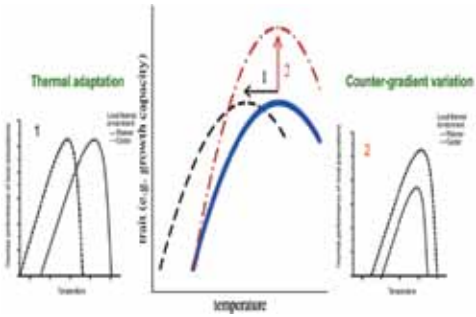

Figure: Theoretical models of thermal adaptation. 1) Local thermal adaptation and 2) countergradient variation. Arrows represent shifts in the thermal optimum and maximum performance in the TPC of natural populations. Gaitan-Espitia *et al.*, 2013. *PloS One*: e70662

Thermal reaction norms of metabolic rates, performance, and behavior: Plasticity integration or modularity?

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Standard metabolic rate (SMR) and maximal metabolic rate (MMR) are fundamental measures in ecology and evolution because they set the scope within which animals can perform activities that directly affect fitness. In ectotherms, both SMR and MMR are greatly influenced by ambient temperature (Ta). Yet, little is known about whether individual differences in SMR and MMR hold across an ecologically relevant range of Ta (i.e., thermal repeatability). Moreover, it is possible that thermal sensitivities of SMR and MMR are important individual attributes, and correlated with one another and other important phenotypic attributes, but virtually nothing is known about this. In this presentation, different study models (salamanders, toads, yabbies) are used to show, first, that SMR and MMR are repeatable across temperature gradients and, second, individuals significantly differ in thermal sensitivity. In other words, there are large inter-individual differences in the intercepts and slopes of thermal reaction norms of SMR and MMR. Moreover, individual differences are found for intercepts and slopes of thermal reaction norms of locomotory performance (toad example) and activity (yabby example). However, in these cases thermal sensitivity of metabolic rate is largely independent of the thermal sensitivity of performance and activity. Thus, there is little evidence for the presence of “plasticity integration” of thermals reaction norms of metabolism, performance, and behaviour – at least at the phenotypic level. Instead, it appears that thermal sensitivities of metabolism, performance, and behaviour are organised into independent modules.

Water-based tradeoffs over pregnancy in a reptile, the Aspic viper (*Vipera aspis*)

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A major goal in ecology is to understand and predict species responses to environmental variations. Clarifying the proximate factors involved is a crucial step to unravel general ecological patterns such as habitat use or species distribution. In this context, the use of an ecophysiological approach can be particularly relevant. Trophic resource and energy tradeoffs attracted considerable interest especially during reproduction, but water-based tradeoffs remain relatively overlooked to date. However water is a critical, often limiting resource that must be considered. The regulation of water balance may have a key influence on physiological and behavioral tradeoffs. We evaluated the importance of water-based tradeoffs over pregnancy in a species characteristic of "ecotone" habitats (hedgerows, edges), the Aspic viper (*Vipera aspis*). This species is viviparous and highly depends on thermal conditions during reproduction. Our general hypothesis is that, as thermal conditions, hydric conditions should also expose individuals to important physiological and behavioral tradeoffs during pregnancy. We combined descriptive (thermoregulation, water losses) and experimental studies (manipulation of water availability) that suggest a significant tradeoff between thermoregulation and water balance regulation. Water-based tradeoffs induce physiological and behavioral adjustments that are relevant to understand reproductive strategies and identify possible transgenerational (mother-embryos) conflicts. As energy or thermal resource, water should therefore also be considered to address ecological or evolutionary questions.

Physiological adjustments of reptile embryos in response to temperature variation

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Most reptile embryos, which are trapped within an immobile egg, are traditionally viewed as thermally passive. Contrary to intuition, our experimental studies show that reptile embryos are capable of physiological adjustments. First, reptile embryos can control their own rates of heating and cooling. Live snake eggs heat more rapidly than they cool, whereas dead or infertile eggs cool down more rapidly than they heat up. Second, HSP70 may promote thermal tolerance of reptile embryos. HSP70 overexpression generated benefits by enhancing embryo heat tolerance and hatching success, but subsequently imposed costs by decreasing heat tolerance of surviving hatchlings. Third, embryos may adjust mitochondrial respiration and metabolic enzyme activities in response to developmental temperature to achieve a high developmental rate at high temperatures. Our data suggest that reptiles may actively adjust physiological processes to cope with temperature variation even at an early life-history stage.

Maximal performance versus energy savaholics - different thermal strategies in two sympatric lizards

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The thermal biology of ectotherms is limited by physiological constraints, i.e. critical minimum and critical maximum body temperature, but also by what is ecologically obtainable, i.e. the operative environmental temperature. The preferred body temperature (T_{pref}) of a species is the temperature level where performance is optimal for activity (but not necessarily at a maximal level). T_{pref} is usually determined in lab experiments where lizards choose their body temperature along a continuous temperature gradient without disturbing extrinsic factors. This value is often used as a basis for further analyses (e.g., geographic distribution, climate warming and vulnerability of species). However, some lizards do not reach their T_{pref} in nature. It is known that nocturnal geckos are active at body temperatures well below their experimentally determined T_{pref} because the environmental temperature is too low. Moreover, even if attainment was theoretically possible, heliotherm and thigmotherm lizards often function away from their T_{pref}. In these cases, the ecological niche affects the thermal niche in a way that is difficult to measure in the lab. To gain more detailed information about the thermal niche under natural conditions, we investigated the temperature profiles of two sympatric heliotherm Malagasy lizard species (*Oplurus quadrimaculatus* and *Zonosaurus laticaudatus*) using external temperature data loggers in the field. Our results show significant differences in thermal preferences between the species and clear differences in temperature patterns. *Oplurus quadrimaculatus* maintains T_{pref} for as long and accurately as possible to allow for maximal performance even though at higher energetic costs. Body temperatures slightly below T_{pref} result in immediate changes in behaviour and finally inactivity. On the contrary, *Z. laticaudatus* reaches its T_{pref} for only a few hours per day and shows normal feeding behaviour at body temperatures well below T_{pref}. This pattern saves energy but implies activity at suboptimal performance. Albeit lab experiments reveal important information on critical and preferred temperatures and acclimation capacity, we emphasize the importance to study the ecology and behaviour under natural conditions to determine thermal requirements and consequences of climatic changes.

Genome size and low temperature lead to larger cell and body size in the edible frog (*Pelophylax esculentus*)

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Genome size correlates positively with cell size that has been clearly demonstrated in polyploids. Larger cells may have crucial consequences for whole organisms, including lower growth rate or larger body size. Indeed, polyploidy tends to increase body size in invertebrates but no clear effect of polyploidy on body size has been found in vertebrates. Differences in cell size may also explain patterns of geographic variation of body size in diploid animals; at low temperatures majority of ectotherms are built of larger cells and grow larger (the temperature-size rule) and consequently larger organisms inhabit colder environments at higher latitudes (Bergmann's rule). This paper shows the relationship between temperature, cell size, growth rate and body size in diploid and polyploid vertebrates. As a study model, we used edible frog (*Pelophylax esculentus)* reared as tadpoles at water temperatures of 19°C and 24°C. We found that the size of erythrocytes and epidermal cells was larger in both diploid and triploid tadpoles at low temperature, and triploids had larger cells at both temperatures. In froglets, the effect of temperature, in which they developed as tadpoles, was non-significant on the size of their erythrocytes and hepatocytes, and triploid froglets still had larger cells. Triploid tadpoles grew faster than diploids at 19°C while there was no clear difference between ploidies in growth rate at 24°C. At metamorphosis, tadpoles of both ploidies reached larger body mass at low temperature and triploids were larger at both temperatures. Triploid tadpoles, reared at low temperature, built of the largest cells, were therefore the largest. To our knowledge, our study is the first to report on the increase of body mass of a polyploid vertebrate caused by low temperature and we show that the increase of cell size contributes to the increased body mass. We suggest that large body mass of triploids may provide selective advantage especially in colder environments that may explain the prevalence of triploids in the northern parts of the geographic range of *P. esculentus*.

When to switch between being small or large? – temperature-size rule in a rotifer

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Vast majority of ectotherm species were observed to follow the Temperature-Size Rule (TSR). It means that they grow faster but for a shorter time and achieve a smaller size at higher temperatures, while showing the opposite growth pattern at low temperature. It is not identified what is the developmental stage at which growing larger in cold vs. growing smaller in warm is determined.

We examined the timing of TSR in the rotifer *Lecane inermis* by exposing different life stages to 15°C, 20°C or 25°C. We tested whether the TSR in offspring is controlled by the mother (during her development, or during egg laying period) or, alternatively, directly by the offspring (during development of the egg or hatchlings). We observed the two-point TSR control: by the mother and at the egg stage.

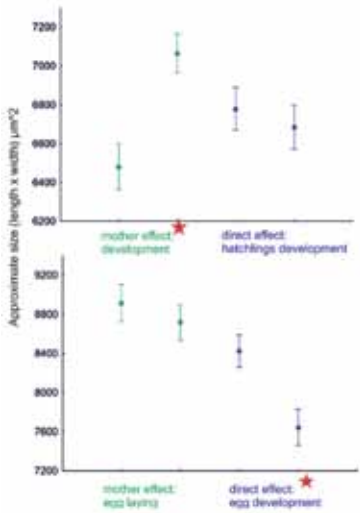

Figure: The results of contrast analysis for differences in progeny size as a response to exposure to low or high temperature at different life stages, in two separate experimental sets. The asterisks denote the stage in which the effect of temperature on offspring size was significant. This effect was observed for the mother effect and for egg development.

Walczyńska A, Sobczyk M., Czarnoleski M., Kozłowski J. 2015. The temperature-size rule in a rotifer is determined by the mother and at the egg stage. *Evolutionary Ecology*.

An altitudinal metabolic syndrome in ectotherms? A transversal analysis with ground beetles, amphibians and reptiles

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Closely related and ecologically similar species with opposite patterns in the altitudinal distribution are ideal models for comparative eco-physiological and evolutionary studies. Metabolic rates reflect organisms' life functions, having repercussions on fitness and, hence, on species survival potential and distribution. To assess the role of metabolism as a driving force in altitudinal distribution of terrestrial ectotherms, we selected three species groups: ground beetles, amphibians and reptiles. In each group we compared between one high-altitude specialists and one low-altitude generalist. We experimentally determined metabolic rates and potential metabolic activity (enzymatic capacity). Thermal dependence of metabolic rates was investigated using a design of measured metabolic rates at three temperatures (T) in 5°C intervals around species preferred body T. Furthermore, we assessed the realized metabolism, obtained from the ratio of potential metabolic activity and metabolic rate. All individuals tested were collected from populations at a mid-altitude zone of species distribution overlap, to avoid possible effect of acclimation to low or high altitudes. Parallelism in differences found between high-mountain specialists and low-altitude generalists across different ectotherm taxa will provide insight into the role of metabolism subjacent to ectotherm distributional patterns such as altitudinal segregation. Due to importance of metabolism in organisms' performance (physiological, behavioural, morphological, and life history traits), several correlated responses are expected and can be tested in future. Further work on mechanistic niche modelling should incorporate metabolic traits to better understand geographic distributional patterns of ectotherm species.

Cold in the tropics: Metabolic compensation in tropical lizards

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Changing environmental conditions can have drastic effects on the performance of ectotherms due to the strong temperature dependence of their biology. Behavioural thermoregulation and physiological plasticity are two rapid and reversible mechanisms that allow animals to buffer the impact of climatic changes. Physiological plasticity has long been predicted to increase with increasing latitude, implying only a very limited capacity of tropical ectotherms to acclimate their performance. However, this theory is contradicted by recent macrophysiological analyses. To investigate the potential for thermal compensation in tropical ectotherms, we conducted field measurements of energy expenditure in two sympatric lizards from Madagascar along a steep environmental gradient from dry spiny forest to rainforest and across seasons. The climatic extremes of the gradient differ by mean (~2°C) and maximum (~8°C) ambient temperature. *Oplurus quadrimaculatus* is an obligate saxicolous species and thus ecologically forced to perform at high body temperatures. This is reflected in a lower metabolic rate compared to the larger species *Zonosaurus laticaudatus* with a rather shade based activity. Coupled with lower body temperatures during activity, *Z. laticaudatus* seems to perform at its thermal limit in the hot spiny forest but shows neither spatial nor temporal acclimatization effects. In contrast, *O. quadrimaculatus* appears to be well fitted for a life in the heat and does indeed show no metabolic acclimatization in the hot spiny forest. In the colder rainforest environment however, seasonal limitations to behavioural thermoregulation result in lower body temperatures, which are compensated through an increase in metabolic rate. Complementing recent studies about thermal tolerance limits in tropical ectotherms, these results suggest that metabolic acclimatization may act as a compensatory mechanism if the capacity for behavioural thermoregulation is limited.

Thermal dependence of developmental rate and energy use by embryonic geckos (*Paroedura picta*)

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In ectotherms, environmental temperature is the most prominent abiotic factor that modulates life-history traits. Despite this, we often do not know the proximate mechanisms that underlie the thermal reaction norms of key traits, such as body size. Here, we report the effects of three constant temperatures (24, 27 and 30°C) on the rate of embryonic development, the energetic cost of incubation and body size at hatching in the Madagascar ground gecko (*Paroedura picta*).

The cost of incubation was estimated as the difference between the energy content of an egg and energy content of the hatchling that emerged from that egg. As expected, the duration of incubation depended strongly on incubation temperature, with mean incubation periods being 107 days at 24°C, 68 days at 27°C and 51 days at 30°C. Hatchlings from eggs incubated at 24°C were significantly smaller than those from eggs incubated 27°C or 30°C. Interestingly, the energy density of hatchlings was significantly lower at 24°C in comparison to 27 and 30°.

Based on our estimates of the energetic contents of eggs and hatchlings, the energetic cost of incubation at 24°C exceeded the cost at other higher temperatures. Therefore, the difference in body size at hatching resulted from a difference in the way that embryos used energy.

Nutrient and temperature effects on growth rate and body composition of the slug *Arion vulgaris*

Karolina Naumiec[1], Szymon Drobniak[1], Elzbieta Szulinska[2], Jesus M. Txurruka[3], Grazyna Wilczek[2], Paulina Kramarz[1]

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Temperature and food are two of the most important abiotic factors influencing an animal's biology. However, relatively little attention has been paid to how these the two factors interact (e.g., exposure to sub-optimal temperatures coupled with an unbalanced diet) in affecting an organism's physiology.

Juvenile slugs were raised in optimal (15°C) or one of two suboptimal temperatures (10°C or 20°C) for one month. They were provided one of five different diets with the following protein:carbohydrate (P:C) ratios (in %): 10:50, 20:40, 30:30, 40:20, 50:10, supplemented with cellulose (31.9%), oil (8%) and vitamins (0.1%). We measured growth rate, dry mass and concentrations of proteins, carbohydrates and fat in the body wall and hepatopancreas.

Growth rate was highest in the optimal temperature regime (15°C) and in the balanced diet treatment (30:30 P:C ratio). Surprisingly, dry weight was highest in slugs from the 10:50 P:C/20°C treatment. Only macronutrient ratio influenced body composition. The most striking result was an increase in glycogen concentration in the body wall of slugs fed a high-carbohydrate diet (Fig. 1).

Our results indicate that temperature may change the response of an animal to differing nutritional values of food. This has important implications in studies of differences between geographic populations, in which both factors can vary.

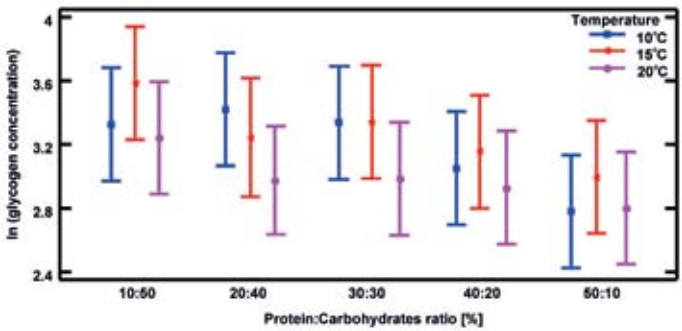

Figure 1. Concentration of glycogen (µg mg^{-1}) in body wall; mean and 95% confidence intervals are shown.

Quantitative genetics of thermal reaction norms for development time and body mass of *Tribolium castaneum*

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It is generally assumed that development time (DT) decreases with increasing temperature, which leads to smaller body size (BS) in higher thermal conditions. This can generate a negative thermal reaction norm (TRN) for both DT and final BS. Both traits are likely to be correlated, however animals have size regulating mechanisms that result in BS that is fairly independent of DT (1). Furthermore, fecundity selection on females and sexual selection on males are among the major sources of selection influencing DT and BS in insects (2).

Using a quantitative genetics approach (isofamilies), we explored the genetic basis for variability in TRNs for DT and BS (here body mass) in *Tribolium castaneum.* In addition, as sexual dimorphism is often neglected in thermal biology studies, we also tested for sex-specific differences in TRNs.

Both BS and DT were influenced by substantial genetic effects. However, the additive genetic effect was much smaller than the interaction of the genetic effects from particular combinations of parental lines. Likewise, we found small but positive genetic interaction components in TRNs for both DT and BS in both sexes. Interestingly, the effect of the interaction of genetic variance on TRNs was higher in females in the case of BS, and higher in males in the case of DT. This was further confirmed by a decomposition of effects analysis performed using the Cockerham-Weir diallel model. For both BS and DT, the extranuclear interaction effect was dominant and responsible for the majority of variance.

Our results indicate that the genetics of TRNs are not governed by simple additive genetic effects and are instead influenced by complex effects involving genetic interactions. Variance of such genetic effects seems to be sex-specific likely due to variation in selection among sexes (2). It is also dependent on the trait type probably because DT and BS are determined by another traits such as growth rate and hormonal regulation which are themselves complex traits that are the result of interactions among genetic and environmental variables (1).

1) Nijhout *et al.* 2010 *Phil. Trans. R. Soc. B* 365:567–575; 2) Stillwell *et al.* 2010 *Annu. Rev. Entomol*. 55:227–245

Genetic correlations between development time and body mass at different temperatures and sexes in *Tribolium castaneum*

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In insects, final body size, which has significant fitness consequences, strongly correlates with development time, and both traits depend on environmental conditions. The phenotypic correlation (r_P) between traits comprises an environmental (r_E) and a genetic (r_A) correlation. In cases of size-related traits, r_P as well as r_A should be positive in favorable *vs*. less-favorable environments, but a negative correlation could reflect a trade-off between two traits.

We exposed progeny of half-sib families of *Tribolium castaneum* to stable and fluctuating regimes of benign (30°C) and stressful (35°C) thermal conditions, Measured traits were: development time (DT) from egg-to-pupa and pupa-to adult (thus also from egg-to-adult) as well as body mass (BM) of pupa and adult of both sexes.

Correlations between adult BM and both pupa-to-adult and egg-to-adult DT had significant environmental components: egg-to-adult DT was negatively correlated with adult BM, whereas pupa-to-adult DT was negatively correlated with adult BM in stable conditions, and positively correlated with adult BM in fluctuating conditions. None of these correlations contained substantial, significant genetic components. In contrast, correlations between adult and pupal BM had a strong, positive genetic component, independent of the experimental group. All these effects were largely sex-independent, with the exception of the correlation between pupa-to-adult DT and adult BM, in which there were significant dam effects (genetic and maternal effects combined), but only in females.

Our results indicate that DT (particularly between pupal and adult stages) is not genetically linked to adult BM and is therefore ecologically flexible, as seen in the sensitivity of these environmental correlations to experimental conditions (stable vs. fluctuating temperatures). On the other hand, the BM of the intermediate (pupal) stage is tightly genetically linked with adult BM and thus cannot be easily adjusted by ecological conditions and physiological plasticity without incurring costs in terms of (fitness-related) adult BM.

Does female control over the Temperature-size rule during early ontogeny in terrestrial isopod *Porcellio scaber?*

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Temperature-size rule (TSR) describes an inverse relationship between developmental temperature and final body size. Although TSR is the most observed pattern among ectotherms, the underlying mechanism remains unclear. One of the proposed mechanisms is the mismatch between oxygen demands and oxygen availability. Because oxygen requirements increase with the body size stronger at higher temperature compared to lower temperature, individuals may respond to such oxygen limitations by adjusting body size. TSR has been shown to be more pronounced in aquatic environment, in which oxygen is less available than in air. Here we used two-factorial design to examine the effect of temperature (15°C and 22°C) and oxygen level (10% and 22%) on duration and body mass gain during early ontogenetic development in terrestrial isopod *Porcellio scaber*. Early development occurs within the maternal brood pouch and the progeny experience a change from aqueous to gaseous environment shortly before hatching. Females supply the progeny with oxygen only during aqueous phase. We found that developmental time in both phases was two times shorter at 22°C compared to 15°C. However, individuals from 15°C and 22°C did not differ in body mass at hatching. Oxygen concentration had a differential effect in aqueous and gaseous phase but only at 15°C. Hypoxic conditions significantly increased mortality of early stages during aqueous phase (two times higher compared to other conditions), but not during gaseous phase. Although hypoxic conditions significantly prolonged the developmental time in gaseous, but not in aqueous phase, body mass at hatching did not differ. Our results suggest that female may control the early development by supplying the oxygen during aqueous phase, but at the cost of higher mortality. TSR thus may not apply to life-stages that are associated with mother´s environment. The strategy of early development of progeny to take place in a motherly brood pouch might have been an important evolutionary milestone for the invasion of land by terrestrial isopods.

Does high temperature and low oxygen lead to smaller cells? The case of common rough woodlouse (*Porcellio scaber*)

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How cell size contributes to body size of organisms and may explain scaling of metabolism with body mass is still not fully understood. Following the concept of optimal cell size small cells are more efficient in transporting nutrients to cytosol and oxygen throughout the tissue, but they incur high cost associated with cell membrane maintenance. Here, we report results of an experiment on the woodlice *Porcellio scaber* which examined whether small cells are developed in warm and hypoxic environments (compared to cold and hyperoxic) in which metabolic demands are high but supply of oxygen is low. We placed adult woodlice for mating and egg laying in two temperatures (15 and 22°C) and two oxygen levels (10 and 22%). Following offspring release from the motherly brood pouch the juveniles were further maintained in these conditions. we sampled animals during development at a standardised body mass to measure and count ommatidia. An ommatidium is built from a defined number of cells, so we use the average size of ommatidia in an eye as a proxy of cell size. We found that warm environment produced woodlice with smaller ommatidia ($p=0.001$). The size of ommatidia did not differ between oxygen treatments ($p=0.19$) and interaction between temperature and oxygen was excluded from analysis by stepwise procedure. Oxygen and temperature had an interactive effect on the number of ommatidia ($p<0.05$): woodlice had highest number of ommatidia in the warm and hypoxic environment. The effect of temperature on the size of ommatidia is consistent with predictions of the theory of optimal cell size, but we did not find predicted effects of hypoxia. It is likely that the effects of oxygen would be more pronounced at later stages of ontogeny in this ongoing experiment. Alternatively, our low-oxygen treatment may not represent oxygen limitations for this species, which naturally occurs in oxygen-deficient habitats.

Aquaporin 9 in the freeze tolerance mechanism of Cope's Gray Treefrogs

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Cope's Gray Treefrogs *Hyla chrysoscelis* accumulate glycerol in response to cold as part of their freeze tolerance mechanism. This glycerol may derive from glucose stores in the liver that are broken down and released through glycerol transporters. We therefore hypothesized that these treefrogs would express aquaporin 9 (AQP9), a transmembrane protein from the aquaglyceroporin family that facilitates rapid glycerol transport, predominately in the liver. We also hypothesized that this protein would be up-regulated during cold acclimation to promote glycerol permeability. To test this hypothesis, we sequenced the AQP9 homologue HC-9, analyzed RNA and protein expression, and characterized water and glycerol permeability through a *Xenopus* oocyte expression system.

HC-9 has 315 amino acids which include 2 conserved NPA motifs unique to aquaporins, 5 conserved residues typical of glyceroporins, and a high homology (69%) to human AQP9. Furthermore, characterization using the *Xenopus* expression system suggests an enhancement in glycerol permeability.

In warm-acclimated animals, HC-9 mRNA (RT-PCR) and protein (western blotting) were expressed in many tissues, with some of the highest levels in liver, stomach, ventral skin, and muscle. Across most tissues, realtime mRNA expression (qRT-PCR) decreased in cold-acclimated (5°C) and frozen (-2.5 °C) treefrogs, with recovery in treefrogs returned to 5°C for 24 hours. Meanwhile, protein expression increased in the liver and ventral skin of cold and frozen treefrogs, but remained unchanged or decreased in all other tissues.

HC-9 expression is clearly regulated as a part of the Cope's Gray Treefrogs freeze tolerance mechanism. Contrary to our hypothesis, most tissues have decreased expression, which may reduce glycerol permeability to protect cells during the freeze process. Liver is a notable exception by increasing protein expression, which may facilitate the re-uptake of glycerol during ice formation. Liver appears to play a critical role in the distribution of the cryoprotectant glycerol, and this study suggests HC-9 facilitates in this role.

Supported by NSF IOS-1121457.

Understanding adaptive gene-expression during estivation in a terrestrial snail

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One of the most generalized adaptive responses of organisms to environmental stress is physiological dormancy: hibernation and torpor as a response to cold; diapause and estivation as a response to water scarcity. During estivation, animals reduce metabolic rate and physiological functions to a minimum, with a concomitant up- and down-regulation of several gene clusters. These are related with tissue protection from prolonged inactivity such as inhibition of muscle wasting, pro-apoptotic signalling and post-translational protein modifications. Although most of this information comes from vertebrate models, data from invertebrates are becoming available and suggest that several cellular and molecular processes are evolutionarily conserved. In this study, we analysed the transcriptome (hepatopancreas) of garden snails (*Cornu aspersum*) during aestivation, and compared it with active individuals. We used RNA-seq and Illumina platforms, and sequenced 6 libraries, obtaining 10.5 M of clean reads in average. We detected a total of 128.340 transcripts corresponding to 110.096 genes. In general, we found that estivating snails over-expressed more than 600 transcripts, whereas about 300 were down regulated, compared with active snails. It is particularly interesting that we found strong differences in expression between treatments, detecting 500-fold overexpressed transcripts. Also, we report key genes for snail estivation, including genes involved in response to stress, oxidation-reduction process and regulation of peptidase activity.

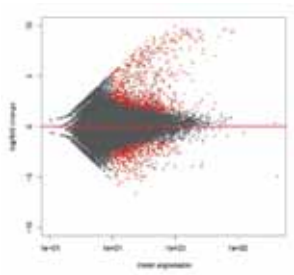

Figure: Log fold-change expression versus normalized gene-expression. Each point represents an individual transcript. Transcripts with positive log fold-change are over-expressed during estivation. Significantly over-expressed transcripts are shown in red.

Effect of developmental temperature on the cells size in the Madagascar ground gecko (*Paroedura picta*)

Anna Maria Labecka[1], Marcin Czarnoleski[1], Zuzana Starostová[2], Anna Sikorska[1], Elżbieta Bonda-Ostaszewska[4], Lukáš Kubička[3], Lukáš Kratochvíl[3], Jan Kozlowski[1]

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Thermal conditions during development affect many aspects of ectotherms' biology including development, adult size and metabolic rate, but thermal-dependence of cell size is not well studied. Relatively large area of plasma membranes and short transport distances within cytoplasm associated with small cells should speed up resource processing in warm conditions, when the demand for resources is high. On the other hand, small cells require more resources to maintain their plasma membranes, and this cost should favor large cells.

Testing predictions of this theory, we raised Madagascar geckos *Paroedura picta* at three temperatures (24, 27 and 30°C). Next, we examined a thermal-dependence of body size and cell size in several types of cells (erythrocytes, chondrocytes in trachea, cells of striated muscles in tail, hepatocytes in liver, epithelial cells in kidney proximal tubules, duodenum and skin). In general, we show that our thermal treatments induced changes in development and adult size of geckos, but males responded differently than females. Along with body size, geckos developed different cell size according to rearing conditions, but not all cell types responded in the same manner. We discuss how our results can help to understand fitness consequences of cell size changes in different tissues.

Longitudinal and latitudinal size variation of erythrocytes in the North American lizard *Sceloporus undulatus*

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We compared the size of erythrocytes in eight populations of the Northern American lizard *Sceloporus undulatus*. The populations originated from four phylogenetically distinct clades distributed from the east to the west, and each clade was represented by two sister populations, one in the north and one in the south of the North American continent. We measured snout-vent length of each lizard and the area of erythrocytes. Lizards were generally larger in the northern populations, in accordance with Bergmann's rule. Within populations the size of erythrocytes increased with body size which indicates that changes in cell size contributed to individual differences in the size of lizards. Erythrocytes were generally larger in two most eastern clades and in most cases in southern populations within clades. We discuss our results in the context of the theory of optimal cell size. According to this concept cells can undergo plastic or evolutionary changes in size according to the costs and benefits associated with the area of cell membranes. On the one hand, small cells provide relatively large membrane area for oxygen and nutrients transport which can help to meet increased demand for resources in warm or thermally fluctuating environments. On the other hand, small cells spend more resources on the maintenance of cell membranes, which makes large cells more profitable in resource-deficient environments.

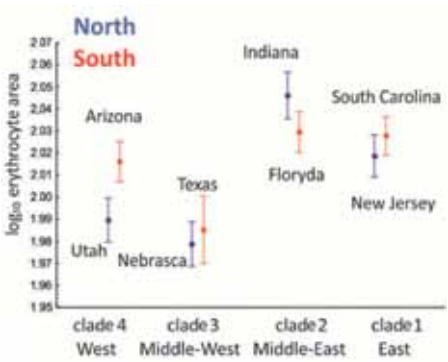

Figure: Erythrocyte size area varies among populations. Bars represents 0.95 confidence intervals.

Phenotypic Flexibility of Energetics in a Seasonal World

Organizers:

Michał S. Wojciechowski, *Nicolaus Copernicus University, Poland*
Małgorzata Jefimow, *Nicolaus Copernicus University, Poland*

Sponsored by *Society for Integrative & Comparative Biology, Division of CPB*

The effect of seasonal variation in climate on phenotypic flexibility: an assessment of the climatic variability hypothesis in different biological systems

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Recent studies indicate that phenotypic plasticity could be the main mechanism for species persistence under a scenario of climate change. However, standard models aimed to predict the effect of climate change on future species distribution do not allow for the inclusion of differences in plastic responses among populations or species. Obviously, this is not a capricious constraint: the quantification of plasticity for different traits in different populations or species is an arduous task. An encouraging way to solve this problem is the identification of general patterns in phenotypic plasticity, which (if any) could be easily incorporated into the models. Within this context, the hypothesis of climate variability (CVH) is particularly relevant because it directly connects phenotypic plasticity with climatic and geographic variables at a global scale. Basically, the CVH states that phenotypic plasticity should increase with climate variability (e.g., seasonal changes in temperature), and therefore, it should also increase with geographic latitude. However, given that the direct evidence supporting the CVH mainly comes from data on thermal tolerance ranges in ectothermic animals, it is hard to know if this hypothesis really provides a general pattern for phenotypic plasticity. Thus, the main aim of the present dissertation is to review some recent studies evaluating latitudinal pattern in plasticity for non-energetic traits (e.g., digestive morphology, energy storage, life history), and using other types of organisms (e.g., endotherms, plants). The main conclusion of this revision is that while it seems clear that phenotypic plasticity increases with latitude, many more studies are needed to establish the existence of other regularities on this general pattern, for example, in relation to the kinds of traits, organisms, and environments.

Mechanistic underpinnings of seasonal metabolic flexibility in birds

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Small birds show flexible physiological adjustments to changing energy demands throughout the annual cycle, with increments of organismal metabolic capacities in response to periods of elevated energy expenditure. Because organismal metabolic capacities are primarily functions of skeletal muscle metabolism, two major mechanisms exist for expanding organismal metabolic capacities – increases in muscle size or cellular metabolic intensity. These changes may also be accompanied by upregulation of fuel or oxygen supply pathways to muscles. Elevated pectoralis and heart masses often accompany increases in organismal metabolic capacities. Expression patterns of the muscle growth inhibitor myostatin and its metalloproteinase activators (TLL-1 and TLL-2) are generally, although not without exception, regulated in a manner consistent with a role for myostatin in mediating these changes in muscle or heart masses. Citrate synthase or cytochrome c oxidase activities, common metrics of cellular metabolic intensity, correlate positively with energy demands and metabolic capacities in some species, but not in others. Thus, cellular metabolic intensity is an inconsistent target for flexible metabolic responses in birds. Lipid transport and oxidation capacities are often upregulated with increasing organismal metabolic capacities. However, the specific regulatory steps targeted for adjustment are often not consistent among species. Perhaps the most consistent contributor is cytosolic fatty acid binding protein, which is generally upregulated under conditions of elevated energy demand in birds. Variation in the specific mechanisms underlying increased metabolic capacities among species and years appears to be the rule in birds. Such variation in underlying mechanisms likely imparts a considerable capacity for variation in phenotypically flexible responses, such that birds can respond to changing energy demands over a variety of time scales.

Intraspecific variation in the thermal physiology of a southern African passerine bird

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Most comparative studies of avian thermal physiology have focused on variation among species; far fewer have examined intraspecific variation along climatic gradients. In the last few years, we have examined intraspecific variation in a range of physiological variables in a common, widespread southern African ploceid passerine, the white-browed sparrow-weaver (*Plocepasser mahali*). Body temperature and patterns of heat dissipation behavior (HDB) differed significantly between two arid-zone populations. Birds at a hotter, more arid site maintained a body temperature (T_b) setpoint approximately 1.3 °C higher than that of conspecifics at a cooler, more mesic site, and showed a greater magnitude of daily heterothermy. This variation in T_b was mirrored by differences in HDB, with birds at the hotter site commencing panting at higher air temperatures than conspecifics at the milder site. More recently, we have examined seasonal variation in upper and lower thermal limits among populations from three sites varying in maximum and minimum air temperatures (T_a). Heat tolerance and evaporative cooling capacity varied among sites, with birds at a hot desert site exhibiting lower T_b and evaporative water loss (EWL) at $T_a > T_b$ during summer compared to winter, whereas conspecifics at cooler sites did not show any seasonal change. Birds at the hot site also showed a significantly higher heat tolerance limit (taken as the T_a at which T_b = 44 °C) in summer compared to winter. Summit metabolism (M_{sum}; maximum resting thermogenesis) differed by ~60 % among populations, and was significantly higher in birds inhabiting a seasonally colder site compared to birds at a milder site. The magnitude and direction of seasonal changes in basal metabolic rate (BMR) also varied among populations, ranging from winter increases of ~50 % to slight winter decreases. Collectively, these data reveal substantial among-population variation in the thermal physiology of *P. mahali*, making this species a suitable model for exploring the contributions of phenotypic plasticity, adaptation and epigenetic effects to intraspecific physiological variation.

Testing the uncoupling of physiological maintenance costs and maximal thermogenic capacity in birds facing different thermal regimes

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In birds, seasonal acclimatization to cold is associated with increases in maintenance costs, measured as basal metabolic rate (BMR), and shivering cold endurance, measured as summit metabolic rate (M_{sum}). These parameters vary in tandem but recent evidence suggests that they may not be functionally dependent. Increases in M_{sum} are known to reflect changes in skeletal muscle, which become larger in the cold, while increased food consumption in winter would lead to high BMR through larger digestive organs.

In this study, we attempted to uncouple BMR and M_{sum} in captive White-throated sparrows (*Zonothricia albicollis*) by manipulating food quality (expected effect on food intake and BMR) and temperature (expected effect on muscle size and M_{sum}). Birds were acclimated to either -8°C or 28°C and received, in each temperature treatments, a "regular" diet (pellets for passerines) or a "diluted" diet (regular diet + 30% cellulose).

Results showed that birds consumed 100% more food in the cold than in the warm treatment, while food intake was 40% higher on the diluted diet than on the regular diet. Most internal organs were heavier (10-39%) in cold acclimated birds. The mass of pectoral muscles, gizzard and kidneys were influenced by diet only, while intestine mass was correlated with food intake. Results also showed that BMR increased with food intake, but was not affected by temperature, while size-independent M_{sum} was 18% higher in the cold but remained independent from diet or food intake. Our results therefore support the separate influence of food consumption on BMR and temperature on M_{sum}. Results on links between body composition and metabolic performance will also be discussed.

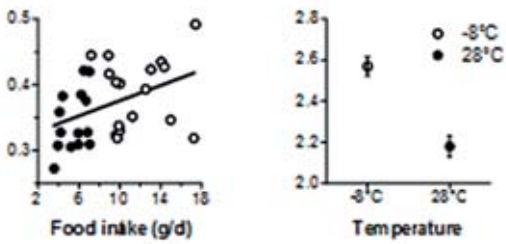

Figure: Effects of food intake and temperature on BMR and M_{sum}.

Increasing maximal metabolic rate improves intra-winter survival in small endotherms

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Small resident bird species living at northern latitudes increase their metabolism in winter and this is widely assumed to improve their chances of survival. However, the relationship between winter metabolic performance and survival has yet to be demonstrated. Using capture-mark-recapture, we followed a population of free-living Black-capped chickadees (*Poecile atricapillus*) over three years and evaluated their survival probability within and among winters. We also measured the size-independent body mass (M_s), hæmatocrit (Hct), basal metabolic rate (BMR) and maximal thermogenic capacity (M_{sum}) and investigated how these parameters influenced survival within and among winters. Results showed that survival probability was high and constant both within (0.92) and among (0.96) winters. They also showed that, while M_s, Hct and BMR had no significant influence, survival was positively related to M_{sum}, following a sigmoid relationship, within but not among winter. Birds expressing a M_{sum} below 1.26 W (*i.e.* similar to summer level) had less than 50% chance of survival while birds with a M_{sum} above 1.35 W had at least 90% chance of surviving through the winter. Our data therefore suggest that individuals that are either too slow or unable to adjust their phenotype from summer to winter have little chances of survival and thus that seasonal upregulation of metabolic performance is highly beneficial. This study is the first to document in an avian system the relationship between thermogenic capacity and winter survival, a proxy of fitness.

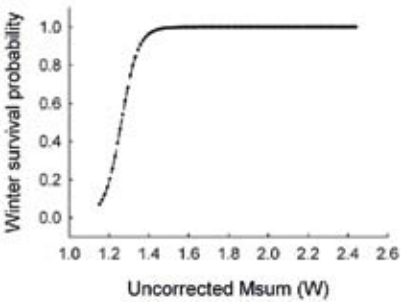

Figure: Relationship between winter apparent survival and winter uncorrected M_{sum}, with survival controlled for the effect of hæmatocrit

Flexibility of basal metabolic rate in response to short-term thermal acclimations and its within- and between-seasonal repeatability in Siberian hamster

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Basal metabolic rate (BMR) is a repeatable trait in most of animals studied, but we do not know whether its flexibility in response to changes in ambient temperature (T_a) is also repeatable and thus, whether it can be considered a trait responding to natural selection. BMR is adjusted in response to changes in T_a and its change affects the costs of living. We hypothesized that, if individual variations in physiological reaction norms are evolutionarily important, then flexibility of a trait should show consistent individual differences which might be selected for. We predicted that both BMR and its flexibility due to expositions to different T_as are repeatable within and between seasons. Male and female Siberian hamsters (*Phodopus sungorus*) were acclimated for 100 days to winter-like, and then to summer-like conditions, and after each acclimation were repeatedly exposed to 10 and 28°C for ~2 weeks. BMR was measured after each acclimation and exposition. Within-seasonal repeatabilities were estimated using intra- and inter-individual variance obtained from models adjusted for different T_a or not. Plasticity was calculated by subtracting repeatability coefficients obtained from models without adjusting for T_a from models with T_a as a factor. We tested if changes in BMR were reversible by correlating its absolute values. Between-seasonal repeatability of BMR and of its flexibility were estimated in models adjusted for body mass (m_b). BMR was repeatable within each season, and repeatability was higher in winter (r=0.59) than in summer (r=0.42). When adjusted for m_b, BMR was repeatable between seasons (r=0.34 after acclimation to 10°C; r=0.38 after acclimation to 28°C). Phenotypic flexibility was higher in summer (~25%) than in winter (<10%), yet it was repeatable between seasons (r=0.20) when adjusted for seasonal changes in m_b. The results support our hypothesis and show that not only BMR but also its flexibility is repeatable and may be a target for selection. We also suggest that BMR of seasonal rodents is more flexible in summer but more repeatable in winter, when energy conservation is most essential.

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Seasonal changes of the phenotypic flexibility of energetics in small mammals and a putative mechanism of its control

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Animal energy budgets comprise a complex of flexible traits, which are reversibly adjusted in response to changes in energy demands. We tested the hypothesis that flexibility in basal metabolic rate (BMR) varies seasonally because it is affected by seasonal changes in the animal's physiology. For example, at the onset of winter the body mass (m_b) and BMR of many small mammals decreases, their gonads regress, they grow a winter coat and some become heterothermic. These changes may affect the way animals react to changes in their thermal environment. We exposed three species of small rodents to ambient temperatures between 10 and 28°C after they were acclimated to winter and, subsequently, to summer-like conditions. In support of our hypothesis we found that energetics of Siberian hamsters (*Phodopus sungorus*) and house mice (*Mus musculus*) acclimated to winter was less flexible in response to changes in T_a than when acclimated to summer. Conversely, flexibility of BMR in Syrian hamsters (*Mesocricetus auratus*) did not differ between seasons. Body mass of all species was lower in winter than in summer. We propose two possible, mutually nonexclusive hypotheses to explain the seasonality of the observed flexibility. One is that increased secretion of melatonin (MEL) in winter leads to an increase in baseline levels of corticosteroid hormones (CORT). Increased baseline CORT levels would either reduce the possibility to respond to stress caused by changes in thermal conditions, or seasonally increased CORT would lower their secretion in response to the thermal stress. The second is that seasonal changes in *m*b, which should correlate with changes in energy use and optimize energy expenditure, reduce the potential responses to variations in thermal environment in winter. Data from the literature support the first hypothesis, since the physiology of Syrian hamsters, which do not show seasonal changes in flexibility, depends on MEL to a lesser extent than the physiology of Siberian hamsters and house mice. Similar seasonal changes of m_b in all three species, despite differences in flexibility of energy metabolism, also support the first hypothesis.

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Changes in blood parameters in response to short intense exercise in zebra finches

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Maximum aerobic performance is linked to high oxygen requirements that are typically met by immediate physiological adjustments, like for example sudden changes in blood parameters. Domesticated mammals, like dogs and horses, show higher hemoglobin concentration, larger hematocrit and red blood cell number immediately after short bouts of intensive exercise. In birds little is known about dynamics of hematological parameters in response to short but intense activity. Here we present a study on zebra finches (*Taeniopygia guttata*) that aimed at investigating the differences in hematological parameters between maximal performance and unexercised conditions. We analyzed hematological parameters and assessed BMR and MMR at flying for 5 minutes in flight hover wheel in 27 adult birds. We analyzed hemoglobin concentration, hematocrit, red blood cells number and cell area. Maximum oxygen uptake during flight exercise appeared to be 8 times higher on average in comparison to basal metabolic state of birds indicating a severe physical activity imposed by our treatment. Short intense exercise significantly lowered the hemoglobin concentration measured immediately after performance in the flight hover wheel ($p=0.02$). Hematocrit, red blood cells number and cell area were not affected, but tended to be lower in performing intense exercise ($p>0,06$ for all). In mammals, the increase in hematological parameters after intensive exercise is usually interpreted in terms of the so called 'splenic intervention', a release of a large number of stored red blood cells into the cardiovascular system. Our results suggest that birds may respond differently to short intensive exercise and possibly do not employ splenic intervention like proposed for mammals. Our data may rather be interpreted in terms of increased plasma levels and thus higher blood dilution possibly by shifts of interstitial fluid. Our results promote that flying birds may employ different mechanisms compared to running mammals to meet increased oxygen requirements during intense exercise.

Sponsored by Physiological and Biochemical Zoology

Immune function and physiology of a wild bird faced with food unpredictability

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Food supplementation to wild birds occurs across landscapes and over every season. However, natural food abundances may sometimes not be adequate. In winter, especially, natural food availability might be unpredictable whereas the availability of human provided food remains more constant. This variability in resources might then have a negative impact on immune function, as might occur if adequate energy and nutrients for mounting an immune response is not acquired. To test this idea we compared immune function and physiology of captive black-capped chickadees (*Poecile atricapillus*) with a constant and unpredictable food supply. Control birds received ad libutum food each day of the experiment; birds assigned to the unpredictable treatment received only 80% of their daily energy requirements on random days and ad libitum food on other days. Throughout the course of the study we examined multiple aspects of the immune system (constitutive and induced), body mass, fat and lean mass. Treatment birds maintained higher mass over the course of the study and had significantly higher fat mass compared to control birds. Preliminary results show that treatment birds mounted a weaker immune response to phytohemagglutinin and tended to have lower hematocrit compared with control birds. Results from this study demonstrate that during times of variable and uncertain food availability, like extreme weather events or long winters, birds might have compromised or weaker immune systems even if they maintain more body fat as a hedge against uncertainty.

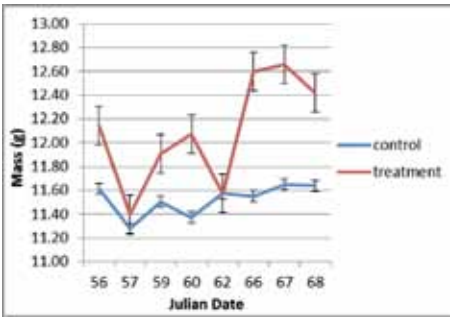

Figure: Mass (g) of control and treatment birds during the course of the study.

Repeatability of resting metabolic rate in *Myotis daubentonii*

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Physiological traits like e.g. resting metabolic rate (RMR), are often measured once and assumed to represent a far longer time span than covered by the actual measurement. This assumption is pivotal to relate RMR to major life history traits or environmental factors. The prerequisite for this is the repeatability of RMR over time where the repeatability describes the individual's consistency over time. A very short time span might lead to a high repeatability but probably only because the time was too short for the metabolism to change. Bats are exceptional small mammals as they are found at the fast end of the metabolic continuum but occupy the slow end of the life-history range. Repeatability of Chiropteran RMR would allow deep insights into their ecology, physiology and behaviour. In this study we assessed repeatability of RMR in *Myotis daubentonii* (n=55) using Pearson's product-moment correlation coefficient between subsequent measurements of an individual's RMR. Measurements were either short term (<20, 21-40, 41-60 or 61-80 days between measurements) or long-term (110-320, 355-1160 days between measurements). RMR was corrected for mass changes by regression analysis and residuals were used for further analysis. Up to 60 days RMR was repeatable ($p<0.05$). For longer time spans no correlation could be found. Depending on the flexibility of a trait, the repeatability would generally be expected to decrease with an increase in the time period over which it is measured as has been found here. Most studies of repeatability are conducted in the laboratory which might inflates estimates of reputability due to stable conditions. Repeatability measurements with wild animals are few but essential for the relation of RMR to life history and the environment.

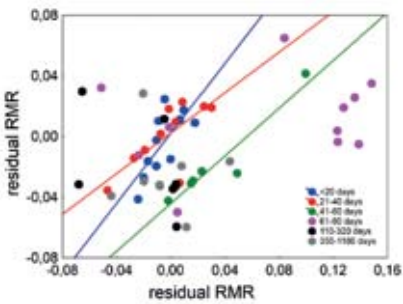

Figure: Repeatability of body mass-corrected resting metabolic rate (RMR) estimated as Pearson's correlation.

Black or white, that is the question: roost selection and the expression of heterothermy in microbats

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Small scale variations in climate and temperature can strongly impact the behaviour and thermal biology of small mammals, particularly in terms of nest/roost choice. Microbats inhabit a variety of ecological niches and use a range of roosts that have very different thermal characteristics in order to fulfil varying requirements. However, little is known about whether tree-roosting bats actively choose roosts based on thermal characteristics of the roost and the potential energetic benefits of this behaviour. We quantified the thermal biology of a captive Australian microbat, *Nyctophilus gouldi* (n=7, ~10g) using temperature telemetry, to determine how roost choice influences use of heterothermy. All individuals were housed together in an aviary exposed to natural fluctuations of ambient temperature and offered the choice of black- or white-coloured roost boxes that were well insulated and otherwise identical. Black boxes were on average 4˚C (maximum 7.5°C) warmer than white boxes at their maximum daytime temperature. Bats chose black boxes on most nights (90.7%); only two individuals chose a white box on one night over the course of the study, immediately returning to a black box on the following evening. To allow for a comparison in expression of heterothermy use between black and white boxes, black roosts were removed after 8 days. Bats rewarmed to normothermia at midday on 78% of days when using black boxes, and only on 65% of days when using white boxes. Independent of box colour, bats always used passive rewarming before they actively aroused in the day. Bats in black boxes began active arousal at a higher temperature (19.2˚C ± 3.2˚C) than bats in white boxes (16.2˚C ± 2.3˚C) and 30 min earlier (Black*:* 11:43 hours ± 21 min, White*:* 12:13 hours ± 21 min). Our study demonstrates that when offered food *ad libitum,* this microbat selects warmer roosts in order to passively rewarm to higher body temperatures and thus save more energy prior to active midday arousals.

Sponsored by Physiological and Biochemical Zoology

Adult phenotypic plasticity in thermogenesis: A comparative study using high and low altitude deer mice.

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High altitude is one of the most extreme environments experienced by terrestrial mammals due to both low ambient temperatures and oxygen availability. Deer mice native to high altitude have a greater thermogenic capacity in hypoxia compared to a lowland population, likely as a consequence of both genetic adaptations and phenotypic plasticity[1]. To understand the adaptive variation in phenotypic plasticity, F1 generation lab-reared mice were acclimated to chronic hypoxia, cold, and hypoxia+cold.

Acclimation led to equal increases in thermogenic capacity in hypoxia for all stressors in high altitude deer mice. The resulting VO_2 summit after acclimation was equivalent to wild mice previously measured *in situ*[1]. Low altitude mice also increased their thermogenic capacity after acclimation to hypoxia. This heightened ability is equal to that of the unacclimated high altitude mice. Together these findings suggest that the increased thermogenic capacity of the wild mice is based both on differences in phenotypic plasticity, and on differences in genotype.

VO_2 summit in hypoxia was supported by lipids in deer mice even though carbohydrates would provide an oxygen saving advantage. Also, rates of lipid oxidation increased after acclimation to cold, and cold combined with hypoxia.

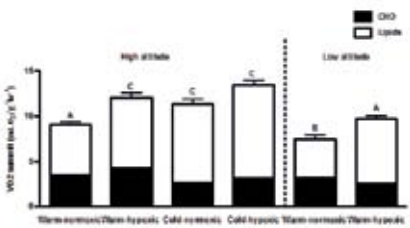

Figure 1. Summit VO_2 in cold and the contributions of lipids and glucose to total fuel use during VO_2 summit, tested at 12% oxygen. Letters denote changes in VO_2 summit, columns sharing a letter are not significantly different.

[1]Cheviron, Z.A., Connaty, A.D., McClelland, G.B., & Storz, J.F. (2013). Functional genomics of adaptation to hypoxic cold-stress in high-altitude deer mice: Transcriptomic plasticity and thermogenic performance. *Evol*. 68, 48-62.

King penguins rewarm their skin temperature to normothermia during the night at sea: A descriptive study in condition of captivity

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Pelagic diving birds of high latitudes experience high energetic challenges during their foraging trips at sea and even greater expenditure during their periods of inactivity. The king penguin (*Aptenodytes patagonicus*) spends more energy during the night, when it is resting, than during the day, when it is hunting for prey. This species is also known to spare energy during diving, thanks to a general hypothermia, in both deep and peripheral tissues. However at sunset a rewarming to normothermia, that concerns also the skin, contributes to increase heat-loss during all the night

To explain this apparent paradoxical strategy during night at sea, we hypothesize the existence of an energetic conflict between thermoregulatory and digestive processes. During daylight, birds are likely unable to assimilate the free fatty acids (FAT, the end product of prey digestion) in their peripheral subcutaneous adipose tissue (SAT). It is the expected consequence of the reflex reduced peripheral blood perfusion that contributes to reduce heat-loss. In contrast, during the night, a reconnection of blood circulation to peripheral tissues may be the necessary way to allow the incorporation of FFA inside the SAT. The inevitable consequence would be a local rewarming and a dramatic increase of peripheral heat loss.

In a first step, we have tried to mimic the conditions of a resting night at sea and events of rewarming skin temperature, using a water tank in which feed king penguins equipped with four internal temperature tags were maintained 48 hours. In a second step, we have tested a generalisation of our hypothesis studying body temperature variations on penguins fat and feed or in situation of long-term fasting.

Differences in growth, fattening and energetic strategies between early and late-born juvenile garden dormice (*Eliomys quercinus*)

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Timing of breeding is a key of reproductive success in seasonal environments. The decline of food resources and ambient temperatures over summer, conflicts with the needs of offspring for growth, especially in late-breeders. One adaptive strategy for late-born offspring is to exhibit higher-than-normal growth rates. Prior to winter, while ensuring their structural growth, juvenile hibernators have to accumulate sufficient fat reserves to ensure their overwintering survival. In particular, late-born juveniles can use short bouts of torpor to counteract intermittent periods of fasting and to ensure optimal fattening prior to hibernation.

In this study, we aimed to investigate differences in (i) rate of growth and pre-hibernation fattening, (ii) body mass and body size prior to hibernation, and (iii) energy-saving strategies such as use of short torpor bouts, between early-born (mid-Mai) and late-born (mid-July) female juvenile garden dormice according to food availability. Juveniles were either intermittently fasted or fed *ad-libitum*, and kept individually under natural photoperiod and ambient temperature. We found that late-born juveniles grew and gained mass twice faster than early-born individuals. Despite being smaller at the start of the experiments, late-born juveniles reached similar maximal body size, but a 10% lower body mass prior to hibernation, suggesting lower pre-hibernation fat reserves, compared to early-born animals. Interestingly, neither early nor late-born juveniles entered torpor during the first weeks of growth, but torpor use substantially increased in late pre-hibernation fattening. However, fasted late-born juveniles, but not well-fed individuals, extensively increased their use of torpor from the start of their growth period, when exposed to low ambient temperatures.

We conclude that late-born juveniles prioritize structural size over body (fat) mass prior to hibernation. Further, the use of torpor seems to conflict with the process of growth, but is increased in response to declined ambient temperatures and food availability.

Torpid in the city: hibernation ecology of the European hedgehog in urban habitat

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We have very little understanding as to why some species thrive in urban environments and others perish. Those successfully tolerating urbanisation likely take advantage of various biological traits that allow a quick response to environmental change and disturbance. Torpor is the most powerful tool used to adjust energy expenditure but its role in the success of small mammals in urban habitat is unknown. To address this knowledge gap we investigated the hibernation ecology of the European hedgehog *Erinaceus europaeus* in a large city in northern Germany. We monitored torpor patterns and nest site use with temperature-sensitive transmitters and data loggers. Preliminary results show that hibernation lasted from November to May (mean ambient temperature 5.5°C ± 4.6°C) and hedgehogs usually chose nest sites located under bushes or piles of leaves in private gardens adjacent to parks. Individuals' torpor patterns differed considerably despite the close proximity of their hibernation sites; we measured both large daily fluctuations in skin temperature as well as more stable torpid temperature profiles. Periodic arousals commenced around 22:00 h with a rewarming rate of 0.08°C/min, followed by a normothermic period of about six hours, before torpor was entered again at a cooling rate of 0.01°C/min. Hedgehogs changed nest sites during early and late hibernation and roads were routinely crossed. In late hibernation south-facing nests were chosen, probably to profit from the energetic benefits of passive rewarming. Our data indicate that urban hedgehogs show a long hibernation season with marked individual differences in torpid skin temperature patterns and change their nest sites occasionally. Flexibility in torpor use is therefore likely an important trait for the success of hedgehogs in the city as it allows the physiological adjustment of energy expenditure in a highly artificial environment.

How are birds dealing with hot environments?

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Global climate has been changing and it is becoming increasingly unpredictable. For example, by 2050, temperatures in Africa are predicted to increase by 1.5 to 3°C, and are projected to continue to increase beyond this time. Ambient temperature is a very important factor in an ecological context and a shift in mean temperatures can potentially be challenging for many populations of different animal species. Any increase in temperature above the upper critical temperature of the thermoneutral zone would require energetically costly processes to remain in the thermal comfort zone. This is in accordance with the Heat Dissipation Limits (HDL) theory that suggests a trade-off between the cost of down-regulating body temperature and fitness costs of operating at supra-optimal body temperatures. In the context of global warming the ability of animals to dissipate heat might be a crucial trait leading to increased fitness. This problem is especially important in the case of birds which produce a lot of extra heat when flying.

We tested core (using a thermocouple) and peripheral (eye temperature, using infrared camera) body temperature (Tb) of zebra finches *(Taeniopygia guttata)* kept for two weeks in three different temperatures: 5 °C, 20 °C and 35 °C degrees. The core T_b differed significantly between treatments and the difference in mean body temperature between the coldest and the hottest treatment was around 2°C. We found a significant correlation between core and peripheral temperature values, which suggests that eye temperature is a good proxy of internal body temperature. In 35 °C, birds were not able to down-regulate their body temperature below 42°C. The range of T_b in the hottest treatment was also much narrower which suggests stronger metabolic constrains in this environment. A consequence of an inability to cool down below 42.5 °C would be accelerated biochemical reactions caused by thermal conduction. Accelerated biochemical reactions may potentially lead to increased MR and subsequently higher temperature as a side effect. This shows that maintaining the optimal body temperature can be very challenging for birds in hot environments and might influence their fitness.

Energy budgets across a climatic gradient in two sportive lemur species (*L. leucopus* and *L. ruficaudatus*)

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Consequences of global climate change are expected to be most pronounced in extreme habitats such as the dry regions of southern and western Madagascar, with increasing temperatures and decreasing precipitations. Across this area there is already a strong north-south gradient with increasingly harsh dry periods and more unpredictable precipitation together with a decrease in food quality in the south. All habitats are characterized by high diurnal and seasonal fluctuations in ambient temperature. Animals show different adaptions to cope with these environmental conditions e. g. by lowering their metabolic rates, and our study aimed to elucidate potential within-genus differences in adaptive potential in Malagasy lemurs. *Lepilemur leucopus* (500 – 700g) the smallest member of the Lepilemuridae family is living in the spiny forest of the very south of Madagascar, the bigger sister species *L. ruficaudatus* (600 – 900g) is distributed across the deciduous dry forest of West-Madagascar. Using indirect calorimetry we analysed relationships between ambient temperature and metabolic rates on wild-caught individuals during the – physiologically more challenging – dry season (July – August 2014). *L. leucopus* and *L. ruficaudatus* show one of the lowest weight-specific metabolic rates measured so far for mammals, presumably due to their folivorous diet and as an adaptation to their unpredictable habitats. Corresponding to the general physiology allometric correlation, the smaller *L. leucopus* has a higher weight-specific metabolic rate than *L. ruficaudatus*. We did not detect any signs of heterothermic episodes in either species. Our data highlight the importance of careful considerations when drawing conclusions between – even closely related –species and ultimately could be used to create a model to predict the future of these species and support their conservation especially in the light of climate change and global warming.

The importance of phenotypic plasticity for locomotion and thermogenesis in high altitude deer mice

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Effective locomotion or thermogenesis in cold and hypoxia at high altitude are the result of genetic adaptations and adaptive variation in phenotypic plasticity. We used F1 generation low and high altitude (LA and HA) deer mice to test the hypothesis that acclimation to hypoxia, cold or both induces phenotypic plasticity, distinct with altitude ancestry. We measured exercise- and cold-induced aerobic capacity (VO_2max) and submaximal exercise fuel use. Skeletal muscle, brown adipose tissue (BAT) and white adipose tissue (WAT) were assessed for phenotypic plasticity in response to acclimation.

Acclimations had distinct effects on whole-animal performance measures and on subordinate traits in both populations of mice. We found that acclimation to hypoxia increased reliance on carbohydrate oxidation during submaximal running in HA mice. This was associated with increased in skeletal muscle hexokinase activity and in transcripts levels for GLUT transporters. Hypoxia, cold and both combined increased cold-induced VO_2max and non-shivering thermogenesis, but did not affect brown adipose tissue mass in LA and HA mice. However, the expression in uncoupling protein (UCP)-1 was increased in BAT and significant plasticity was also induced in WAT of HA mice. These data show that phenotypic plasticity is affected by altitude ancestry and is an important component of adaptation to this extreme environment in deer mice.

Phenotypic flexibility and thermal tolerance in the Chilean rodent *Phyllotis darwini* from populations inhabiting the species geographical range boundaries

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Several studies have shown an important increase of approximately 0.7 °C in average global temperatures over the past century, and predict substantial acceleration in future warming rates. In turn, it has been suggested that species that have evolved the greatest absolute thermal tolerances have done so at the expense of maintaining the acclimatory ability of this tolerance. Thus, the extant more thermo tolerant species would paradoxically be most likely to be at threat by increasing temperatures. Despite the analysis of physiological tolerances along geographical gradients that mimic anticipated warming trends, can illuminate how organisms might respond physiologically to climate change, the measures of physiological tolerance has been mainly restricted to ectothermic species. Due to their dependence with environmental temperatures and influence on the distribution boundaries in endothermic species, we measured two physiological variables related to animals' tolerance: thermal tolerance (CTmin, CTmax) and metabolic rates (BMR, Msum). These variables were measured in rodents (*Phyllotis darwini*) from the northernmost and the southernmost limit of their distribution in Chile. We found differences between populations in the metabolic scope and limits of thermal tolerance. These results suggest that more studies are needed to know the mechanistic causes of climate-related distribution patterns.

Seasonal Control of Mammalian Energy Balance

Organizers (*Deutsche Zoologische Gesellschaft*):

Annika Herwig, University of Hamburg, Germany
Martin Jastroch, *German Research Center for Environmental Health, Germany*

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Hibernation and torpor – control and plasticity of metabolic depression in mammals

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Torpor is a powerful behaviour for reduction of energy expenses in mammals and birds. It is characterized by a pronounced reduction of metabolic rate (plus heart and ventilation rate) and is adjusted to a fraction of basal metabolic rate. Entrance into torpor is initiated by metabolic rate depression and remains under thermoregulatory control. This indicates that torpid animals remain to be endotherms, but can alternate between a euthermic tachymetabolic state and a torpid bradymetabolic state with largely reduced body functions.

Hibernation is characterized by multi-day torpor bouts and a suspension of all behavioural activities during the hibernation season, thereby reducing energy requirements by more than 95%. This is contrasted by daily torpor which may reduce energy requirements by about 60%. However in contrast to hibernation daily torpor allows maintenance all social, territorial and other behavioural activities of a small mammal.

The biochemical mechanisms underlying metabolic depression are not fully resolved yet. Several studies suggest rather uniform reductions of cellular metabolism in hibernation and daily torpor, focusing on depression of glycolysis and depression of transcription, translation and protein degradation. Thus, the turnover of cellular proteins is slowed down and cell proliferation and differentiation comes to a halt.

The environmental cues for the induction of torpor behaviour as well as the endocrine signaling of metabolic depression are still a mystery. Daily torpor in small mammals, esp. laboratory mice, often occurs as an immediate response to food withdrawal. This provided first incidence that the neuroendocrine circuitry involved in food regulation (NPY, ghrelin), sympathetic activity, and the control of thyroid hormone activity are involved in the control of torpor behaviour and metabolic depression.

Energetics of torpor and hibernation: Mechanisms and beyond

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Many small mammals and birds have developed specific mechanisms of energy saving, achieved by active and controlled reduction of metabolic rate and hence body temperature (T_b), i.e. daily torpor and hibernation. Despite two centuries of research on torpor, physiological mechanisms that regulate this overwintering strategy are still poorly understood. Several factors are known to affect the expression of torpor. Among intrinsic ones, gut-produced hormones are of increasing interest for their potential roles in regulating energy and lipid balances. For instance, glucagon-like petide-1 (GLP-1) is though to be related to the modulation of torpor expression. Plasma GLP-1 was negatively associated with torpid minimal T_b in food-restricted mouse lemurs in summer. No correlations were found in individuals in winter. Among extrinsic factors, dietary intakes of polyunsaturated fatty acids (PUFA) have strong impacts on torpor bout duration, minimal T_b tolerated and energy savings. Because of their high proneness to peroxidation, heterotherms would face a trade-off between beneficial PUFA effects on membrane function at low T_b and cellular damage due to lipid peroxidation. Under food restriction, mouse lemurs in winter shifted the dietary fatty acid oxidation toward saturates and spared PUFA, without increase of oxidative damage, during increased expression of torpor. Conversely, such shift was not observed in summer individuals, which limited their use of torpor. At the molecular level, PUFA are thought to act via effects of the membrane fatty acid composition on sarcoplasmic reticulum (SR) Calcium ATPase (SERCA) in the hibernator's heart. This trans-membrane pump is responsible for removing calcium into the SR and hence for continued cardiac function even at extremely low Tb. We found evidences for up-regulation of SERCA activity by higher proportions of Linoleic acid and lower amounts of Docosahexanoic acid in torpid Syrian hamsters, compared to summer and winter euthermic individuals. Animals with increased SERCA activity also reached lower T_b in torpor. Further work is now ongoing to determine how PUFA act on torpor bout duration and what the consequences on ageing processes during winter are.

Photoperiod, season, torpor and fat

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Seasonal changes in thermal physiology and torpor expression of many heterothermic mammals are regulated by photoperiod. Torpor is characterized by substantial, controlled reductions in metabolic rate (MR) and body temperature (T_b) and clearly differs from hypothermia in having a reversed hysteresis between T_b and MR during cooling and rewarming. As function at low T_b during torpor requires changes in tissue lipid composition, we examined whether and how fatty acids are affected by photoperiod acclimation in hamsters, *Phodopus sungorus*, a strongly photoperiodic species, and how compositional changes in fatty acids are related to changes in morphology and thermal biology. Hamsters under short photoperiod had smaller reproductive organs and most had a reduced body mass in comparison to long photoperiod hamsters. Fur colour of hamsters under short photoperiod was almost white, whereas that of long photoperiod hamsters was brown. Short photoperiod acclimation resulted in regular (28% of days) spontaneous torpor use by all individuals even at a mild ambient temperature of 18°C, whereas all hamsters in long photoperiod remained normothermic. The composition of total fatty acids differed between acclimation groups for brown adipose tissue (5 of 8 fatty acids), heart muscle (4 of 7 fatty acids) and leg muscle (3 of 11 fatty acids). Moreover, the percent composition of 54% of all fatty acid detected was correlated with the mean skin temperature of individuals. While some of the compositional changes of fatty acids were consistent with a homeoviscous response, this was not the case for all fatty acids, and the proportions of saturated and unsaturated fatty acids did not differ between acclimation groups. Our study confirms that the seasonal change in morphology and thermal biology of the species is largely controlled by photoperiod and provides new evidence that some of the functional changes are linked to the fatty acid composition of tissues and organs.

The somatostatin agonist pasireotide reveals a pivotal role for the pituitary in seasonal body weight change and energy metabolism

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The Siberian hamster is an exemplar for the range of physiological adaptations made by seasonal mammals. The hamster reduces body weight and undergoes involution of the reproductive organs when transferred from long days (LD, 16h light:8h dark) to short days (SD, 8h dark:16h light). Body weight loss is composed of a reduction in both lean and fat tissue which includes the weight of internal organs such as the liver and brown adipose tissue (BAT). Upon return to LD hamsters re-establish LD physiology. At around 12 weeks in SD, hamsters initiate spontaneous torpor, a strategy to reduce energy expenditure. The mechanisms underlying seasonal physiology are poorly understood. We hypothesised that changes in body weight is governed by regulation of the growth hormone (GH) axis.

Male hamsters were administered with long acting release pasireotide a somatosatin agonist designed to inhibit GH release from the pituitary. Pasireotide administered to LD hamsters caused a gradual reduction in body weight over 7 weeks with reduction in lean and fat tissue mass including testes and BAT. Pasireotide administered to hamsters previously acclimated to SD and switched back to LD retarded the increase in body weight. In SD hamsters, pasireotide increased the frequency of torpor bouts from 2-3 bouts per week to 5-6 bouts per week. Pasireotide treatment reduced circulating IGF-1 and resulted in increased growth hormone releasing hormone mRNA expression in the arcuate nucleus and reduced expression of somatostatin mRNA in the periventricular nucleus, indicative of reduced GH feedback to the hypothalamus. These data suggest neuroendocrine regulation of the GH axis may be involved in regulation of seasonal body weight. In addition the seasonal hypothalamic-neuroendocrine axis may have a role in regulating peripheral fat masses including BAT and torpor frequency.

The metabolic response to season of Eurasian red squirrels (*Sciurus vulgaris*) in semi-urban habitat

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Small homeothermic species inhabiting seasonal environments need to find cost-effective means of balancing energy budgets while maintaining a high body temperature throughout the year. Often these strategies include changes in body mass and metabolism and to cope with the energetic bottlenecks of winter individuals store energy as fat or in food caches. While annual fluctuations in ambient temperature and food resource availability demand physiologcial flexibility of species living in natural habitat, extremes are often buffered in urban environments. Therefore, we investigated whether Eurasian red squirrels (*Sciurus vulgaris*; 360 g) balance the energetic challenges of changing seasons with adjustments in thermal physiology. Using open-flow respirometry we measured resting metabolic rates (RMR) in each season of squirrels inhabiting a large city park, where natural and human-provided food is available year-round. Our results indicate BMR was higher and the lower critical temperature of the thermal neutral zone was lower in winter than in summer, however thermal conductance varied little among seasons. Interestingly, body mass did not change throughout the year. This physiological response suggests that seasonal ambient temperature fluctuations and food availability trigger changes in the squirrels' thermal energetics. They likely balance energy expenditure by reducing activity during colder times, which enables them to maintain a high body mass year-round. Such a response probably benefits squirrels and their adaptation to urban habitat and is potentially common among species that are successful in modified environments.

Energy budgets of *Lepilemur leucopus*: Physiological adaptation to extreme seasonality

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Energy is the currency for all biological processes and thus fundamental for the understanding and prediction of abundance and distribution of species. Particularly folivorous small mammals have to balance between energy uptake and expenditure, due to a diet low in nutrition and a costly homeostasis of body temperature. Hence, many species use physiological energy saving strategies like daily torpor or hibernation during unfavorable seasons. This study presents first data on physiological seasonal adaptations of the white-footed sportive lemur (*Lepilemur leucopus*) in the dry season (Malagasy winter), which is usually accompanied by colder ambient temperatures and food shortage. *L. leucopous* is a nocturnal, small bodied and strictly folivorous primate. The species is endemic to Madagascar and lives in one of the driest and climatically most unpredictable parts of the country. The resting metabolic rate (RMR) of 14 free ranging individuals was measured in the wet and dry season using indirect calorimetry. Although RMR in the dry season was continuously higher than in the wet season, no daily torpor or hibernation was found. In the wet season, the thermoneutral zone (TNZ) was well defined between 25 and 30° C and the species showed only 50 % of the mass-specific prediction value. In the dry season, however, RMR accounted to 92 % of the prediction value without showing a distinct TNZ. Thus, *L. leucopus* does not seem to be able to use physiological energy saving strategies to overcome unfavorable or unpredictable periods, and might thus be particularly vulnerable to degrading conditions due to climate change and consequences in resource availability and quality. Studying the physiological response to different seasons under natural conditions assists in drawing general conclusions regarding the survival of this endangered lemur species.

Seasonal regulation of physiological, biochemical and hormonal parameters in a Neotropical marsupial, *Dromiciops gliroides*

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The endotherm organisms that periodically face high energy demands and nutritional bottlenecks require of an integrated programming in the regulation of their seasonal physiological activity. This regulatory strategy includes the variation in gene expression, metabolic and biochemical metabolites and hormonal changes related to energy use. Our study represents the first evidence of seasonal torpor changes operating on a Neotropical marsupial using different approaches; physiological, biochemical and hormonal. In particular, we determine seasonal changes in body mass (B_m), leptin, biochemical parameters, metabolism and respiratory quotient (Rq) of *D. gliroides* under controlled conditions (T_{env} = 20 °C, natural photoperiod) and semi-natural (T_{env} and natural photoperiod). The results suggest that *D. gliroides* exhibits short periods of torpor, and its duration and depth depend mainly on the T_{env}. The B_m showed a significant positive correlation with leptin, and a strong seasonal effect, mainly during summer. Proteins and lipid metabolites such as beta-hydroxybutyrate and cholesterol showed similar patterns (reduction in summer). Furthermore, creatinine showed an inverse pattern with higher values occurring in summer. Finally, metabolism also had higher seasonal effect: only RMR, the slope and Rq, showed treatment effect. In conclusion, the ability of *D. gliroides* to metabolize different nutrients appears to be not completely determined by the physiological state of the animal and certainly there is an endogenous rhythm in the use of metabolic substrates used as nutrients. In general, the effect of photoperiod on the incidence of torpor was more evident that the influence of T_{env}, confirming some patterns observed in hibernating eutherians.

Influence of gonadal hormones on adrenocortical function in the sand rat *Psammomys obesus*

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The adrenal cortex exerts a wide range of biological effects, particularly with regard to the stress response and regulation of overall metabolism. The wild sand rat *Psammomys obesus,* presented seasonal variations of testicular activity in synergy with the adrenal cortex activity. The present study was undertaken to evaluate the effect of testicular androgens on adrenal cortex function, apoptosis process and androgen receptor immunolocalization. Three groups of animals were used in this study: castrated animals, castrated animals subjected to testosterone replacement and intact males as a control group. Adrenal apoptosis was analysed by *in situ* end labelling of fragmented apoptotic nuclei DNA and immunohistochemistry by using androgen receptor antibody. Our results indicate that the highest apoptotic index was detected in the inner zones mainly in the *zona reticularis*. However, androgen deprivation upregulated the incidence of apoptosis within the *zona fasciculata* and decreased it within the *zona reticularis*. In control animals, high androgen receptor immunostaining was observed in *zona glomerulosa* and outer *zona fasciculata*. Nevertheless, androgen deprivation induced an increase in the labelling index in the *zona fasciculata*. Testosterone replacement performed in castrated sand rats restored most of control observations. These results indicate that, in the sand rat, male sexual hormones have a modulatory effect mediated by androgen receptors on the adrenocortical activity. This modulation served as a mechanism to maximize reproductive fitness and survival of this species in the harsh desert environment.

Transcriptome analysis of hypothalamic gene expression during torpor entrance in Djungarian hamsters

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In habitats with pronounced annual changes in environmental conditions, animals have to develop strategies to cope with extreme requirements like low ambient temperature and reduced food availability. Therefore Djungarian hamsters exhibit multiple adaptions to winter. The most extreme measure of this animal to maintain energy balance in times of food shortage is the expression of spontaneous daily torpor. Daily torpor is a voluntary, normoxic hypometabolism that reduces energy requirements by up to 70% as compared to hamsters that remain active all the time. The hypothalamus has been proposed to play a role in the regulation of daily torpor. However, the endocrine and neurological signals, which initiate the metabolic depression during the entrance into torpor, are still unknown. To uncover genes that are involved in the initiation of daily torpor, we investigated the hypothalamic transcriptomes of torpid hamsters during the entrance into torpor (ZT 1, $T_b \leq 31$ °C) using Illumina sequencing. About 28 000 genes were detectable (RPKM ≥ 0.1) in the hypothalamus of Djungarian hamsters. The expression of 284 genes was significantly altered during entrance into the torpid state. 181 genes were up- and 103 down-regulated. Analysis of gene ontology patterns and most strongly regulated transcripts show evidence of remodeling and plasticity during torpor entrance. Five of the twenty most strongly up-regulated genes were collagens which are major extracellular matrix structural constituts. Additionally Dnha2, Micalcl and Myo15a, components of the cytoskeleton, rank among the most strongly up-regulated genes. Furthermore five zinc finger proteins belong to the most down-regulated genes. Zink finger proteins are proposed to be involved in transcriptional regulation. These results provide an overview of gene expression pattern during the initiation of torpor and support the hypothesis that the hypothalamus plays a key role in the regulation of this extreme physiological phenomenon.

The spatial ecology of Eurasian red squirrels is adjusted in relation to food type and abundance

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The energy budget of an animal species is closely linked to its ecology and balancing energy expenditure with energy acquisition is key for survival. Therefore, biotic and abiotic environmental changes, whether natural e.g., season or anthropogenic e.g., urbanisation, can be challenging. While some species decline in numbers or disappear altogether from urban habitat, the Eurasian red squirrel (*Sciurus vulgaris*) successfully lives in cities. Since squirrel space use is influenced by the distribution and abundance of major food resources, changes in movement patterns are likely to reflect responses to modifications in environmental conditions affecting these resources. To investigate these relationships, home ranges and habitat use of squirrels inhabiting a large city park in northern Germany were measured via radio-telemetry and related to both natural and anthropogenically-provided food sources. The squirrels' home ranges encompassed areas with year-round supply of supplemented food but they adjusted their activity and shifted home range core areas closer to natural food in seasons where supplemented food supply was less reliable. Consequently, both food type and abundance had a measureable effect on the space use of individuals. Additionally, heavier individuals' core areas were located closer to supplemented food, whereas the core areas of lighter animals were closer to natural food resources, potentially indicating an effect of food energy content on body condition. The results of our study suggest that squirrels' movement patterns and feeding habits, and hence energy balance, can be directly affected by human activity. They also illustrate that the consequences of urbanisation are not always detrimental for native animal species and thus an improved knowledge of available food resources and their effect on habitat use is important for understanding the ecology and energetics of urban wildlife.

Effects of wintertime fasting and seasonal adaptation on AMPK and ACC in hypothalamus, adipose tissue and liver of the raccoon dog (*Nyctereutes procyonoides*)

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The raccoon dog (*Nyctereutes procyonoides*) is a canid with autumnal fattening and superficial winter sleep. We used farm-bred raccoon dogs to examine the effects of wintertime fasting and seasonality on AMP-activated protein kinase (AMPK), a regulator of energy balance, and its target protein, acetyl-CoA carboxylase (ACC). Total AMPK, phosphorylated AMPK (pAMPK), total ACC and phosphorylated ACC (pACC) were measured by Western blot from hypothalamus, liver, intra-abdominal and subcutaneous white adipose tissues (aWAT and sWAT) of three experimental groups: winter fasted, winter fed and autumn fed. Plasma leptin, ghrelin, insulin and adiponectin levels were also measured. AMPK expression was lower in hypothalamus, sWAT and aWAT of both winter groups, with a parallel decline in pAMPK levels in adipose tissues. pACC level was higher in hypothalamus and lower in sWAT of the winter groups compared to the autumn fed group. Liver pAMPK was lower in the winter fasted group, with a parallel decrease in ACC and pACC. Plasma leptin levels followed a seasonal pattern with lower levels in the winter groups. Insulin and ghrelin concentrations were lower in the winter fasted group. It seems that the functions of AMPK and ACC are regulated in a season-dependent manner in the raccoon dog. Furthermore, the central regulation of ACC might be disconnected from AMPK. The responses of AMPK and ACC to wintertime fasting were quite opposite to the effects observed previously in non-seasonal mammals and also differed from hibernating species. The distinctive effects of prolonged fasting and seasonal adaptation on AMPK–ACC pathway could be characteristic of the wintering strategy of the raccoon dog.

Role of the 5-HT2B/2C serotonin receptor agonist in the cross-talk between ghrelin and serotonin in sheep: Altering effects of photoperiod and nutritional status

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In temperate latitudes, sheep are seasonal breeders whose reproductive activity and food intake are regulated mainly by photoperiod. Ghrelin and serotonin are hormones that regulate appetite and energetic homeostasis and also interact with melatonin. It is presumed that serotonin and its 5-HT2B/2C receptor modulate ghrelin secretion. To verify this hypothesis in vivo experiments were conducted using 5-HT2B/2C receptor angonist (m-CPP). The experiments were carried out on 16 ewes of the Polish Longwool Sheep breed, ovarectomized with subcutaneously inserted estradiol implants (fasted for 24-h and fed *at libitum*). Intravenous treatments consisted of saline - Control group (CG1); ghrelin (2.5 µg/kg b.w., GHRL) – Exp. group 1 (Gr 1); m-CPP (2.5 mg/kg b.w.) – Exp. group 2 (Gr 2) and GHRL and after 15 minutes m-CPP – Exp. group 3 (Gr 3). The experiment was carried out during the short and long photoperiod. Blood samples were collected every 15 minutes during 3 hrs. Blood plasma GHRL was assayed using ELISA kit. In the group of fed sheep i.v. injection of mCPP strongly decreased ($P<0.01$) GHRL concentration during SD and LD compared to CG1 and Gr1 groups. After GHRL and mCPP infusion concentration of GHRL was significantly ($P<0.01$) decreased compared to Gr1 and CG1 during SD and LD seasons. During LD photoperiod in fasted group of sheep GHRL caused significant ($P<0.01$) increased in GHRL concentration compared to other groups. Injection of mCPP decreased ($P<0.01$) GHRL concentration compared to CG1 and Gr1 groups. The increased ($P<0.01$) in GHRL concentration was observed after GHRL and mCPP injections compared to Control group. Study demonstrated that activation of 5-HT2B/2C receptor inhibits GHRL secretion in fed ewes during long and short day. In SD activation of 5-HT2B/2C receptor inhibits GHRL secretion regardless of metabolic status. To conclude: GHRL secretion is regulated by 5-HT2B/2C receptor in seasonally breeding ewes depending on photoperiod and metabolic status.

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The advantage of gluttony: Digestive efficiency in insectivorous bats

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The digestive tract is the functional link between food as an energy source and metabolizable energy. Digestive efficiency may change due to variable organ size, structure, enzyme activity, or retention time in response to energetic demands of the animal. Efficient digestive tracts may be better at processing food but require higher energetic investments for maintenance even when post-absorptive. Digestive flexibility has been proposed as a widely used physiological adjustment in response to increases in energy demand, for example during pregnancy and lactation. In this study we hypothesized: 1) the amount of consumed food correlates positively with digestive efficiency and 2) a longer retention time incurs a higher digestive efficiency. We used male, female, and juvenile *Myotis daubentonii* as study organisms. Bats were fed with mealworms of which one was marked with metabolically inert colour pigments to record retention time. Feeding trials were conducted in two consecutive nights. In the first night food was limited while in the second trial food was offered *ad libitum*. Digestive efficiency correlated positively with the consumed amount of food. This suggests that maintenance of a very efficient digestive system is more costly than the net input energy when food is limited leading to a negative energy balance. As this needs to be a very flexible mechanism the probability of anatomical changes is low but a change in retention time might increase digestive efficiency. However, we could not find a correlation between digestive efficiency and retention time suggesting that enzymatic activity is regulated to the amount eaten. This study underlines the tight energetic budget of bats and their flexible regulation mechanisms.

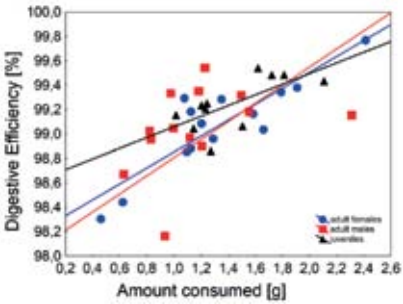

Figure: Positive correlation between digestive efficiency and the amount of consumed mealworms.

Cellular Stress Tolerance, Longevity, and Nutrition

Organizers (*Deutsche Zoologische Gesellschaft*):

Rüdiger J. Paul, *University of Münster, Germany*
Thomas Roeder, *University of Kiel, Germany*

Endogenous functions of cytochromes P450 and stress response in the nematode *C. elegans*

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The genome of the nematode *Caenorhabditis elegans* contains 75 full length cytochrome P450 (CYP) genes whose individual enzymatic and biological functions are largely unknown yet. Using microsomes isolated from adult worms, we found that *C. elegans* indeed produces spectrally active CYP proteins that display typical CO-difference spectra. To identify the CYP components of specific monooxygenase systems, we performed systematic gene silencing by RNAi as well as heterologous expression experiments.

Besides exogenous functions, CYPs of *C. elegans* are part of endogenous signaling pathways by synthesizing eicosanoids. Eicosanoids are sets of regio-isomeric epoxy- and hydroxy-derivatives derived from 20-carbon polyunsatu-rated fatty acids (PUFA). These signaling molecules known to modulate the contractility of cardiomyocytes and vascular smooth muscle in mammals serve as regulators of muscle activity in the nematode, too. **CYP-13A12** and **CYP-33E2**, expressed in the marginal cells of the pharynx, were identified as major isoforms contributing to eicosanoid biosynthesis. Its loss blocks *C. elegans*' behavioral response to reoxygenation (after anoxia), characterized by a rapidly increased locomotion speed, called the "O2-ON response" and the main-tenance of high pumping frequency, respectively. Co-expression of CYP-13A12 and CYP-33E2, respectively, with a CYP-reductase in insect cells resulted in the reconstitution of an active microsomal monooxygenase system that metabolized different PUFAs to specific sets of regioisomeric epoxy- and hydroxy-derivatives. The main product was in both cases 17,18-epoxy-eicosatetraenoic acid (EEQ). Already short-term incubation with 17,18-EEQ was sufficient to rescue both impaired locomotion and pharyngeal activity of the PUFA-deficient mutant strains.

Based on these results, we suggest that CYP-dependent eicosanoids function as signaling molecules in the regulation of the O2-ON response of *C. elegans*. Moreover, these signaling molecules are also required as regulators of pharyngeal activity and feeding behavior in *C. elegans*.

Age-dependent response to environmental change in the long-lived Antarctic clam *Laternula elliptica*: A bivalve model of cellular aging

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The Antarctic mud clam *Laternula elliptica* is one of the best studied "benthic models" and keystone in West Antarctic coastal ecosystems. At constant cold temperatures, these bivalves grow to large body size and live longer (~40y) than related temperate and warm water species, with older females being the most important reproducers in the populations. While Antarctic animals are generally of low stress tolerance, the bivalves do survive heat exposure of up to 10°C in short-term experimental studies, and protect their proteins from oxidation and unfolding through redox buffering the tissues (glutathione) and by activating transcription of both, antioxidants and heat shock proteins. On first sight the clams appear to be of striking resilience to the current rapid rise of water temperature in the West Antarctic, but it now turns out that older specimens are strongly impacted by the consequences of climate warming. They reduce aerobic metabolism when exposed to coastal sediment run-off and show higher mortality and less immune protection upon injury from ice growlers. Hypoxic conditions in Antarctic coastal sediments intensify as more organic matter is produced on newly ice-free areas, and older *L. elliptica* fail to conserve energy charge and to prevent protein oxidation and apoptotic cell death in gills during prolonged hypoxic exposure (16 days). Food limitations caused by enhanced sediment erosion and reduced phytoplankton productivity under turbid meltwater plumes draw on energy reserves, and young animals induce autophagic and cell cycling factors in starvation experiments in support of cell renewal and repair. On the contrary, old clams fail to activate autophagic genes and oxidized proteins as well as age pigments accumulate in slow proliferating tissues. If climate change proceeds at its current speed, this is bound to alter population age structure of this habitat structuring key species. I will summarize current knowledge of the age-related ecological and molecular stress response in this long-lived Antarctic ectotherm.

Role of adipokinetic hormone in the stress response of *Drosophila melanogaster*

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Adipokinetic hormone is also called “insect glucagon” referring to its insulin-antagonizing action in promoting the mobilization of body energy stores during periods of negative energy balance. Next to this canonical role in energy homeostasis, an ever-growing number of gain-of-functions studies in a variety of insect species had implicated Adipokinetic hormone in a puzzling diversity of additional functions including stress response regulation. However, the lack of Adipokinetic hormone mutant insects had so far precluded systematic loss-of-function studies on this neuropeptide hormone.

We have generated Drosophila AKH mutants and provide evidence that the pre-adult development of the vinegar fly proceeds remarkably independent from Adipokinetic hormone under controlled laboratory conditions. In contrast, energy homeostasis of adult Drosophila and the response of the fly to nutritional and other environmental stressors is under control of Adipokinetic hormone signalling.

Our data support the view that - in contrast to insulin - Adipokinetic hormone function is non-essential for Drosophila development. Rather, Adipokinetic hormone signalling increases the adaptiveness to environmental variability, which is characteristic for the natural habitat of insects.

Iron homeostasis in Mediterranean mussel *Mytilus galloprovincialis* (Lamarck, 1819)

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Iron is required for normal cell growth and proliferation. However, excess iron is potentially harmful, as it can catalyse the formation of toxic reactive oxygen species via Fenton chemistry. Ferritin plays a central role in the maintenance of intracellular balance of iron (Fe^{2+}/Fe^{3+}). Ferritin is almost ubiquitous and largely conserved from bacteria to human, although the role in the regulation of iron trafficking varies in different organisms. The metal chelator, ferritin molecule is a protein shell composed of 24 protein chains arranged in 2,3,4 point symmetry, with the capacity to sequester up to 4500 atoms of iron in a ferrihydrite mineral core. In this study we characterize full length *M. galloprovincialis* ferritin cDNA and gene sequence. The ferritin cDNA transcript with the 5' and 3' untranslated regions is 791 bp long. The putative open reading frame (114-638 bp) encodes a polypeptide of 174 amino acids, with a theoretical pI/Mw 4.88/ 20.114 kDa and three conserved domains: ferroxidase diiron center, ferrihydrite nucleation center and iron ion channel. According to the bioinformatics analysis, this ferritin is likely to be a homolog of vertebrate H-ferritin. The ferritin expression is regulated at both transcriptional and translational levels. The iron levels in the cell regulate the ferritin translation, removing the interaction between iron regulatory protein (IRP) and iron response element (IRE; 17-44 bp). In response to oxidative stress, the cells activate a defence strategy using ferritin to restrict the metal availability, so the ferritin as an anti/pro-oxidant agent in mussel *M. galloprovincialis* has a potential application as biomarker of oxidative stress.

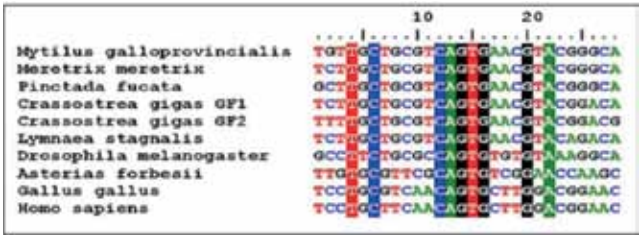

Figure: The highly conserved iron response element: alignment of several different species. The homologous residues are shaded (Wang et al., 2009)

Wang X. *et al.* 2009, Cloning, characterization and expression of ferritin subunit from clam *M. meretrix* in different larval stages. *Comp. Biochem. Physiol. Part B* 154:12-16.

Transplanted to the cooling pond of power plant mussels as a model organisms for the study of climate changing

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Bivalve mollusks from the cooling ponds of the power stations represent excellent model systems to study the long-term (years to decades) effects of elevated temperature of freshwater organisms (Falfushynska *et al.*, 2014). However, the ability of transplanted mussels to reflect the health status of the habitants of these ponds is unknown. In this study the molecular responses of residential and transplanted to the cooling pond of thermoelectric power plants in West Ukraine were compared. Male residential specimens of *Unio tumidus* from cooling pond (NP-group), pristine area (C-group) and mussels transplanted from pristine area to the cooling pond for 14 days (TrP-group) were compared by their molecular responses of stress and activities of detoxification systems. It was shown plural similarities between the indices of the mussels from NP- and TrP-groups that distinguished them from the C-group, particularly in digestive gland. Both exposures to the heated environment led to oxidative stress response (including upregulation of superoxide dismutase, lower levels of reduced glutathione and glutathione-*S*-transferase and elevated level of oxidized glutathione), elevated activity and release of cathepsin D from lysosomes, the decrease in lysosomal membrane stability. The transplanted mussels demonstrated more signs of the lesions due to decreased choline esterase activity and elevated level of vitellogenin (sign of endocrine disruption). The levels of pyruvate and lactate were also different between the NP- and TP-groups. Depletion of metallothionein was detected only in the NP-group showing the deeper depression of this stress-related and metal-buffering protein. This study shows that transplanted mussels can serve as excellent bioindicators of the environmental impact of the power plants.

Falfushynska H *et al.* 2014 *Science of the Total Environment* 500-501: 339-350.

The variable roles of PMK-1/SKN-1 and DAF-2/DAF-16 signaling in *Caenorhabditis elegans* in protecting against heat and heavy metal stress

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DAF-2 and p38 MAPK/PMK-1 signaling and associated transcription factors (DAF-16, SKN-1) play pivotal roles in the cellular response of *C. elegans* towards environmental stressors. PMK-1 was shown, for instance, to be involved in heat stress responses by its heat-induced shuttling into cell nuclei above 32°C, which takes place in parallel to the nuclear translocation of DAF-16. Survival at 34-35°C was also worse among mutants of the PMK-1 pathway in comparison to wild type (Mertenskötter *et al.* 2012). Gene expression analyses by RNA-Seq and RT-sqPCR revealed PMK-1-mediated support for the expression of genes (e.g., via SKN-1) coding for chaperones or related to protein biosynthesis/degradation. However, the behavior of PMK-1/SKN-1 and DAF-2/DAF-16 differs when studying heavy metal stress in *C. elegans* (Keshet *et al.* 2015). This study revealed a reduced cadmium (Cd) tolerance of *pmk-1*Δ, but an improved Cd tolerance of *daf-2*Δ and *daf-16*Δ (probably due to an enhanced expression of MRP-1). From RNA-Seq studies, the reduced tolerance of *pmk-1*Δ to Cd stress was related to a reduced expression of SKN-1 target genes as well as a weakening of antioxidant defense and detoxification mechanisms. Surprisingly, *pmk-1*Δ also showed a reduced expression of DAF-16 target genes (e.g., chaperonines). This impedes additionally the stress tolerance of the mutant and could be explained by a feedback loop involving target of rapamycin (TOR) signaling. Because of DAF-16's essential function as key transcription factor in stress tolerance mechanisms, current experiments focus on details of its stress-induced cytoplasmic-nuclear shuttling using transgenic strains carrying reporter gene constructs and a new microscopical setup. This method enables a quantitative and time-resolved determination of the activation state of DAF-16 in living animals. Additional RNAi treatments serve for the detection of cellular stress control mechanisms.

1) Mertenskötter *et al.* 2012. *Cell Stress and Chaperones* 18: 293-306; 2) Keshet *et al.* 2015. *Cell Stress and Chaperones* (in prep).

The heat shock protein 90 (HSP90) stress response of the microcrustacean *Daphnia pulex*

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In all organisms, stress sensing and stress signal processing are indispensable for the cellular stress response (CSR), which is essential to survive initial stress periods and to regain cell homeostasis via physiological adaptation. Particularly, ectothermic animals are challenged by severe changes in temperature. We studied first the relationships between acute heat or hypoxia and stress signals such as fluctuations in ROS level and glutathione redox state in *Daphnia pulex*. These signals were linked to the levels of the transcription factors HIF (hypoxia-inducible factor) and HSF (heat shock factor), which play important roles for the CSR, and to the expression of HSP90 genes. The corresponding HSP90 proteins are molecular chaperones more specific in their substrate spectrum than other HSPs. During non-stress conditions, they are preferentially engaged in the maturation/maintenance of proteins, which are involved in signal transduction. Up-regulation of HSP90 gene expression upon cellular stress ensures the ongoing protection of signaling pathways or proteasomes (maintenance of the 'HSP90 capacitor'), despite an increased burden of protecting other types of proteins. The quantification of HSP90 expression (RT-qPCR) showed a much faster response to heat stress in starved animals than in well-fed animals. These differences were due to different glutathione-based antioxidant capacities, which affect ROS fluctuations as an important signal for stress gene expression. Testing artificial ROS signals revealed dependence of the time point of stress gene induction on the concentration of applied hydrogen peroxide, with higher H_2O_2 concentration inducing faster and stronger HSP90 expressions and vice versa. In conclusion, HSP90 expression during the stress response of *Daphnia pulex* is likely controlled by ROS signals, which are primarily influenced by the glutathione system.

Acute heat-stress induces diencephalic neuropeptide Y mRNA expression and alters brain concentrations of free amino acids in chicks

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Heat-stress increases body temperature and reduces food intake in chickens. Neuropeptides and amino acids play a vital role in the regulation of food intake. However, the responses of neuropeptides and amino acids to heat-stress-induced food-intake regulation are poorly understood. In this study, the diencephalic mRNA expression of some neuropeptides related to food intake and the content of free amino acids in the brain and plasma were examined in young chicks exposed to a high ambient temperature (HT; 40 ± 1°C for 2 or 5 h) or to a control thermoneutral temperature (CT; 30 ± 1°C). HT caused a significant increment in rectal temperature and plasma corticosterone level and suppression in food intake. HT further increased the expression of neuropeptide Y (NPY) and agouti-signaling protein (ASIP) precursor mRNA, while no change was observed in pro-opiomelanocortin, cholecystokinin, ghrelin, or corticotropin-releasing hormone precursor mRNA. It was further found that the diencephalic content of free amino acids – namely, tryptophan, branched-chain amino acids (leucine, isoleucine, valine) and serine – was significantly higher in HT chicks in comparison with CT chicks (Ito *et al.*, 2015). The induction of NPY and ASIP expressions and the alterations of free amino acid during HT suggest that these changes can be the results or causes the suppression of food intake.

Ito K., Bahry M. A., Yang Y., Furuse M., Chowdhury V.S. 2015. Acute heat stress up-regulates neuropeptide Y precursor mRNA expression and alters brain and plasma concentrations of free amino acids in chicks, *Comp. Biochem. Physiol. A, Mol. Integr. Physiol.* 187: 13–19.

Quantification of cadmium clearance by different detoxification mechanisms in the nematode *Caenorhabditis elegans*

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As a soil-dwelling organism the nematode *C. elegans* is exposed to natural as well as to anthropogenic pollutions with heavy metals. The heavy metal cadmium (Cd) is one of the most toxic stressors, and chronic Cd exposure is associated with reduced life expectancy and serious diseases in many organisms. Previous studies have shown that *C. elegans* uses multiple lines of protection and various stress response systems to counteract cadmium-induced risks and damages. The most prominent stress-responsive elements are ABC transporters such as MRP-1 and HMT-1, with the latter exporting cadmium-phytochelatin-complexes. Following a new experimental approach, we quantified the Cd clearance rate mediated by different ABC transporters in *C. elegans*: in wild type (WT) worms and different ABC transporter knockout strains as well as in other Cd-related mutants (*pcs-1Δ*, *mtl-2Δ*), we measured cadmium import and export directly using ICP-MS (Inductively Coupled Plasma Mass Spectrometry). First results showed *hmt-1Δ* and *pcs-1Δ* to strongly deviate from WT in Cd import and export rates. Surprisingly, *mrp-1Δ* showed the lowest functional deviation from WT, which may have resulted from compensatory effects. In contrast to previous approaches, which measured “fitness” (survival and/or reproduction) under cadmium stress, the new approach enables to detect directly cadmium-relevant mechanisms in living animals.

HIF, HSF, and the cellular stress response (CSR) of *Daphnia pulex*

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Heat stress poses an extraordinary challenge to ectothermic organisms, which requires various stress response mechanism to survive the initial stress period and to regain cell homeostasis via physiological adaptation. The transcription factors HIF (hypoxia-inducible factor) and HSF (heat shock factor) play essential roles for the cellular stress response (CSR). We are studying CSR in the water flea Daphnia pulex upon the exposure to different stressors (e.g., heat, hydrogen peroxide, hypoxia) by measuring ROS (reactive oxygen species) and (glutathione) redox signals together with the cellular localization of HIF and HSF (Western blots using both cytoplasmic and nuclear extracts). These data are related then to the expression of HIF target genes on the mRNA level. Of special interest are the dynamic relationships between stress signals and responses. We detected, for instance, dependence of the speed of stress responses on glutathione redox buffer capacity or a direct link between the point in time, when ROS reaches maximal levels (ROS peak) and the moment, when (stress) gene expression starts. Ongoing studies include further stress-relevant factors such as catalase activity and the determination of stress responses also on the protein level.

A novel approach to quantify transcription factor activity in *Caenorhabditis elegans* in a time-resolved manner

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Transcription factors (TFs) are essential for the regulation of gene expression and act downstream of signaling cascades. Under stress conditions, they translocate into cell nuclei and initiate the expression of target genes, which are required to generate a cellular stress response. Co-expressed reporter genes or proteins (e.g., green fluorescent protein, GFP) enables the direct observation and cellular localization of these factors. We developed a novel technique to quantify the cytoplasmic-nuclear shuttling of the GFP-tagged transcription factor DAF-16/FoxO in a time-resolved manner in the nematode *Caenorhabditis elegans*. This approach uses changes in overall fluorescence intensity when DAF-16::GFP enters the cell nuclei, with the nuclear translocation of the TF indicating cellular stress. As standard, changes in the cytoplasmic/nuclear localization of DAF-16 were determined as function of the applied oxygen partial pressure ("characteristic oxygen curve", COC). Additional RNA interference (RNAi) was then applied to knock down cellular control factors of DAF-16 such as LET-363 (TOR) or the AMP kinase AAK-2 in order to study the consequences for the COC. Specific changes (increase/decrease in nuclear DAF-16 localization) were verified by measuring the expression of DAF-16 target genes. First results indicate ROS-induced nuclear DAF-16 translocations in context with elevated mitochondrial activity and protein biosynthesis. This new method may be used with any tagged transcription factor not only at varying oxygen partial pressures but also at varying intensities of other stressors.

Comparative analysis of quercetin effects in human erythroleukemia cell lines

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The cellular microenvironment is an important territory to the beginning of the studies of cell physiology. The cancer may be considered a failure, not detected and corrected, inside this cellular microenvironment in homeostasis. Multidrug resistance (MDR) is the primary cause of cancer–related mortality. A number of different natural compounds have been studied in the hopes of identifying treatments that overcome the mechanisms of drug resistance. The aim of this study was to analyze the effects of Quercetin (QUE), a flavonol with potential anticancer activity, on MDR chronic myeloid leukemia cells (Lucena-1) and their non-MDR parental cell line (K562). The cell lines were maintained in RPMI-1640 with 10% fetal bovine serum, 1% antibiotic and antimycotic, in cell culture flasks at 37 °C. The viability of K562 and Lucena-1 cells exposed to quercetin was performed trough three independent experiments containing triplicates of samples (n=9). For each experiment, the cells were centrifuged, washed twice with PBS, resuspended in medium to a final concentration of 5x105 cells/mL and treated with different concentrations of quercetin (5, 10, 50, 100, and 500 µg/mL). Control group received the same volume of sterile water. Cell viability was analyzed by the technique trypan blue exclusion after exposure to quercetin. The ability to induce cell death by apoptosis or necrosis was measure using a epifluorescence microscope, its ability to produce oxidative stress (ROS) was quantified by fluorimeter, and DNA damage was evaluated by comet assay. The effects on the ABCB1 activity was mensured by rhodamine-123 and ABCB1 gene expression that encoding this protein was analised by real-time PCR. Our study demonstrates that QUE had antiproliferative and cytotoxic effects on the cells. QUE induced apoptosis, necrosis, DNA damage, and inhibited the activity of the ABCB1. Our results suggest that the effect of QUE on cell viability might be the result of DNA damage, and inhibition of ABCB1 pump activity. We propose that QUE has an anti-MDR effect in this model of MDR human leukemia.

Effect of TCDD, PCB 126 and PCB 153 on granulosa layer proliferation in chicken ovarian follicles

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Dioxins and polychlorinated biphenyls (PCBs), the globally distributed persistent organic pollutants, accumulate in the food chain, mainly in the adipose tissue of animals. The role of these xenobiotics in the function of the avian ovary in not fully understood. The aim of this study was to evaluate the effect of TCDD and two different PCB congeners (PCB 126 and PCB 153) on cell proliferation in the granulosa layer of white non-hierarchical and yellow hierarchical follicles of the laying hen.

Hy-Line hens (25-weeks old, n=15) were decapitated 2 h after ovulation. The granulosa layers isolated from the white (1-4 mm and 4-8 mm) and yellow (15-25 mm) ovarian follicles were incubated for 24 h (38°C) in Eagle's medium in the three trials: a) control, TCDD (1, 10 or 100 nM), hrEGF (human recombinant EGF; 100 nM), TCDD (10 nM) + hrEGF (100 nM) (n=5); b) control, PCB 126 (1, 10 or 100 nM), hrEGF (100 nM), PCB 126 (10 nM)+hrEGF (100 nM) (n=5); c) control, PCB 153 (1, 10 or 100 µM), hrEGF (100 nM), PCB 153 (10 µM)+hrEGF (100 nM) (n=5). Granulosa cell proliferation was measured by [3H]-thymidine incorporation (McElroy *et al.*, 2004).

TCDD and PCB 126 inhibited basal and EGF-stimulated granulosa cell proliferation in all investigated ovarian follicles; the effect of PCB 153 was opposite ($p<0,05$).

Dioxins and PCBs via effects on proliferation in white non-hierarchical follicles may affect the process of the follicle selection into preovulatory hierarchy, while in the yellow hierarchical follicles it may affect their rapid growth in the period before ovulation.

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Specific tasks and interactions of the PMK-1 and DAF-2 pathways and ABC transporters in *C. elegans* during cadmium stress

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PMK-1 and DAF-2 signaling control the highly stress-relevant transcription factors SKN-1 and DAF-16. We studied specific tasks and interactions of these signaling pathways and ABC transporters in C. elegans wildtype (WT), signaling mutants, and transgenic strains during severe cadmium stress. pmk-1 deletion (*pmk-1D*) caused survival under cadmium stress to decrease, whereas daf-2 and daf-16 deletion just as PMK-1 and DAF-16 overexpression led to its increase. Deletion of the ABC transporter genes mrp-1 and mrp-4 reduced cadmium tolerance as well. Transcriptome profiling via RNA-seq of WT and *pmk-1D* under control and cadmium stress conditions showed, for instance, promoting effects of PMK-1 on SKN-1 target genes, chaperone genes and genes for protein biosynthesis. Surprisingly, nuclear DAF-16 translocation and DAF-16 target gene expressions were found to be lower in stressed *pmk-1D* than in stressed WT, which is a further disadvantage for the mutant. Time-resolved RT-PCR also showed higher expression levels of *daf-15* (a negatively controlled DAF-16 target gene encoding 'raptor' of the TOR complex) and *mrp-1* in stressed *daf-16D* and *pmk-1D* in comparison to stressed WT. The up-regulated *daf-15* expression may be part of an compensation mechanism to substitute PMK-1 promoted TOR activation for protein biosynthesis (in WT) by gene expressions for TOR complexes. Up-regulation of *mrp-1* may be the reason for the elevated cadmium tolerance of *daf-16D*, with its negative control by DAF-16 likely related to the shutdown of secretion in dauer larvae. There are also indications for PMK-1, SKN-1, and DAF-16 targets to be essential elements of the first line of defense against acute cadmium stress, with ABC transporters providing permanent protection against this stressor.

Protein, lipid and sugar content in larvae and pupae of *Spodoptera exigua* from control and cadmium line after cadmium exposure

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Differences in defence effectiveness between lines of *Spodoptera exigua* selected by cadmium treatment have been earlier noted. The effectiveness of defence may depend on biomolecules stores. In this study, we compared the survival rate of different developmental stages (larvae and pupae) and effect of cadmium exposure on protein, lipid, total sugar and glycogen content in selected stages of *Spodoptera exigua* from two lines with and without cadmium treatment (120-generational selection). We established the following experimental groups: control (individuals from control line – not exposed to cadmium), KCd (individuals from control line and exposed to cadmium) and CdCd (individuals from cadmium line continuously exposed to cadmium). The contents of biomolecules were measured in whole bodies of 1-day-old larvae of 3rd, 4th and 5th stage and in 1-day-old pupae (female and male, separately).

The survival rate was disturbed only in individuals from control line exposed to cadmium (KCd group: 26% and 53% lower than in control group, in the cases of larvae and pupae, respectively).

Differences in biomolecules stores dependently on line origination were observed. Individuals from cadmium line contained more protein, sugar, glycogen stores. It was especially distinct in the last larval stage and in pupal female (Tab. 1).

Experimental groups	3rd larval stage	4th larval stage	5th larval stage	Pupal female	Pupal male
Protein content					
KCd	↑	↑			
CdCd			↑	↑	
Sugar content					
KCd	↑				
CdCd			↑		
Glycogen content					
KCd				↑	↑
CdCd				↑	
Lipid content					
KCd	↓				
CdCd	↓	↓		↓	

Table 1: Biomolecules content tendency in larvae and pupae of *S. exigua* after exposure to cadmium. Experimental groups (KCd and CdCd) were compared to control line. Statistical significant differences ↑increase ↓decrease; Kruskal-Wallis test ($p<0.05$).

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Infanticide and siblicide in maize-fed common hamsters: The nicotinamid deficiency responsible

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Energy and protein limitations are two major factors that affect reproduction and hence life-history evolution in mammals. With agriculture intensification and the expansion of monoculture, farmland species are often constrained by the lack of food diversity and quality. In the context of conservation biology, we designed an experimental study to understand how food modulates the reproduction of the farmland common hamster (*Cricetus cricetus*). We submitted 30 reproductive females to corn- or wheat-based diets, complemented with proteins (earthworms or clover) after mating and until weaning. Results revealed that females fed with maize-based diets displayed hyperactivity and aggressive behaviors compared to females fed with wheat-based diets. They also showed a reduction of up to 75% of their reproductive success, mainly caused by a high rate of infanticide during the first day after parturition. Finally, surviving pups in the maize groups displayed growth-retards and two cases of siblicides were observed. We found no significant differences in macronutrients, energy or minerals contents of the diets that could explain these results. This indicates that, independently of the protein, lipid and carbohydrates contents of diets, maternal investment can be constrained in maize-based diets. This strongly suggests that either a toxic compound present in maize kernels or an amino acid (or vitamin) deficiency is in cause. We proved that the tryptophan and vitamin B3 deficiencies in maize and earthworms are responsible of these results. Given the importance of this vitamin in red-ox reactions (through its implication in NAD and NADP), we expect that, despite their reduced maternal investment, maize-fed females would display higher oxidative stress levels and ultimately higher ageing rates than wheat-fed females. Given the rate of maize monoculture intensification, associated with a decrease in food diversity and an increase in the contamination of food items, these results could be of particular interest to explain the dynamics and the decline of farmland species.

Effect of feeding and digestion on oxidative stress in rainbow trout

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Feeding is fundamental to all animals and involves several elements such as hunting/foraging, ingestion, mechanical processing, digestion, nutrient absorption, assimilation, growth, excretion etc. Feeding is considered a major energetic challenge for fish, and owing to the magnitude of this post prandial enhancement of metabolism it has been proposed as a significant mechanism of cellular death and ageing (Sohal and Weindruch, 1996). The production of reactive oxygen species (ROS) post-feeding should therefore affect longevity. To our knowledge, however, ROS damage and antioxidant defences have not been studies during a feeding cycle in fish. We used adult rainbow trout at 15 °C and voluntarily feeding on a 3 % single ration per week, to quantify ROS damage and antioxidant defenses 24 h before feeding and after 1.5, 6, 24, 48, 72 and 96 h post-feeding in gills, stomach, intestine and liver. The enzymatic activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) were determined as a proxy for antioxidant defenses, while lipid peroxidation (LPO), protein oxidation (PO) in the same tissues, and DNA damage (in red blood cells only) were determined as a proxy of oxidative damage. Tissue specific differences were found in the antioxidant defenses, with liver and intestine showing the highest and gills showing the lowest. Overall our results show no major changes in the ROS damage or in the antioxidant defenses during a feeding cycle. DNA damage in red blood cells, however, showed lowest damage between 48 to 96 h post feeding, increasing between 24 h pre-feeding and 24 post feeding. Our results show no evidence of the proposed mechanism by which feeding would have a major influence on longevity via the induction of oxidative stress.

Sohal RS, Weindruch R. 1996. Oxidative stress, caloric restriction, and aging. Science 5271, 59-63.

Links between Oxidative Stress and Life Histories

Organizers:

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Oxidative stress and life-history trade-offs: Is it evolutionary stable to have single physiological currencies?

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Traditionally, life-history trade-offs have been based on differential allocation of limited energy to different traits depending on the prevailing circumstances. Recently, oxidative stress has been proposed to be a "new" currency in life-history trade-offs, replacing energy. However, there is substantial reason to believe that multiple trade-off currencies operate simultaneously, which can affect the relative importance of specific currencies in certain environments. Here we explore this idea using a stochastic simulation approach. Our main question is whether the presence of a single "physiological" trade-off currency (i.e. like oxidative stress) between reproduction and survival, or of two such currencies that could interact in one of three ways (multiplicative effect, maximal effect, or minimal effect) change evolutionary outcomes produced by natural selection. We used a wide array of parameter values, and compared the state of the two physiological currencies as well as three main life history traits: fertility rates, average age at death, and lifetime reproductive success at the end of the simulations. Final values of all examined traits differed in most cases between the one- and two-trade-off scenarios. Two currencies generally produced higher final lifetime reproductive success, implying potential selection for organisms to evolve multiple trade-off currencies. Future studies should investigate whether our theoretical model has any empirical support by conducting factorial experiments of single and multiple physiological currencies. This would bring light to when oxidative stress (and other currencies) are more or less significant for shaping life-history traits.

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The interacting consequences of ATP turnover, bioenergetics and mitochondrial ROS metabolism: Should increasing ATP demand increase mitochondrial ROS production?

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Mitochondria are well recognized for their capacity to produce reactive oxygen species (ROS). The excess release of ROS from the mitochondrion should lead to macromolecular damage, or cellular level oxidative stress. Combined, these concepts have fostered a view, mostly through inference, that mitochondria are the major source of ROS leading to cellular oxidative stress. A corollary concept is that during periods of increased cellular energy demand, such as during growth, mitochondrial ROS production will be elevated in concert with increasing mitochondrial respiration. But is it appropriate to connect increasing respiration to enhanced mitochondrial ROS production?

The relationship between mitochondrial bioenergetics and the formation of ROS does not support a simple linkage between rates of respiration and mitochondrial ROS production. Increasing cellular ATP turnover leads to increasing mitochondrial oxygen consumption. However, in response to increased ADP availability, the mitochondrial bioenergetic state will shift towards lower capacity to form ROS; we see this with mitochondria from mammals and fishes. Meanwhile the enzymatic antioxidant systems appear relatively unaffected by this shift in energetic state. Thus, at the mitochondrial level, elevated cellular ATP demand should reduce ROS production with concomitant increasing rates of oxygen consumption. In contrast to simply being a source of ROS, the metabolism of the membrane permeant ROS hydrogen peroxide implicates mitochondria as regulators of ROS levels. This potentially challenges how mitochondria should be considered with regards to their role in cellular oxidant metabolism. However, and important to their role in cellular oxidative stress, failure to regulate or maintain the cellular environment (i.e. pH, oxygen levels or changing temperature) may shift the balance of mitochondrial ROS metabolism, which could then lead to altered cellular oxidant stress.

Mitochondrial function and life history variation in the house mouse

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A central tenet of biology is that the costs of reproduction contribute to earlier senescence. Because reproduction is energetically demanding, it has been assumed that reproduction stimulates the production of reactive oxygen species that damage cells and hasten aging. Yet, several studies have shown that oxidative damage is typically unchanged or reduced in reproductive individuals when compared to non-reproductive controls. To evaluate the relative cost of reproduction, we quantified mitochondrial function in the liver of house mice (*Mus musculus*) that bred several times verses those that never bred. Mice were maintained in enclosures that mimic the conditions of free-living mice. Reproductive females had the opportunity to breed for 8 months, while non-reproductive females were maintained for a similar duration with infertile male(s). At 10.5 months, the males were removed to curtail breeding. Females were sacrificed at 12 months of age and liver mitochondria were isolated by differential centrifugation and used to measure respiratory control ratio (RCR) and protein levels of oxidative damage and endogenous antioxidants. The RCR of reproductive females was not different compared to non-reproductive females (4.18±0.13; 3.64±0.30; respectively, $p=0.09$). Oxidative damage of liver mitochondria, as measured by 4 hydroxynonenal (4HNE), was higher in reproductive mice compared to non-reproductive mice (11.9±0.8; 8.19±1.60; respectively, $p=0.05$), despite having higher mitochondrial protein levels of antioxidants. Specifically, liver SOD1 protein levels in reproductive mice were 11.9±1.3 and in non-reproductive were 2.18±1.18 ($p<0.05$). Also, liver catalase protein levels in reproductive mice were 24.3±4.3 and in non-reproductive were 5.30±0.56 ($p<0.05$). Our data indicate that enhanced liver mitochondria SOD1 and catalase protein levels have been insufficient to prevent oxidative damage to liver. But interestingly, enhanced oxidative damage did not negatively impact mitochondrial respiratory function suggesting that increased oxidative damage may not contribute to a reduction in RCR that could contribute to aging. Additional work is needed to elucidate how mitochondrial function changes with parity and differs among metabolically active tissues.

Reproduction is not costly in terms of oxidative stress

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The trade-off between current and future reproduction is one of the fundamental assumptions of life-history theory. Investment in current reproduction is associated with costs which often appear as decreased survival to the next reproductive attempt or lower future reproductive success. We conducted three studies to test whether oxidative stress is the proximate mechanism of these costs of reproduction. We measured oxidative damage (OD) to lipids and proteins in different organs as well as the level of antioxidants (total glutathione and catalase) in breeding and non-breeding bank voles. In laboratory experiments we used females from lines selected for high aerobic metabolism and from non-selected control lines, and manipulated their reproductive effort by allowing them to wean 1 or 2 litters (first experiment) or decreasing or increasing litter size in four consecutive litters (second experiment). We also measured OD and antioxidants in bank voles trapped in their natural environment before and after the breeding season. Contrary to the predictions of oxidative stress theory, the OD in reproducing females was decreased or unchanged as compared to non-breeding voles. Moreover, in laboratory experiments, OD did not differ between females from the selected and control lines. In the first experiment, OD to lipids in kidneys and muscles was lower in breeding than in non-breeding voles. In the second experiment, OD to lipids and proteins in liver and to proteins in the heart was lower in females that weaned enlarged litters than in non-breeding ones. We found negative relationship between the oxidative damage and activity of catalase in kidneys that could provide evidence of protective action of antioxidants. In the third study, females after reproduction had in most cases lower or unchanged level of OD to lipids and proteins compared to before breeding.

In conclusion, our studies did not confirm the hypothesis that oxidative stress is the crucial element in the proximate physiological mechanism underlying the fundamental life-history trade-off between current and future reproduction.

Evidence for cost of reproduction in humans: Women with high lifetime reproductive effort have increased levels of oxidative stress in postmenopausal period

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Recent studies on the trade-off between reproduction and longevity indicate that the detrimental effect of oxidative stress may be a central component of the cost that reproductive effort incurs on lifespan. Increases in metabolism related to reproductive effort cause major rises in oxidative metabolism that in turn may elevate oxidative stress. It might be thus expected that maternal aging will be driven by reproduction-associated increases in oxidative stress. To date, this theoretical prediction has rarely been verified in humans.

To test the hypothesis that reproductive effort is associated with increased oxidative stress in humans we conducted a study of 100 postmenopausal women from the Mogielica Human Ecology Study Site in Poland. In these women high reproductive effort is accompanied by high energy expenditure associated with physical activity. We found that independently of age, postmenopausal women with high reproductive effort (>= 4 pregnancies) during their lifetime had higher levels of oxidative damage to DNA, indicated by 20% increase in levels of 8-OHdG and higher levels of oxidative defence, indicated by 60% increased production of Cu-Zn SOD when compared to women with low reproductive effort (<4 pregnancies). Moreover, we found linear associations between levels of all biomarkers of oxidative stress (8-OHdG, Cu-Zn SOD and TBARS) and the number of pregnancies during the lifetime suggesting a dose-response association between reproductive effort and oxidative stress. This study presents the first clear evidence for oxidative stress as a cost of reproduction in humans.

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To deal or not to deal with oxidative stress: A key component for the coexistence of competing species?

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Oxidative constraints are believed to drive life-history evolution. However, the occurrence of a causal link between oxidative stress and life history remains controversial. If such evolutionary link exists, it should be assessable, not only at inter-individual or intra-population level, but also at inter-specific level. To address this issue we measured oxidative stress marker (TBARS) and antioxidant capacity (superoxide dismutase and catalase activity, total antioxidant capacity) in wild-caught females from two sibling weevil species (*Curculio elephas, C. glandium*) that coexist and compete for a fluctuating resource. We correlated these markers to ecological and physiological traits such as longevity, reproductive effort or flying capacity and activity of a pace-making mitochondrial enzyme (citrate synthase). We found that the short-lived *C. elephas* sacrifices red-ox homeostasis for immediate reproduction upon adult emergence as characterized by low antioxidant defences and elevated oxidative damage. Comparatively, *C. glandium* massively invests in antioxidant and maintains low oxidative damage, which may contribute to sustain their extended lifespan, long immature pre-reproductive period and highly-performing flying capacity. Intriguingly, in the long-lived *C glandium*, we also reveal an reactivation of antioxidant defences at the time of their intensive oogenesis period while oxidative damages remained unchanged. Our results thus support the existence of a strong but complex relationship between oxidative stress and life history evolution and highlight the need for a finer-scale picture of antioxidant strategies. We propose that such diversification of strategies toward oxidative stress would favor ecological niche partitioning of competing species, and constitute a physiological prerequisite for their coexistence.

Oxidative shielding and the cost of reproduction

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Life-history theory assumes that reproduction and lifespan are constrained by trade-offs which prevent their simultaneous increase. Recently, there has been considerable interest in the possibility that this cost of reproduction is mediated by oxidative stress. However, empirical tests of this theory have yielded equivocal support. Using meta-analysis we show that oxidative damage is positively associated with reproductive effort across females of various species. Yet paradoxically, categorical comparisons of breeders *versus* non-breeders reveal that transition to the reproductive state is associated with a step-change reduction in oxidative damage in certain tissues and markers. Developing offspring may be particularly sensitive to harm caused by oxidative damage in mothers. Therefore, such reductions could potentially function to shield reproducing mothers, gametes and developing offspring from oxidative insults that inevitably increase as a consequence of reproductive effort. We explored this possibility using data from a study of wild banded mongooses (*Mungos mungo*) in Uganda. Females had reduced oxidative damage during pregnancy, compared with pre- and post-breeding periods. Furthermore, ultrasound scans showed that females with higher levels of oxidative damage carried fewer foetuses, and they had lower offspring survival to emergence from the den, as expected if there are transgenerational impacts of maternal oxidative damage. According to this perspective, we hypothesise that the cost of reproduction is mediated by dual impacts of maternally-derived oxidative damage on mothers and offspring, and that mothers may be selected to diminish such damage. Such oxidative shielding may explain why many existing studies have concluded that reproduction has little or no oxidative cost. Future advance in life-history theory needs to take account of potential transgenerational impacts of the mechanisms underlying life-history trade-offs.

Thermogenesis, fasting and oxidative stress: New insights from model and non-model animals

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Wild birds and mammals often experience ambient temperatures below their thermoneutral comfort zone, and periods of food shortage (*i.e.* fasting). Despite oxidative stress being increasingly recognized as a mediator of life histories, we still have little information on the impact of environmental constraints such as cold exposure or fasting on oxidative homeostasis. However, both constraints are known to affect mitochondrial bioenergetics (*i.e.* the main producer of reactive oxygen species), and at a broader scale energy expenditure.

First, I will present novel insights linking thermogenesis, mitochondrial functioning and oxidative stress in two model animals, namely transgenic mouse lacking the uncoupling protein 1 (UCP1; Stier *et al.* 2014, J Exp Biol) and captive zebra finches (*Taeniopygia guttata*) treated with the mitochondrial uncoupler *2,4-dinitrophenol* (Stier *et al.* 2014, J Comp Physiol B). In the absence of non-shivering thermogenesis (linked to UCP1 activity), prolonged exposure to low ambiant temperatures (4 weeks at 12°C) increased oxidative damage levels in transgenic mice, but not in wild-type mice that exhibited an up-regulation of antioxidant defences. In zebra finches, an acute exposure to cold (24h - 12°C) increased DNA oxidative damage, while prolonged exposure (4 weeks - 12°C) did not. Further, the mitochondrial uncoupling treatment was able to prevent acute cold-induced oxidative stress.

Second, I will present novel insights linking long-term fasting, mitochondrial functioning and oxidative stress in wild king penguins (*Aptenodytes patagonicus*). Here, we found that fasting had an impact on mitochondrial functioning and significantly increased oxidative damage levels but not antioxidant defences (Schull & Stier, in prep).

Altogether, our results indicate that ambient temperature and fasting may shape life histories of wild birds and mammals through their impact on mitochondrial functioning and oxidative balance.

Oxidative status during reproduction in Magellanic penguins (*Spheniscus magellanicus*): Incubation versus chick rearing

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Magellanic penguins (*Spheniscus magellanicus*) breed colonially coinciding with improved environmental conditions. Their breeding cycle is comprised of periods (courtship and nest building, incubation, guard phase and crèche) which require different allocations of energy. Energy release is directly related with oxidative balance. Our aim therefore is to examine whether oxidative status in blood plasma (total plasma antioxidant capacity, oxidant components and total non-protein thiol groups) differs between incubation and the guard phase. Magellanic penguins perform longer foraging trips during the incubation period than during the guard phase probably in response to energy loss during pre-incubation activities. Therefore, we expected oxidative status to be higher in the former than in the latter. Fieldwork was conducted in a Magellanic penguin colony located in Estancia San Lorenzo (42º 05'S, 63º 49'W), Peninsula Valdes, Argentina. All penguins sampled were nesting under shrubs incubating two eggs or rearing two chicks (to avoid variation in parental effort). Our results support the prediction as incubating adults had significantly lower antioxidant levels for the total plasma antioxidant capacity and for the total non-protein thiol groups. In contrast, oxidant components did not show significant variation between the two breeding periods. We did not find differences in oxidative status between sexes. In addition, adults were significantly heavier during incubation which could be related to a higher foraging activity in this stage for self-maintenance.

The effects of silvering and infection with *Anguillicola crassus* on the capability to deal with radical oxygen species in swimbladder tissue of the European eel (*Anguilla anguilla*)

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The European eel has a very peculiar lifecycle which begins far off the US east coast in the Sargasso Sea. Transported by ocean currents larvae reach European coasts, enter freshwater systems and spend most of their life as so called yellow eels in rivers and lakes. After several years in freshwater, eels return to the coast and in a process called silvering prepare for their second long-distance migration back to the Sargasso Sea for reproduction. During this journey, eels perform extended diel vertical migrations, and the concomitant changes in hydrostatic pressure significantly affect the swimbladder, functioning as a buoyancy organ. As the swimbladder is primarily filled with oxygen, the tissue has to cope with extreme hyperoxic conditions, which typically are accompanied by the generation of radical oxygen species (ROS). In addition, since the introduction of the parasitic nematode *Anguillicola crassus* in the early 1980's, swimbladder function of most of the European eels is impaired by the infection with this parasite.

To answer the question how the swimbladder tissue can cope with hyperoxic conditions and if silvering or the infection with *A. crassus* affect this capability, we analyzed muscle and swimbladder tissue from uninfected yellow, infected yellow, uninfected silver and infected silver eels for the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), the metabolite glutathione (GSH/GSSG) and additionally the level of lipid peroxidation which increases with ROS stress. In swimbladder tissue we found increased concentrations of GSH/GSSG as well as higher activities of SOD, GPx and GR, suggesting SOD and the glutathione cycle to be of high importance for ROS detoxification. Comparing uninfected yellow with uninfected silver eels, the concentration of GSH/GSSG and the activity of SOD were elevated after silvering, corresponding with lower levels of lipid peroxidation. While in yellow eels the infection with *A. crassus* had no effect, in silver eels the capacity to cope with ROS was significantly impaired. (supported by the FWF P26363-B25)

DNA damage in the beet armyworm (*Spodoptera exigua*) after 14 years of selection to cadmium resistance

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The existence of an animal under permanent, mild stress requires a kind of trade-off. The enhancement of the protective/repairing mechanisms to minimize negative effects of harmful substances is beneficial for an individual. Natural selection can happen in the case of pests inhabiting polluted areas e.g. with metals. In our laboratory an unique line of *S. exigua* has been selected for 14 years. In this study we try to answer a question: does long lasting (for 120 generations) contact of *S. exigua* with cadmium (44 $\mu g \cdot g^{-1}$ dry weigh of food) contribute to the selection of animals with better defensive strategies in comparison to the non-selected insects. The question about DNA stability and susceptibility to damage of the insects from such unique, preselected line is especially important to resistance mechanisms in insects. We applied a comet assay to assess the level of DNA damage in hemoctyes of *S. exigua* from cadmium and control lines. Cells after isolation were treated H_2O_2 to induce oxidative stress. The level of DNA damage was measured in different period times after treatment. The basal level of damage was slightly higher in cadmium line. After H_2O_2 application fast regeneration of the damage was observed only in cadmium line, which could indicate presence of repairing mechanisms existing permanently in the cells. This might indicate a form of adaptation in insect from cadmium line, however, the background of this phenomena and its stability needs further research.

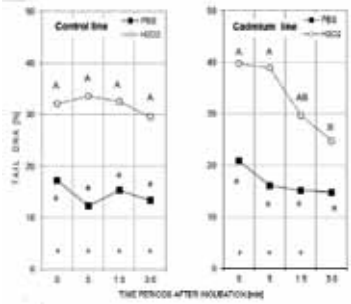

Figure: Tail DNA (%) in nuclei of hemocytes of 5th instar of *S. exigua* from control and cadmium lines. ○ or ■ – mean of medians of fifty nuclei measured in each slides; * – differences for time period (0, 5, 15 or 30 min) between reference and treated groups within each line; the same letters -homogenous time-groups within a line.

This project was supported by National Science Centre, Poland (decision DEC-2013/09/B/NZ9/01754)

Role of Free Radicals in the Survival Machinery of Animals that Endure the Hardships of the Environment

Organizer (*South American Society for CPB*):

Marcelo Hermes-Lima, *University of Brasilia, Brazil*

Decoding the molecular machinery controlling metabolic rate depression

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The "Edges of Life" have fascinated thinkers from the time of Aristotle. Environmental extremes trigger extreme animal responses. Stresses ranging from drying to freezing to oxygen deprivation to extreme heat or cold can all trigger descent into the realm of "the living dead" – states of hypometabolism where animals are alive but show few signs of normal life. For example, from our own perspective as homeotherms that are severely injured if core body temperature drops below ~25°C, it is amazing to consider how some mammals descend into deep torpor and chill to near 0°C during winter hibernation. Similarly, we cannot survive without oxygen for more than a few minutes, but many turtles can endure 3-4 months submerged in cold water without breathing oxygen. Nature has set in place crucial mechanisms to allow animals to "turn down the fires of life". The basic framework for this is in every organism but discovering the underlying biochemical secrets is the challenge. My lab has identified multiple adaptive strategies that control metabolic rate depression, reprioritize fuel/energy use under torpid or anoxic states, and provide protection to cells during long term hypometabolism in animal models including hibernating mammals, anoxia-tolerant turtles, and estivating or freeze tolerant frogs. These include altered cell signaling, differential gene expression under the control of stress-responsive transcription factors and microRNA action, regulated suppression of energy-expensive cell functions, changes to enzyme properties to alter their function during hypometabolism, enhanced antioxidant defenses, and protein protection by chaperones. Startling similarities exist in the basic mechanisms of hypometabolism across phylogeny and between phenomena including torpor, hibernation, estivation, anaerobiosis, anhydrobiosis and freeze tolerance. Overall, it is apparent that the capacity for hypometabolism is a stem characteristic of animal lineages but has been lost or marginalized in various species, including humans who have largely "forgotten" this skill. An appreciation of the common elements of hypometabolism is crucial for comparative biochemists as we continue to explore the molecular unity and diversity of animal life on Earth. Studies in my lab are supported by NSERC Canada. For more information go to www.carleton.ca/~kbstorey.

Understanding how marine invertebrates handle the redox stress of hypoxia and reoxygenation

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Maximum performance, such as highest respiration and heart rates, or pumping activities are often interpreted as marking the range of "optimal physiological performance" in marine species. Mollusks and especially some bivalves are hypoxia-adapted to the point where they maintain extremely low oxygen partial pressure (<4 kPa pO_2) in shell water and hemolymph over prolonged periods of several hours *against* normoxic (21 kPa) "outside" media (water). This results in low metabolic rate and may contribute to the extreme longevity of some bivalve species. Shell water pO_2 is, however, not constantly low, but oscillates between high and low values at species specific patterns in mobile and sessile bivalves, and at least the gills experience these fluctuations. Alternations of tissue pO_2 between hypoxia and reoxygenation produce oxidative stress in vertebrate models. Hypoxia tolerant ectotherms (frogs, turtles) up-regulate antioxidants during hypoxia to avoid oxidative damage during reoxygenation. Low *a priori* metabolic rates in mollusks and the induction of an alternative oxidase (AOX) that shortcuts ETS after complex 1 and reduces phosphorylation rates and mitochondrial membrane potential both curtail the risk of ROS production during reoxygenation. Therefore, induction of antioxidant levels during hypoxic exposure is often not detectable, and presumably not necessary. Furthermore, lower shell water pO_2 enables constitutive protein levels of hypoxia-inducible factor (HIF) in molluscan tissues that reinforces expression of genes supporting anoxic survival. Environmental adaptation also strongly shapes the response of the oxygen dependent subunit HIF-α at the level of mRNA expression. Intertidal species or populations are faster in raising HIF-α mRNA levels during experimental hypoxia/anoxia exposure than related subtidal species or populations exposed to the same stress scenario.

In my talk I will show bivalve and gastropod examples in support of these low oxygen strategies in evolutionary early non-model organisms, which can be so different from all we perceive as "normal".

Revisiting the concept of "preparation for oxidative stress" in hypoxia and post-hypoxic stress

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Organisms that tolerate wide variations in oxygen availability, especially to hypoxia, usually face harsh environmental conditions during their lives. Such conditions are, for example, lack of food and/or water, low or high temperatures and reduced oxygen availability. In contrast to an expected strong suppression of protein synthesis, a great number of these animals present increased levels of endogenous antioxidant defenses during oxygen deprivation. These observations have puzzled researchers for more than twenty years. Initially, two predominant ideas seemed to be irreconcilable, one was that there would be an assured reduction of reactive oxygen species (ROS) production in these animals during hypoxia, while the other was that the induction of antioxidant enzymes would require the overproduction of ROS. This induction of antioxidant enzymes during hypoxia was viewed as a way to prepare animals for oxidative damage that may happen ultimately during reoxygenation. The term "preparation for oxidative stress" (POS) was coined (in 1998) based on such premise. However, there are many cases of increased oxidative damage in several hypoxia tolerant organisms exposed to hypoxia, or other conditions of low oxygen stress, that are freezing exposure, severe dehydration, estivation and air exposure of water breathing animals. In addition, over the years, the idea of a reduction of ROS formation under hypoxia was dismissed. Actually, several findings indicate the opposite. Therefore, it became possible to provide a molecular explanation for the POS process in animals under low oxygen stress. We propose that under reduced oxygenation ROS are overproduced and induce changes in the activity of antioxidant enzymes most probably by regulating specific transcription factors (FoxOs, Nrf2, HIF-1α, NF-κB and p53) and posttranslational mechanisms. We also propose herein that the observed increased levels of endogenous antioxidant defenses in several torpid mammalian hibernators should also be regulated by the molecular POS mechanisms that resemble those of hypoxic-tolerant species, even though there is no evidence that torpid hibernators experience hypoxia.

Temperature and hypoxia influence on oxidative stress markers of aquatic animals exposed to environmental pollutants.

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Hypoxia and temperature variation represents important stressful conditions to aquatic organisms. Some of the physiological adjustments to these conditions are related to oxidative stress, since it has been known that these conditions can lead to increases in the production of reactive oxygen species that instigates alterations in antioxidant defence systems. These adjustments are very intricate and require an adequate health status of the organism, and any disturbance on the physiology of organisms, such as pollutant exposure, can have a relevant impact on how the animal endures such environmental insults. The aim of this work is to present results from studies carried out by our group on mussels *Perna perna* subjected to hypoxia in combination or not to diesel oil exposure, and on anuran tadpoles exposed to herbicides under different temperatures. Mussels maintained in clear seawater or diesel-contaminated seawater were exposed to air (hypoxia) for different periods of time, and then re-submersed (reperfusion) in clear or diesel-contaminated seawater. Oxidative stress parameters (lipid peroxidation, antioxidant defenses and lysosomal stability) were measured. In brief, hypoxia decreased the activities of some antioxidant enzymes, turning the mussels more susceptible to oxidative stress, although no increase was observed in lipid peroxidation. However when mussels were subjected to reperfusion in diesel-contaminated water, a substantial increase in lipid peroxidation was observed, indicating that the presence of pollutants in seawater can negatively affect the oxidative stress responses of mussels to hypoxia/reperfusion in natural environments. With regard to temperature effects on tadpoles, the animals (*Rhinella schneideri*) were exposed to two different herbicides (sulfentrazone and chlomazone), at three different concentrations, and maintained at 28, 32 and 38 ^{o}C. In general, oxidative stress was more evident as higher the temperature, and this effect was substantially more drastic in tadpoles exposed to the herbicides, highlighting the implication of pollutants on disrupting the responses of aquatic organisms to changes in water temperature.

Adaptive response to oxidative stress: From bacteria to human

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Under aerobic conditions, living organisms are continuously exposed to reactive oxygen species (ROS) of both, endogenous and exogenous origin. Owing to existence of finely regulated antioxidant systems they are able to maintain certain steady-state ROS levels. However, under certain circumstances, the balance between ROS generation and elimination is disturbed leading to enhanced ROS level with certain structural and functional consequences called “oxidative stress”. Here I am going to characterize principal mechanisms of regulation of protection against oxidative stress – upregulation of gene expression of antioxidant enzymes. To date, many mechanisms responsive to increased ROS concentrations have been characterized to different extent. There are at least two known mechanisms of sensing and transduction of signals from ROS to transcription machinery. The first one, is found only in bacteria and is based on reversible oxidation of [Fe,S]-clusters in SoxR sensor protein with further transmission of the signal to corresponding targets. The second mechanism is based on reversible oxidation of cysteine residues in specific regulatory proteins. The latter mechanism is realized in bacterial OxyR regulon, yeast H_2O_2-stimulon, plant NPR1/TGA and Rap2.4a systems, and animal Keap1/Nrf2, NF-κB and AP-1, and others. The “animal” group is known to be presented in humans as well. Although hundreds studies have been carried out in the field with different taxa, the comparative analysis of adaptive response is quite incomplete and therefore, this paper aims to cover a plethora of phylogenetic groups to delineate common mechanisms. Potential future directions of this research will be discussed. The comparative approach is used to shed light on fundamental principles and mechanisms of regulation of antioxidant systems. The idea is to provide starting points from which we can develop novel tools and hypothesis to facilitate meaningful investigations in the physiology and biochemistry of organisms’ response to oxidative stress. Under environmental stress the mechanism discussed are not only the basis for survival, but also are useful to occupy new ecological niches.

Determination of free radical formation under environmental stress in aquatic invertebrates

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In oxidative and nitrosative stress studies, the quantification of antioxidant defenses and oxidative damage are often not easily interpretable without a proper determination of reactive oxygen (ROS) and nitrogen (RNS) species production. The only quantitative way to measure ROS production is electron paramagnetic resonance (EPR). As EPR is often not available, live-imaging (LIT) techniques using ROS sensitive fluorescent dyes have become popular in free radical research. Using life imaging the cellular source structures of ROS formation can be identified and the underlying physiological processes can be experimentally determined. We studied the oxidative metabolism related to environmental change (notably salinity variations and hypoxia-reoxygenation) in invertebrate models. In our talk we provide an overview of results ranging from fluorometry of crab hemocytes, to confocal analysis of complete platyhelminths and *ex-vivo* bivalve gills. Based on our experience, we will also make an overview of the pros and cons of using such techniques, a matter which is strongly debated since some years.

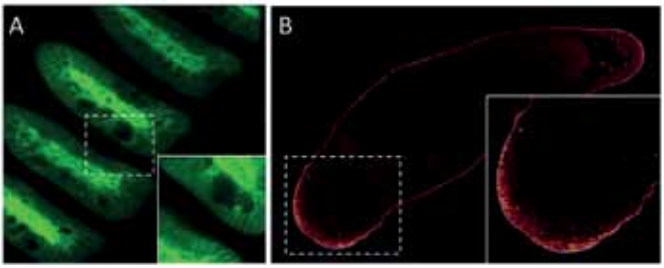

Figure: LIT applied to different study models. A) DCF fluorescence in *Mytilus edulis* excised gills. B) MitoSOX staining of living *Macrostomum lignano.*

Redox balance, endogenous antioxidants and metabolic adjustments during tropical diapause in the sunflower caterpillar *Chlosyne lacinia* (Lepidoptera: Nymphalidae).

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Many studies have focused on the modulation of endogenous antioxidants during situations of metabolic depression. However, few have studied the redox metabolism during the diapause of tropical insects. Thus, our aim was to identify adaptations of the redox metabolism associated with the tropical diapause of the caterpillar *Chlosyne lacinia* during the dry season in Central Brazil. We measured the activity of metabolic and antioxidant enzymes, as well as glutathione levels, in whole body homogenates. Experimental groups were active animals (control), diapausing animals for 0-24 hours, 20, 40, 60 and 120 days, and post-diapause active animals. Citrate synthase activity decreased (70%) at diapause beginning and returned to control levels at the end of diapause. The activities of ascorbate peroxidase (64%), catalase (51%) and glutathione peroxidase (43%) also decreased during diapause. Lipid peroxidation levels were 60% lower in diapausing animals. On the other hand, isocitrate dehydrogenase (ICDH) and glutathione transferase (GST) activities rose sharply during the first hours of diapause by 360% and 370% respectively. The concentrations of total glutathione (GSH-eq), reduced glutathione and disulfide glutathione (GSSG) all fell during diapause and returned to control levels after diapause. Therefore, the GSSG/GSH-eq ratio was unaltered. Increased ICDH and GST activities stand as important adjustments for the diapause phenotype. Moreover, the maintenance of redox balance (i.e. GSSG/GSH-eq ratio) indicates that *C. lacinia* presents efficient biochemical adaptations during tropical diapause.

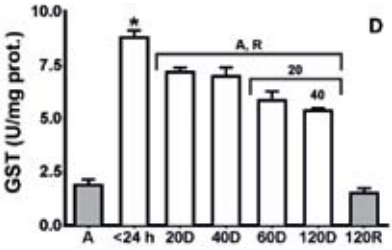

Figure: GST activity from active (A); diapausing for less than 24 hours (<24 h), 20, 40, 60 and 120 days; and post-diapause larvae. Statistical differences are: A – *versus* active; 20 or 40 – *versus* diapause for 20 or 40 d; R – *versus* post-diapause; * – *versus* to all other groups. N=7.

Temperature affects oxidative stress, resulting damage and its defense in *Daphnia*

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Poikilothermic aquatic organisms are challenged by changes of ambient temperature conditions. In response to altered temperatures, metabolic rate, respiration and oxygen transport capacity are increased in water fleas of the genus *Daphnia*. Mitochondrial energy production, however, is a major source of reactive oxygen species (ROS). Their potential danger to cellular macromolecules is defeated by specific enzymatic and redox buffer mechanisms.

In daphnids acclimated to temperatures in the range of 10°C to 30°C or challenged by acute heat stress, these interrelations have been studied. Oxidative stress as indicated by ROS production rate was measured with a fluorometric assay, and the amount of lipid damage was determined with the TBARS method. Moreover, components of oxidative defense were analysed, monitoring the glutathione system and ROS-detoxifying enzymes like catalase.

Acclimation temperature was found to affect ROS levels to a greater extent than acute heat stress, which may be the result of adjusted mitochondrial density. Similarly, macromolecular damage showed larger differences comparing animals from varying acclimation temperature than in response to acute exposure to elevated temperatures. Here, a higher level of lipid damage in cold-acclimated animals was observed. Finally, ROS defense systems (catalase, glutathione) also showed characteristic responses to temperature changes, suggesting a role in limiting oxidative damage as well as for signalling processes. All in all, the observed phenotypic plasticity, adjusting metabolic characteristics to the different needs at altered temperatures, seems to result in enhanced vulnerability of cold-acclimated animals towards oxidative stress.

Role of catalase in the redox control of hypoxic stress in tilapia and land gastropods

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Humans and most mammals suffer severe damage when exposed to an ischemia reperfusion episode due to an overproduction of reactive oxygen species (ROS). In contrast, several hypoxia/anoxia tolerant animals survive very similar situations, an ability that has been attributed to an efficient antioxidant system. However, the specific contribution of each antioxidant enzyme to protection against the reoxygenation-associated oxidative stress after periods of hypoxia/anoxia is not well understood. We evaluated the role of catalase in cycles of wide and abrupt change in oxygen availability (expected to cause a rise in ROS production) in two animals by its *in vivo* inhibition with 3-amino-1,2,4-triazole (ATZ): hypoxia tolerant Tilapia fish and pulmonate and anoxia tolerant snails, *Helix aspersa*. Neither a cycle of hypoxia/anoxia-reoxygenation nor the inhibition of catalase resulted in significant changes in many parameters in the three analyzed tissues (liver, hepatopancreas and foot muscle): activity of various antioxidant enzymes, levels of glutathione and oxidative stress markers (GSSG:GSH-eq ratio, TBARS and carbonyl protein). In fish, the disulfide/reduced glutathione ratio increased during reoxygenation only in ATZ-injected fish, demonstrating a redox imbalance caused by catalase suppression. This result indicates that catalase is relevant for the control of hepatic ROS formation in fish under hypoxia/reoxygenation. In contrast, in snails, post-anoxia reoxygenation did not cause changes in parameters that are known to become changed in different animals by either catalase suppression or reoxygenation and that indicate redox imbalance and oxidative stress (levels of GSSG/GSH, TBARS and carbonyl protein). Anoxia exposure caused an increase in selenium-dependent glutathione peroxidase (Se-GPX) activity in the foot muscle of both saline- and ATZ- injected snails (by 2.0 and 1.7 fold, respectively). The exceptional resistance to A/R stress of *H. aspersa* may be explained by the increase in Se-GPX activity in foot muscle and the maintenance of other antioxidant parameters in foot and hepatopancreas.

Air exposure behavior of the semiterrestrial crab *Neohelice granulata* allows tolerance to severe hypoxia but not prevent oxidative damage due to hypoxia-reoxygenation cycle

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The air exposure behavior of the semi-terrestrial crab *N. granulata* during severe hypoxia was studied to verify if it in fact allow increase of tolerance to severe hypoxia and does not induce oxidative damage, caused by hypoxia and reoxygenation cycles. The lethal time for 50% of the crabs subjected to severe hypoxia (0.5 $mgO_2.L^{-1}$) with free access to air during 96h was compared to that of crabs subjected to severe hypoxia without access to air. In other experiment, crabs were placed in aquaria divided into three zones (water, intermediate, land) for 270 minutes, and the time spent in each zone was recorded. Lipid peroxidation (LPO) damage to the walking leg muscles was determined for the following four experimental conditions: normoxic water with free access to air (C); hypoxic water without access to air (H); hypoxic water followed by normoxic water without air access (H/R); and hypoxic water with free access to air (H/A). When exposed to hypoxic water, *N. granulata* spent significantly more time on land, approximately 135min, whereas animals exposed to normoxic water spent more time submerged, approximately 187 min. By this behavior, *N. granulata* was able to maintain a 100% survival rate when exposed to severe hypoxia. However, *N. granulata* still return to water after periods of air exposure (~14 min), causing a sequence of hypoxia and reoxygenation events. In the LPO analysis, the control and hypoxia groups had different ($p < 0.05$) LPO levels than the hypoxia/re-oxygenation and hypoxia/air exposure groups (Figure). Therefore, despite increasing the survival rate, hypoxia with air access does not decrease the lipid peroxidation damage caused by the hypoxia and reoxygenation cycle experienced by these crabs.

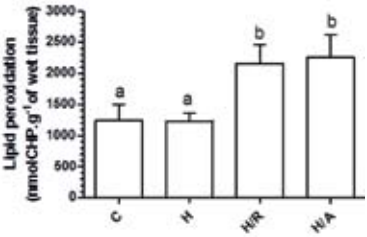

Figure: Muscle lipid peroxidation levels in the crab *N. granulata*. Data are mean ± s.e. for three experiments with five individuals each. Different letters represent differences ($p < 0.05$) between groups.

How do osmoregulating tissues cope with oxidative stress? A Mediterranean crab as a study model

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Green crabs are one of the most osmotically tolerant species in estuaries, and thus represent an interesting physiological model in osmoregulation studies. The Mediterranean crab *Carcinus aestuarii,* possess two groups of spatially segregated gills: anterior gills (pairs 1-6) serve purely respiratory purposes, while posterior gills (pairs 7-9) contain osmoregulatory structures. The presence of similar tissues serving partially different purposes provides a unique opportunity to study differential adaptation, in terms of free radical metabolism, upon salinity change. *C. aestuarii* were immersed for two weeks in sea water (SW) (37ppm), diluted SW (dSW, 10ppm) and concentrated SW (cSW, 45ppm). Measurements of hemolymph osmotic pressure indicate that the species is an osmoconformer down to 25ppm and hyper-regulates below this point. Animals acclimated at cSW show the lowest respiration rates (RR) and reactive oxygen species (ROS) formation (measured fluorometrically though C-H_2DFFDA on hemolymph and through electron spin resonance, EPR, on tissues). Exposure to decreased environmental salinity leads to increased RR, reduced tolerance to hypoxia and increased circulating ROS levels. Further analyses on excised tissues show how posterior gills are responsible for the high RR and also ROS production (EPR). However, these posterior gills are able to avoid cellular damage by enhancing antioxidant activities (catalase and superoxide dismutase). Anterior gills, on the other hand, show no differences in antioxidant capacity among treatments which translates into a dramatic increase in caspase 3/7 activity (which for dSW-acclimated animals is 2.4-fold and 5-fold higher than those placed in SW and cSW, respectively). Our results support the hypothesis that a “preparation for oxidative stress” mechanism is put in place in osmoregulating gill tissues to avoid the negative consequences of FR formation derived from the increased metabolic rates fueling active ion pumping. Further research will be directed towards identifying which molecules are involved in signaling and triggering such an adaptation.

Effects on total antioxidant capacity and lipid peroxidation *in vivo* exposure of golden mussel (*Limnoperna fortuneii*) to graphene oxide carbon nanomateiral.

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In recent years the use of materials at the nanoscale, which have different properties than at the macro scale, has been intense due to their technological applications. Several nanomaterials (NM) posses distinct properties such as electrical, flexibility, transparency, etc. The carbon NM graphene and its derivatives have been introduced recently in manufacturing processes. In the present study we evaluated the potential toxic effects of two concentrations of graphene oxide (GO): one low (GO1) of 0,25 mg/l and other high (GO2) of 2,5 mg/l, through exposure *in vivo* in fresh water bivalve in *Limnoperna fortuneii* (golden mussel). After 96 hour of exposure oxidative stress parameters were evaluated as total antioxidant capacity (ACAP) and lipid peroxidation (TBARS) in four different organs of the mussel: gills, mantle, adductor muscle and digestive gland. The results showed that there was no significant difference between the control and treatments in the gills, mantle and digestive gland. Adductor muscle of mussels exposed to GO2 presented a reduction of total antioxidant capacity ($p<0.05$). When analyzed lipid peroxidation by TBARS it was found significant differences ($p<0.05$) between control and treatment in all organs. There was an increase of peroxidation in treatment of higher concentration (GO2) in mantle and adductor muscle and in the treatment of lower concentration (GO1) in gill. On the other hand, in the digestive gland, at both GO concentrations it was observed lower TBARS levels than the control group.Thus, it is clear that GO present in the water column is able to be internalized by sessile animals and that regardless of their concentration, this NM is able to induce damage and compromise the capacity antioxidant in the different organs of *L. fortunei*.

Climate Change and Associated Challenges: How Fish Will Cope

Organizers (*South American Society for CPB*):

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The lips of tambaqui: Adaptation or limitation under climate change scenarios

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Colossoma macropomum is a common fish of the Amazon where it is locally known as tambaqui. Tambaqui regularly faces periods of extreme hypoxia in its habitat along the rivers of the region. As many fish species, it can improve many adjustments to safeguard oxygen transfer to tissue during those extremely poor oxygen conditions. One of these adjustments is the expansion of lower lips to skim the water surface that is richer in dissolved oxygen. Below 10% oxygen saturation almost all animals expand the lips in about two hours. Similarly, the lips are retracted in about two hours when normoxia is restored. Earlier studies from our lab showed that specimens of hypoxic tambaqui drown if denied access to the water surface, presenting near 60% decrease in blood oxygenation. Recent studies shed light on the mechanisms behind lips expansion in tambaqui. These studies showed that several genes are up-regulated while others are down-regulated, clearly suggesting that lip expansion in tambaqui is a complex mechanism genetically encoded. Our previous study (Groff *et al.*, 2010, Journal of Photochemistry and Photobiology B: Biology 99: 93–99) showed that hypoxic tambaqui exposed to UV radiation for different periods presented clear dose-response in DNA damage profile, despite a significant damage repair was observed 24h after cessation of UV exposure. The climate change scenarios foreseen by Intergovernmental Panel for Climate Change for the near future include among others a “savanization” of part of the Amazon, increased water temperature, acidification and even lower dissolved oxygen. These conditions would significantly affect tambaqui that will respond to lower oxygen using the lips to skim the water surface that will be more exposed to sunlight and so, to UV. As many other fish species in the Amazon depend on water surface to maintain regular oxygen supply to tissues, they are all vulnerable to the foreseen climate change scenarios. The same can be projected for tambaqui rose in open surface ponds what would drastically affect fish production in the Amazon.

INCT ADAPTA/CNPq/FAPEAM)

Mitochondrial plasticity as a determinant of surviving climate change

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The thermal limit of cardiac mitochondrial efficiency could be a major determinant of species distribution (Iftikar *et al*., 2014). The effect of high temperature on brain mitochondria is less well explored. We examined the effect of 6 temperatures (20°C, 25°C, 30°C, 37°C, 40°C and 45°C) on brain mitochondrial function in homogenates from two Orectolobiform sharks: the Epaulette shark (*Hemiscyllium ocellatum*), which undergoes metabolic depression in response to anoxia, and the Grey carpet shark (*Chilloscyllium punctatum*) which does not. Yet the latter can withstand comparable periods of anoxia to the Epaulette shark. Both of these sharks can occupy shallow reef flats and estuarine habitats potentially exposing them to temperature stress during summer low tides. We measured the effect of each temperature on: i) mitochondrial coupling efficiency; ii) non-phosphorylating proton leak from mitochondria; and iii) changes in substrate utilisation for complex I and complex II.

High resolution oximetry with a multiple substrate-inhibitor protocol revealed that for both species: mitochondrial coupling (efficiency) was greatest at 25°C, and maintained at 30°C but was 25% lower at 37°C and 50% lower at 40°C. Mitochondria in both species were totally uncoupled at 45°C.

Despite an exponential increased in proton leak as temperature increased, Epaulette mitochondria maintained their electron transport system in coupled mitochondria at 25-37°C, while Grey carpet shark mitochondria showed a 30% decrease in mitochondrial efficiency at 37°C compared to 25°C. Examination of substrate utilisation revealed that Epaulette shark mitochondria had a more stable complex 1 utilisation than Grey carpet sharks, especially at 37°C.

These two species of Orectolobiform sharks maintained mitochondrial coupling efficiency over a wide range of thermal challenges but failed at temperatures higher than they currently encounter in their natural environment.

Iftikar *et al.*, (2014) J. Exp. Biol. 217, 2348-2357.

Effects of increasing temperature on the activity of key metabolic enzymes in liver of the Antarctic fish *Notothenia coriiceps*

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The Antarctic Peninsula has experienced a continuous rise in water temperature. Therefore, investigating how and at what extent the activities of metabolic enzymes of Antarctic stenothermal fishes are affected by exposure to increasing temperatures is of great interest. In fact, alterations in the activity of enzymes associated with the aerobic capacity of mitochondria have been linked to changes in the limits of thermal tolerance in fish. In the present study, we analyzed the effect of increasing temperature on the activity of enzymes involved in glycolysis [hexokinase (HK); pyruvate kinase (PK); lactate dehydrogenase (LDH)], Krebs cycle [citrate synthase (CS)] and the pentose-phosphate pathway [glycose-6-phosphate dehydrogenase (G6PDH)] in the liver of the Antarctic fish *Notothenia coriiceps*. Enzyme activity was determined in field-collected and laboratory-acclimated fishes. In laboratory, fishes were acclimated to 0°C and kept at 0°C (control) or exposed to 4°C for 1 and 6 days. The HK activity was significantly reduced in fish exposed to 4°C for 1 and 6 days, while the PK activity was decreased only in fish exposed to 4°C for 6 days. The LDH activity was not affected by the temperature increase. However, there was a reduction in the enzyme activity in fish acclimated to 0°C for 6 days when compared to the field-collected fish. There was a decrease in CS activity in fish kept in 0°C or 4°C for 6 days when compared to those maintained under the same conditions for 1 day. The G6PDH activity was increased in fish exposed to 4°C for 1 and 6 days. These findings indicate that the activity of enzymes involved in energy production is affected by water temperature changes. The increased G6PDH activity suggests the involvement of the pentose-phosphate pathway in the use of liver glucose, thus pointing its role in the maintenance of the cellular redox status under stressing conditions. In turn, the lower activity of glycolytic enzymes observed in fish exposed for 6 days indicates a reduced energy availability via glucose metabolism, thus limiting the capacity of *N. coriiceps* to reorganize the energy metabolism and its thermal plasticity in response to increasing water temperature.

Greater flexibility in metabolic rate confers a growth advantage under changing food availability

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Phenotypic flexibility in physiological, morphological and behavioural traits can allow organisms to cope with new and changing environments. Flexibility in standard metabolic rate (SMR) may be particularly important since SMR reflects the minimal energetic cost of living and is one of the primary traits underlying organismal performance. SMR can increase or decrease in response to food availability, but consequences of these changes for growth rates and other fitness components have not been evaluated. Evidence suggests that individuals with a higher SMR grow faster under high food levels, while a lower SMR can be beneficial to growth at low food levels. If these are causal relationships, there could be significant growth advantages for those individuals that are better able to modify their SMR under changing food levels of food availability.

We examined individual variation in metabolic flexibility in response to changing food levels and its consequences for somatic growth in juvenile wild-origin brown trout (Salmo trutta). Like many other organisms, larger body size often confers an advantage in competitive interactions and survival in young brown trout, so early growth rates can have important consequences for fitness. However, food availability can exhibit marked spatial and temporal variation in the freshwater streams they inhabit, so flexibility in their metabolic rates may be critical to growth.

We found that SMR increased when individuals were switched to a high food ration and decreased when they were switched to a low food regime. Shifts in SMR, in turn, were linked with individual differences in somatic growth; individuals that increased their SMR more in response to elevated food levels grew fastest while individuals that depressed their SMR more in response to lowered food levels fared better under those conditions. These results suggest that flexibility in standard metabolic rate is a key mechanism that allows organisms to cope with variable environments.

Long-term overnight hypoxia is compensated for by similar growth rates in tambaqui (*Colossoma macropomum*, Cuvier 1818)

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Overnight hypoxia is a common event in Amazon waters. Aquaculture pond presents these characteristics also. Fish of the Amazon are adapted to these conditions, although hypoxia may affect their metabolism and impair adequate growth. We have analyzed the effects of overnight hypoxia over 90 days on growth rate, biochemical and physiological parameters of *C. macropomum*. The results revealed that tambaqui exposed to overnight hypoxia over 90 days presented no major changes of hematological parameters, suggesting an effective mechanism for adjustment to overnight hypoxia. However, during the first 15 days fishes lost weight and presented low specific growth rates, reflecting a possible reduction in metabolism, probably in the attempt to minimize energy expenditure with digestive processes. After this period, they presented a gradual increase in weight and growth rate, reaching, at the end of three months, 81% of weight gain compared to the animals kept under normoxia. The increased blood lactate and liver and muscle LDH levels explain these remarkable recover growth rates. Simultaneously, decreased aerobic rates were observed; muscle CS and HOAD had reduced levels during fish growth period. Interestingly, liver and muscle MDH increased in hypoxic fishes, suggesting higher rates of gluconeogenesis. The present findings suggest that tambaqui is adapted to chronic overnight hypoxia and grow over time by adjusting its physiological and biochemical parameters, reducing energy expenditure by the increase of anaerobic/aerobic rates

INCT ADAPTA – CNPQ and FAPEAM

Thermal sensitivity of cell metabolism of different Antarctic fish species mirrors organism temperature tolerance

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The Antarctic fish fauna has evolved distinctive physiological specialization to conquer this unique environment, and will act quite sensitive to ocean warming. Here we test if thermal sensitivity of cell metabolism, in particular energy expenditure for protein synthesis mirrors organism thermal tolerance by comparing different Antarctic fish species: the circumpolar distributed Antarctic eelpout, *Pachycara brachycephalum* after long-term warm-acclimation (0°C vs. 5°C acclimation), and, along a latitudinal gradient, the sub-Antarctic nototheniid fish, *Lepidonotothen squamifrons* and the high-Antarctic white-blooded icefish, *Chiondodraco hamatus* (sub- vs. high-Antarctic).

Warming-induced rise in hepatic cell respiration was similar between acclimation as well as latitudinal groups whereas temperature-dependent rates of protein synthesis differed between species. Despite long-term acclimation, energy expenditure for protein synthesis was similar in hepatocytes from 0°C and 5°C acclimated *P. brachycephalum* and decreased after acute warming from 5 to 10°C. A finding which is in line with reduced organism growth rates observed at temperatures beyond 5°C. In *L. squamifrons* a rise from 0 to 5°C doubled the fraction of protein synthesis to overall cellular respiration while it remained unaffected in the high-Antarctic icefish *C. hamatus*.

Observed temperature dependent cellular energy expenditure for protein synthesis mirrors organism thermal growth performance suggesting that thermal sensitivity is manifested at the cellular level thereby paralleling the degree of cold adaptation of the investigated fish species. The data suggest further that sub-Antarctic fish might benefit from warmer waters contrary to high Antarctic fish like *C. hamatus*.

In conclusion, cellular thermal sensitivity can be linked with organism temperature tolerance making cell cultures a useful tool in studying environmental impact on marine ectotherms.

Ionoregulation in two Amazonian erythrinid teleosts: Air-breathing compromises branchial ion uptake

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The traira (*Hoplias malabaricus*) and the jeju (*Hoplerythrinus unitaeniatus*) are both members of the erythrinid family living in the Rio Negro black water. The traira is an obligate water breather with large gills and a poorly vascularized air-bladder, whereas the jeju is a facultative air-breather using its highly vascularized air-bladder for aerial oxygen uptake. Total gill surface area, however, is significantly reduced in the jeju. We examined net ion fluxes (Na^+, Cl^-, and K^+) as well as ammonia and urea excretion under normoxia, severe hypoxia (1 mg O_2/L) and hyperoxia (30 mg O_2/L). The rate of urea excretion was elevated in the jeju as compared to the traira, but the N/Q ratio was not significantly different. Net Na^+, Cl^- (positive in traira, negative in jeju) and K^+ fluxes between the fish and the Rio Negro water were significantly different between the two species, indicating an impairment of branchial ion uptake in the air-breathing jeju. Neither hypoxia nor hyperoxia had a significant effect on net ion fluxes in the jeju, while in traira Na^+ and Cl^- uptake rates were reduced under these conditions. To assess the possible contribution of the gut to ion homeostasis we analyzed fluxes and transport capacities for Na^+, Cl^-, K^+, ammonia, and glucose of the anterior, mid, and posterior intestine in gut sac preparations *in vitro*. In anterior gut sections of the jeju, the area specific Na^+, Cl^-, and K^+ uptake rates were significantly higher than in anterior gut sections of the traira. Similarly, the overall transport capacities of the jeju gut for these three ions as well as for ammonia and glucose by far exceeded the capacities calculated for traira. We conclude that in the transition to air-breathing, there is a shift of ionoregulatory capacity from the gills to the gut in freshwater fish, and in consequence branchial ionoregulation becomes less impacted by variations in environmental O2 levels.

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The Evolution of Innate and Acquired Immunity

Organizer:

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Constraint and adaptation in salamander immune genes

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Acute die-offs of amphibian populations worldwide have been linked to the emergence of viral and fungal diseases. Inter- and intra-specific immunogenetic differences may influence the outcome of infection, but selective landscape of amphibian genes involved in immune response is poorly understood. We used targeted re-sequencing and high-throughput amplicon re-sequencing to scan almost 650 immunological genes in two closely related species of newts (*Lissotriton montandoni* and *L. vulgaris graecus*) for signals of adaptive evolution with special emphasis on balancing selection. The studied newts are members of a complex of recently diverged, morphologically distinguishable taxa, characterized by a history of gene flow. We used several population genetic approaches to identify plausible targets of positive and balancing selection. To minimize the incidence of false positives and maximize power of the scan a sampling scheme accounting for the effects of population structure was applied and a demographic model estimated from the data was used to generate neutral expectations. We present a set of genes which have likely evolved adaptively in the recent evolutionary history of the *Lissotriton* newts and discuss their relevance to the understanding of immune response and utility in conservation. We put special emphasis on Toll-like receptors (TLRs) which are an essential component of innate immunity and also prime acquired defenses. We find evidence for purifying and positive selection acting on TLRs in both species. Overall, both forms of selection have been stronger in *L. v. graecus*, while constraint on most TLR genes in *L. montandoni* appears relaxed. We infer that TLR genes undergo distinct trajectories of adaptive evolution in closely related amphibian lineages, highlight the potential of TLRs to capture the signatures of different assemblages of pathogenic microorganisms, and speculate about possible differences between lineages in the relative roles of innate and acquired immunity.

Immune and neuroendocrine regulation of inflammation in teleost fish

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The neuroendocrine- and immune systems interact in a bi-directional fashion, pathogen recognition is communicated to the brain and the immune response is influenced by physiological changes. We hypothesize that this is essential for homeostasis during stress and infection to create a balanced immune response. The network includes corticosteroids, classical pituitary hormones, cytokines, chemokines, neuropeptides and neural pathways. Leukocytes may also produce many neuro-endocrine factors, e.g. corticotropic releasing factor (CRF), opioids, melatonin, as well as hormones belonging to cytokine type I family: growth hormone (GH) and prolactin (PRL). Neuroendocrine-immune interaction is a phylogenetically conserved phenomenon. Teleost fish form nice and well established models that bridge the gap between moral concerns in using higher vertebrates and the undoubted advantages of a complete vertebrate model comprising a fully developed immune system. We studied the role of chemokine-, opioid-, adrenergic- and melatonin G-protein coupled receptors (GPCR) on carp (*Cyprinus carpio*) leukocytes during inflammation. Ligand interaction by morphine, adrenaline and melatonin both *in vitro* and *in vivo* considerably decreased chemotaxis and expression of CXC chemokines and/or their receptors. This may have substantial influence on the process of inflammation, the efficacy of which is crucial for an effective immune response and is highly dependent on leukocyte recruitment by chemokines. Different families of GPCRs interact through receptor desensitization and heterodimerization. Both phenomena may have important physiological consequences including changes in receptor pharmacology, downstream signalling and receptor trafficking or internalization. For mammals it is hypothesized that in this way the analgesic effect of opioid receptors might be weakened to enhance pain perception at inflammatory sites where chemokines and chemokine receptors are abundantly present. Vice versa opioid binding to their receptors might significantly reduce the migratory properties towards chemokines. This phylogenetically conserved mechanism may serve to alert for inflammatory danger while safeguarding the animal for excessive, thus harmful, inflammatory responses.

Expression of estrogen receptors in/on carp leukocytes: indications for neuroendocrine-immune interaction via estrogens

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Estrogens are well-known for their reproductive function but they also modulate the immune response. It is well documented for mammals that estrogen-induced immunoregulation is mediated via nuclear (ERs) or membrane (GPR30) receptors, expressed differentially on/in leukocyte populations. Expression of estrogen receptors on/in fish leukocytes and their involvement in immune regulation and host defence are less explored and poorly understood. The present study aimed to measure the expression of estrogen receptors in the lymphoid organs and on/in leukocyte populations of common carp and to identify effects of their agonists/antagonists on leukocyte activity. We found that ERa, ERb and GPR30 genes are constitutively expressed in lymphoid organs (spleen, thymus, head kidney) and in lymphocytes, monocytes/macrophages and neutrophilic granulocytes. In lymphoid organs the GPR30 gene showed lower expression than ERa and ERb. Genes for both ERa and ERb showed a similar level of expression in the thymus while in spleen and head kidney, expression of ERb was higher than ERa. Moreover, the expression of ERa in the thymus was higher than in the spleen. Compared to the ERb and GPR30 genes, in leukocytes the expression of the ERa gene was higher. Interestingly, immunocytochemical studies suggest that lymphocyte populations differ in ERb expression, therefore further studies are needed to clarify which population of lymphocytes does not express ERb. 17-b estradiol and G1 (GPR30 agonists) increased PMA-induced production of reactive oxygen species. This was down-regulated with the GPR30 antagonist - G15 or wortmannin (inhibitor of PI3Ks) which suggests that estrogen acts via the membrane GPR30 receptor and that PI3K is involved in this process. In summary, estrogen receptors are expressed in carp leukocytes and immune organs and participate in the regulation of the immune response.

Extracellular traps as a conservative mechanism of phagocyte defense towards pathogens

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All organisms are being constantly exposed to or actively attacked by pathogens present in the surrounding milieu. For this, independently of their complexity, all animals, but also plants, developed means to defend themselves. Some of the mechanisms are universal, including phagocytosis, oxidative burst, and toxic killing by endogenous proteases and antimicrobial proteins. And just a decade ago, a new common mechanism was described that is called Extracellular Traps (ET). ETs were originally reported to be formed by neutrophils and such NETs were shown to catch, immobilize and (in some cases) kill the pathogens. NETs consist of decondansated DNA which serves as a back-bone to which granular (e.g. neutrophil elastase, NE) and nuclear (e.g. histones) components of neutrophils are attached. First *in vitro* studies reported on NADPH-dependent reactive oxygen species (ROS) being involved in NET formation, and revealed a significant role of NE and peptidylarginine deiminases type IV (PAD4) in this process. While the latter facilitates histone citrullination leading to DNA decondensation, NE degrades histones. Our recent studies applying intravital microscopy in mice revealed that *in vivo* NET formation also depends on NE and PAD4, but not on ROS, during bacterial systemic inflammation. Furthermore, we showed that DNase, while very effective *in vitro*, hardly removes the NET components, other than DNA, from the vasculature. This is because they secondarily attach to glycocalyx, including von Willebrand factor. Moreover, we showed that when NETs persist in vasculature or tissue, they cause collateral damage to the host.

As phagocytosing cells are widely spread throughout the animal kingdom, we have also verified ET formation in other animal groups, fish (carp) and invertebrates (earthworms). Also in these animals, ETs were seen to catch bacteria, and ET formation only weakly, if at all, was NADPH-dependent upon stimulation with physiological (bacterial, fungal or viral) factors. However, ETs of fish and earthworms are also characterized by some distinctive features. All these aspects of ETs will be discussed during the presentation.

Does the local inflammation can be still defined as local?

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Peritonitis is an inflammatory reaction characterized mainly by the recruitment of macrophages and polymorphonuclear leukocytes to the peritoneal cavity as well as chemokine/cytokine production by residential and accumulated there immune cells. Although this process develops locally inflammation and cytokines have been shown to modulate the functions of the neuroendocrine glands. One of them is the pineal gland which circadian and circannual pattern of melatonin synthesis is considered as a chemical transducer of information between environmental lighting conditions and the internal milieu. For years our research group have been investigating the mechanisms involved in the effects exerted by the experimental peritoneal inflammation on the chicken pineal biosynthetic activity. In *in vivo* studies we shown that developing peritonitis caused an inhibition of melatonin biosynthesis leading to the disappearance of its circadian rhythm. The decrease in melatonin biosynthesis is a result of transcriptional downregulation of the *arylalkylamine N-acetyltransferase* (*Aanat*) gene, followed by reduced synthesis and activity of the encoded enzyme. In *in vitro* model the IL-6 and IL-18 have been proven to be the main mediators in the pineal – immune axis. Feedback of the immune system on the pineal gland is indicated also by reports of accumulations of lymphocytes in the pineal. To support the role of this lymphocytes in our *in vivo* experimental model we shown the expression of IL-18, IL-1beta, IL-6 as well as Toll-like receptors (TLR 4, TLR 15, TLR 21) in the pineal gland. To summarize we propose that locally developing peritonitis inhibits pineal melatonin biosynthesis via 3 possible mechanisms: 1) activated peripheral blood leukocytes release IL-6 which affects pineal gland, 2) cytokines expressed in the pineal gland control melatonin biosynthesis in a paracrine way 3) direct activation by pathogens of TLRs triggers changes in the *Aanat* transcription. Therefore the hypothesis that the inflammation is only local process has to be redefined.

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Galleria mellonella as an insect model to study host-pathogen interactions

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Insects possess only an innate immune system, which allows them to cope efficiently with many kinds of infections. Despite their evolutionary distance from mammals, their innate immune mechanisms are, in many aspects, similar to innate immune response of mammals. They serve as good models to study virulence mechanisms of pathogens. The main aspects of insect immune response are cellular and humoral branches. The cellular response involves insect's blood cells – haemocytes, whose main function is phagocytosis of foreign bodies or sequestration thereof from the insect body in structures called capsules or nodules. As a part of humoral response, insects synthesise antimicrobial peptides (AMPs). The main organ responsible for systemic synthesis of AMPs and their secretion to the hemolymph is the fat body, which is an analogue of the mammalian liver. In addition, other cells or tissues such as haemocytes, lymph glands, and epithelium synthesise defence peptides. These peptides possess antibacterial and/or antifungal activity. Their mode of action is mainly destabilisation of microbial membranes but some of them can also interfere with other aspects of cellular physiology. Despite the absence of antibodies, T and B cells, the immune system of insects can be *primed* depending of their previous infection.

Galleria mellonella is a moth, whose larvae feed on the honeycomb inside bee nests and feed with wax and pollen. They cause a disease called "galleriose". It is a generally accepted and widely used insect model to study many aspects of innate immunity. We present how immune response is activated in time after development of septicaemia caused by the intrahemocoelic injection of gram-positive bacteria *Bacillus thuringiensis* and after natural infection with the filamentous fungus *Beauveria bassiana*. Further, we show how heat shock, applied directly before or during infection development, influences the host-pathogen interaction. Expression of insect immune-relevant genes, defence activity of the hemolymph, and susceptibility of insect immune-relevant proteins to virulence factors secreted by pathogens is taken into account.

Melatonin regulates chemotaxis of carp phagocytes

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The "hormone of darkness", melatonin is a crucial mediator responsible for synchronization of many physiological processes including the immune response. Interestingly, it was demonstrated that in mammals and birds leukocytes express melatonin receptors MT1/MT2 and moreover express all key enzymes necessary for melatonin synthesis. Very little is known about the involvement of melatonin in the regulation of the immune response in teleost fish.

Therefore, we focused on its role in the regulation of innate immunity in common carp (*Cyprinus carpio* L.).

We found that the MT1 receptor gene is expressed in lymphoid organs, as well as in neutrophilic granulocytes and monocytes/macrophages, the key players in inflammation. Moreover, melatonin *in vitro* and *in vivo* (during acute peritonitis) regulates the activity of leukocytes. Most robust effects of melatonin were observed for leukocyte migration. Melatonin *in vitro* in dose-dependent manner reduced leukocyte chemotaxis towards CXC chemokines, while *in vivo* it decreased migration of neutrophilic granulocytes towards the focus of inflammation. The latter phenomenon, was most probably connected with melatonin-induced down-regulation of gene expression of CXCL chemokines. These data suggest a potent anti-inflammatory function of melatonin. However, our previous observation of melatonin-induced inhibition of leukocyte apoptosis suggests a dual function for this hormone.

Therefore, we can conclude that in teleost fish, melatonin may perform a pleiotropic and extrapineal function. It implies moreover an evolutionary conserved role for melatonin in the process of neuroendocrine-immune interaction.

Is there any association between interferon-β and procalcitonin in patients with multiple sclerosis

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Interferon-beta (IFN-B) treatment reduces the relapse rate in multiple sclerosis (MS). It also increases the expression of CD73, an ectoenzyme, which produces adenosine from adenosine monophosphate (AMP) precursor by enzymatic dephosphorylation. AMP is known to be abundantly present at sites of inflammation, and more importantly adenosine, the product of CD73, is known to possess both anti-inflammatory and neuroprotective characteristics. Procalcitonin (PCT) is a peptide precursor of the hormone calcitonin. Measurement of procalcitonin can be used as an indicator of severe sepsis caused by bacteria. What is the probable relationship between IFN-B and procalcitonin in patients with multiple sclerosis? Thirty patients, diagnosed with MS (24 females, 6 males, mean ages was 28.20±7.35), were studied. Newly diagnosed patients were enrolled in this study before beginning any treatments. Patients with primary and secondary progressive disease, who were on treatments with IFN-B (Rebif), were also included. Thirty healthy people without any inflammatory or autoimmune disease were selected as normal control subjects (22 female, 8 male; mean age was 28.64±8.43). Serum levels of procalcitonin were detected in all subjects by ELISA method. Collected data were analyzed by SPSS software. Statistical significance was calculated using the Independent Student's t test for comparison between case and control groups and Pearson correlation test helped evaluating the relationships. The level of significance in all cases was set to a two-tailed $p<0.05$.According to the results, there was a significant difference in serum levels of melatonin between case and control groups. The mean values of serum procalcitonin concentrations were 0.19±0.01 and 0.24±0.02 ng/ml in case and control groups, respectively. Results also showed significant difference in concentrations of WBC between case and control groups. While the mean value for WBC was 7156±597.33 in case group, it was 8400±316.50 per ml in controls. Finally, we concluded that PCT is not a valid of sepsis inpatients with MS.

Potential response to selection of HSP70 as a component of innate immunity of the abalone *Haliotis rufescens*

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Disease resistance is reflective of immune function and can be measured by assessing the components of the immune system. In invertebrates, heat shock proteins (HSPs) are immune effectors and have been described as potent activators of the innate immune response. Several diseases have become a threat to abalone farming worldwide, therefore, increasing disease resistance is considered to be a long-term goal for breeding programs. A trait will respond to selection only if it is determined partially by additive genetic variation. The aim of this study was to estimate the heritability (h^2) and the additive genetic coefficient of variation (CV_A) of HSP70 as a component of innate immunity of the abalone *Haliotis rufescens*, in order to assess its potential response to selection. These genetic components were estimated for the variations in the intracellular (in haemocytes) and extracellular (serum) protein levels of HSP70 in response to an immunostimulant agent in 60 full-sib families of *H. rufescens*. Levels of HSP70 were measured twice in the same individuals first when they were young and next when they were pre-harvest adults, in order to estimate the repeatability (R) and the variation in the potential response to selection of this trait during the abalone lifespan. High HSP70 levels were observed in abalones subjected to immunostimulation in both the intracellular and extracellular haemolymph fractions. This is the first time that changes in serum levels of HSP70 have been reported in response to an immune challenge in molluscs. HSP70 levels in both fractions and at both ages showed low h^2 and R, with values that were not significantly different from zero. However, HSP70 induced levels had a CV_A of 13.3-16.2% in young adults and of 2.7-8.1% in pre-harvest adults. Thus, despite its low h^2, HSP70 synthesis in response to an immune challenge in red abalone has the potential to evolve through selection because of its large phenotypic variation and the presence of additive genetic variance, especially in young animals.

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Preferential temperature and metabolic rates of toads (*Rhinella icterica*) challenged with lipopolysaccharides of gram-negative bacteria (LPS)

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The ability to mount an immune response during infection at different temperatures constitutes an important element in the evolution of life history strategies. This aspect must be particularly relevant for ectotherms, such as amphibians. We studied the effect of lipopolysaccharide of gram-negative bacteria (LPS) injection on preferred temperatures of toads (*Rhinella icterica*), as well as on their metabolic rates measured at mean (A) operative temperature in the field; B) preferential temperature before LPS challenge; and C) preferential temperature after LPS challenged. Temperatures (A) were measured with an infrared thermometer and temperatures (B) and (C) were measured in a thermic gradient with a thermographic camera. Metabolic rates at different temperatures were measured through intermittent respirometry, in toads injected with LPS or saline. Operative temperatures were 17 ± 1.52 °C, and preferred temperatures were 22 ± 3.28 °C and 26± 2.34 °C, respectively before and after LPS challenge. Standard rates of oxygen consumption increased linearly with temperature. LPS injection significantly increased rates of oxygen consumption at 22°C, and toads injected with LPS showed equivalent rates of oxygen consumption at 22°C and 26°C. Our results show that: (1) higid toads prefer temperatures higher than their operative temperatures in the field, and LPS challenge evokes behavioral fever. (2) LPS injection do not cause significant metabolic responses at operative temperatures in the field; but (3) preferential temperatures by LPS challenged toads decrease metabolic scope of reaction to infection.

Cadmium accumulation in *Eisenia andrei* earthworms inhibits maturation, reproduction and growth with little effects on regeneration of depleted coelomocytes and amputated posterior segments

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Lumbricid earthworms are often exposed to polluted soil. They are also commonly subjected to the stress from attack by diverse predators that induce extrusion of coelomocyte-containing coelomic fluid via dorsal pores or the loss of body segments; in both cases a restorative period ensues involving the renewal of the immune-competent cells and lost tissues/organs. The aim of our investigations was to test the hypothesis that exposure of juvenile earthworms (Eisenia andrei) to cadmium-polluted soil, combined with electrostimulation-induced depletion of coelomocytes or the surgical amputation of posterior segments, will have adverse effects on earthworm maturation, reproductive output, and regenerative processes. Non-clitellate individuals of E. andrei of similar body weights were: (i) untreated; (ii)subjected to coelomocyte extrusion by the electric shock; or (iii) posterior segments amputated. Worms from each treatment group were maintained for 3, 5 and 7 weeks on either unpolluted soil or soil spiked with cadmium chloride (500 mg/kg air-dried soil). Cadmium exposure/accumulation inhibited growth; coelomocyte depletion/restoration did not disturb growth. Sexual maturation (indicated by clitellum formation) was delayed and cocoon production inhibited in Cd-exposed worms. Coelomocytes were significantly depleted by electrostimulation, and the kinetics of their recovery was similar in worms kept in clean and cadmium polluted soil. Eleocyte numbers and riboflavin content were similar in untreated (i.e. intact) worms maintained on clean and polluted soils, respectively. In contrast, amoebocyte numbers in worms maintained on cadmium-spiked soil were significantly higher during 3-5 weeks of exposure compared with their counterparts maintained on unpolluted soil. Soil cadmium had a negligible effect on regeneration of caudal segments. In conclusion, exposure of juvenile E. andrei to cadmium polluted soil delayed their maturation and inhibited reproduction but with little effects on either the restoration of experimentally depleted coelomocytes or the regeneration of amputated posterior segments.
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The influence of food composition and temperature on the immunological response of the slug *Arion vulgaris*

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The immunological response of an animal can be modulated by many environmental factors; among these, temperature and the nutritional value of food are of paramount importance. In our study we aimed to measure the influence of both factors on the activity of phenoloxidase (PO), an enzyme involved in melanization. PO activity is also often used as an indicator of the physiological performance of an individual under given conditions.

Juveniles of *Arion vulgaris* were raised in optimal (15°C) or one of two suboptimal temperature regimes (10°C or 20°C). Animals were provided one of five different diets with the following protein:carbohydrate ratios: 10%:50%, 20%:40%, 30%:30%, 40%:20%, 50%:10%, supplemented with cellulose (31.9%), olive oil (8%) and vitamins (0.1%). After one month we measured activity of PO in samples of haemolymph.

PO activity was affected only by temperature: it was enhanced in both suboptimal temperature regimes (Fig. 1). The increased PO in 10°C and 20°C activity indicates that suboptimal ambient temperatures may affects immunological response of an animal. At the same time, the lack of change in PO activity across different nutritional treatments do not necessarily indicate a lack of immune response. Future studies of other components of *A. vulgaris*' immune response that are complementary to PO activity, such as lysozyme activity and haemocyte numbers, will help to address this question more fully.

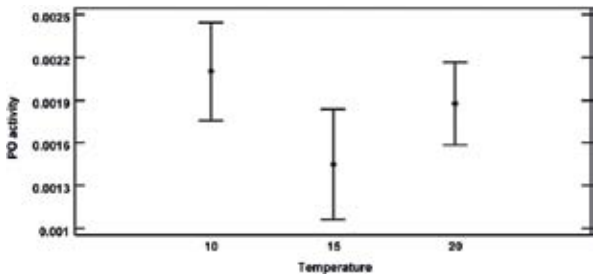

Figure 1. Phenoloxidase activity in *Arion vulgaris*. Mean and 95% confidence intervals are shown.

Red Queen processes drive positive selection on Major Histocompatibility Complex (MHC) genes

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Major Histocompatibility Complex (MHC) genes code for proteins involved in the incitation of the adaptive immune response in vertebrates, which is achieved through binding antigens of pathogenic origin. An excess amount of nonsynonymous substitutions routinely observed at antigen binding sites (ABS) of MHC have made MHC genes a paradigm for positive selection. This positive selection is attributed to parasite-driven balancing selection. However, the nature of the selection remains controversial. Two mechanisms of balancing selection are most often mentioned: advantage of MHC heterozygotes, which should be able to bind wider spectrum of pathogen-derived antigens, and host-parasite arms race (Red Queen process), that imposes frequency-dependent selection favoring rare MHC alleles.

We used individual-based computer simulations to investigate the roles of heterozygote advantage and Red Queen processes in maintaining MHC polymorphism.

In contrast to predominating overarching view, we found that novel mutations at ABSs are strongly favored by the Red Queen process but not by heterozygote advantage. Our simulations revealed also that levels of MHC polymorphism were high and driven mostly by the Red Queen process at a high parasite mutation rate, but were low and driven mostly by heterozygote advantage when the parasite mutation rate was low. Thus, when parasites evolve quickly, the Red Queen is capable of explaining both positive selection and high polymorphism of MHC genes.

Open session

Organizer (*European Society for CPB*):

Martin R. Lindley, *Loughborough University, UK*

Reversibility of developmental acclimation in *Drosophila melanogaster*

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Insect species are found under most environmental conditions. A key aspect of the success of insects is their ability to cope with both high and low temperatures through adaptive plasticity, which has allowed various species to thrive under a variety of thermal conditions, including some of the harshest environments on earth. Adaptive plasticity in thermal tolerance is found within Drosophilids, which have a tropical origin, but have colonised subtropical and temperate environments. Studies on thermal acclimation often distinguish between developmental and adult acclimation. Developmental acclimation can affect body size which is a non-reversible plastic response. Adult acclimation, on the other hand, often induces reversible physiological responses. In Drosophilids several studies have found that short-time acclimation to a given stress can enhance an organism's survival in both juvenile and adult life stages to subsequent stresses. However, the similarity of the mechanisms activated at different life stages, the interaction between developmental and adult acclimation, and the dynamics of reversibility of thermal tolerance are yet to be fully explored. Our aim was to investigate the reversibility of developmental acclimation effects in the adult stage and study potential carry-over effects from developmental acclimation in *Drosophila melanogaster*.

We found that high and low temperature acclimation during development led to acclimation benefits on respectively, the upper and lower critical thermal limits, and that the developmental acclimation effects were only partly and non-linearly reversible. The dynamics of the reversibility were very different for high and low temperature acclimation. Finally, we observed a tendency for flies from developmental acclimation at low temperature to maintain long lasting benefits in traits related to senescence. The non-linear reversibility coupled with the different effects of cold and heat acclimation suggest that the trajectory of the phenotypic plasticity for organisms thriving in variable thermal environments is highly complex and a result of different delays in phenotypic adjustments across traits.

Phenotypic plasticity and local adaptation of colour contribute to bearded dragons' ability to camouflage, a function which is prioritised over thermoregulation in the wild

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One of the most important functions of animal colouration is that of camouflage. This can be achieved either through colour change or through local adaptation of colour that leads to improved background matching. However, the relative importance of phenotypic plasticity and geographic adaptation of animal colouration in colour changing species is poorly understood. In this study, we examined the importance of local colour adaptation and colour change in camouflage in a laboratory setting. Then we looked at how bearded dragons deal with potentially conflicting functions of colour (camouflage and thermoregulation) in the wild. We examined the capacity for colour change and its implications for camouflage in two populations of bearded dragons representing the extremes in body colouration and geographical range. Dragons changed colour in response to background colour, allowing them to better match their backgrounds. Local adaptation of skin colour was also important to the degree of background matching; lizards of each population matched the backgrounds characteristic of their local habitats better than dragons from the foreign population. When studied in the field, the colour of individual, radio-tracked bearded dragons correlated strongly with background colour and less strongly, but significantly, with temperature. We found no evidence that individuals simultaneously optimise camouflage and thermoregulation by choosing light backgrounds when hot or dark backgrounds when cold. We conclude that both phenotypic plasticity and local adaptation of colouration contribute to an increased capacity of bearded dragons for background matching. When confronted with the conflicting requirements of thermoregulation and camouflage, bearded dragons prioritize the latter in the wild, consistent with the greater immediate threat of compromising camouflage.

Staying put: Torpor use during a severe storm

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Storms provide an extreme environmental challenge to many organisms and are predicted to increase in frequency and intensity due to climate change. Apart from a few observations reporting escape behaviour prior severe storms, there are no quantitative data on what animals do during storms and how small mammals with high energy demands survive these challenging periods. We studied a population of Australian sugar gliders, *Petaurus breviceps* (body mass ~120 g), in a coastal, subtropical habitat during spring when a storm with heavy rain and category 1 cyclone wind speeds passed the area. This arboreal marsupial is vulnerable to inclement weather due to their small body size and mode of locomotion. All twelve individuals remained in their established home ranges during the storm and either stayed inactive in their tree hollows or reduced foraging times during the cyclone. Although this species uses torpor only irregularly most sugar gliders entered deep and prolonged torpor during the storm to minimize energy demands. All animals survived the storm and continued normal foraging activity during the following night(s). It thus appears that torpor use may be an important survival strategy for small, non-flying vertebrates that cannot easily flee severe weather events such as storms. We can therefore assume that heterothermic mammals with the ability to use torpor have a crucial adaptive advantage over homeothermic species that cannot as the former outlast challenging environmental conditions by reducing metabolism and thus energetic needs.

Relax, there will be better times: sexual hormones in a small arboreal hibernator

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Edible dormice (*Glis glis*) are arboreal hibernators, highly dependent on beech seeds as food. Since beech mast seeding fluctuates and most trees are unable to flower in consecutive years, dormice reproduce and the males develop testes only in years of high or intermediate mast events. We addressed the question of whether and how this pattern is reflected by the hormonal status of the animals. Between 2012 and 2014 there were different mast conditions at our study site in the Vienna Woods. 2012 and 2014 were non-mast years and 2013 a year with full mast. In 2014 we conducted a supplemental feeding experiment by providing animals on one half of the area with high caloric seeds. Sex hormone levels in feces samples were determined using enzyme immunoassay analysis (EIAs). We found that in years when reproduction occurred, testosterone levels in males were significantly higher, and that providing supplemental food at the beginning of the season led to elevated testosterone levels. Since female dormice give birth relatively late in the season (end of July/August) both gestagen and estrogen levels were higher towards reproduction in 2013 and supplemental feeding in 2014, and increased with the mass of the animals. In 2012 (non-mast year) sex hormone levels in males and in females were very low. Supplemental feeding had no detectable influence on either gestagen or estrogen levels in females. We found elevated levels in both sexual hormones in 2014, irrespective of supplemental food availability, but still lower than in the mast year 2013. Therefore, we hypothesize that sex hormone levels in females are regulated by factors beyond the presence of food with high energy density. We conclude that mast events influence the hormonal status of males, which can be mimicked by supplemental feeding. Increases in female dormice sex hormones seem to be independent of food availability. Further research is required to clarify the mechanisms governing reproductive decisions in dormice.

Occurrence of aberrant mitochondrial DNA in long-lived *Arctica islandica*: possible impact on fitness and longevity

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Mitochondrial DNA (mtDNA) is normally maternally inherited and encodes for subunits of respiratory chain complexes and ATP synthase, among many other processes. The integrity of mtDNA is crucial for cellular energetic and redox homeostasis, and mtDNA mutations have been associated with individual well being and longevity. Bivalves are the only zoological group in which Doubly Uniparental Inheritance (DUI), characterized by the presence of 2 divergent mtDNAs within different tissues of male individuals, is frequently observed. The F-mtDNA, maternally inherited, is found in somatic tissue and female gonads whereas the M-mtDNA is usually found in male gonadic tissue only. Our recent investigations highlighted the existence of this particular mtDNA inheritance system in long-lived populations of *Arctica islandica*. This clam is distributed throughout the North Atlantic shelf regions. Due to different environmental regimes (salinity, temperature, oxygen), the maximum lifespan of its populations varies between >500 years around Iceland and 35 years in the Baltic Sea. Our new analyses of mitochondrial marker gene sequences in somatic tissues indicate North Atlantic populations to be genetically homogenous, but reveal the existence of a "divergent" mtDNA haplotype with a genetic difference >8% in some individuals, male and female, belonging to the Icelandic population. This "divergent" mtDNA, similar to the M-mtDNA previously found in *Arctica islandica* male gonads, occurs in somatic tissues from 20% of animals living around Iceland. In association with transcriptomic and biochemical data, we will discuss the possible impacts of this uncommon mtDNA haplotype on *Arctica islandica* biology and cellular physiology. This study will enhance the understanding of the role of DUI and mtDNA in general for fitness, aging and adaptation of bivalves.

Effect of reduced food intake on spontaneous locomotor activity in laboratory mice selected for high and low level of basal metabolic rate (BMR)

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Spontaneous physical activity represents an important component of daily energy expenditures in both animals and humans. Differences in physical activity may be related to susceptibility to metabolic disease or obesity. In particular, reduced physical activity under conditions of limited food availability may conserve energy and thus prevent loss of body mass and fat mass ('thrifty genotype hypothesis'). However, physical activity and its changes during food restriction show wide inter-individual variation.

We studied the effect of 30% caloric restriction (CR) on spontaneous locomotor activity (SLA) in laboratory mice, divergently selected for high (H-BMR) and low (L-BMR) level of basal metabolic rate. Selection increased initial SLA in the H-BMR line but did not change it in the L-BMR mice. Body mass loss during CR did not differ between both lines. CR increased SLA in both lines and significantly modified its temporal variation. However, the initial between-line difference in SLA was not affected by CR.

Our results show that the H-BMR mice can maintain their genetically-determined high SLA under conditions of reduced food intake without sacrificing their body mass. We hypothesize that this pattern may reflect higher flexibility of energy budget in the H-BMR line: the results of our earlier experiment show that mice from this line reduce their BMR during CR. This energy saving may allow for sustaining elevated SLA in spite of reduced food intake. We conclude that although initial variation in SLA between the lines is maintained under CR, it is not linked to different levels of metabolic thriftiness.

Mean corpuscular volume is not correlated to erythrocyte size and does not predict metabolic rate

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Cell size is hypothesized to be negatively related to whole organism basal metabolic rate. According to this hypothesis smaller cells are associated with higher metabolic rate in comparison to large cells due to lower cell surface to volume ratio with the membrane bound metabolic processes as the underlying mechanism. Several recent studies on ectotherms are in agreement with this hypothesis, but data for endotherms are scant. Many studies use mean corpuscular volume (MCV) as a proxy of erythrocyte size, but the link between MCV and metabolic rate is not established. MCV is estimated from two commonly measured hematological parameters, namely hematocrit (HCT) and the number of red blood cells (RBC count), but is it a good proxy of cell size? We investigated whether MCV does reflect erythrocyte size measured directly under light microscopy. To test the hypothesis that cell size predicts metabolic rate we estimated both MCV and erythrocyte area and measured BMR in 33 zebra finches (*Taeniopygia guttata*).

Surprisingly, measurements of MCV and erythrocyte area were not correlated (R=-0.122, P=0.50). The coefficient of variation for MCV was twice that of erythrocyte area. In contrast to the cell metabolism hypothesis erythrocyte area was positively related to BMR (F=8.63, P=0.007), while MCV was not related to BMR (F=0.39, P=0.537). A negative relationship between erythrocyte size and BMR has been confirmed for several ectotherms, but the relationship has so far not been investigated in in birds.

Our data suggest that MCV may not constitute a good proxy for erythrocyte size. MCV shows substantial variation that may originate from the fact that this variable is calculated from two other variables, both with significant measurement error. This error may multiply when calculating MCV. We conclude that MCV should be used with caution or be avoided, and direct measures of erythrocyte size should be used for investigations on red blood cell size.

The physiology and biochemistry of omega-3 polyunsaturated fatty acids and their bioactive derivatives

Martin R. Lindley[1], Alex J. Wadley[1], Lynsey Wilson[1], Sarabjit S. Mastana[1]

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Over the past three decades there has been substantial interest in the therapeutic potential of fish oils for various inflammatory conditions such as rheumatoid arthritis, inflammatory bowel diseases, and asthma. In addition, fish oil, rich in omega-3 polyunsaturated fatty acids (PUFAs) exerts anti-inflammatory and immune-modulatory effects and may be useful as a nutritional countermeasure to exercise-induced inflammation and immune dysfunction. Initial interest in the cardiovascular benefits of EPA and DHA was provoked by investigations of Greenland Eskimos who have remarkably low incidences of cardiovascular diseases. Both experimental and epidemiological studies have since been conducted to examine the effect of consuming fish oil, which contains high amount of EPA and DHA. The omega-3 PUFAs, (EPA and DHA) found in fish oil, appear to have additional anti-inflammatory properties primarily through their effects on the neutrophil and macrophage component of the inflammatory response.

We will introduce long chain omega-3 polyunsaturated fatty acids to the audience and attempt to elucidate the mechanism by which these fatty acids achieve their purported effects. We will show that the dietary behaviour of migratory birds may shed some light on the possible ergogenic effects of omega-3 fatty acids whilst also identifying the different potential mechanism by which these fatty acids impact inflammatory pathways. Work from our research group and other international groups will be used to highlight possible mechanisms of action and therefore possible therapeutic roles of dietary manipulation and supplementation.

The potential of omega-3 polyunsaturated fatty acids to suppress post-exercise oxidative stress and inflammation

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Acute exercise is associated with a transient increase in reactive oxygen species (ROS) production. ROS have various roles in controlling tissue function during exercise, as well as mediating post-exercise metabolic adaptations via intricate redox signalling pathways. Increases in exercise-induced ROS are also associated with post-exercise oxidative stress and inflammation; two intrinsically linked processes that may contribute towards delayed onset muscle soreness. Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) are regularly used by athletes, despite any clear evidence of their antioxidant or anti-inflammatory cellular actions following acute exercise in humans. The attraction of ω-3 PUFAs is in their properties to resolve, rather than block post-exercise inflammation, via incorporation into the cell membranes of various active tissues. Furthermore, recent work has highlighted the potential for docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), both ω-3 PUFAs, to exert antioxidant effects. In particular, the striking conformation (all cis-double bonds) and intra-cellular location of DHA may allow essential buffering of ROS at cellular regions of intense oxygen consumption, such as during exercise.

We explored the potential of ω-3 PUFAs to suppress post-exercise oxidative stress and inflammation, in a double-blind, placebo controlled, and crossover ω-3 PUFA supplementation study in trained male cyclists. Before and after two 4-week supplementation phases (daily dosage of ω-3 PUFAs (EPA: 4.08g and DHA: 1.62g) or placebo (olive oil: 6g)), participants engaged in a cycling test to exhaustion. Blood samples were taken over an extensive time-course to monitor parameters of plasma and peripheral blood mononuclear cell (PBMC) oxidative stress in response to exercise. Furthermore, exercise-induced inflammation (plasma interleukins and immune cell subsets) was assessed. These changes were substantiated alongside the degree of individual ω-3 PUFA incorporation into PBMC membranes as a result of the two 4-week supplementation phases.

Sponsored by Physiological and Biochemical Zoology

Effect of *Artemia* nauplii bioencapsulation with PUFA on fatty acids profile of *Acipenser oxyrinchus* larvae

Olga Kushniryk[1], Maja Prusinska[2], Oleksii Khudyi[1], Lidiia Khuda[1], Ryszard Kolman[2], Arkadiusz Duda[2], Grzegorz Wiszniewski[2], Mykhailo Marchenko[1]

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The disappearance of *A. oxyrinchus* from ichthyofauna of the Baltic Sea was caused by human activity in the late 20th century. The implementation of technologies for artificial reproduction of this species is the key to successful re-introduction. It is well known that sturgeons from a natural environment, unlike those grown within artificial conditions, have a higher content of essential polyunsaturated fatty acids (PUFA's). One of the most critical stages of fish ontogenesis is the beginning of exogenous feeding. Use of starter live food, enriched with biologically active substances, can promote the larvae survival rate at this stage. *Artemia* nauplii can be used as the aforementioned food; however they have a relatively low content of PUFA's. To optimize the nutritional value of *Artemia* we applied S. presso® («INVE Aquaculture», Belgium), which contains 25 fatty acids, 5 monosaturated fatty acids (MUFA) and 7 PUFA's. The mass fraction of PUFA in the product is 70.7%. The use of this emulsion provides the accumulation of 2% eicosapentaenoic acid(EPA) in *Artemia*, while the content of docosahexaenoic acid (DHA) becomes 4 times higher in enriched brine shrimp compared to non-enriched. Before the external feeding the oleic acid (28%), palmitic acid (21%) and DHA (16%) prevailed in the fatty acid composition of *A. oxyrinchus* larvae. After almost 3 weeks of larvae feeding on *Artemia* the share of oleic acid remained highest, but the content of linolenic acid significantly increased (from 1.4% to > 20%), which may be due to its high content (about 30%) in brine shrimp. In sturgeon larvae fed on enriched *Artemia* the level of EPA was 1.7 times higher and the level of DHA was 3 times higher than in larvae fed on non-enriched brine shrimp. In general, the ratio of DHA / EPA in the body of sturgeon larvae was 1.22 when fed on enriched *Artemia* and it was 0.72 when fed on non-enriched live food. In addition, bioencapsulation of essential fatty acids into *Artemia* nauplii allows the reduction of the mortality of fish larvae and the acceleration of their growth. Such approach may help to restore the population of *A. oxyrinchus* in the Baltic Sea.

Possible causes and consequences of different hibernation patterns in dwarf lemur species (*Cheirogaleus*) – Mitovy fatsy sahala

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All dwarf lemur species (*Cheirogaleus* spp.) hibernate in their natural habitats, decreasing energy and water demands drastically to survive the harsh seasons of the Malagasy winter, from the eastern rainforests to the western dry forests. However, the different species are very flexible in the *modus operandi* and timing of hibernation, and even within species differences are considerable, depending on habitat and climatic parameters. We compare hibernation aspects of the different dwarf lemur species and discuss possible causes of consequences of different hibernation patterns. Whereas dry forest species hibernate in tree hollows of various insulation properties, rainforest species retreat underground for hibernation. These different strategies of hibernaculum choice have extensive consequences on hibernation parameters and patterns. As expected, the colder the habitat is during winter, the greater the energy savings by hibernation are. There is evidence suggesting that more stable environments in the hibernacula are preferred and thus possibly represent the ancestral condition, indicating that the hibernation machinery was originally adapted to and runs best at mostly constant T_a. However, it is also conceivable that hibernation in tree hollows could have been the original *modus operandi* in the ancestral *Cheirogaleus*, implying that hypometabolism in some species is truly independent of T_a, and fluctuating T_a might even be advantageous, allowing for extended, continuous bouts of hypometabolism by avoiding interruptions by active arousals.

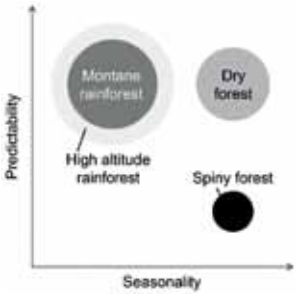

Figure: Hibernation hyperspace: Schematic diagram of the profitability of hibernation in Malagasy Cheirogaleidae in relation to the seasonality and climatic predictability of their habitats. The darker the colour of the circles, the warmer the habitat is during winter (mean ambient temperature); the larger the circles, the more profitable hibernation in this habitat is.

Water vapor should be measured, not removed, for metabolic measurement

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Because the dilution effect of water vapor on respiratory gasses causes unacceptable inaccuracy, especially with O_2, it is almost universally scrubbed from air streams prior to analysis. Unfortunately, chemical scrubbers can interact with CO_2 and must be replaced frequently. And, thermal scrubbers are unable to remove water vapor below ~0.65 kPa, are relatively unreliable and the condensed water within them can act as a CO_2 capacitor. Both approaches also add significant internal volume, slowing response times. We contend that if water vapor pressure and total (barometric) pressure are measured, the dilution effect of water vapor can be removed mathematically, and several other benefits can be derived: an important source of data on water flux rates can be obtained, metabolic water production can be estimated, other physiological parameters such as respiratory water loss can be measured, and system reliability can be significantly improved. These principles apply equally to all animals regardless of their mass scaling. To demonstrate the efficacy of retaining and utilizing water vapor data, using real-world gas exchange data, we show >99% removal of O_2 concentration fluctuations caused by water vapor, and also quantify water flux rates and metabolic water production in rats using a continuous three-gas (1 sec temporal resolution) Promethion metabolic phenotyping system. We also demonstrate, using both mouse and rat data, that the common assumption that water vapor concentration within different animal cages is approximately constant is incorrect. Water vapor data should be embraced, not discarded.

Actin and Actin Binding Proteins: A Dynamic Framework in Different Organisms from Bacteria to Human

Organizers (*Japanese Society for CPB*):

Hitoshi Tatsumi, *Nagoya University, Japan*
Kimihide Hayakawa, *Nagoya University, Japan*
Shoichiro Ono, *Emory University, USA*
Pekka Lappalainen, *University of Helsinki, Finland*

Mechano-sensing by actin filament

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Cells are continuously exposed to various mechanical stimuli arising from osmotic pressure, fluid flow, muscle contraction, distension of visceral organs, etc. which initiate a variety of cellular responses. Rearrangement of actin filaments is one of such responses, playing a crucial role in successive cellular events including shape change and migration. However, the mechanosensor that initiates the filament rearrangement had not been identified. Using a single molecule imaging and manipulation technique, we have recently found that the actin filament itself acts as a mechanosensor. Single actin filaments were manipulated by optical tweezers so as to tense the filament in the presense of the actin severing protein cofilin; relaxed actin filaments were severed by cofilin in less than ca. 20 s, whereas tensed ones were not severed or severed with a longer delay. Single molecule imaging and analysis of cofilin bindings to actin filaments showed that the on-rate of cofilin binding to an actin filament was decreased in tensed filaments in comparison with relaxed ones, suggesting that tension in the filament affects the apparent severing activity of cofilin by decreasing the on-rate of cofilin binding. This accounts for the observations that actin filaments with less tension are disassembled, whereas tensed ones are not in live mammalian endothelial cells (Fig1). We discuss the possible involvement of such a tension-dependent regulation of actin dynamics in cells derived from different species and genera.

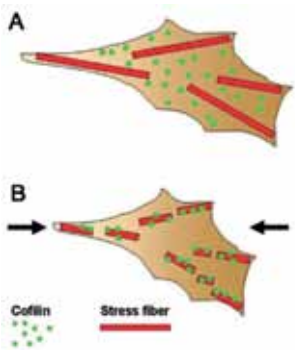

Figure 1. (A) Actin stress fibers generate contractile force in adherent cells, which prevents the binding of cofilin to the fibers. (B) When the tension declines by relaxing the cell substratum or by decreasing the contractile force, cofilin binds to and severs stress fibers.

Single molecule imaging and analysis of cooperative binding of cofilin to actin filaments

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Cooperative binding is a phenomenon displayed by oligomer proteins in which the affinity of the binding sites for a ligand is increased (positive cooperativity) or decreased (negative cooperativity), upon the initial binding of a ligand to a binding site, as observed typically in oxygen binding to hemoglobin. Cooperative binding is essential to understanding the regulatory mechanism of enzyme and receptor activities. However, dynamic processes of cooperative binding in solution have not been directly imaged and analyzed at the single molecular level. We performed super-high resolution real-time single molecule imaging of binding/unbinding of the actin-filament severing protein of cofilin to/from single actin filaments. Results show that relatively a long-lasting binding (>0.4 s) of a single cofilin molecule increased the affinity of other cofilin binding sites in a way dependent on the distance from the initial biding site. This effect decreases in a single exponential fashion with respect to the distance with a space constant of 65 nm (across 24 actin subunits). We propose a biophysical mechanism underlying this characteristic cooperative phenomenon and discuss its role in the severing action of cofilin as well as in cell functions.

Regulation of actin filament turnover by cyclase-associated protein and ADF/cofilin

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The actin cytoskeleton is often dynamic and undergoes regulated rearrangement in a number of cell biological events. Although actin can polymerize and depolymerize by itself, the rate of spontaneous actin filament turnover is very slow and needs to be regulated by actin-binding proteins to support dynamic cellular processes. Some of the actin-binding proteins are conserved among eukaryotes and serve as core regulators of actin filament dynamics. Actin depolymerizing factor (ADF)/cofilin is one of such actin regulators, which enhances actin filament turnover by severing actin filaments and promoting actin monomer dissociation from the pointed ends of filaments. Recent studies have identified additional proteins including actin-interacting protein 1 (AIP1), coronin, and cyclase-associated protein (CAP), that closely cooperate with ADF/cofilin to promote actin filament turnover to even faster rates. CAP is a multi-domain protein, and its N-terminal part enhances actin filament disassembly, while the C-terminal part promotes recycling of ADF/cofilin-depolymerized actin monomers for new rounds of actin polymerization (Ono, 2013). Our group has identified two CAP genes, CAS-1 and CAS-2, in the nematode *Caenorhabditis elegans*. CAS-1 is expressed in striated muscle and required for sarcomere assembly (Nomura, et al. 2012). Although expression patterns of CAS-2 is still to be determined, CAS-2 null mutant worms show disorganized actin cytoskeleton in the distal gonads where germline development occurs. Both CAS-1 and CAS-2 proteins promote recycling of ADF/cofilin-depolymerized actin monomers in vitro. Importantly, the C-terminal part of CAS-2 was necessary and sufficient for promoting recharging of actin monomers with ATP to dissociate ADF/cofilin (Nomura and Ono, 2013). Using site-directed mutagenesis, we obtained biochemical evidence that dimerization of CAS-2 through the very C-terminal region is essential for actin-monomer recycling activity. Interestingly dimerization is not required for its binding to actin monomers, suggesting that binding of the CAS-2 C-terminus is not sufficient to promote nucleotide exchange of actin monomers. The results strongly suggest functional significance of CAP dimerization.

Generation of contractile stress fibers depends on mechanosensitive actin filament assembly and disassembly

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Adhesion and morphogenesis of many non-muscle cells are guided by contractile actomyosin bundles called ventral stress fibers. While it is well established that stress fibers are mechanosensitive structures, physical mechanisms by which they assemble, align, and mature have remained elusive. Here we show that arcs, which serve as precursors for ventral stress fibers, undergo lateral fusion during their centripetal flow to form thick actomyosin bundles that apply tension to focal adhesions at their ends. Importantly, this myosin II-derived force inhibits vectorial actin polymerization at focal adhesions through AMPK-mediated phosphorylation of VASP, and thereby halts stress fiber elongation and ensures their proper contractility. Stress fiber maturation additionally requires ADF/cofilin-mediated disassembly of non-contractile stress fibers, whereas contractile fibers are protected from severing. Taken together, these data reveal that myosin-derived tension precisely controls both actin filament assembly and disassembly to ensure generation and proper alignment of contractile stress fibers in migrating cells.

MreB forms curved sheets of filaments that nucleate on membranes *in vitro*

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Using super resolution fluorescence microscopy, bacterial cytoskeletal element MreB has been shown to form up to 3.4 µm long filaments underneath the cell membrane in *Bacillus subtilis* cells and in other bacteria. Filaments move along various angles perpendicular to the cell's long axis and affect the architecture of the cell wall. It is not clear how MreB filaments assemble in a defined pattern, and how they nucleate. MreB filaments have intrinsic affinity to the cell membrane, we therefore used a flat membrane system to study the polymerization of fluorescently labeled MreB *in vitro*. Nucleation occurred efficiently at the membrane, dependent on divalent ions, and filaments extended within a range of seconds. Most filaments were curved, and frequently branched. New monomers could be added to the lateral side of filaments, and electron microscopy revealed that MreB filament consist of sheets of protofilaments, from which filaments can branch off, or sheets can fuse. Monovalent kations inhibited the formation of filaments, suggesting that the mixture of mono – and divalent cations inside cells cause MreB filaments to feature an average of 1.7 µm, whose curvature may cause the preferential circumferential localization. Our data show that many properties of MreB filaments *in vivo* can be explained by their intrinsic biochemical properties *in vitro*. To investigate how many MreB molecules are located within the filaments, and how many are freely diffusive, we have employed single molecule fluorescence microscopy. We show that a large fraction of MreB is bound to the filaments, and that many molecules move along the membrane, facilitating their association with existing filaments. Membrane proteins MreC and MreD are also essential for cell shape maintenance and show similar diffusive behavior like MreB. A model is presented how MreB molecules associate with filaments through mostly lateral association with filament sheets, which is a different mode of assembly than actin.

Statistical Approaches to Physiological Data Processing

Organizers:

Teresa G. Valencak, *University of Veterinary Medicine, Austria*

John R. Speakman, *Chinese Academy of Sciences, China & University of Aberdeen, UK*

Comparative metabolomics: Mapping physiology to phylogeny

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Felsenstein's groundbreaking 1985 paper on the comparative method sparked tremendous growth in the breadth and rigor of comparative studies. Over the past decade, we have also witnessed a revolution in the instrumentation and computational power applied to molecular studies. Advances in these two fields present us with a considerable statistical challenge: How should we map thousands to tens of thousands of traits (levels of metabolites or mRNA) onto a phylogeny, and use these data to ask compelling questions about the evolutionary causes and consequences of physiological variation among species. We have used liquid chromatography-mass spectroscopy to identify and quantify thousands of metabolites in the fruit fly genus, Drosophila. I will present our recent published and unpublished findings, and discuss the challenges, opportunities, and some possible solutions as we try to bring a phylogenetic perspective to high-dimensional physiologically relevant data.

Covariates, random effects, false discovery rates and beyond

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Comparative and evolutionary physiology studies have a multitude of experimental conditions and/or variables that can be compounded when using genomics (i.e., transcriptomics or metabolomics) due to an extremely high number of variables (i.e., >10K transcripts, or >200 metabolites). These types of physiology studies require complex statistical approaches. Generalized linear mixed models (GLMMs) are flexible for analyzing normal and non-normal data when random effects are present. Hence GLMMs work well for comparative and evolutionary physiology studies (Figure), whereas projection methods, such as sparse partial least squares-discriminant analysis (sPLS-DA) or sparse generalized canonical correlation analysis (sGCCA) work well when correlating physiological traits with genomic data that are high dimensional. We review the use of GLMMs in comparative and evolutionary physiology and biochemistry studies, and discuss what is considered a fixed or random effect and the procedures for a GLMM analysis. In addition, we discuss the use of sPLS-DA and/or sGCCA in physiology studies that use genomic approaches.

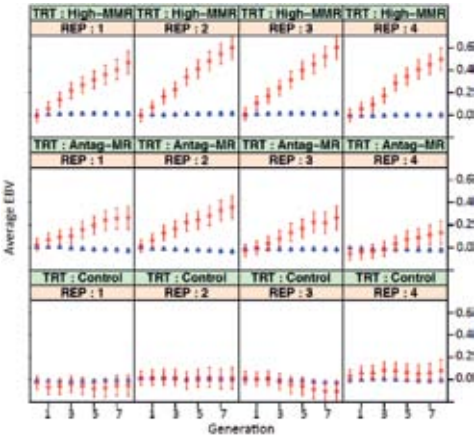

Figure: GLMM (animal model) of response to selection for increased maximal metabolic rate (MMR) in laboratory mice. The single analysis (joint analysis) and the results are shown for each treatment and replicate. EBV - estimated breeding value; TRT - treatment; High-MMR - directional selection for increased mass-independent MMR; Antag-MR - antagonistic selection for increased mass-independent MMR and decreased mass-independent basal metabolic rate (BMR). Blue—BMR, red—MMR.

Diverse body-size scaling of the pace of life complicates body-size adjusted comparisons of rates of metabolism and other biological processes

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The most important intrinsic factor related to the variation of many biological traits is body size. Therefore, detection of behavioral, ecological and taxonomic effects on interspecific variation of biological traits often begins with statistical adjustments to differences in body size. However, these body-size corrections ignore recent findings indicating that the body-size scaling of the rates of metabolism and other biological processes making up the "pace of life" is itself affected by various ecological factors and other lifestyle or taxonomic differences. Therefore, I argue that new methods should be devised to untangle the relative effects of body size and other factors on trait variation. I will discuss three possible, not completely satisfactory methods that attempt to mitigate this problem, at least in part: (1) minimizing co-variation between body size and taxonomy, by calculating separate scaling relationships for each lower taxon of a higher taxon that has a sufficient sample size, and then using each of these relationships to factor out the effect of body size, (2) minimizing co-variation between body size and various ecological factors, by calculating separate referential scaling relationships for species with specific ecological lifestyles or from specific environments, and (3) multivariate scaling of a trait against body size and other selected factors simultaneously. Using these and other possible methods could have profound effects on our understanding of trait variation and co-variation.

An analysis of variation of continuous, quantitative characters in a phylogenetic, behavioral, and environmental context

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A variety of methods have been used to analyze the factors responsible for the variation in comparative physiological functions. A phylogenetic-based analysis is concerned with the origin, evolution, and distribution of qualitative character states. A multifactorial ANCOVA approach examines the impact of continuous factors, such as body mass, on the level of quantitative functions, as modified by discrete behavioral character states and conditions in the environment. Species' characters include food habits, torpor, activity, and migration. Ecological factors involve climate, substrate, altitude, and habitat. The analysis of quantitative functions must incorporate both the species' behaviors and environmental factors without which a species' uniqueness is lost and the analysis ineffective. The demonstration of correlations of energy expenditure with behavioral and ecological factors is inadequate unless it indicates the quantitative impact of these factors on energy expenditure. The impact of mass defines the scaling relationships of physiological functions, whereas qualitative behavioral and environmental factors are principally responsible for the residual variation around a scaling curve. When these factors are included in an analysis, multifactorial analyses can account for nearly all of the quantitative variation in physiological functions. This ability permits an examination of the quantitative consequences for life under a variety of conditions. The interaction of the quantitative impacts of character states with conditions in the environment is the basis for the evolution of physiological functions. The two approaches to the analysis of character states are asking different questions, which prevent them from being effectively combined.

In vivo oxygen metabolism and hemodynamic changes measured in awake mouse brain with an optical imaging system

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Cerebral blood flow (CBF), cerebral blood volume (CBV) and cerebral metabolic rate of oxygen ($CMRO_2$) are important parameters for the diagnosis of brain diseases, such as stroke and dementia. In this study, we developed a new optical imaging system that can simultaneously measure CBF and CBV changes, as well as oxygen metabolism, in the same brain area of an awake mouse. CBF and CBV were simultaneously measured with laser speckle imaging (LSI) and intrinsic optical signal imaging (IOSI), which was verified with laser–Doppler flowmetry (LDF). Moreover, to measure oxygen metabolism, flavoprotein autofluorescence imaging (FAI) was performed for the same brain area as the LSI and IOSI measurements. The change in CBF according to LSI was correlated with that of LDF. Similarly, the change in CBV obtained by IOSI was correlated with RBC concentration changes measured with LDF. The change in oxygen metabolism observed with FAI was correlated with the LSI CBF changes, although the change in CBF was proportionately greater than that for oxygen metabolism. We found that the relationship between oxygen metabolism and CBF as measured by our system was in good agreement with the relationship between $CMRO_2$ and CBF measured in human PET studies and animal LDF studies.

Brain functional imaging in a mouse model of cerebrovascular disease (right common carotid artery occlusion) was performed using the system, and attenuation of hemodynamic response to sensory stimulation was observed. In conclusion, our simultaneous CBF, CBV and oxygen metabolism measurement system is not only useful for studying neurovascular coupling and the mechanism of brain disease.

Open Session: Ideas and Discoveries in Comparative Physiology and Biochemistry

Organizers (*American Physiological Society, Comparative and Evolutionary Physiology Section*):

Berry Pinshow, *Ben-Gurion University of the Negev, Israel*
Harvey B. Lillywhite, *University of Florida, Gainesville, USA*

Is cooling and freezing an appropriate euthanasia method for ectothermic vertebrates?

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Cooling to freezing temperatures are commonly experienced by various ectothermic vertebrates living in temperate habitats; indeed, activity and dormancy often occur at near-freezing temperatures. Thus, many vertebrates are cold- or even freeze-tolerant. Yet cooling followed by freezing has been banned as a method of euthanasia by institutional animal care and use committees (IACUCs) and ethics committees worldwide, based largely in the assumption that ice crystals cause pain when they form in tissue. Several considerations prompt the need for re-examination of this policy, which should include the flexibility for exemptions in compelling cases. Key considerations are: (1) Nociception in various vertebrates is not comparable to "pain" as perceived by humans. (2) Excitable membranes undergo cold block of action potentials at temperatures of 0 C or above. (3) When ice crystals form in tissues, presumably these would disrupt any activity of excitable membranes and thus inhibit neural transmission of "pain." (4) Transmission velocities for neuronal action potentials fall rapidly at low temperatures, and nociceptive neurons cease functioning before those transmitting other signals such as touch. (5) Cold is anesthetic. (6) Normal brain function ceases at low temperatures in many ectotherms. (7) Current guidelines for IACUCs and ethics committees neglect the natural histories of ectotherms, as well as evolutionary considerations. (8) Delivery of chemical anesthetics can be painful, and the effects are highly variable in different species. The use of chemical anesthetics with minimal "pain" requires species-specific training and expertise to do it properly. (9) Freezing provides a humane and practical means of euthanasia for ectothermic vertebrates that are often studied in remote field situations where chemical anesthetics are not available. These considerations advocate a need for increased communication among physiologists, ecologists and veterinarians with the purpose of reassessing the current euthanasia guidelines for ectothermic vertebrates.

Animal burrow ventilation – new ideas about ancient phenomena

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Burrows are the living environment of species of many taxa, ranging in size from ants to aardvarks and burrow morphology is a product of natural selection on the animals that build them. Yet little is known about how burrow environments meet the physiological needs of their occupant(s). For example, while some animals construct burrows that promote ventilation by penetration of air gusts (eddies) from the turbulent surface boundary layer, others animal may create burrows that impede eddy capture and limit ventilation. We are exploring this concept using burrows of three species of scorpion from Israel and Namibia. We hypothesized that the burrow acts as an extension of the animal's physiology, limiting ventilation and regulating fluxes of heat between the occupant and the ambient environment. We cast 30 natural burrows of *Scorpio maurus palmatus*, 19 burrows of *Opistophthalmus wahlbergii,* and four of *O. setifrons* with molten aluminum and used a 3D scanner to quantify the burrows' linear measurements, angles, and volumes. We found that burrows of all three species had several structural features in common, in particular a horizontal platform just below the entrance and near the surface, where the scorpion may warm before exiting to hunt at night. All burrows also had at least two bends, ending in an enlarged terminal chamber that is dark, damp and cool. Burrows reach depths where soil temperature is adequately stable, providing refuge from hot surface conditions. *S. m. palmatus* can apparently influence their internal body temperature by tracking temperature gradients within their burrows. They also modify their burrow structure in response to soil moisture, digging deeper and more tortuous burrows in dry conditions. We found *S. m. palmatus* burrows not to be ventilated by eddies and that entrance size, rather than tortuosity, prevented eddies from penetrating.

Energy metabolism and hydration state affect discontinuous gas exchange cycle properties in desert locusts under combined dehydration and starvation stress

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The classic "hygric hypothesis" for the evolution of discontinuous gas exchange cycles (DGC) in insects posits that it serves to restrict respiratory water loss. However, dehydrated grasshoppers abandon DGC when conditions are expected to favor strict management of body water stores. The two density-dependent phases of the desert locust (*Schistocerca gregaria*) were used to investigate the mechanistic basis for changes in DGC properties during exposure to combined dehydration and starvation stress, followed by rehydration. We expected the stress-resistant gregarious locusts to maintain DGC longer than solitarious locusts under stressful conditions, and locusts of both phases to recover DGC when rehydrated. We found no phase-dependent variation in body water content, water loss rates, metabolic rates or time to DGC abolishment. When rehydrated, locusts of both phases reemployed DGC after ingesting the same amount of water. Nevertheless, dehydrated solitarious locusts had significantly shorter interburst durations, suggesting that their ability to buffer CO_2 was reduced. Only the gregarious locusts recovered their initial capacity for CO_2 accumulation when rehydrated, indicating a role for factors other than hemolymph volume in the control of cycle characteristics. Hemolymph protein concentration measurements and calculated respiratory quotients suggest that catabolism of hemolymph proteins in solitarious locusts may be responsible for the reduced hemolymph buffering capacity and consequently compromised ability for CO_2 accumulation during the interburst. Nevertheless, in both phases DGC was lost at similar hydration states, suggesting inadequate tissue oxygen supply as the major factor limiting DGC.

Physiological and behavioural strategies for survival of fire by small mammals

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Globally, climate change is already causing an increase in the occurrence of destructive wildfires, changing the physical and biological characteristics of many ecosystems. While fires are an important natural phenomenon that can rejuvenate landscapes and increase biodiversity, many species may not be able to cope with a change in fire regime. Data on how populations change in response to fire are available to some extent, but we currently have little understanding of the physiological and behavioural adjustments that may enable an individual to survive a fire and its aftermath. Therefore, we examined whether the persistence of small mammals after a fire, when ground cover providing shelter from predators and food availability are reduced, is aided by torpor that affords energy savings and in turn lessens foraging requirements. Physiological and behavioural data were collected on free-ranging female brown antechinus (*Antechinus stuartii*) in Australia before (pre-fire group) and after a prescribed fire (post-fire group) and also at a control site nearby (control group). The dense ground cover of the site was obliterated by the fire and while before the fire no predators were detected on trail cameras, after the fire, foxes, feral cats and wild dogs were recorded. The antechinus that survived the fire had preferred resting high in large trees, whereas one individual perished sheltering under shallow rocks. Interestingly, individuals in the post-fire group did not move out of their original sites even though unburned habitat was nearby. Importantly, in the post-fire group torpor was observed twice as often, mean torpor bout duration doubled, minimum T_b was 3.9°C lower and daily activity was significantly reduced in comparison to the other groups. Our results reveal that antechinus increase both the depth and duration of torpor bouts and decrease activity in response to a reduction in ground cover and a probable increase in predation pressure resulting from a prescribed fire. Therefore, the use of torpor likely increases the chance of survival for small mammals by saving energy in a post-fire landscape where resources are reduced.

Febrile birds die at high ambient temperatures because the magnitude of the febrile response is unregulated

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Although there is considerable overlap in the physiological mechanisms that underlie febrile mediation in birds and mammals, recent studies identified important differences in the regulation of body temperature during the fever response. The significance of these differences is still poorly understood and there have, thus far, been no studies to evaluate the characteristics of febrile mediation in birds and in mammals in a comparable experimental model. We investigated the metabolic cost and the magnitude of lipopolysaccharide induced fevers in New Zealand White rabbits (n = 8) and Pekin ducks (n = 8) exposed to a range of environmental temperatures (3°C to 35°C). We also assessed whether hot environmental conditions would cause varying degrees of heat stress in LPS treated birds and mammals by exposing animals to conditions that simulate a heatwave. Ducks developed dangerously high febrile peaks in hot environments and significantly lower febrile peaks in cold conditions, with the energetic cost of fever remaining constant, irrespective of the ambient temperature. In contrast, the metabolic cost of fever in rabbits was influenced by the ambient temperature, but the febrile peaks remained constant. Unlike rabbits, ducks did not seem to employ regulatory mechanisms that control the amount of metabolic energy channeled to the febrile response or the magnitude of fever and in ducks, the febrile peak temperatures became life-threatening or lead to death. The absence of a physiological control mechanism to modulate the fever response in birds very likely contributes to the vulnerability of birds in conditions of climate change.

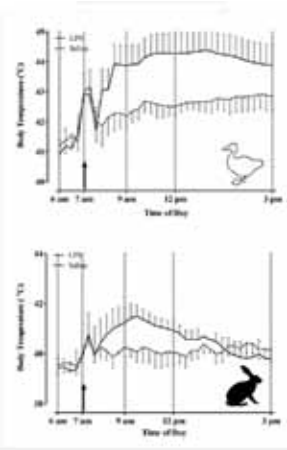

Figure 1: The body temperatures of ducks and bunnies in a hot ambient temperature (38°C after injection of LPS and saline

Evolution of thermogenic adipose tissue in mammals

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Brown adipose tissue (BAT) is a unique evolutionary outcome enabling adaptive thermoregulation in eutherian mammals. BAT releases energy as heat, in contrast to energy-storing white adipose tissue (WAT). Heat production is catalysed by the BAT-specific mitochondrial uncoupling protein 1 (UCP1). Recently, the molecular signature of a third adipocyte type expressing UCP1, termed the beige or brite (brown-in-white) cell, was characterized in rodents and humans. However, the physiological role, the contribution to systemic thermoregulation and the evolutionary origins of the beige/brite cell are unknown. In marsupials, which diverged from eutherian mammals about 140 MYA, we identified expression of UCP1 in adipose tissue of 'brownish' appearance, but noradrenaline did not trigger the classical adaptive excitation of thermogenesis. We have determined UCP1 gene expression levels during juvenile development of the grey short-tailed opossum, *Monodelphis domestica* and found the emergence of UCP1 in three different adipose tissue depots that coincides with a shift of fat storage from the liver towards distinct anatomical adipose tissue sites. Despite progressive increases in UCP1 levels, histological analysis identified no significant remodelling into typical multilocular lipid droplets of BAT. To determine the molecular identity of UCP1-positive adipocytes in marsupials for cellular classification and functional implications, we performed RNA sequencing of the interscapular adipose tissue and characterized global gene program changes associating with UCP1 gene expression. We annotated gene products to the respective modern eutherian orthologs to answer the question which pathways were already wiring UCP1 biology in adipocytes about 140 million years ago.

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Constant protein degradation protects skeletal muscles from disuse atrophy in hibernating Daurian ground squirrels

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Understanding the mechanisms that protect against or limit disuse muscle atrophy in hibernators during prolonged inactivity has important implications for its treatment. We examined whether external factors influence the pathways regulating protein synthesis and degradation, leading to muscle atrophy prevention in Daurian ground squirrels (*Spermophilus dauricus*). We investigated the effects of hindlimb-unloading (HU) in different seasons and hibernation on the soleus (SOL) muscle-to-body mass ratio, fiber cross sectional area (CSA), fiber distribution and muscle ultrastructure. We also measured changes in protein expressions and activation states of Akt, mTOR and FoxO1 and the mRNA expressions of atrogin-1 and MuRF1. Compared with the control groups, autumn and winter HU significantly lowered the SOL muscle-to-body mass ratio, decreased type I and II CSA and induced ultrastructural anomalies. However, these indices were unchanged between pre-hibernation and post-hibernation. Our findings indicate that the squirrel SOL was significantly atrophied after autumn and winter HU, but was protected during hibernation. Furthermore, phosphorylation levels of Akt and mTOR significantly decreased, while the phosphorylation level of FoxO1 and mRNA expressions of atrogin-1 and MuRF1 increased after HU. During hibernation, the phosphorylation levels of Akt and mTOR significantly decreased, but the phosphorylation level of FoxO1 and mRNA expressions of atrogin-1 and MuRF1 remained unchanged. These results suggest that the protective mechanism was initiated by a combination of external factors, including muscle disuse, fat storage and short photoperiod. Relatively constant protein degradation during hibernation appeared to be an important molecular mechanism by which disuse muscle atrophy was prevented in these hibernators.

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Fibroblast growth factor-23 (FGF-23) upregulates the expression of osteoclastogenic factors in osteoblast-like UMR106 cells

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Fibroblast growth factors (FGFs) are polypeptides that have been identified in multicellular organisms from nematodes to mice and humans. Most FGFs are mitogenic; however, bone-derived FGF-23 possesses an endocrine function with unique ionoregulatory functions. FGF-23 produced by osteoblasts and osteocytes is primarily responsible for urinary phosphate excretion and suppression of $1,25(OH)_2D_3$ production in mammalian kidney. Recent investigation revealed that FGF-23 also inhibits bone mineralization, but whether it can modulate osteoclast-mediated bone resorption was not known. As a source of FGF-23 production, osteoblasts not only control bone formation, but also secrete osteoclastogenic factors to activate osteoclasts and bone resorption. We determined the mRNA levels of osteoclastogenic factors i.e., macrophage colony-stimulating factor (MCSF), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-alpha) by quantitative real-time PCR in osteoblast-like UMR106 cells treated with 0.1, 1, 10, or 100 ng/mL recombinant FGF-23. We found that UMR106 cells abundantly expressed several subtypes of FGF receptors, i.e., FGFR1, 2, 3 and 4, as well as klotho that is the co-receptor for FGF-23. All concentrations of FGF-23 significantly upregulated MCSF expression, while the lower concentrations of 0.1–10 ng/mL could upregulate MCP, IL-6 and TNF-alpha. In addition, cell proliferation was reduced after direct exposure to FGF-23, as determined by 5-bromo-2'-deoxyuridine assay. The present study thus corroborated the possible role of FGF-23 in the regulation of osteoclastogenic factor expression in osteoblasts, which might, in turn, modulate bone remodelling.

A suckling-induced prolactin surge helps lactating mammals supply calcium for bone growth in neonates

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Prolactin, an important lactogenic hormone in mammals, has long been recognized as a potent hypercalcemic hormone in fish, amphibians and reptiles. Previously, we demonstrated that prolactin was responsible for enhanced calcium absorption in lactating rats. We therefore hypothesized that prolactin-induced calcium absorption helps supply calcium for milk production, leading to higher milk calcium content and better bone calcium accretion in the offspring. The characteristics of prolactin surge were first determined in breastfeeding humans and rats. Specifically, lactation led to hyperprolactinemia (baseline prolactin levels of ~200 ng/mL), and prolactin levels abruptly rose within 10 min by 10-min of breast pumping or suckling, peaked within 20–30 min (~400 ng/mL), and returned to the baseline within 60–90 min. Oral calcium supplementation, by gavage, at 90 min prior to suckling, which allows a large amount of calcium to reach the duodenum and proximal jejunum, caused an increase in bone mineral density (BMD) and milk calcium content in lactating rats, whereas no BMD change was observed when calcium supplementation was not synchronized with the suckling-induced prolactin surge. Interestingly, 11-week-old offspring, previously nursed by calcium-supplemented dams, exhibited better bone elongation (femur and tibia) as well as higher trabecular volumetric BMD, as determined by micro-computed tomography. We tentatively conclude that the conserved calciotropic action of prolactin is essential to supply calcium for lactating mammals. It probably helps increase milk calcium content, which, in turn, promotes bone growth in neonates. This study has been approved by the institutional ethics committees.

Effects of fasting and prey ingestion on the hormonal levels and metabolism of carbohydrates, lipids and ketone bodies in the South American rattlesnake *Crotalus durissus* (Serpentes, Viperidae)

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The metabolic increment that follows meal ingestion has been well characterized at cellular, organismal, and ecological levels for many ectotherm vertebrates, especially on those characterized by infrequent ingestion of large meals. Thus, snakes have become naturally the paramount model of investigation in many comparative studies focused on digestive physiology. Indeed, the impressive changes in energy demand and the associated morphological and physiological adjustments observed after meal ingestion are well documented in many species. On the other hand, the concurrent adjustments on the activity of enzymes involved in different metabolic pathways remain unknown, as well as the influence of fasting and prey ingestion in hormones related to the metabolic control. In this work, we hypothesized that the post-prandial period of the rattlesnake Crotalus durissus is followed by an up-regulation of the activities of enzymes of the glyconeogenic pathways and lipids and carbohydrates catabolism in order to support the stores deposition after meal ingestion. Also we expect that the levels of the thyroid hormones and leptin are elevated during the post-prandial period of rise in metabolic rate. For this purpose, we performed measurement on maximum activity of fructose bisphosphatase (FBPase), glycogen synthase (GS), b-hydroxibutirate dehydrogenase (HBDH), succinilCoA ketotransferase (SKT) and b-hydroxyacylCoA dehydrogenase (HOAD) in several organs, as well as measurements of T3, T4 and leptin levels in plasma of snakes kept in fasting and after meal ingestion. Our results indicate that the transition from fasting to the post-prandial period in rattlesnakes is not followed by changes in hormones levels in plasma. On the other hand, we observed an increase in the activity of enzymes that promote the carbohydrates deposition after feeding, with minor modifications in lipids and ketone bodies metabolism in all organs analyzed, except in the heart. The activity of SKT was increased in the heart of fasting snakes.

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Methylene blue cytotoxicity in a zebrafish cell line: A comparative study of its toxicity and phototoxicity

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We compared the mechanisms of action of methylene blue (MB) in a zebrafish cell line, both in the absence (MB alone) and in the presence of photosynthetically active radiation (MB+PAR). Cells were exposed to equitoxic concentrations of MB in each treatment. There was a significant increase in the levels of reactive oxygen and nitrogen species 3 h after MB treatment, while this increase was only observed 12 h after treatment with MB+PAR. All treatments with MB resulted in an increase in DNA damage after 3 and 6 h. However, cell death by apoptosis was observed from 6 h after treatment with MB + PAR and 12 h after treatment with MB alone. The expression of genes related to cell cycle arrest and apoptosis was altered after MB+PAR treatment. Thus, this zebrafish cell line is sensitive to the photodynamic action of MB, and MB is able to generate DNA damage and to induce apoptosis in this cell line both alone and in the presence of PAR. However, the pathways leading to apoptosis in this model appear to be dependent on the type of MB exposure (in the presence or absence of PAR).

The effect of ghrelin on body temperature during LPS-induced systemic inflammation in five-day-old chicks

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Ghrelin is a peptide predominantly produced in stomach that can act on the brain affecting food intake. In mammals it stimulates food intake and inhibits fever induced by endotoxin. In contrast, in birds, ghrelin has an anorexigenic effect, but no data exist about its modulation of body temperature (T_b) in euthermy as well as during systemic inflammation. Thus, the aim of the present study was to investigate the effect of intracerebroventricular (icv) injection of ghrelin on T_b and food intake of five-day-old chicks with and without systemic inflammation induced by lipopolysaccharide (LPS; endotoxin from gram-negative bacteria). Body temperature of chicks was measured with a sensor that was inserted through the cloaca inside the animal's rectum after icv injection of ghrelin (0.005, 0.05, 0.1, 0.5, 1 µg/µL) or vehicle (1% Evans Blue solution; injection volume 1mL). The concentrations of 0.05, 0.1, 0.5 and 1 µg/µL, but not 0.005 mg/mL, decreased T_b 1-2h after injection. The lower dose of ghrelin was chosen to verify the effect on T_b and food intake after systemic inflammation induced by intramuscular (im) injection of LPS (2 and 100 µg/kg). LPS at the dose of 2 µg/kg induced fever while the higher dose induced *T*b decrease in the first hour and fever 3-5 hours after injection. Ghrelin did not alter the fever induced by 2 µg/kg of LPS while it caused an increase in the latency to fever development after initial decrease in T_b induced by LPS 100 µg/kg. Both doses of LPS reduced food intake, but the effect was more pronounced with the higher dose. Ghrelin decreased food intake per se, but did not alter the effect of LPS on this variable. These results indicate that exogenous ghrelin, acting on the brain, may have an antipyretic effect in more severe inflammation without influencing the anorexigenic effect of LPS in five-day-old chicks.

Stability properties of myoglobin in the Argentinian hoki (*Macruronus magellanicus*)

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Introduction: The structural and stability properties of fish myoglobin (Mb) have been investigated mainly in fish with red muscles such as tuna and mackerel. Only few studies, however, have reported on Mb from fish white muscles. In a comparative study, we isolated Mb from the cardiac muscle of the Argentinian hoki (*Macruronus magellanicus*), one of the most important commercial fish species with white muscle. We did a mechanistic analysis of the pH dependence of hoki oxymyoglobin (MbO_2). In addition, the complete amino acid sequence of hoki Mb was determined.

Ferrous hoki Mb was separated from its ferric met-form on an anion exchange column chromatography. Autoxidation of hoki MbO_2 was examined over the wide range of pH 5-11 in 0.1 M buffer at 25˚C. The autoxidation rates of hoki MbO_2 to its met-form were more than 3 times higher than those of bigeye tuna (*Thunnus obesus*) oxyMb over the whole range of pH values studied. Its pH dependence was analysed in terms of an "acid-catalyzed two-state model" (Figure). The resulting rate constants for nucleophilic and proton-catalyzed displacement process by H_2O were 2-6 times higher than the for bigeye tuna oxyMb. On the other hand, hoki Mb showed 72 % homology in amino acid sequence to yellowfin tuna (*Thunnus albacares*) Mb. However, there was a region of low homology to tuna Mb. This corresponds to the loop between C-helix and E-helix. The present findings suggest that the environment around heme iron of hoki Mb is less hydrophobic and accessibility of the solvent water into the heme pocket plays a key role in the stability properties of hoki MbO_2.

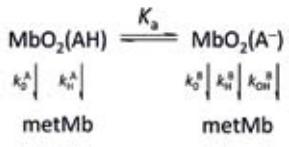

Figure: Acid-catalysed two-state model. k_0 is the rate constant for nucleophilic displacement by H_2O, k_H for proton-catalysed displacement by H_2O, *k*OH for displacement by O_H^-.

Kobayashi *et al.* (2014) *Fukushima J Med Sci*, 60:31-34.

Matrix metalloproteinase-2 and -9 participate in regression and recrudescence of the chicken oviduct

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The avian oviduct undergoes multihormonally-controlled extensive tissue remodelling during reproductive cycle covering the development, regression and recrudescence. Such processes require cyclic turnover of the extracellular matrix (ECM) components. The matrix metalloproteinase (MMP) system is known to play a key role in ECM turnover in several types of tissues, including reproductive ones. Unfortunately, very little information is currently available on the role of MMPs in the oviduct of birds during different physiological processes. Therefore, the aim of the study was to determine expression, localization and activity of MMP-2 and MMP-9 in the chicken oviduct during pause in egg laying.

Hy-Line Brown hens (n=18) were fed *ad libitum* (n=18; control) or subjected to induced pause in laying by food deprivation for 5 days, followed by feeding every second day and from day 10 fed *ad libitum* (n=18). All parts of the oviduct i.e. the infundibulum, magnum, isthmus, shell gland and vagina were isolated from birds on the day 6 (when hens stopped egg laying and oviducts were regressed by 36.7%), 13 (during oviduct recrudescence) and 17-20 (after resumption of egg laying) of experiment.

Real-time PCR, Western blot and activity assay revealed section-dependent expression and activity of both examined MMPs. On the protein level two forms of MMP-2 and MMP-9, latent and active, were detected. In the wall of the oviduct MMP-2 was localized mainly in the luminal epithelium, while MMP-9 was present in the connective tissue. In experimental chickens gene expression and activity of MMPs was elevated in most of the oviductal parts on days 6 and 13 when compared to the control. However, there were no changes in the amount of MMP-2 protein in the oviduct during pause in laying.

The results obtained indicate that MMP-2 and MMP-9 are involved in regression and rejuvenation of the chicken oviduct during pause in laying.

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High stocking density and food deprivation affect AVT, IT and stress pathways in the gilthead sea bream (*Sparus aurata*)

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The influence of chronic stress situation induced by high stocking density (HSD) and/or food deprivation (FD) was assessed on hypothalamic AVT, IT and stress pathways in gilthead sea bream (*Sparus aurata*). Fish were randomly assigned to one of the following treatments: (1) fed at low stocking density (LSD; 5 kg·m^{-3}); (2) fed at high stocking density (HSD, 40 kg· m^{3}); (3) food-deprived at LSD (LSD-FD); and (4) food-deprived at HSD (HSD-FD). After 21 days, samples of hypothalamus and plasma were obtained. Cortisol clearly increased due to food-deprivation at both stocking densities, although only the combination of HSD and FD seemed to be controlled by the CRH/CRH-BP axis. In addition, increased AVT and IT plasma levels were observed in both fed and starved specimens under HSD conditions (Mancera *et al.*, 2008. Together with challenges in pro-VT and pro-IT gene expression, as well as in AVTR V1a2-type mRNA levels, suggest that vasotocinergic and isotocinergic systems could be involved in the regulation of stress pathways at the hypothalamic level. However, food deprivation alone did not affect AVT and IT receptor expression levels in the hypothalamus. Our study shows that both vasotocinergic and isotocinergic pathways are involved in physiological changes induced by food deprivation and/or stocking density, suggesting that different stressors (FD or HSD) are handled through different stress pathways in *S. aurata*.

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Determination of minerals and amino acids in fish scale and chicken eggshell and the potential of amino acid-enhanced intestinal calcium absorption

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Calcium and collagen are important as key structural components of bone. Natural calcium sources, such as fish scale and chicken eggshell, may bring greater benefits than formulated calcium salt because they contain other minerals such as P, Zn and Mg, and proteins, which may bring a positive effect to bone metabolism. Moreover, certain nutrients, such as monosaccharides and sodium salts were reported to improve calcium absorption via solvent drag phenomenon. Since the sodium-dependent absorption of amino acid is similar to monosaccharide absorption, it was hypothesized that fish scale-derived amino acids, especially glycine and proline, could also facilitate calcium absorption. This study aims to evaluate the possible use of fish scale (FS) and chicken eggshell (CE) as a calcium supplement or animal foods. Mineral compositions in FS (mainly *Labeo rohita)* and CE were determined by ICP-MS. It was found that FS and CE contained comparable amount of Ca (434.16 mg/g for FS vs. 473.29 mg/g for CE) and P (166.50 mg/g for FS vs. 116.41 for CE) and levels of Pb, As, Cd, Sb and Hg were lower than WHO food standards. By using Ussing chamber technique to investigate whether amino acids stimulate calcium absorption, duodenal segments of Sprague-Dawley rats were exposed to either normal bathing solution or glycine-containing bathing solution. The result showed glycine stimulated calcium absorption. In conclusion, fish scale and chicken eggshell were potential raw material for calcium supplement or animal feed since they were rich in calcium and/or amino acids which were building block for bone formation.

Immunoexpression of MMP-9 in the seminal vesicle of the Libyan jird (*Meriones libycus*) during the seasonal reproductive cycle and after castration

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An immunohistochemical study of the MMP-9 was performed on seminal vesicles of the Libyan jird in order to verify its involvement in the seasonal activity and physiology of this gland. The Libyan jird, a nocturnal Saharan rodent belonging to the gerbillidae family was captured in the Beni-Abbes area in the Algerian Sahara. Castration was performed in the spring with bilateral removal of the testicles after ether anesthesia and it was the indirect method of immunohistochemistry with amplification with streptavidin biotin peroxidase that was used. In the breeding period (spring and early summer) the immunostaining of MMP-9 was intense and occupies the entire cytoplasm of epithelial cells and smooth muscle cells (SMCs); no immuno-response was observed in the extracellular matrix (ECM) and secretion. In the resting phase (late summer, autumn, late winter) and in the thirty days castrated animals immunohistochemical signal substantially decreases in epithelial cells and appears with great intensity in the ECM. Immunoreaction was maintained in SMCs with high intensity and remains still absent in the secretion. After a prolonged orchiectomy (50 and 90 days) the immuno-response disappears from the ECM and intensifies in epithelial cells. These results show a seasonal fluctuation in the expression of MMP-9 and suggest the modulating effect of testosterone on MMP-9 and the involvement of this enzyme in the physiology of the Libyan jird seminal vesicles and in its atrophy process and its seasonal tissue remodeling.

Tetramethylpyazine suppresses disuse-induced cytoplasm calcium overloading in skeletal muscle fibers, by a modified measuring approach

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[Aim]To test the hypothesis that tetramethylpyrazine may suppress elevated cytoplasm calcium concentration in skeletal muscle fibers of hindlimb unloading (HLU) rats. [Method] Hindlimb unloading rats were used as a muscle atrophy model in this study. The intracellular calcium concentration of single skeletal muscle fiber was measured by fluorescent Ca^{2+} indicator Fluo-3/AM with a modified approach. Twenty-four female Sprague-Dawley rats were divided into 3 groups: control group (CON), hindlimb unloading + water group (HLU+W) and hindlimb unloading + 60.0mg·kg^{-1} tetramethylpyrazine group (HLU+Tmp). [Results] After 14-day hindlimb unloading, the concentration of Ca^{2+} in single gastrocnemius muscle fibers increased by 189.6% (P<0.01) compared with CON group, however, as compared with hindlimb unloading rats, treatment with tetramethylpyrazine significantly decreased the level of intracellular Ca^{2+} by 54.4%（P<0.01）in single gastrocnemius muscle fibers. [Conclusion] 14 days hindlimb unloading causes cytoplasm calcium overloading in gastrocnemius, and tetramethylpyrazine can retard the increase to a certain extent.

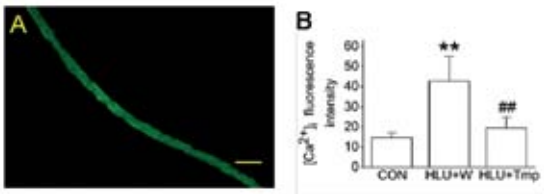

Figure: A: Representative fluorescence image of single fiber from gastrocnemius of CON group loaded by fluo-3/AM for 20 min. Bar = 100 µm. B: Bar graph depicting the changes in the mean intensity of cytoplasm Ca^{2+}. Five fibers were analyzed in each sample. Values are mean±SD, **P <0.01 versus CON, ##P <0.01 versus HLU+W.

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Evolutionary novelty: symbiosis of nudibranch mollusk with denitrifying and diazotrophic bacteria

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Symbioses involving bacteria and invertebrates contribute to the biological diversity of environments. A new symbiosis between the dorid nudibranch *Rostanga alisae* (Mollusca: Opisthobranchia) and the members of the bacterial groups Rhodobacterales and Rhizobiales, known for their ability to denitrify under anaerobic conditions and for nitrogen fixation and also displaying antimicrobial activity has reported for the first time. Transmission electron microscopy revealed a presence of dense clusters of rod-shaped Gram-negative bacteria enclosed with a membrane within bacteriocytes located in the epithelium of the nudibranch foot. The number of bacteria in bacteriocytes varied from one item to a few dozens. There were three main morphotypes of bacteria corresponding to the stages of lifecycle of endosymbionts. The presence of three transitional forms, monomorphism of bacteria within each bacteriocyte, and the presence of dividing bacteria suggest that the revealed bacteria are symbionts of the nudibranch. Molecular phylogenetic analyses revealed the bacteriocyte-associated bacteria to be dominated by α-*Proteobacteria* (*Labrenzia* and *Maritalea* clones), and gram-positive Actinobacteria (*Iamia* and *Ilumatobacter*) were found as single clones. Revealed in bacteriocytes β-*Proteobacteria* (*Achromobacte*r) and γ-*Proteobacteria* (*Lysobacter* and *Stenotrophomonas*) were detected also in the intestine and in amicrobic tissues, correspondently, indicating the nonspecificity of these bacteria. Fluorescence *in situ* microscopy confirmed the presence of *Labrenzia* clones in bacteriocytes. Fatty acid analyses suggest that endosymbionts are likely supply the host with nutrients. The abundance of specific bacterial fatty acids in the lipids may serve as an indicator of the presence of symbionts in the mollusk tissues.

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Mouse Schwann cell culture and the expression of L-MAG in Schwann cells and in myelinating cocultures

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The knowledge of the control of SC proliferation is essential for understanding how the PNS nerve develops, how the myelin sheath is formed by SCs, and what happens during the degeneration of myelin sheath. Here we investigated the effects of several growth factors with the aim of improving the efficiency of our published protocol for expanding mouse SCs (Honkanen et al, 2007). We studied the expression of Large Myelin-Associated Glycoprotein (L-MAG) in Schwann cells alone, and in cocultures with neuronal axons in an attempt to better understand the role of L-MAG during PNS myelination.

Studies of mouse Schwann cell culture growth factors that are essential for rodent SC survival in culture include heregulin-β1 and forskolin. The proliferation of mouse-derived SCs in culture requires the presence of other growth factors. We studied effect of several factors known to be expressed in SCs after nerve injury on mouse SC proliferation. These included FGFb, PE, EGF, PDGF-BB, and TG-β1. We found that heregulin-β1 and forskolin are essential for mouse SC to survive in culture, FGF and PE were the best combination of growth factors promoting the mouse SC proliferation, and that neuronal axons provide effective mitogenic environment for mouse SCs.

The expression of the Large Myelin-Associated Glycoprotein in PNS was studied in SCs cultured alone and in cocultures of SCs and DRGNs. The expression of L-MAG in nuclear area, Golgi and in endoplasmic reticulum is different depending whether the SCs are contacted with neuronal axons.

Honkanen *et al.*, *European Journal of Neuroscience*, Vol. 26, pp. 953–964, 2007.

The effect of light exposure on the activities of certain transporters and enzymes involved in light-enhanced calcification in the giant clam, *Tridacna squamosa*

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The giant clam *Tridacna squamosa* undergoes light-enhanced calcification, a process which facilitates their growth. This study aimed to examine several transporters and enzyme activities related to light-enhanced calcification in *T. squamosa* exposed to 12 h of darkness (control) and 12 h of light. One of the transporters studied was Na^+/K^+-ATPase (NKA), which can transport NH_4^+ in substitution for K^+. Deposition of $CaCO_3$ during light-enhanced calcification releases H^+. H^+ can react with NH_3 to form NH_4^+ in the extrapallial fluid, and NH_4^+ can be transported through NKA into the adjacent inner mantle. Indeed, there was a significant increase in Na^+/NH_4^+-activated-NKA activity in in the inner mantle of *T. squamosa* exposed to 12 h of light compared to that exposed to 12 h of darkness (control). The inner mantle of *T. squamosa* exposed to 12 h of light also had increased activity of glutamine synthetase, an enzyme which forms glutamine from glutamate and NH_4^+. Results obtained from this study support observations reported elsewhere on a significant increase in pH and a significant decrease in the ammonia concentration in the extrapallial fluid, with a significant increase in the glutamine concentrations in the inner mantle of *T. squamosa* exposed to light. Furthermore, changes in activities of other transporters like N-ethylmaleimide(NEM)-sensitive-V-H^+-ATPase, Na^+/K^+-activated-NKA and H^+/NH_4^+-activated-H^+/K^+-ATPase in the inner mantle and the ctenidia indicate that light-enhanced calcification may perturb the acid/base balance and Na^+ homeostasis in the hemolymph.

Space use and behaviour of Eurasian red squirrels inhabiting semi-natural and urban environments

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With cities growing at a rapid pace animal species must either retreat to patches of natural habitat or adapt to novel conditions in urban areas. These adaptations can be related to changes in diet, population density, movement patterns, reproductive strategy and disease avoidance/resistance, all of which influence daily energy expenditure. A small mammal species known to succeed in urban environments is the Eurasian red squirrel (*Sciurus vulgaris).* Therefore, we investigated differences in space use and behaviour, specifically home range size, home range overlap and activity patterns of free-ranging individuals living in a semi-natural and an urban habitat within a large city in northern Germany. Squirrels were radio-tracked during the same time period. Preliminary results suggest that urban squirrels have smaller home ranges, increased home range overlap among individuals, shorter travelled distances and reduced activity compared to conspecifics in the semi-natural study site. Interestingly, body mass at the time of capture did not differ between habitats ($t_7 = 0.12$, $p = 0.909$). The dissimilarities we identified are possibly explained by denser and more dependable supplementary food sources in the urban habitat. Hence, it appears urban squirrels are able to gain sufficient energy with shorter foraging times and decreased travelled distances. The flexibility in activity patterns and concomitant maintenance of body mass displayed by *S. vulgaris* likely contributes to the species' success in cities. We stress the importance of ascertaining which species are potentially able to benefit from supplemented food sources in order to predict their sensitivity to urbanisation, and suggest that combining physiological and ecological data is essential for this process.

The possible role of SGPP-5 in reproduction in the desert locust *Schistocerca gregaria*

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In 1987, the first member of a new family of serine protease inhibitors was discovered in the crayfish *Pacifastacus leniusculus*. These pacifastin like peptides belong to a family of Arthropoda specific inhibitors, of which the endogenous functions haven't been fully detected so far. Nevertheless, there are indications that they may play a role in several regulatory pathways in the desert locust, *Schistocerca gregaria*. Pacifastin like inhibitors appear to inhibit digestive enzymes *in vitro* in diverse insect species. It has already been shown that SGPI-2 is a potent and specific inhibitors for bovine chymotrypsin, whereas others more specifically inhibit trypsins.

Preliminary results indicated that the fifth precursor of the pacifastins like inhibitors, SGPP-5, has an influence on the reproduction of adult locusts. Analysis of possible copulation and post copulation effects after RNA interference of SGPP-5 will help us to verify if there is indeed a regulatory link between the pacifastins like inhibitor and the reproductive cycle in adult *S. gregaria*.

Energy budget is not enough: stoichiometry helps to explain the life history evolution

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It is widely accepted that the major factor affecting the evolutionary optimization of animal life histories is energy balance, therefore studies focus on the energy costs and benefits of adaptations, the efficiency of energy acquisition and investment, and limits to energy budgets. However, in heterotrophs, equally important seems to be the problem of maintaining stoichiometric balance. The mismatch between chemical compositions of consumer tissues and their resources strongly affects major life history traits (growth rates, body size, reproduction strategies, survival). Therefore, proportions of elements in food and in various tissues may be a currency of choice in life history optimization studies. We present two examples of herbivore life histories shaped by the stoichiometries of their food, representing the extremes of the feeding strategies: (1) nutrient deficient dead wood exploited by xylophages, (2) nutrient rich pollen used by bees. Xylophages are extremely limited nutritionally, especially by N, P, Cu, K and Na (concentrations 50 – 1000 times higher in their bodies than in the dead wood), with females at higher stoichiometric mismatch than males. In spite of the poor digestibility of wood, this resource is not limiting energetically and it is available in great excess. The nutritional constraints are offset only in part with extended development time (possible due to reduced mortality inside of tree trunks). The unusually high variance in size at maturity in xylophages may be explained by the combined effect of prolonged development time due to limiting stoichiometry and discrete time course in a seasonal climate. (2) In contrast, the pollen eaten by bees is nutritionally very rich but it costs a lot of time and effort to collect sufficient amount. Stoichiometrically, it is not well balanced either (limiting are the elements S, Cu, K and Na, with concentrations approx. 10 times lower than in bee tissues) and highly variable among taxons and seasons. This affects the feeding strategy, patterns of reproduction and mortality risk of pollen eaters. Dependence of life history traits on the food stoichiometry should be taken into consideration while discussing life histories evolution.

Cardiac physiological hypertrophy in response to cold in Red Junglefowl

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In response to cold environmental temperatures (T_a) some avian species respond with an enhanced aerobic scope characterized by increased shivering thermogenesis due to skeletal muscle hypertrophy, raised blood oxygen transport via increased red blood cell count and hemoglobin levels and enlarged cardiac output due to hypertrophy of the heart muscle. Red Jungle fowl hatchlings raised outdoors by attentive mother hens (mean T_a 11.3 °C instead of 30 °C as common indoor temperature) also show the characteristic signs of cold acclimatization. Skeletal muscle hypertrophy was seen in the left gastrocnemius muscle (0.95±0.11 SD % vs 0.63±0.06% in indoor age matched individuals) but not in the pectoral muscle. Hematocrit and hemoglobin concentration were 43±3% and 127±7 g l^{-1} in outdoor animals, significantly larger than 35±3% and 111±7 g l^{-1} in the age matched indoor group. Heart mass in the outdoor group was more than double the size of the indoor group, 0.97±0.08% vs. 0.40±0.05%. From these results I hypothesize that outdoor raised individuals have an enhanced aerobic scope supported by an orchestrated acclimatization response of the oxygen transport system. Simultaneously, the possibility to induce a cold acclimatization phenotype in Red Jungle fowl opens up for the analysis of the genetic architecture of seasonal cardiac hypertrophy taking advantage of the well annotated chicken genome.

Salinity acclimation enhances desiccation tolerance in the aquatic beetle *Nebrioporus baeticus* (Dytiscidae), but not *vice versa*

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Cross resistance and trade-off patterns are expected to emerge when the physiological mechanisms to deal with different stressors are shared. Salinity and desiccation are key natural stressors for water beetles living in temporary inland saline streams in the Mediterranean regions, whose adults periodically leave the aquatic medium to disperse to other habitats. Osmotic stress, imposed by salinity, triggers similar physiological responses to desiccation stress, activating mechanisms to avoid water loss and the increase of internal osmotic concentration. The aim of this study was to identify crossed patterns (trade-off or cross resistance) between salinity and desiccation responses in adults of the aquatic beetle *Nebrioporus baeticus*, an endemic species of saline streams from the southeast of Spain. We conducted two experiments to address the following questions: 1) does desiccation stress increase tolerance to salinity?, and 2) does acclimation to stressful salinity confer higher tolerance to desiccation?. In the first experiment, beetles were previously exposed to moderate (40% RH) or extreme low (10% RH) relative humidity at 20°C and then to a stressful salinity (50 gl^{-1}; 50 g of marine salt, Ocean Prodac, dissolved in distilled water). In the second one, specimens were acclimated at stressful (50 gl^{-1}) and not stressful (12 gl^{-1}) salinities and then subjected to moderate desiccation (40% RH). The pre-exposure to desiccation decreased survival under salinity stress (trade-off). In contrast, acclimation at sublethal salinity led to a higher tolerance to desiccation (cross-resistance). Therefore, our results suggest that crossed patterns between both stressors depend on the direction of exposure. Water loss during desiccation likely resulted in a significant increase of haemolymph osmotic concentration and involved a high energetic cost, severely affecting individual performance and impairing osmoregulation mechanisms during the subsequent exposure to salinity. However, we hypothesize that when exposure to stressors occurs in the inverse direction, the activation of osmoregulation mechanisms at a sublethal salinity, minimizes water loss during the initial hours of exposure to desiccation, enhancing organism´ survival.

Using the reaction norm approach to study individual variation in glucocorticoid responses to temperature

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Environments, and ambient temperatures (T_a) in particular, are currently changing at unprecedented rates. Glucocorticoids (GC) have long been hypothesized to be one of the main endocrine mechanisms orchestrating organismal responses to variations in T_a. We performed an extensive literature review to investigate whether a consistent vertebrate response of GC to changes in temperature exists. Out of 88 studies that investigated the GC response to a decrease in T_a, 77% reported an increase in GC. Similarly, out of 52 studies that investigated the GC response to an increase in T_a, 86% reported an increase in GC. These patterns were consistent across both short (hours to days) and long term (weeks to months) periods. This supports the hypothesis that GCs modulate the responses to changes in T_a. However, GC assessment is often based on point sampling. While such a single GC sample is thought to provide an indication of the status of the individual at a specific point in time, it is important to remember that GC responses are extremely plastic and highly dependent on context. Such an approach therefore does not permit assessment of individual responsiveness of the GC system, which is arguably a more consistent reflection of a hormonal trait. Recently an alternative approach has been suggested, which considers plasticity an important characteristic of an individual's phenotype[1]. When changes in a trait are plotted along an environmental gradient, the resulting relationship is called a reaction norm[2], a concept developed in ecology[3], but still rarely applied to endocrine systems. Using such a reaction norm approach can be an important tool to further investigate whether GCs function as individually-consistent hormonal traits, and ultimately critical in increasing our understanding of the evolution of hormonal systems. We will therefore assess individual GC reaction norms across a T_a gradient in captive great tits (*Parus major*).

1) Williams, TD 2008 *Philos. Trans. R. Soc. Lond., B*, 363(1497), 1687–1698; 2) Lessells, CKM 2008 *Phil. Trans. R. Soc. B,* 363(1497), 1589–1598; 3) Dingemanse, NJ, *et al.* 2010 *Trends. Ecol. Evol.*, 25(2), 81–89.

Intra- and interspecific patterns of desiccation resistance across congeneric saline water beetles with different habitat preferences

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Insects have evolved diverse physiological adaptations to deal with desiccation, such as increasing water content and reducing water loss through the cuticle. For aquatic beetles living in inland waters in arid regions, the ability to resist drought stress together with dispersal capacity are expected to be keys to their vulnerability to global climate change. We measured different traits related to resistance and tolerance to desiccation (i.e. body mass (m_b), water loss rate (m_{H20}/h), water content (m_{H20}/m_b), cuticle content ($m_{cuticle}/m_b$), survival and recovery capacity) in adults of four saline aquatic beetle species of the genus *Enochrus* exposed to desiccation. We explored this set of traits at intra and interspecific levels with two aims: i) to understand the physiological mechanisms underlying desiccation resistance of beetles and to identify whether the relationships among these traits are consistent in the studied species, and ii) to determine if differences in desiccation traits among the species reflect adaptations to different aquatic habitats (lotic vs. lentic and hyposaline vs. hypersaline). At the intraspecific level, m_b was not clearly correlated with any of the other traits; thus, contrary to what is found in other insect groups, desiccation resistance appears not to be mediated by changes in size in these species. We found a consistent positive relationship between initial water content and water loss rates in all the studied species, suggesting that desiccation rate is regulated as a function of individual water content, i.e. the mechanisms to minimize water loss could be activated when a critical level of water loss is reached. Cuticle content seemed not to be a good proxy for cuticle permeability since it was related neither to water loss rate nor to water content. Species living in more saline habitats were more tolerant to desiccation, which seems to be accomplished by reducing water loss rates and increasing water content. However, no differences were found between beetles from lotic and lentic habitats. The relationship between tolerance to salinity and desiccation in beetles is of great interest for future studies on insect adaptions to osmotic stress.

Temperature-dependent sex determination ruled out in the Chinese soft-shelled turtle (*Pelodiscus sinensis*) via molecular cytogenetics and incubation experiments across populations

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The sex determination mechanism for the Chinese soft-shelled turtle (*Pelodiscus sinensis*) is subject to controversy. Some populations have been shown to possess sex chromosomes and thus genotypic sex determination (GSD), while others were reported to exhibit temperature-dependent sex determination (TSD). To test whether TSD and GSD coexist in this species or whether populations differ in their sex-determining system, we conducted egg incubation experiments to investigate how temperature influences hatchling sex in a wide range of populations of this species in China. In parallel, we used comparative genome hybridization (CGH) to study the micro-sex chromosomes of adult *P. sinensis* in the 2 populations that were previously identified to be TSD. The incubation experiments showed that temperature did not affect hatchling sex in any of the studied populations. CGH indicated that turtles have micro-sex chromosomes of the female heterogametic (ZZ/ZW) system in the 2 disputed populations. These results indicate that *P. sinensis* is a GSD rather than a TSD species. Thus, the apparent coexistence of TSD and GSD in this species is the result of previous misdiagnosis in purportedly TSD populations.

Identification of Fos expression in medulla oblongata areas of the African catfish, *Clarias gariepinus* submitted to hypoxia

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The c-fos gene is part of a family of genes expressed early, the IEG's (Immediate Early genes). This gene encodes Fos, which is used in the verification of the neuronal activation after various types of stimuli. In the present study we analyzed this protein expression in the bulbar region of the African catfish, *Clarias gariepinus*, a facultative air-breathing fish. The expression of Fos was evaluated after acute exposure to hypoxia. The experiments were conducted individually with four groups (N = 5): 1. Group exposed to normoxic water without access to air; 2. Group exposed to hypoxic water without access to air; 3. Group exposed to normoxia with access to air; 4. Group exposed to hypoxia with access to air. Five days before the experiments, the fish were transferred to tanks where they were acclimated to normoxia. Fos expression were located in the reticular formation and vagal motor nuclei in all groups. A tendency of decreased Fos expression was observed in the hypoxic group without access to air. This probably occurred because the fish underwent to hypometabolism and, consequently, neuronal reduction of the putative areas involved in the respiratory rhythm generators and maintenance of breathing.

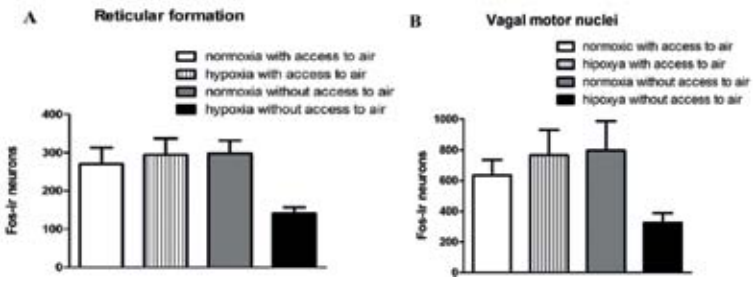

Figure 1: Number of Fos-immunoreactive in Reticular formation (A) e Vagal motor nuclei (B) after normoxic and hypoxic exposure, with and without access to air. Bars are mean ± SEM.

cDNA cloning of two crustacean female sex hormones from the kuruma prawn *Marsupenaeus japonicus*.

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The sex differentiation in crustacean is well known to be controlled by an androgenic gland hormone. Recently, another hormone, crustacean female sex hormone (CFSH), has been discovered. This novel hormone was purified from the female eyestalk of the blue crab *Callinectes sapidus* (Cas-CFSH). Gene knockdown of Cas-CFSH by RNA interference was shown to inhibit the appearance of the female reproductive characteristics. Therefore, it has been thought that CFSH controls female secondary sex characteristics. In order to accumulate knowledge on CFSH, here we cloned two CFSH cDNAs from the kuruma prawn *Marsupenaeus japonicus.* An eyestalk CFSH (Maj-CFSH_ES) and an ovarian CFSH (Maj-CFSH_OV) cDNAs were cloned by 5'- and 3'-RACE. The Maj-CFSH_ES cDNA consisted of 1,050 bp including a 5'-untranslated region (UTR) (23 bp), an open reading frame (ORF) (735 bp), and a 3'-UTR (292 bp). The ORF was conceptually translated into a putative prepropeptide comprising 244 amino acid residues, consisting of a signal peptide (SP) (34 residues), a CFSH-precursor-related peptide (CPRP) (44 residues), a processing signal (2 residues) and Maj-CFSH_ES (164 residues). Maj-CFSH_OV cDNA consisted of 942 bp including a 5'-UTR (130 bp), an ORF (678 bp), and a 3'-UTR (134 bp). The ORF encoded a putative prepropeptide comprising 225 amino acid residues, consisting of a SP (24 residues), a CPRP (35 residues), a processing signal (2 residues) and Maj-CFSH_OV (164 residues). Although both of mature Maj-CFSH_ES and Maj-CFSH_OV showed low amino acid sequence identities to Cas-CFSH (38%), eight conserved Cys residues were observed in the two molecules. In the analysis of the tissue-specific gene expression by RT-PCR, Maj-CFSH_ES was detected in female and male eyestalks, suggesting that Maj-CFSH_ES might have roles except for the female sex hormone in *M. japonicus*. On the other hand, Maj-CFSH_OV was expressed strongly in the ovary but weakly in the testis. This result indicates that Maj-CFSH_OV might be one of the female sex hormones in *M. japonicus*.

Intermittent feeding during broiler breeder rearing affects tibia strength and rigidity by altering the morphology of cortical bone

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Broiler breeder hens raised on the same commercial farm and originating from the same grand-parental flock were either fed daily (ED) throughout rearing or habituated to an intermittent feeding schedule with two non-consecutive fasting days weekly (5:2) from 6 weeks of age. Feed amounts were adjusted so that both treatments received the same amount of feed weekly. The birds were raised in separate pens based on body size (small: S or normal/large: L) from 4 weeks of age to reduce feed competition and improve body mass homogeneity. At 12 weeks of age, 10 birds from each feeding*size group were euthanized and had their long bones dissected. The left tibia from each bird was measured with digital calipers, subjected to a three-point bending test caudocranially and cross-sectioned to determine cortical bone area, which was significantly larger in large birds but unaffected by feeding treatment.

Tibiae from birds fed intermittently withstood higher levels of stress at both yield and break points (Figure 1). This difference was particularly evident in large birds. Tibiae were also significantly more elliptical in large, daily-fed birds, probably constituting a less rigid shape. Mediolaterally broader bones may be the result of a functional demand as the birds grow heavier and assume a more waddling gait, which may also increase the risk of leg weakness (1). Sampled birds were matched by body weight between feeding treatments, but may have experienced different growth rates earlier during development.

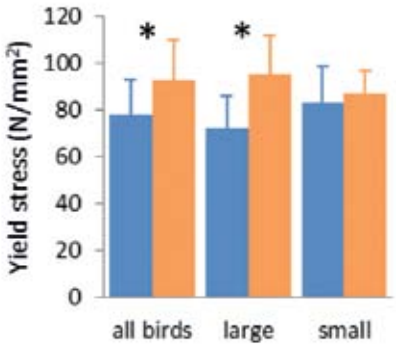

Figure 1: Average stress at yield in tibiae from daily-fed (blue) or 5:2-fed (orange) chickens in a three-point bending test. Error bars denote standard deviations.

1) Paxton H, Daley MA, Corr SA & Hutchinson JR 2013 *The Journal of Experimental Biology* 216:3237-3248

Impact of extensive low intensity aerobic exercise on heart force contraction of the migratory Neotropical teleost fish, *Brycon amazonicus* (Characidae).

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In most fish species, extracellular Ca^{2+} influx (L-type channels and/or Na^{+}/Ca^{2+} exchanger -NCX) is enough to activate myocardium contraction. Some species, however, depends on sarcoplasmic reticulum (SR) for supplying Ca^{2+} and little is known about the effects of long exercise periods on fish heart Ca^{2+}. *Brycon amazonicus,* a fast swimming and migratory species was chosen to investigate the effects of extensive exercise on the *in vitro* twitch force (TF-mN.mm^{-2}) of ventricular strips under crescent stimulation frequencies (0.2-2.6Hz). Two experimental groups (EX: exercised - 2 mth at 5 cm/s of artificial stream, and CT: control - 2 mth without stream). The relative roles of NCX and SR were estimated by blockade with lithium (Li) and ryanodine (Ry), respectively. Results are shown in the table bellow. The results demonstrated that exercise training remarkably improved excitation-contraction coupling and both, NCX and SR, are essential for Ca^{2+} cycling in this species.

Frequency (Hz)	TF (mN.mm^{-2})					
	CT	EX	CT-Li	EX-Li	CT-Ry	EX-Ry
0.2	3.8 ± 0.5	5.7 ± 0.6 a	4.0 ± 0.5	4.5 ± 2.4	2.0 ± 0.3 a	3.7 ± 0.9 b
0.4	3.8 ± 0.5	5.7 ± 0.6 a	3.8 ± 0.5	4.4 ± 2.4	1.9 ± 0.3 a	3.7 ± 0.9 b
0.6	3.6 ± 0.5	5.6 ± 0.6 a	3.0 ± 0.5	3.9 ± 2.2	1.9 ± 0.3 a	3.7 ± 0.9 b
0.8	3.5 ± 0.5	5.6 ± 0.5 a	2.5 ± 0.5	3.1 ± 1.8 b	1.9 ± 0.3 a	3.7 ± 0.9 b
1.0	3.4 ± 0.5	5.7 ± 0.6 a	1.9 ± 0.4 a	-	1.9 ± 0.3 a	3.8 ± 0.9 b,c
1.2	3.4 ± 0.5	5.9 ± 0.6 a	1.6 ± 0.2 a	-	2.0 ± 0.3 a	3.9 ± 0.8 b,c
1.4	3.1 ± 0.5	5.9 ± 0.6 a	1.2 ± 0.2 a	-	2.0 ± 0.3 a	4.1 ± 0.8 b,c
1.6	2.9 ± 0.5	5.9 ± 0.6 a	0.9 ± 0.1 a	-	2.0 ± 0.3	4.1 ± 0.8 b,c
1.8	2.8 ± 0.5	5.9 ± 0.7 a	0.7 ± 0.2 a	-	2.0 ± 0.3	4.0 ± 0.8
2.0	2.8 ± 0.4	5.1 ± 0.7 a	-	-	2.3 ± 0.3	3.7 ± 0.8
2.2	2.7 ± 0.6	4.9 ± 0.7 a	-	-	2.2 ± 0.4	3.6 ± 0.8
2.4	2.2 ± 0.6	4.5 ± 0.7 a	-	-	2.1 ± 0.4	3.3 ± 0.8
2.6	-	2.8 ± 0.6	-	-	-	-

Significant differences ($p<0.05$): **a** - in relation to CT; **b** - in relation to EX; **c** - in relation to CT-Ry

Early differentiation of the pancreatic islets in grass snake *Natrix natrix* L. (Lepidosauria, serpentes) embryos

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Pancreatic endocrine cells of grass snake (*Natrix natrix* L.) embryos were investigated in different developmental stages by electron microscopy using conventional methods. The age of embryos was calculated with the developmental table of this snake species (Rupik, 2002). The material was fixed and stained with using standard methods in electron microscopy. The ultrastructural observations have shown that four types of endocrine cells with different granules are present in the embryonic endocrine pancreas of the grass snake. These cells were distinguished by size, shape and electron-density of their granules. The first type of endocrine cells was identified by largest granules of various size and generally round in profile. Sometimes, these granules had highly electron-dense inner core and moderately electron-dense outer zone. The second type of cells was less frequently observed than the first type. Their granules were smaller and characterized by different shape and size, and their content was often separated from the limiting membrane by distinct electron-lucent zone. The third type was rarely observed with granules smaller than in the first and the second type, generally round, occasionally irregular in shape and electron-dense content. The fourth type of cells was detected at the periphery of islets with the smallest and round in shape granules. On basis of these results, one can assume that these cells correspond with the A-, B- and D-cells in adult reptilian species. The fourth type of endocrine cells has not been identified in the embryonic pancreas of the grass snake by its ultrastructural features.

Rupik W. 2002. Normal developmental table in early development of adrenal glands in grass snake Natrix natrix L. *Advances in Anatomy Embryology and Cell Biology* 164: 24–32.

Effect of thermal physiology on differential success of invasive and native coccinelids in a climate change scenario

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Several hypotheses have been postulated to explain the success of invasive species in the recipient community. However, physiological and life history traits are usually ignored and could be playing an important role. In this context, we assessed a theoretical model proposed by Zerebecki & Sorte (2011) who predicted that invasive species would be more eurythermic than native ones. Faced with an environmental temperature increase in a climate change scenario the thermal tolerance of native species would exceed their limits before those of invasive ones. We tested these predictions using invasive (*Harmonia axyridis*, *Hippodamia variegata*) and native (*Eriopis chilensis)* coccinellid species in central Chile after acclimating them for two weeks according to environmental temperatures they experience in the field (7, 15 and 26 °C). Specifically we tested and compared 1) Upper and lower lethal temperatures 2) Critical thermal maxima and minima and 3) Life history traits. Upper and lower Lethal temperatures were not significantly different between species and none incremented their lethal limits following acclimation. Our results suggest a trade-off between breath limits and the ability to acclimate of those limits. However their critical temperatures were different. *H. axyridis* that is considered a harmful pest all over the world has a lower critical thermal maximum than the other two species, which accords with aestivation behavior observed in the field. Also, this species has a higher critical thermal minimum (tolerance to lower temperature) than the other two species which also matches with its rapid, ongoing invasion of the southern part of the country. In addition, *H. axyridis* has better egg laying, egg to adult viability and survival than the other two species. We conclude that physiological and life history traits are playing a role in the invasive success of this coccinelid and should certainly be accounted for.

Zerebecki RA & Sorte CJB 2011 *Plos one* 6 e14806.

Personality and collective behavior in the soldier crab

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The soldier crab (*Mictyris guinotae*) appears in great numbers and feeds while wandering en masse during daytime low tide. The mechanism of formation of mass wandering has not been clarified. In this study, to investigate if the soldier crabs use visual images of neighbours as a stimulus for wandering, a dummy crab was presented to crabs and their reactions were observed. The subjects were 60 crabs that had been collected from the tidal flat of Iriomote Island in Okinawa, Japan. They were divided into two groups of 30 subjects each. All subjects were tested once in apparatus cut from 3 mm thick blue Perspex. It was 2 cm wide, 10 cm deep and 13 cm long straight, one-side open-ended runway, and sat on a cork sheet on which was printed a 5 cm radius circle. The center of the runway exit was positioned in the center of the circle thereby enabling each subject's angle of emergence to be measured. Each crab was introduced into the dead end of the runway. After emerging from the runway, the point at which the subject crossed the 5 cm radius circle was marked with a pencil. The angle of emergence from the center of the runway exit was later measured. For the half of the subjects, a dummy crab was displayed on the left or right side of the open area. The position of the dummy was altered for each subject. For another half, no dummy was displayed. In the dummy experiment, half of the subjects moved toward the dummy while the other half did away from it. In the experiment without dummy the subjects moved on the center of the open area. These results suggest that each crab has individual preference for the neighbouring individual. It is interesting that uniformity of the mass wandering in the soldier crab results not from uniformity but from variation of personality of each individual.

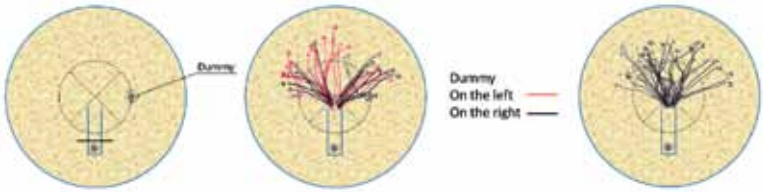

Figure: Left; Set-up for the dummy experiment. Center; Traces of individuals' motion in the dummy experiment. Right; Traces in the experiment without dummy.

Cytoskeletal molecular biomarkers of chemical pollution impact in fish and snake brain

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Development of molecular biomarkers, which adequately and reliably describe the cells' functional state and, indirectly, an organism as a whole, is important for the estimation of early effect of ecotoxicants. Glial fibrillary acidic protein (GFAP) is considered to be a reliable neurospecific biomarker of pollutant influence. Through pronounced biomagnification effect the hydrobionts are much more subjected to environmental pollutants as compared with terrestrial animals. The purpose of our study was to estimate the validity of GFAP as a biomarker in common species of fish and snake. We studied the Prussian carp *Carassius gibelio* and the dice snake *Natrix tessellata* inhabited both industrially polluted site and control (non-polluted) one. For the GFAP analysis we used the western blot. The lipid peroxidation (LPO) was determined with malonic dialdehyde level in a brain tissue for the valuation of negative pollution effect. Our research found the significant upregulation of GFAP (1.9-2.1 times), which was observed in the brain of fish and snake from polluted areas as compared to a control group. GFAP over expression was accompanied with the increase of amount of the degraded polypeptide fragments of GFAP (Figure). It demonstrates well-determined protein degradation down to 40 kDa in both fish and snake brain. Observed changes should be a result of upregulation of the proteolysis of glial cytoskeleton. Moreover, in animal brain we observed significant elevation of LPO (45 % as compared to a control). Thus, the industrial pollution leads to astrogliosis in the brain tissue of fish and snake. That makes them an option for both environmental and population assessment with reliable neurospecific molecular biomarkers.

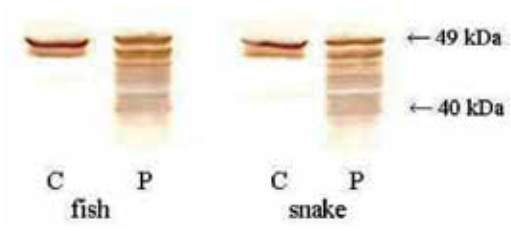

Figure: Immunoblotting of the brain cytoskeletal proteins of the Prussian carp and dice snake under ecotoxicants influence: C – control site, P – polluted site.

Temperature effects on baroreflex and cardiorespiratory interactions in the toad *Rhinella schneideri*

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For an adequate blood supply to support metabolic demands, vertebrates regulate blood pressure, maintaining sufficient perfusion and avoiding ischemia. Terrestrial amphibians are subjected to a wide range of environmental changes, and temperature is one of the most important factors that directly affect all physiological and biochemical processes; among them, cardiorespiratory function. Interactions between cardiovascular and respiratory systems operate coordinately to match oxygen delivery to demand in many ectotherms and thereby increased oxygen consumption can be achieved at high environmental temperatures. Temperature changes seem to influence resting heart rate (f_H) more than blood pressure (BP) in amphibians; however, little is known about thermal influences on the baroreflex regulation in these animals. It has been suggested that ventilation may also be involved in the BP feedback loop regulation in amphibians; however, no data exist regarding different metabolic demands imposed by temperature changes in this relationship. Using a pharmacological approach to change arterial BP (phenylephrine and sodium nitroprusside), we investigated the effect of temperature on baroreflex sensitivity and cardiorespiratory interactions in the South American Schneider's toad, *Rhinella schneideri*. We found that baroreflex sensitivity in *R. schneideri* is temperature dependent, shifting the operating point of the baroreflex-f_H relationship up across temperature (from 15 to 30 °C), keeping the absent changes on cardiac reflex to hypotension rather than hypertension. In addition, highly significant relationships between BP regulation and ventilation, independent of temperature, might result from integrated information from baroreceptors on the central nervous system affecting respiratory centers.

Thermoregulatory behavior is widespread in the embryos of reptiles and birds

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Recent studies have demonstrated that thermoregulatory behavior occurs not only in post-hatching turtles but also in turtles prior to hatching. However, (i) do embryos move away from dangerously high temperatures as well as towards warm temperatures? and (ii) is such embryonic movement due to active thermoregulation, or (more simply) to passive embryonic repositioning caused by local heat-induced changes in viscosity of fluids within the egg? (iii) does thermoregulatory behavior also occur in the embryos of other reptile and bird species? We found that embryos of an emydid turtle (*Chinemys reevesii*) avoid dangerously high temperatures by moving to cooler regions of the egg. The repositioning of embryos is an active rather than passive process. Live embryos move towards a heat source, whereas dead ones do not. Further, we found that such behavior is widespread, but not universal, in reptile and bird embryos. We recorded repositioning within the egg, in response to thermal gradients, in the embryos of three species of snakes (*Xenochrophis piscator*, *Elaphe bimaculata*, and *Zaocys dhumnades*), two turtles (*Chelydra serpentina* and *Ocadia sinensis*), one crocodile (*Alligator sinensis*), and four birds (*Coturnix coturnix*, *Gallus gallus domesticus*, *Columba livia domestica*, and *Anas platyrhynchos domestica*). However, we detected no significant thermoregulation by the embryos of two lizard species (*Takydromus septentrionalis* and *Phrynocephalus frontalis*). Overall, embryonic thermoregulatory behavior seems to be widespread in reptile as well as bird species, but may be unimportant in the small eggs laid by most lizards.

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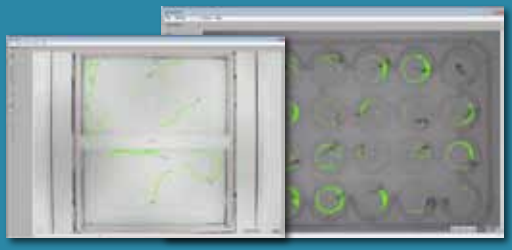

// Multiple free swimming zebrafish larvae

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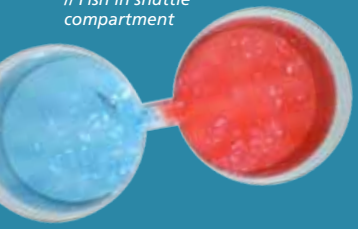

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