Thermal Tolerance and Cadmium Susceptibility of Amphipods Endemic to Lake Baikal

Temperaturtoleranz und Cadmiumsensitivität endemischer Amphipoden des Baikalsees

Dissertation
zur Erlangung des akademischen Grades
– Dr. rer. nat. –

Dem Fachbereich 2 Biologie/Chemie
der Universität Bremen
vorgelegt von

Lena Jakob
Diplom-Biologin

Bremen

Gutachter

Prof. Dr. Hans-Otto Pörtner
Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research
Department of Integrative Ecophysiology
Am Handelshafen 12
27570 Bremerhaven, Germany

Prof. Dr. Rolf Altenburger
UFZ – Helmholtz Centre for Environmental Research
Department of Bioanalytical Ecotoxicology
Permoser Str. 15
04318 Leipzig, Germany

Prüfer

Prof. Dr. Martin Zimmer
Leibniz-Center for Tropical Marine Ecology (ZMT)
Department of Mangrove Ecology
Fahrenheitstrasse 6
28359 Bremen, Germany

Dr. Franz Josef Sartoris
Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research
Department of Integrative Ecophysiology
Am Handelshafen 12
27570 Bremerhaven, Germany

Tag des Promotionskolloquiums: 31.10.2016

Abbreviations

AR5	Fifth Assessment Report of the Intergovernmental Panel on Climate Change
BPT	Breakpoint temperature
CO_2-eq/yr	Carbon dioxide equivalents per year
COX	Cytochrome c oxidase
CS	Citrate synthase
DOC	Dissolved Organic Carbon
EPA	United States Environmental Protection Agency
FW	Fresh weight
GDH	Glutamate dehydrogenase
GHG	Greenhouse gas
Gt	Gigatonne
HADH	Hydroxyacyl-coenzyme A dehydrogenase
HDP	Heatdenaturable proteins
HSP	Heat stable proteins
Hsp70	70 kilodalton heat shock proteins
IPCC	Intergovernmental Panel on Climate Change
LC1	Lethal concentration for 1% of the test group in a given setup
LC50	Lethal concentration for 50% of the test group in a given setup
LDH	Lactate dehydrogenase
LTemp50	Lethal temperature for 50% of the test group in a given setup
LTime50	Time until 50% of the test group has died
MRG	Metal rich granules
MRG+exo	Metal rich granules + exoskeleton (subcellular compartment)
MSF	Metal sensitive fraction
MT	Metallothionein
MTLP	Metallothionein-like proteins
OCLTT	Oxygen- and capacity-limitation of thermal tolerance
OECD	Organization for Economic Co-operation and Development
OSPAR	The Convention for the Protection of the Marine Environment of the North-East Atlantic
PAH	Polycyclic aromatic hydrocarbon
pHe	Hemolymph (extracellular) pH
PK	Pyruvate kinase
POP	Persistant organic pollutant
RCP	Representative concentration pathway
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals (European Union Regulation No 1907/2006)
RMR	Routine metabolic rate
RNA	Ribonucleic acid
SPM	Subcellular partitioning model
T_c	Critical temperature
T_d	Denaturation temperature
T_p	Pejus (= getting worse) temperature
UNESCO	United Nations Educational, Scientific and Cultural Organization
UNFCCC	United Nations Framework Convention on Climate Change

Die Dissertation weist keinerlei inhaltliche Änderungen im Vergleich zur der beim Prüfungsamt eingereichten Version auf. Geringfügige Abweichungen von der eingereichten Version beschränken sich lediglich auf Sprache und Form auf den Seiten v, vi und 61, die nach Genehmigung des Prüfungsausschussvorsitzenden vorgenommen wurden.

TABLE OF CONTENTS

Summary

Lake Baikal, the world's most ancient and by volume largest freshwater body on earth, is affected by global change and regional human activities; this concerns littoral regions of the lake in particular. The direct and indirect effects of rising temperatures and pollution on the unique endemic littoral amphipod fauna of Lake Baikal are as yet unresolved. Thus, the aim of this thesis is to provide insights into the physiological processes determining thermal tolerance and toxicant susceptibility in two of the most abundant littoral amphipod species (*Eulimnogammarus verrucosus* and *Eulimnogammarus cyaneus*) in comparison with the related gammarid *Gammarus lacustris*, which is ubiquitously spread in the Holarctic. The three species experience different temperature regimes. *G. lacustris* experiences the highest thermal fluctuations as it inhabits shallow water habitats, followed by *E. cyaneus*, which stays in the upper littoral of Lake Baikal throughout the year. By contrast, *E. verrucosus* migrates to sublittoral areas when temperatures in the upper littoral of Lake Baikal rise in summer. Further, the species differ in body size (body length from the rostrum to the uropods); *E. verrucosus* (3.5 - 4 cm) is about 3 - 4 times larger than *E. cyaneus* (ca. 1 cm). *G. lacustris* (ca. 1 - 1.5 cm) is only slightly larger than *E. cyaneus*. It inhabits some isolated bays of Lake Baikal but is not part of the typical littoral amphipod community. Whether global change will promote a widespread invasion of the non-endemic *G. lacustris* from isolated shallow bays into Lake Baikal is yet unknown.

In a comparative framework thermal plasticity of physiological performance parameters was studied on the whole animal, biochemical and molecular level in all three amphipod species under progressive temperature increase (0.8°C d^{-1}; start: 6°C). Toxicant susceptibility was investigated by measuring cadmium (Cd^{2+}) uptake, subcellular cadmium compartmentalization, concentration-mortality relationships and physiological responses to low biologically effective concentrations derived from concentration-mortality relationships.

Ventilation rates were limited at lower temperatures in *E. verrucosus* (10.6°C) than in *E. cyaneus* (19.1°C) and *G. lacustris* (21.1°C). These so-called breakpoint temperatures (BPTs) were correlated with migration of *E. verrucosus* from the upper littoral to deeper and cooler areas. Moreover, there was strong indication that the BPTs of ventilation correspond to the maximal habitat temperature of *E. cyaneus* and *G. lacustris*. Thus, within the framework of oxygen- and capacity-limitation of thermal tolerance (OCLTT), it was suggested that the BPTs of ventilation reflect the first level of thermal limitation, i.e. the pejus ("getting worse") temperatures of the species. Like ventilation, oxygen consumption was constrained at lower temperatures in *E. verrucosus* (15.0°C) than in *E. cyaneus* (25.2°C) and *G. lacustris* (23.6°C). Surpassing the BPTs of oxygen consumption led to exponentially increasing mortality. Consequently, the BPTs of oxygen consumption were proposed to correspond to the critical temperatures of the studied species.

Temperature-dependent changes in activities of key metabolic enzymes were correlated with those in oxygen consumption rates in all three amphipod species, however, the shapes of curves representing these changes differed between the species. In *E. verrucosus*, maximal activities of aerobic enzymes in response to changing temperatures followed hyperbolic or peak-shaped curves and, like oxygen consumption rates, decreased at a breakpoint of around 15°C. Only lactate dehydrogenase, which is involved in anaerobic processes increased significantly beyond 15°C. Simultaneously, transcriptional levels of genes coding for enzymes involved in aerobic metabolic processes were down-regulated and genes involved in the response to hypoxia simultaneously up-regulated in *E. verrucosus*. By contrast, no breakpoint was observed for aerobic enzyme activities of *E. cyaneus* and *G. lacustris*. Enzyme acitivities increased exponentially under elevated temperature. While *E. cyaneus* showed slight thermal compensation through progressively decreasing RNA transcript levels of many enzymes with rising temperature, no thermal compensation was observed for *G. lacustris*. Consequently, Q_{10}-relationships of enzyme activities at high temperatures were lower in *E. cyaneus* than in *G. lacustris*.

In contrast to *E. verrucosus,* smaller-sized *E. cyaneus* and *G. lacustris* had faster cadmium uptake rates and thus lower lethal concentrations, likely due to their higher ratio of permeable body surface area to body volume. Subsequent effect-scaled experiments (exposure to species-specific LC1 of $CdCl_2$; *G. lacustris* had to be excluded due to high cannibalism) revealed that more cadmium was bound to heat stable proteins in *E. cyaneus* than in *E. verrucosus*, congruent to its higher cellular stress response capacity. In contrast, exposure to their species-specific LC1, led to similar concentrations of cadmium associated with the metal sensitive fraction (contains cadmium bound to subcellular fractions which includes heat denaturable proteins and cell organelles) in the two species, however, with species-specific physiological responses of the oxygen supply system. Sublethal cadmium concentrations resulted in metabolic depression and reduced ventilation in *E. verrucosus* but not in *E. cyaneus*. Furthermore, the combination of cadmium (sublethal concentration at 6°C) and increased temperature was shown to be more deleterious than each single factor alone reflected by elevated mortality in both species.

In conclusion, thermal constraints on the oxygen supply system in *E. verrucosus*, *E. cyaneus* and *G. lacustris* may shape the upper temperature limits to the thermal habitats of the species, in line with the theory of an allometry of thermal tolerance and the hypothesis of a systemic to molecular hierarchy of thermal tolerance with the tightest constraints at the highest hierarchical level (whole animal). Concomitant changes at different organizational levels observed for *E. verrucosus* suggest a tightly regulated system in response to decreasing systemic oxygen availability caused by elevated temperatures. The results presented here underline that both physiological and behavioral responses to changing environmental conditions may determine a species' success under global change. *E. verrucosus* has a high behavioral plasticity (mediated by its migration behavior) but low physiological plasticity to cope with rising temperatures. By contrast, *E. cyaneus* has a higher capacity to tolerate current thermal fluctuations in the upper littoral. However, present summer temperatures may already touch the pejus range of *E. cyaneus*. Consequently, despite the higher thermal tolerance of *E. cyaneus* it might be more severely affected by future global change than *E. verrucosus*. Furthermore, despite the fact that lethal concentrations of cadmium were much lower for *E. cyaneus*, *E. verrucosus* showed a stronger physiological response (metabolic depression) to concentrations far below lethal ones. Consequently, the extremely sensitive reaction of *E. verrucosus* to low levels of a chemical stressor underlines that sublethal effects may not necessarily mirror sensitivity rankings based on mortality data. These findings underscore the necessity of water management strategies strictly avoiding chemical contamination of Lake Baikal waters. Rising temperatures likely enhance the deleterious effects of chemical stressors as shown here for cadmium.

Whether global change will provide a competitive advantage for *G. lacustris* in comparison to Baikal endemics and, by extension, promote the widespread invasion of this non-endemic species, could not be elucidated. *G. lacustris* showed only slightly non-significantly higher heat tolerance than the Baikal endemic *E. cyaneus* and showed a similar cadmium susceptibility as *E. cyaneus*. Secondary effects of global change such as eutrophication, which has been found in the littoral of Lake Baikal, are important factors that need to be considered in future studies. Organisms which are sensitive to hypoxic conditions are likely the first to be affected by such changes.

Zusammenfassung

Der globale Wandel wirkt sich auch auf den größten und ältesten Süßwassersee der Erde, den Baikalsee, aus. Insbesondere in der litoralen Zone des Sees sind Veränderungen messbar. Über die direkten und indirekten Einflüsse steigender Temperaturen und Umweltgiftbelastung auf die artenreiche endemische Amphipodenfauna des Baikalsees ist bisher wenig bekannt.

Vor diesem Hintergrund war es das Ziel dieser Arbeit, Parameter und Mechanismen zu entschlüsseln, welche die Temperaturtoleranz und die Sensitivität gegenüber Umweltgiften in Amphipoden bestimmen, und ihre ökologische Bedeutung zu diskutieren. *Eulimnogammarus verrucosus* und *Eulimnogammarus cyaneus*, zwei der abundantesten endemischen Arten der bentholitoralen Fauna wurden mit *Gammarus lacustris*, einer in der Holarktis ubiquitär verbreiteten Amphipodenart, vergleichend untersucht. Die drei Arten besetzen Habitate, in denen unterschiedliche Maximaltemperaturen und Temperaturfluktuationen auftreten. *G. lacustris* ist in kleinen und flachen Gewässern zu finden und daher den größten Temperaturschwankungen und Maximaltemperaturen (> 20°C) ausgesetzt, gefolgt von *E. cyaneus*, der im Litoral des Baikalsees hauptsächlich in Tiefen von 0 - 1.5 m zu finden ist, wo Maximaltemperaturen von 20°C erreicht werden können. *E. verrucosus* hingegen migriert von der Uferzone des Baikalsees in sublitorale Bereiche, wenn die Wassertemperaturen im Sommer ansteigen.

Die Arten unterscheiden sich nicht nur hinsichtlich ihrer Temperaturnische, sondern sind auch von unterschiedlicher Größe. Gemessen vom Rostrum bis zu den Uropoden ist ein adultes Individuum der Art *E. verrucosus* (ca. 3.5 - 4 cm) ca. 3 - 4 mal so lang wie ein adultes Individuum der Art *E. cyaneus* (ca. 1 cm). *G. lacustris* (ca. 1 - 1.5 cm) ist nur wenig größer als *E. cyaneus*, zeigt aber im Gegensatz zu den anderen Arten einen ausgeprägten Geschlechtsdimorphismus; männliche Tiere sind wesentlich größer als weibliche. Diese weitverbreitete Art ist auch in sehr flachen und abgeschlossenen Bereichen des Baikalsees zu finden, nicht jedoch in Vergesellschaftung mit der typischen endemischen Amphipodenfauna. Ob sich aus den abiotischen und biotischen Veränderungen im Zuge des globalen Wandels für *G. lacustris* ein Selektionsvorteil gegenüber endemischen Arten ergeben könnte, ist bisher nicht untersucht.

In dieser vergleichenden Studie wurde die Temperaturplastizität verschiedener physiologischer, biochemischer und molekularer Parameter in den drei Amphipodenarten unter graduellem

Temperaturanstieg analysiert (0.8°C d^{-1}; Start: 6°C). Die Sensitivität gegenüber toxischen Stoffen wurde am Beispiel von Cadmium untersucht. Hier wurden Konzentrations-Mortalitätskurven erstellt und die Aufnahme von im Wasser gelösten Cadmium (Cd^{2+}) bestimmt. Weiterhin wurde die Verteilung von Cadmium auf verschiedene subzelluläre Fraktionen und die Effekte von geringen (subletalen) Konzentrationen auf physiologische Parameter wie z.B. Ventilation und Sauerstoffverbrauch untersucht.

Die Grenzen der Temperaturtoleranz wurden durch Abweichungen der untersuchten biologischen Raten von thermodynamischen Gesetzen über einen nichtlinearen Regressionsansatz bestimmt. Die entsprechenden Temperaturen werden hier als „breakpoint temperatures" (BPT) bezeichnet. BPTs wurden in allen Arten zunächst in der Ventilationsrate festgestellt; bei *E. verrucosus* bei 10.6°C, wohingegen die BPTs der Ventilation bei den kleineren Arten *E. cyaneus* und *G. lacustris* erst bei 19.1 bzw. bei 21.1°C lagen. Im Feld fiel die Abundanz von *E. verrucosus* bei Temperaturen um 10 - 11°C stark ab, da die Tiere in tiefere Bereiche migrierten, wo sich das Wasser weniger stark erwärmt als nahe der Wasserlinie. Die maximale Habitattemperatur von *E. cyaneus* (20°C) liegt ebenfalls sehr nah bei der BPT der Ventilation dieser Art. Auch für *G. lacustris* gibt es Hinweise darauf, dass die BPT der Ventilation die maximale Habitattemperatur darstellt, da bei 22°C im Uferbereich des Tümpels keine adulten Tiere mehr an der Wasserlinie gefunden wurden. Die Daten weisen darauf hin, dass die BPT der Ventilation im Rahmen des Modells zur sauerstoff- und kapazitätslimitierten Temperaturtoleranz (OCLTT) als Pejustemperaturen (T_p) (pejus lt. für „schlechter werden") einzuordnen sind, da sie die ökologische Verbreitung der Arten eingrenzen. Limitierungen im Sauerstoffverbrauch waren wie auch bei der Ventilation zunächst bei *E. verrucosus* detektierbar (15.0°C) und erst bei wesentlich höheren Temperaturen bei *E. cyaneus* (25.2°C) und *G. lacustris* (23.6°C). Eine Überschreitung der BPTs des Sauerstoffverbrauchs führte bei allen Arten zu einem exponentiellen Anstieg der Mortalität. Diese BPTs des Sauerstoffverbrauchs wurden daher innerhalb des OCLTT Modells als kritische Temperaturen (T_c) eingeordnet.

Temperaturabhängige Veränderungen der maximalen Aktivität von metabolischen Schlüsselenzymen folgten den Sauerstoffverbrauchskurven (Temperaturbereich: 6 – 23.6°C). Daher zeigten sich bei allen (aeroben) Enzymen, nicht aber bei der Laktatdehydrogenase in Extrakten von *E. verrucosus* eine Limitierung bei ca. 15°C. Simultan verringerte sich die Expresssion einiger Gene, die Enzyme kodieren, die in aerobe metabolische Prozessen involviert sind, wobei die Expression eines Gens, welches ein Indikator für hypoxische Bedingungen ist, anstieg. Maximale Enzymaktivitäten bei *E. cyaneus* und *G. lacustris* folgten hingegen dem Gesetz von Arrhenius und zeigten daher einen exponentiellen Verlauf.

Die Aufnahmerate von im Wasser gelösten Cadmium (Cd^{2+}) war in *E. verrucosus* geringer als die von *E. cyaneus* und *G. lacustris*. Dieser Befund bestätigt Literaturdaten, welche einen Zusammenhang von Cadmiumtoxizität und Körpergröße in nahe verwandten Arten belegen.
Die Analyse der Verteilung von Cadmium auf subzelluläre Fraktionen ergab, dass bei *E. cyaneus* und *G. lacustris* ein höherer Prozentteil des Cadmiums in der Fraktion mit hitzestabilen Proteinen als bei *E. verrucosus* zu finden war, was sich mit ihrem im Vergleich zu *E. verrucosus* höheren Gehalt an Stressproteinen assoziieren lässt. Wenn *E. verrucosus* und *E. cyaneus* ihrem artspezifischen LC1 (letale Konzentration an Cd^{2+} für 1% der Testpopulation) ausgesetzt wurden, zeigte sich nur bei *E. verrucosus* eine Reduktion der Ventilation- und Sauerstoffverbrauchsrate; bei *E. cyaneus* zeigten Kontrolltiere und Cadmium-exponierte Individuen keine Unterschiede. *G. lacustris* wurde aufgrund starker Dezimierung der Versuchstiere durch Kannibalismus in den Konzentrations-Mortalitäts-Experimenten vom LC1-Experiment ausgeschlossen. Gradueller Temperaturanstieg wirkte sich in Verbindung mit geringen Cadmiumkonzentrationen (LC1) in *E. verrucosus* und *E. cyaneus* stärker negativ aus als die einzelnen Faktoren isoliert betrachtet.

Zusammenfassend lässt sich sagen, dass die Sauerstoffversorgungskapazität eine Schlüsselfunktion bei der Entstehung von physiologisch einschränkenden bzw. lebensbedrohlichen Temperaturen einnimmt. Des Weiteren bestätigen die Daten die Theorie der Allometrie der Temperaturtoleranz und die Theorie, dass die Einschränkungen einer hierarchischen Reihenfolge der biologischen Organisationsstufe folgen und sich Effekte zuerst auf der Ganztierebene zeigen. Bei *E. verrucosus* traten Limitierungen auf mehreren Organisationsstufen parallel auf, was darauf hindeuten könnte, dass physiologische Prozesse auf allen Ebenen über die systemische Sauerstoffverfügbarkeit reguliert werden. Auch die sensitive Reaktion von *E. verrucosus* auf Cadmium könnte sich durch die Sensitivität gegenüber oxidativem Stress erklären lassen, da sowohl steigende Temperatur als auch Cadmium oxidativen Stress auslösen können.

Unter den Einflüssen des globalen Wandels bestimmen sowohl Plastizität im Verhalten als auch die Plastizität physiologischer Parameter den Erfolg einer Art. *E. verrucosus* zeigt eine hohe

Plastizität im Verhalten, da diese Art aktiv ihre bevorzugte Temperaturnische aufsucht, obwohl die Nischen im Litoral des Baikalsees sehr eng besetzt sind. Hingegen toleriert *E. cyaneus* die Temperaturfluktuationen im Uferbereich und zeigt somit eine höhere physiologische Plastizität. Jedoch korreliert die maximale Habitattemperatur mit der Pejustemperatur von *E. cyaneus* und folglich könnten sich steigende Temperaturen im Litoral des Baikalsees negativ auf *E. cyaneus* auswirken, trotz seiner höheren Temperaturtoleranz. Die Ergebnisse der Cadmiumtoxizitätsstudie zeigen, dass *E. verrucosus* weitaus höhere LC50 Werte aufweist als *E. cyaneus* und *G. lacustris*. Dennoch zeigten sich bei *E. verrucosus* signifikante Reduktionen der Ventilation und des Sauerstoffverbrauchs bei subletalen Cadmiumkonzentrationen (LC1), die bei *E. cyaneus* nicht auftraten (Daten für *G. lacustris* fehlen). Die subletalen Effekte spiegeln demnach nicht das wider, was auf der Basis der Konzentrations-Mortalitäts-Studien zu erwarten war. Die Resultate zeigen, dass Baikalorganismen sehr empfindlich auf chemischen Stress reagieren können. Daher sollte der Eintrag von Umweltgiften in den Baikalsee durch entsprechendes Umweltmanagement verhindert werden.

Rückschlüsse darauf, dass der globale Wandel *G. lacustris* im Vergleich zu Baikalamphipoden einen Selektionsvorteil verschaffen könnte, lassen die hier vorgestellten Daten nicht zu, da *G. lacustris* nur eine geringfügig höhere Temperaturtoleranz aufwies als *E. cyaneus* und eine ähnliche Sensitivität gegenüber Cadmium wie *E. cyaneus*. Sekundäre Effekte des globalen Wandels wie z.B. die Eutrophierung von Gewässern, die im Litoral des Baikalsees zu beobachten ist, sind wichtige Faktoren, die in zukünftigen Studien berücksichtigt werden sollten. Ein Anstieg sauerstoffverbrauchender Prozesse im Litoral wird sich wahrscheinlich besonders auf die Arten auswirken, die empfindlich auf sinkende Sauerstoffverfügbarkeit reagieren.

1 Introduction

Lakes are sentinel ecosystems of climate change as physical, chemical and biological lake properties respond rapidly to climate-related changes in comparison to oceanic water bodies (Adrian et al. 2009). Additionally, freshwater ecosystems are particularly affected by pollution because municipal and industrial waste often ends up in rivers and streams. Lake Baikal (south-east Siberia, Russia) contains about 20% of the world's unfrozen freshwater resources and was designated a UNESCO world heritage site in 1996 due to its unique abiotic and biotic characteristics. Surface water temperatures of Lake Baikal are projected to rise substantially with climate change and sources of pollution are progressively increasing in the Baikal region (Moore et al. 2009). However, little is known about the potential effects of rising temperature and pollution on the Baikal ecosystem, which is amongst others famous for its highly diverse endemic amphipod fauna, which constitutes one third of the world's described freshwater amphipod species (Väinölä et al. 2008).

This thesis seeks to provide insights into the physiological processes determining thermal tolerance and toxicant susceptibility in Lake Baikal endemic amphipods in comparison with a related and ubiquitous Holarctic species and interprets its findings in the light of global environmental change.

1.1 Global warming and environmental pollution

The Intergovernmental Panel on Climate Change (IPCC) states that an unprecedented degree of global warming has been monitored during the last decades (IPCC 2014). The globally averaged combined land and ocean surface temperatures have increased by 0.85°C [0.65 -1.06°C] over the period 1880 to 2012 (> 90% likelihood) [1]. Furthermore, the upper 75 m of the ocean warmed by 0.11°C [0.09 - 0.13°C] per decade between 1971 and 2010. Warming of the upper ocean (0 - 700 m) during this period is almost certain (99 - 100% likelihood). Many other phenomena demonstrate the undeniable global warming trend such as mass losses of the Greenland and Antarctic ice sheets (period: 1992 - 2011), glacier shrinking, decrease of annual mean Arctic sea-

---

[1] The IPCC Synthesis Report reports findings using five different qualifiers for the underlying evidence and agreement: very low, low, medium, high, and very high. The assessed likelihood of an outcome or result is reported as virtually certain (99 - 100%), very likely (90 - 100%), likely (66 - 100%), about as likely as not (33 - 66%), unlikely (0 - 33%), very unlikely (0 - 10%), and exceptionally unlikely (0 - 1%). The terms extremely likely (95 - 100%), more likely than not (>50 - 100%), more unlikely than likely (<0 - 50%), and extremely unlikely (0 - 5%) are used in addition whenever appropriate.

ice extent (period: 1979 - 2012) and extent of Northern Hemisphere snow cover, increase of permafrost temperatures since the early 1980s and rise of global mean sea level by 0.19 m [0.17-0.21 m]. A significant increase in the anthropogenic emission of greenhouse gases (GHG), i.e. carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), is extremely likely to be the major cause of global warming since the mid-twentieth century. The current atmospheric concentration of GHG is high and unprecedented in at least 800,000 years. Combustion of fossil fuels and industrial activities are the most important sources of GHG and accounted for 65% of the total GHG emission in 2010. Projected global warming trends in the IPCC reports are based on different GHG emission scenarios termed Representative Concentration Pathways (RCP). The four RCPs (RCP2.6, 4.5, 6.0 and 8.5) represent different rates of increase in radiative forcing in 2100 in comparison to 1850 e.g., RCP2.6 stands for an increase in radiative forcing of 2.6 W/m^2. Although the RCPs vary in terms of the dimension of the increase in radiative forcing, all RCPs project an increase in surface temperatures over the 21st century (Figure 1). Moreover, the number and duration of heat waves and extreme precipitation events is projected to increase. Due to the inexorable momentum of climate change, the United Nations Framework Convention on Climate Change (UNFCCC) invited the IPCC to report on the impacts of global warming of 1.5°C above pre-industrial level and the respective GHG emission in 2018 (IPCC Secretariat 2016).

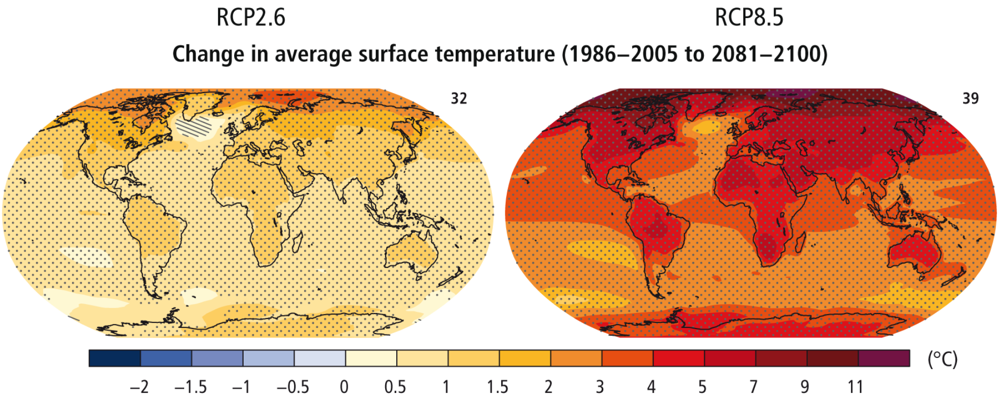


Figure 1: Projected global thermal changes based on different Representative Concentration Pathways (RCP), after IPCC (2014)
Change in average surface temperature based on multi-model mean projections for 2081–2100 relative to 1986–2005 under the RCP2.6 (left) and RCP8.5 (right) scenarios. The number of models used to calculate the multi-model mean is indicated in the upper right corner of each panel. Stippling (i.e., dots) shows regions where the projected change is large compared to natural internal variability and where at least 90% of models agree on the sign of change. Hatching (i.e., diagonal lines) shows regions where the projected change is less than one standard deviation of the natural internal variability.

Concomitant with anthropogenic climate change, the global chemosphere is being heavily impacted by human activities with a significant acceleration in nutrient and material flows (e.g. metals) (Fent 2013). Furthermore, the combustion of fossil fuels does not only lead to a higher level of atmospheric CO_2 but also releases large amounts of persistent organic pollutants (POP), such as perchlorinated aromatic hydrocarbons (PAH), and heavy metals. The total production of chemicals is projected to double from 2000 to 2024, due to the 3% annual increase in the production rate of chemicals (Figure 2) (Wilson and Schwarzman 2009).

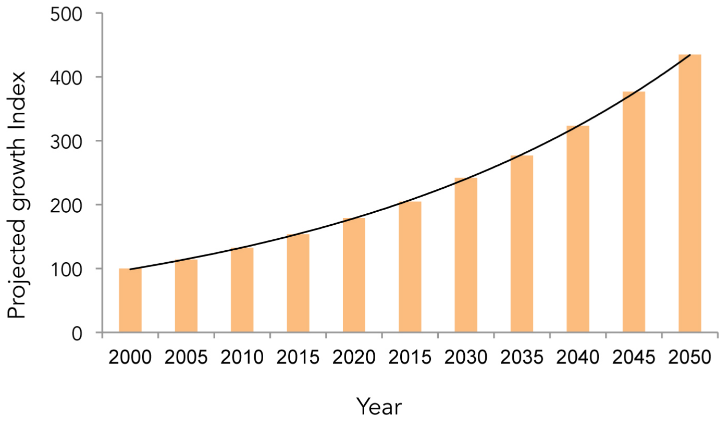


Figure 2:
Projected global chemical production, adapted from Wilson and Schwarzman (2009)
Global chemical production is projected to grow at a rate of 3% per year.

Out of all currently known chemical compounds (about 5 million) 100,000 are in use, out of which 1,000 compounds account for the main share of the yearly production of 400 million tons. In total, 10,000 to 30,000 chemical compounds are of environmental concern (Hartung and Rovida 2009). However, each year, 500 - 1,000 new chemicals are registered (Fent 2013). Thus, researching the effects of pollution and developing policies for chemical industries needs a high strategic effort. The European Union regulation REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) has been established to coordinate research of the effects of chemicals and develop policies for chemical industries. Moreover, priority chemicals have been listed by national and international authorities, like the United States Environmental Protection Agency (EPA) and the OSPAR Commission (The Convention for the Protection of the Marine Environment of the North-East Atlantic), in order to focus on the pollutants of highest environmental concern (OSPAR 2010, EPA 2014).

In conclusion, organisms are currently exposed to a rapidly changing environment involving thermal and chemical changes. The plasticity to adapt to a wide range of environmental conditions will determine a species' success under global change (Somero 2010, Tepolt and

Somero 2014). However, species populations with a wide tolerance range, but which may already live at the edge of that range, are also at risk (Somero 2012). Conversely, tolerant invaders have been shown to conquer new habitats and invasion success was correlated with the degree of stress tolerance (Stachowicz et al. 2002, Grigorovich et al. 2008).

1.2 Lake Baikal – a natural laboratory

Lake Baikal is the most ancient lake on earth with an estimated age of about 25 million years. It is the most voluminous (23,615 km^3) and the world's deepest freshwater body (1,642 m) (Figure 3), with special abiotic and biotic conditions, which have remained stable for the last 2 - 4 million years (Kozhova and Izmest'eva 1998).

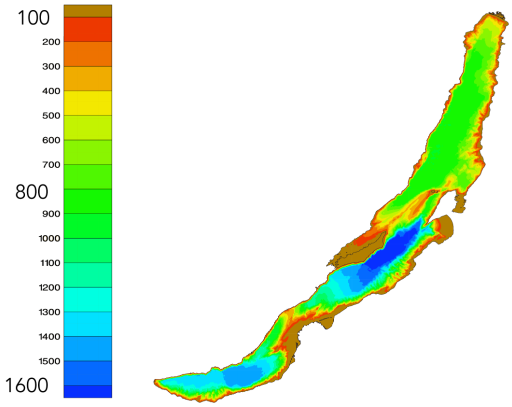


Figure 3:
Bathymetric map of Lake Baikal; from De Batist et al. (2006).

Abiotic peculiarities are (1) high oxygen saturation (12 mg L^{-1}) throughout the entire water column (Martin et al. 1993), (2) stable low temperatures (2.2 - 6.5°C at 100 m depth, Kozhov (1963)) and long seasonal ice-coverage for 4 - 5 months as well as (3) super-oligotrophic conditions. Extremely low electrolyte concentrations of $Na^+ = 155$, $Ca^{2+} = 402$, $Mg^{2+} = 126$, $Cl^- = 12$ and $SO_4^{2-} = 57$ µmol L^{-1} are independent of sampling depth (Zerbst-Boroffka et al. 2000).

The Baikal ecosystem consists of a unique and diverse species inventory which has evolved during its long and independent development, including the only true freshwater pinniped on earth, *Phoca sibirica*. The zooplankton community is dominated by the copepod *Epischura baicalensis* constituting around 90% of total biomass (Kozhov 1963). To date, 2,595 species are described, most of them are endemic (about 80%) (Kozhova and Izmest'eva 1998, Timoshkin 2001). The underlying mechanisms for the "immiscibility barrier" between the Baikal fauna and ubiquitous adjacent Palearctic freshwater faunas are yet unresolved as no insurmountable physical boundaries exist. Potentially, Baikal endemics outcompete invaders as the former are highly adapted to the special conditions prevalent in the lake.

1.2.1 AMPHIPODS OF LAKE BAIKAL

Amphipods of Lake Baikal are virtually 100% endemic and are predominant members of the benthic communities in terms of species numbers and biomass (Rusinek et al. 2012). The spectacular diversity of these endemic amphipods is an exemplary case of adaptive radiation similar to the evolution of Darwin's finches on the Galapagos Islands, cichlid fish species in Lake Tanganyika and notothenoid fish in the Southern Ocean. The amphipod fauna of Lake Baikal is likely polyphyletic, originating from two independent invasions to the lake (Macdonald et al. 2005). Amphipods inhabit all vertical zones and all types of substrates from the shallowest coastal waters down to the greatest depths, with the phylogenetically youngest species being found at the bottom of the lake (Mats et al. 2011). Up to today, 276 species and 78 subspecies of amphipods have been described (Takhteev et al. 2015). The variety of body sizes, colors and habitus is remarkable. Amphipods of Lake Baikal comprise various nutritional niches such as suspension feeders (*Micruropus wahlii*, *Micruropus klukii* and *Micruropus talitroides* (Dybowksi 1874)), algae feeders (*Brachyuropus grewingkii* (Dybowksi 1874)), herbivores (*Gmelinoides fasciatus* (Stebbing 1899), *Pallasea cancelloides* (Gerstfeldt 1858), (*Pallasea cancellus* (Pallas 1776)), detritivores (*Macropereiopus spp.* (Sowinsky 1915)), predators (*Pallasea grubei* and *Micruropus branickii* (Dybowski 1874)), sponge parasites (*Brandtia parasitica* (Dybowski 1874)) and scavengers (*Ommatogammarus albinus* and *Ommatogammarus flavus* (Dybowski 1874)). Due to the large number of closely related amphipod species inhabiting a variety of habitats with different temperature regimes, Lake Baikal is an ideal ecosystem to study the physiological mechanisms underlying thermal adaptation and limitation.

Eulimnogammarus verrucosus (Gerstfeldt 1858) and *Eulimnogammarus cyaneus* (Dybowksi 1874) are omnivorous amphipods of the bentholittoral. *E. verrucosus* is mainly found at depths of 0 - 25 m and includes a higher fraction of plant material in its diet than *E. cyaneus*, which is commonly found at depths of 0 - 1.5 m. *E. verrucosus* shows seasonal and diurnal migration, which has been suggested to correlate with thermal changes (Weinberg and Kamaltynov 1998). Due to its migration behavior, (adult) individuals of this species do not experience the short but intense thermal fluctuation, which has been monitored in the upper littoral of Lake Baikal during summer (Timoshkin 2009). In the beginning of June, temperatures of 5 - 6°C may prevail and may reach up to 20°C in August (Timoshkin 2009). *E. verrucosus* reproduces in winter at temperatures from close to the freezing point to 6°C and was classified as a stenothermal species (Gavrilov 1949). It is active throughout the year and was observed to feed on under-ice algae in winter (M. A. Timofeyev, personal communication). By contrast, about 90% of the *E. cyaneus* population stays in the upper littoral throughout the year and inhabits the thermal habitat with the highest seasonal and diurnal temperature fluctuations (Votintsev 1961). *E. cyaneus* is a more eurythermal species and was even found close to thermal vents in Lake Baikal (D. S. Bedulina, personal communication). It reproduces during summer. In line with their thermal habitats, thermal tolerance was higher in *E. cyaneus* than in *E. verrucosus* in short-term exposures, which is in accordance with their thermal preference ranges (Timofeyev et al. 2001, Timofeyev and Shatilina 2007). Moreover, the constitutive level of Hsp70 was found to be higher in the former species (Bedulina et al. 2013, Protopopova et al. 2014). To date, limits and performances under environmentally relevant scenarios have not been studied. However, this would be essential to estimate the effects of climate change on Baikal amphipods.

Gammarus lacustris, a member of the ubiquitous Holarctic fauna (Takhteev et al. 2015), can be found in some isolated bays of Lake Baikal and has never been reported to co-occur with the typical Baikal littoral amphipod community (Kozhova and Izmest'eva 1998). The isolated bays inhabited by this species are named 'sors' and are characterized by relatively high temperatures in summer, high content of organic matter and comparatively low levels of dissolved oxygen. *G. lacustris* shows a more versatile lifestyle than *E. verrucosus* and *E. cyaneus* and may be part of the pelagic or benthic community depending on habitat conditions. In habitats like Lake-14 it is part of the benthic community, hibernates when cold temperatures prevail and reproduces in summer. In fish-free lakes it can also be pelagic (Zadereev et al. 2010). In short-term experiments *G. lacustris* has been shown to prefer higher temperatures than *E. verrucosus* and *E. cyaneus* (Timofeyev et al. 2001). Whether climate change will promote a widespread invasion of this non-endemic amphipod is as yet unclear.

1.2.2 EFFECTS OF GLOBAL CHANGE ON THE BAIKAL ECOSYSTEM

Continental regions like southeast Siberia are severely affected by climate change due to the absence of oceanic water bodies that could potentially buffer thermal changes (Figure 1). Even though Lake Baikal provides a significant buffering capacity, the annual mean surface water temperature of Lake Baikal has increased by 1.21°C since 1946 (Figure 4). Summer temperatures even exceeded the annual mean increase (Hampton et al. 2008). Furthermore, the ice-free season expanded by 18 days between 1869 and 2000 and between 1949 and 2000 a 12 cm decrease of ice-thickness was recorded in the southern basin (Shimaraev et al. 2002). The summer and fall surface water temperatures of Lake Baikal may increase by more than 4.5°C until 2100 (Moore et al. 2009). In parallel, industrialization, agriculture, tourism and population size of the Baikal region have increased substantially during the last decades, adding potential pollution pressure to the catchment. Lake Baikal lies in the air- and watershed of the industrial corridor of Irkutsk, home to chemical plants and aging industries, and further sources of pollution include the Baikalsk Pulp and Paper Mill and industrial sites in the area of Severobaikalsk. In addition, thawing of permafrost releases stored chemicals and causes ground subsidence, which increases the risk for industrial accidents and the Trans-Siberian Railroad transporting oil (Moore et al. 2009).

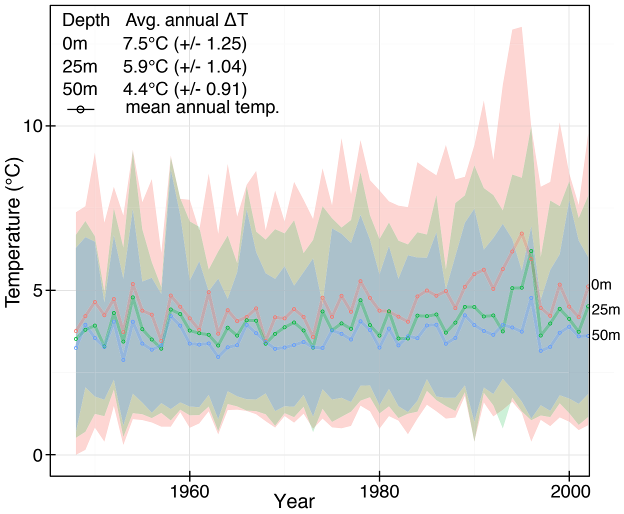


Figure 4: Long-term monitoring of temperature fluctuations in Lake Baikal at different depths; figure adapted from Hampton et al. (2008).

The Angara River is the only outflow of Lake Baikal, while the Selenga River is the major tributary (accounting for about 50% of water inflow) among 400 large and small sources of inflows. The Selenga River flows in from northern Mongolia; it carries the municipal waste of three large Mongolian cities lacking purification facilities. Pollutants that enter the Lake through these sources could build up for centuries to come, as water prevails for 377 - 400 years in Lake Baikal (Gronskaya and Litova 1991). Currently, observed biological effects of global change include a 300% increase in measured chlorophyll-a since 1979, which is correlated with reduced water clarity (Hampton et al. 2008). Algal size distribution shifted from relatively large diatom cells to smaller ones and may affect trophic energy transfer. Moreover, a 335% increase in cladoceran abundance since 1946 has been monitored (Hampton et al. 2008) and mass occurrences of algae, such as *Spirogyra spp.*, have also been observed (Timoshkin et al. 2014). Vertical downward shifts of diatoms were monitored between 1955 and 2000, whereas the density-weighted average depth of most zooplankton groups including cladocerans, rotifers and immature copepods, showed shifts toward shallower positions (Hampton et al. 2014). These phenomena were suggested to be due to reduced mixing and warming surface waters. Changes in ice dynamics are projected to exert most dramatic effects because both primary producers (diatoms) and top predators (seals) of Lake Baikal require ice for reproductive success and population growth (Moore et al. 2009).

Due to the low mineralization of Baikal water, the bioavailability of toxicants is likely high (Wright and Frain 1981) and, moreover, low water temperatures will delay turnover processes of introduced pollutants. Climate change and environmental pollution may synergistically affect aquatic life in Lake Baikal. Ancient freshwater ecosystems, which remained stable for millions of years and are mainly inhabited by endemic species, might be especially vulnerable to a rapidly changing environment. Whether Baikal endemics are able to adapt to the changing conditions or if global change will diminish the competitive advantage of Baikal endemics over species of adjacent faunas is yet to be studied.

1.3 Thermal effects on aquatic ectotherms

The currently observed and projected consequences of global warming are majorly due to the pervasiveness of temperature as an abiotic factor on ectothermic organisms. Virtually any physiological process is influenced by temperature and the increase in temperature results in increased metabolic rates, biochemical rates and almost all other rate of biological activity. The relation between temperature and rates of biological activity has been described over a century ago and mathematically formalized (Arrhenius 1889).

Equation 1:

$$k = Ae^{-\frac{E_A}{RT}}$$

k = Boltzmann’s constant
A = frequency factor
e = exponential factor
E = activation energy
R = gas constant
T = absolute temperature

In animals, this relationship is valid only for the thermal range of normal activity, which is highly species-specific. For most organisms the respective tolerance is found at temperatures of 0 - 40°C (Schmidt-Nielsen 1997) and the tolerance range progressively decreases with organismic complexity. Simple organisms such as bacteria show the widest tolerance ranges (Daufresne et al. 2009, Storch et al. 2014). Numerous temperature tolerance models were built to explain the thermal tolerance ranges of animals and were subsequently revised (Shelford 1931, Fry and Hart 1948, Fry 1971, Elliott 1981). The most recent model, the conceptual model of oxygen- and capacity-limitation of thermal tolerance (OCLTT) (Figure 5) (Pörtner 2010), has successfully explained the temperature sensitivity of many aquatic ectotherms such as sipunculids, annelids, molluscs (bivalves and cephalopods), crustaceans and vertebrates (fishes) (Pörtner 2012).

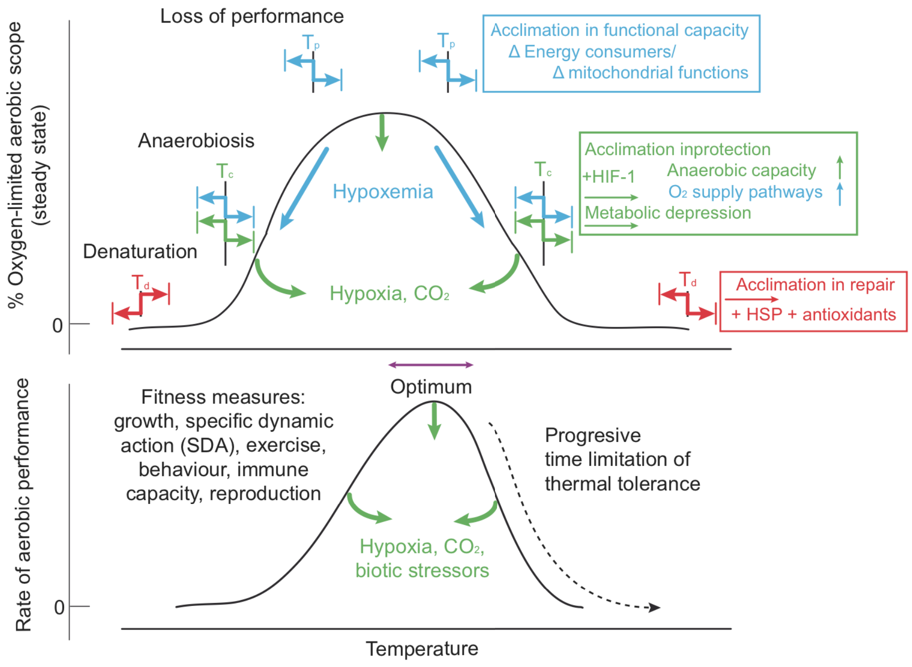


Figure 5: **Conceptual model of oxygen- and capacity-limited thermal tolerance, figure and caption adopted from Pörtner (2010)**

Conceptual model of oxygen- and capacity-limited thermal tolerance and the resulting thermal performance window of a species, of one of its lifestages or its population (modified from Pörtner (2002) and Pörtner and Knust (2007)). Optimized oxygen supply to tissues (reflected in high oxygenation of body fluids) between low and high pejus temperatures (T_p, top) combined with the kinetic stimulation of performance rates by warming supports temperature-dependent performance and a functional optimum (i.e. an optimum of aerobic scope) close to the upper pejus temperature (bottom). This reflects functional capacity in ecosystem-level processes such as competition, foraging, immune response, growth and behavior. The graph depicts acute performance levels and limitations in response to short-term temperature fluctuations. On longer time scales, changing temperatures elicit acclimatization as a limited shift of performance optima and limits on the temperature scale (horizontal arrows). Low and high limits of thermal acclimatization (vertical lines) delineate the thermal niche of a species or its life stage, seen in long-term processes like growth and development. Note that the passive tolerance range, characterized by progressive time limitation, is a relevant component of the niche in those cases when organisms are exposed to extreme temperatures beyond T_c, critical temperatures indicating onset of anaerobic metabolism, possibly even reaching T_d, denaturation temperatures indicating the onset of heat induced loss of structural integrity at the molecular level. As a prominent example, marine invertebrates of the intertidal zone experience combined exposure to temperature extremes, ambient hypoxia or even anoxia as well as transient CO_2 accumulation in body fluids due to loss of gas exchange. On long time scales, ambient hypoxia and elevated CO_2 levels both cause a narrowing of thermal windows and, possibly, lower performance optima through lower functional capacities and reduced systemic oxygen tension.

"Here, performance capacity largely relates to biochemical mechanisms setting aerobic capacity of cells and tissues in general and specifically to the capacity of ventilatory and circulatory organs to ensure sufficient oxygen supply for covering physiological costs above maintenance" (Pörtner 2010). In other words, thermal limitations are due to a thermally restricted functional capacity reflected by progressively decreasing systemic oxygen partial pressures that eventually lead to anaerobic metabolism. Thus, the range of possible oxidative metabolism from rest to maximal steady state exercise, i.e., the aerobic scope, shapes the thermal window of a species. Oxygen limitation occurs at both ends of the thermal window, which is described by three levels of restriction on each side. Pejus (latin for "turning worse") temperatures (T_p) limit the range of optimal performance, the range at which aerobic scope is highest. Surpassing the T_p involves progressively decreasing performance. It represents the ecological boundary of a species as decisive fitness parameters such as feeding performance, growth capacity or reproduction are reduced. Many species stay in their pejus range temporarily and in some cases may only experience optimal temperatures for short periods of time in the course of the seasons. The critical temperature (T_c) defines the onset of anaerobic metabolism. Consequently, anaerobic capacity of a species support short-term survival at temperature extremes and may be paralleled by metabolic depression due to severe hypoxemia. The denaturation temperature (T_d) marks the start of temperature-induced loss of the structural integrity of molecules.

Long-term exposure to certain thermal conditions can cause acclimation and adaptation by shifting the T_p, T_c and T_d, through molecular and biochemical transformations underlying functional, protection and repair capacity, respectively. Thus, the thermal window of a species may shift in response to long-term acclimation, acclimatization to seasonal fluctuations or adaptation to thermal changes along latitudinal gradients. The degree of phenotypic plasticity classifies stenothermal and eurythermal organisms. Stenothermal species specialize on a narrow temperature range whereas eurythermal species have comparatively wide thermal windows. Species that evolved over long time scales under stable thermal conditions may be evolutionary adapted to extremely narrow thermal ranges. For example, *Limopsis marionensis*, a bivalve inhabiting the Weddell Sea, which has an upper T_c of only 2°C (Pörtner et al. 1999).

Thermal limitation may occur at different hierarchical levels, from the molecular to organismic scale, contingent on the level of complexity. Thus, limitations are suggested to occur first at the whole animal level. It has been suggested that OCLTT is mechanistically linked with the capacity and design of molecules and membranes (Pörtner et al. 2007). For example, enzymes of stenothermal and eurythermal species may themselves be tolerant to a narrow or wide temperature range (Guderley and St-Pierre 2008). The three-dimensional structure of proteins may be altered in response to temperature, which affects ligand and substrate binding events

(Somero 1995). Moreover, temperature may affect pore-forming proteins, which may function as ion channels. Functioning of proteins and protein complexes (e.g., enzymes, respiratory pigments and ion channels) may be altered on the transcriptional level (increased/decreased level of transcription or selective transcription of different isoforms with specific thermal characteristics), post-transcriptional (polyadenylation, capping and RNA splicing) and post-translational modifications. The latter covers a large number of covalent modifications of proteins occurring during or after biosynthesis, such as methylation and phosphorylation. Moreover, the structure of membranes may be modified in response to thermal changes directed to maintain a certain fluidity, which is important for rotation and diffusion processes of proteins, thereby affecting their functionality (Somero 1995). Homeoviscous adaptation serves to maintain proper membrane fluidity in response to changed conditions by regulating the ratio of unsaturated to saturated fatty acids (Sinensky 1974).

In summary, a multitude of molecular alterations may constitute the basis for temperature-dependent changes of the physiological status of an organism. Limitations may be found at any hierarchical level but due to the diversity of many interacting factors, they are often initially evident at the highest degree of complexity, the whole animal (Pörtner 2002) like seen in aquatic animals such as mussels (Anestis et al. 2008, Goh and Lai 2014), fish (Pörtner et al. 2004) or amphipods (Publication I and II).

1.4 Abiotic and biotic parameters affecting toxicant susceptibility in aquatic life

A variety of different abiotic and biotic factors influence the ecotoxicological bioavailability of a pollutant. Abiotic factors include pH, ion strength, dissolved organic carbon (DOC), suspended matter, oxygen content and temperature (Fent 2013). Biotic factors cover life stage, age, organism size, sex, health and nutritional status. Factors influencing toxicological bioavailability involve uptake kinetics, detoxification mechanisms, intracellular immobilization and excretion. Induction of protein synthesis (e.g., metalloproteins, heat shock proteins, cytochrome P450 and catalase) may occur in response to exposure to xenobiotics (Fent 2013). Biological responses to toxicants may either be specific or non-specific. The universal stress response system that cells of animals are generally equipped with is activated in response to the damage of biological macromolecules independent of the stressor causing such damage (Kültz 2005). Components of the cellular stress response system mitigate or repair damage of macromolecules and mediate transformation or sequestration of stress causing cellular agents (Kültz 2005). Avoidance strategies can involve physiological and behavioral responses and are directed to reduce the exposure of targets to stress impacts and include, for instance, down-scaling of metabolic activity (metabolic depression, aestivation), leading to a reduced turnover of chemical stressors. However, the short-term reaction is often directed to compensate and is mostly paralleled by increasing metabolic rates (promotion of excretion and detoxification processes) (Calow 1991). The long-term response is likely directed to conserve energy stores, which may be accompanied by a down-regulation of metabolism and/or a decrease of performance parameters like locomotor activity, growth and fecundity.

1.4.1 CADMIUM: A MODEL TOXICANT

Cadmium has recently been listed among the "new top six toxic threats" (Pure Earth (Blacksmith Institute) 2015). Even though direct and indirect use of heavy metals such as cadmium has been regulated by international authorities, they are still of environmental concern as even low concentrations may exert long-term toxic effects on ecosystems and human health. Recently, cadmium has been used for producing nanoparticles for use in solar cells and color displays (EPA 2016). The EPA lately provided a list of priority chemicals (chemicals of highest environmental concern), including cadmium (EPA 2014) and the OSPAR Commission released a review statement for the OSPAR Background Document on cadmium (OSPAR 2010), which states that combustion in power plants and industrial processes make up the largest contribution to cadmium deposition for the marine environment of the North-east Atlantic (85%).

Cadmium has been chosen as a model contaminant in this thesis due to following properties: It is ubiquitous in the environment and emission rates are projected to increase in parallel with industrial combustion. Cadmium has been proven to be highly toxic for all kinds of biota (Currie et al. 1998, Mouneyrac et al. 1999, Larison et al. 2000). Furthermore, it is assimilated and accumulated in animals (Martin et al. 1976, Braeckman et al. 1999). Cadmium is non-metabolizable, and has no known biological function (Hopkin 1989). Only in a single study with diatoms was cadmium shown to have a biological function by ameliorating Zn deficiency (Lane and Morel 2000). Cadmium has no single mode of action, however, many effects are due to the fact that cadmium displaces essential ions (e.g. zinc) from their binding sites in metalloproteins, thereby destroying the biological properties of many enzymes (Jaishankar et al. 2014). Moreover, it substitutes with calcium ions in physiological processes where the ion radius is the determining factor (Simkiss and Taylor 1989). As cadmium also impairs antioxidant enzymes, it provokes ROS accumulation, thereby inducing oxidative stress. On the long run it provokes lipid peroxidation and DNA damage (Nemmiche et al. 2011).

The deleterious effects of toxic agents like cadmium are determined by the capacity of cellular stress defense mechanisms, which are directed to mitigate toxic effects, and by internal sequestration capacity. Only cadmium that is metabolically available exerts toxic effects (Vijver et al. 2004). The sensitivity of aquatic animals, even of closely related species, shows large variation due to differences in uptake kinetics and subcellular fractionation. Organisms take up waterborne cadmium through permeable body surfaces (e.g. gills) sharing uptake routes with calcium (Wright 1980, Wright and Frain 1981, Rainbow 1997). In amphipods, gill surface area increases with body size to a lesser degree than body volume (Moore and Taylor 1984). Consequently, small amphipods have relatively enlarged volume to surface ratios, which influences cadmium uptake and toxicity. Thus, body size is an important factor for cadmium toxicity.

Cadmium was shown to bind to different subcellular compartments (Wallace et al. 2003, Vijver et al. 2004, Buchwalter et al. 2008, Khan et al. 2010). In this subcellular partitioning model (SPM), toxicity was explained best by the fraction of cadmium that was associated with heat labile proteins (mainly enzymes) and cell organelles, which therefore were termed "metal sensitive fraction" (MSF) (Wallace et al. 2003). By contrast, metal that was bound to heat stable proteins such as metallothioneins (MTLPs) was not correlated with toxic effects and is thus referred to as the "biological detoxified fraction" (BDF) (Wallace et al. 2003). The expression of MTLPs and heat shock proteins is part of the universal stress response as these molecules perform chaperone functions (Georgopoulos and Welch 1993, Costello et al. 2004). In consequence, organisms that have a high capacity of the universal stress response system may be comparably tolerant to cadmium as well as to other abiotic stressors such as temperature.

Background concentrations of cadmium in Lake Baikal water sampled near Listvyanka (in about 14 km distance to the here investigated sampling site) were below detection limit whereas in Baikal amphipods 0.0723 – 0.369 µg g (dry weight)$^{-1}$ were determined (Ciesielski et al. 2016).

1.5 Objectives and approaches

Against the background of global warming and progressively increasing industrial pollution, this thesis aimed to characterize the thermal tolerance at systemic and biochemical levels in Lake Baikal endemic amphipods (*E. verrucosus* and *E. cyaneus*) in comparison with a ubiquitous Holarctic potential invader species (*G. lacustris*). Additionally, it intended to identify, which physiological factors, including those limiting thermal tolerance, determine cadmium susceptibility in the studied amphipods.

(i) The first objective was to determine the points at which the temperature-dependence of relevant physiological processes at the highest hierarchical level (whole animal) indicates the onset of limitation. Within the framework of OCLTT, this thesis aimed to identify thermal constraints of routine metabolism, ventilation rate, and extracellular ion regulation in *E. verrucosus*, *E. cyaneus* and *G. lacustris* and intended to integrate the findings with respect to field abundance and mortality.

Therefore, animals were exposed to gradual temperature increases (4 weeks, 0.8°C d^{-1}; 24 hours, 1°C h^{-1}) starting from the reported annual mean temperature of the Baikal littoral (6°C). A nonlinear regression approach was used to determine the points where the temperature response of the aforementioned physiological parameters becomes constrained.

(ii) Secondly, this thesis aimed to investigate the thermal plasticity of key metabolic enzymes. Specifically, the question was asked: Is the thermal plasticity of key metabolic enzymes correlated with organismic thermal tolerance and does metabolic fuel use change with temperature?

Upon exposure of *E. verrucosus*, *E. cyaneus* and *G. lacustris* to gradually increasing temperature (0.8°C d^{-1}), maximal activities of key metabolic enzymes were determined at the respective sampling temperature in freshly prepared crude extracts and their RNA transcripts were quantified to reveal possible changes of gene expression.

(iii) Two main factors influencing cadmium toxicity are (1) the ratio of permeable body surface to body volume and (2) universal stress response capacity. Thus, the third objective was to determine how closely related species that differ with respect to these parameters respond to cadmium exposure regarding uptake, internal metal distribution (cellular compartmentalization) and concentration-mortality relationships. Specifically, the following questions were asked: Do the species show different physiological responses to low biologically effective doses? Can biologically effective doses be related to the internal metal sensitive fraction of cadmium? Furthermore, does cadmium tolerance (on an effect-scaled basis) correlate with thermal tolerance?

Concentration-mortality relationships were determined for *E. verrucosus* and *E. cyaneus* over 4 weeks of exposure at 6°C. Subsequently, they were exposed to their species-specific LC1 (lethal concentration for 1% of the test groups). Subcellular cadmium compartmentalization, oxygen consumption and ventilation rates were monitored weekly in order to reveal progressive physiological changes.

(iv) The fourth objective was to integrate the findings with additional data mainly on the combined effects of temperature and cadmium as well as on cadmium toxicity in *G. lacustris* in comparison with the Baikal endemics species and published literature in order to draw an overall conclusion concerning possible effects of climate change and pollution on Baikal endemic amphipods.

2 Material and Methods

This chapter provides a comprehensive compilation of materials and methods used in this thesis including descriptions of methods applied to obtain data reported under “4 Additional results”.

2.1 Sampling sites

Figure 6 displays a map with the sampling sites. Sampling of endemic Baikal amphipods was conducted in the Southern basin of Lake Baikal at shores in the vicinity of the Bolshie Koty settlement (51° 9137″ N, 105° 0691″ E) at depths of 0 to 1.2 m.

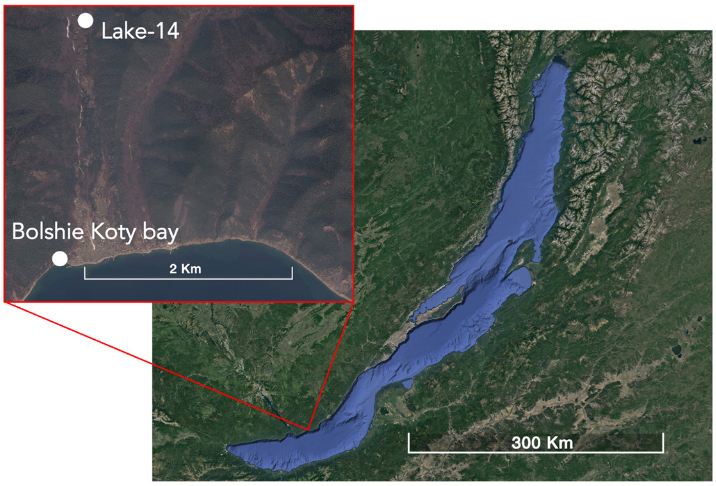


Figure 6:
Sampling sites at Lake Baikal. Baikal species (*Eulimnogammarus verrucosus* and *Eulimnogammarus cyaneus* were sampled at the shoreline of Lake Baikal, close to Bolshie Koty (51° 54′ 11.67″ N, 105° 4′ 7.61″ E). *Gammarus lacustris* was sampled in a small shallow artificial water body named Lake-14 (51° 55′ 14.39″ N, 105° 4′ 19.48″ E).

Referring to the classification of vertical zones of Lake Baikal the sampling site is part of the beach zone, where boulders, pebbles and gravel cover the ground (Kravtsova et al. 2004). Boulders that are partly immersed in sand are vegetated by algae (mainly *Ulothrix zonata*), whereas smaller pebbles and gravel are rarely overgrown as they are continuously in movement due to intensive hydrodynamic activity. Water temperatures of around 5 - 6°C were measured in June and October whereas summer temperatures may reach around 20°C in August (Timoshkin 2009).

G. lacustris were sampled in an artificial pond named Lake-14 (51° 55' 14.39" N, 105° 4' 19.48" E), a relic of gold mining activities in the 19th century. This small eutrophic water body is supplied by ground water, which makes it well-aerated. It is connected to Lake Baikal by a creek and inhabited by ubiquitous species of the Holarctic. *G. lacustris* is the only amphipod species found in Lake-14. The ground of the pond consists of small boulders and gravel, submerged in silty sediment and covered by detritus and macrophytes. Due to the small size of Lake-14 temperatures fluctuate more rapidly in summer and fall than in Lake Baikal. For example, temperatures (25th of August 2007) at 10 cm depth fluctuated from 13 - 17°C between 8 am and 5 pm in Lake Baikal and from 10 - 22°C in a small pond close to Lake Baikal (location not disclosed), respectively. These data were obtained during an international summer school (Bedulina et al. 2007).

Both sampling sites are part of the Pribaikalsky National Park, which was established in 1986, and are thus considered as pristine sites.

2.2 Experimental animals, sampling and maintenance

Experimental animals are depicted in Figure 7.

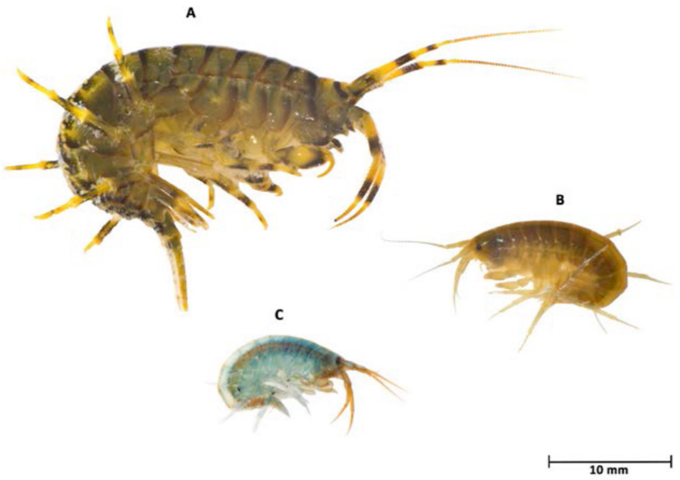


Figure 7:
Adult individuals of *Eulimnogammarus verrucosus* (Gerstfeldt, 1858) **(A)** *Eulimnogammarus cyaneus* (Dybowski, 1874) **(B)** and *Gammarus lacustris* (Sars,1863) **(C)**.

Adult animals were selected with a similar or larger size than those of previous studies in which body length was used to classify adult animals (*E. verrucosus*: 30 mm, *E. cyaneus*: 14 mm and *G. lacustris*: 14 mm) (Timofeyev et al. 2001). Here, animals with a fresh weight of 418 – 942, 19 – 49, and 26 – 145 mg were chosen for *E. verrucosus*, *E. cyaneus*, and *G. lacustris*, respectively. These weights corresponded to the size classes described above. *G. lacustris* shows the highest variability in body weight due to its sexual dimorphism with males being much larger than females (Yu and Chen 2013).

Animals were sampled with a hand net applying the kick sampling technique (Hynes 1961). As this method was developed for sampling in streams the hand net was moved through a figure eight close to the bottom of the lake. In June 5.3 - 7.0°C and 7.2 - 11°C and in September 8.5 - 15°C and 12.0 - 13.3°C were monitored during sampling at depths of 0 - 1.2 m in Lake Baikal and Lake-14, respectively. Similar concentrations of cations were measured in water from Lake Baikal and water from Lake-14 (Table 1).

Table 1: Concentrations of major cations in Lake Baikal and Lake-14

Water body	**Na^+** μmol L^{-1}	**K^+** μmol L^{-1}	**Mg^{2+}** μmol L^{-1}	**Ca^{2+}** μmol L^{-1}
Lake Baikal	220	40	140	450
Lake-14	210	10	240	300

Animals were transported in insulated water containers to maintain sampling temperatures under transportation. Experiments were either performed at the Institute of Biology of Irkutsk State University or at its field station in Bolshie Koty in the months of June - October in 2011 - 2014. Prior to experimentation, animals were sorted to exclude juveniles, injured animals and individuals with leeches that are common parasites of Baikal amphipods.

Animals were kept for at least three days at 6°C in continuously aerated 2 L water tanks (high density polypropylene (PP) of food grade quality or glass) for acclimation to laboratory conditions and to assure that all animals were intact. At least two-third of the tank bottoms (ca.15 x 20 cm) were covered with pebbles to provide shelter for the amphipods. Pebbles were collected at the shore of Lake Baikal and cleaned with boiling Baikal water. Strong illumination was prevented during acclimation and experimental exposures. The species were incubated separately, with one tank containing a maximum of 200 individuals of *E. cyaneus*, 75 of *G. lacustris*, or 25 of *E. verrucosus*, respectively. In 2011 and 2012 animals were fed with commercial food for crustaceans (Tetra, Melle, Germany) whereas a mix of amphipods, water plants, algae and detritus, which was collected in the littoral of Lake Baikal was applied in 2013 and 2014. Prior to application the mix was frozen, air-dried at around 30°C and coarsely shredded. Food was applied *ad libitum* and there was no visible difference in condition of animals fed with either food.

2.3 Experimental setups

Different exposure scenarios were used to investigate the effects of temperature increase and cadmium as well as their combined effects.

2.3.1 GRADUAL TEMPERATURE INCREASE

The experimental protocols were designed to mimic temperature fluctuations during a summer season as well as diurnal changes. In long-term (4 weeks) experiments, water tanks were stored in an incubator (Sanyo MIR 254 (238 L), Osaka, Japan) and the temperature was increased by 0.8°C d^{-1} starting from the temperature of acclimation (6°C). For short-term (24 h) exposures, tanks were placed in water baths, increasing temperature by 1°C h^{-1} by use of a thermostat (WiseCircu, Witeg GmbH, Wertheim Germany). Water was exchanged every third day up to a temperature of 12.4°C, followed by water exchange every other day (13.2 - 18.8°C) and daily water exchanges at the end of the experiment (19.6 - 30.0°C). The experiments were run until reaching 100% mortality. Control animals were kept at 6°C during the respective exposure, which reflects the annual mean temperature reported for the littoral area of Lake Baikal (Falkner et al. 1991, Yoshioka et al. 2002).

2.3.2 CADMIUM EXPOSURES

Two kinds of experiments were performed: (1) Concentration-mortality relationships were studied in order to derive species-specific sublethal concentrations. (2) Physiological performances (ventilation, oxygen consumption, hemolymph cation homeostasis) were studied in LC1 exposures (LC1 = lethal concentration for 1% of the test group). LC1 concentrations are regarded as sublethal concentrations because mortality in LC1 experiments did not deviate from control setups.

2.3.2.1 Concentration-mortality relationships

PP-tanks were filled with Baikal water and were cleaned by exchanging all water every other day. To prevent major losses of cadmium due to adsorption to tank walls, tanks were pre-soaked with water containing cadmium at the respective exposure concentrations for two days. Fifteen individuals of *E. cyaneus* or ten specimens of *E. verrucosus* were placed in each plastic tank with 0.4 and 1.2 L of well-aerated $CdCl_2$-containing Baikal water, respectively. In parallel to a water control without cadmium, treatments were set up with 8.7, 35, 105, 210, 419, 559, 1117 and 2235 nM cadmium (nominal concentrations; three tank replicates per concentration) following the OECD guideline for the testing of chemicals that requires at least five geometric concentrations (OECD 2012). The applied concentrations were based on previously conducted range-finding tests. Tanks were randomly distributed in a laboratory refrigerator (6 ± 1.0°C). Water was exchanged every fourth day during the 4-week exposures. The animals were fed *ad libitum* with a mix of amphipods, algae and water plants collected in the Baikal littoral (frozen, air-dried at ≈30°C and roughly ground). Mortality was monitored daily and dead animals were removed immediately.

2.3.2.2 LC1 exposures

Animals were exposed for four weeks at 6 ± 0.8°C to their species-specific LC1 (lethal concentrations for 1% of the test groups; LC1 for *E. verrucosus* = 115 nM, LC1 for *E. cyaneus* = 18 nM) as sublethal concentration. Water in the tanks was exchanged every fourth day. Oxygen consumption, ventilation and hemolymph pH were measured weekly. Hemolymph pH was only determined in *E. verrucosus*; small size of individuals precluded measurements in the other species. Hemolymph samples for analysis of major cations (Na^+, K^+, Mg^{2+} and Ca^{2+}) were taken at the same time points as whole animal samples for cadmium analysis (frozen in liquid nitrogen and stored at -80°C). In parallel, all parameters were also analyzed in controls.

2.3.3 TEMPERATURE AND CADMIUM

The combined effects of temperature increase and cadmium were investigated in different experimental setups.

2.3.3.1 LC10 exposures (24 h)

E. verrucosus and *E. cyaneus* were exposed to fast gradual warming as described above (1°C h^{-1}) under concomitant exposure to their species-specific LC10 obtained in 24 h experiments at 7°C (Protopopova et al. unpublished). Ventilation was monitored every 6 h.

2.3.3.2 LC1 exposures (24 h)

E. verrucosus and *E. cyaneus* were exposed to slow gradual warming as described above (0.8°C d^{-1}). Every second day, six animals of each species were incubated for 24 h at their respective LC1 in 400 mL plastic vessels containing aerated Cd-containing Baikal water. Control animals were treated the same way in parallel but were transferred to containers with clean Baikal water. Oxygen consumption was investigated throughout the warming treatment.

2.3.3.3 LC1 exposures (14 days)

In 14 day-exposures, *E. verrucosus* and *E. cyaneus* were exposed to both the LC1 of *E. verrucosus* and the LC1 of *E. cyaneus* at 6°C and to temperatures exceeding the preferred thermal range (*E. verrucosus*: 10 - 11°C; *E. cyaneus*: 18°C). These temperatures are close to the suggested pejus temperatures of the two species. Mortality was checked daily and dead animals were removed immediately. Oxygen consumption was investigated at the end of the two-week exposures. Additionally, hemolymph pH was measured (in *E. verrucosus*).

2.4 Physiological, biochemical and molecular parameters

2.4.1 MORTALITY

Mortality was determined in long-term exposures and is always expressed as cumulative mortality. In case of reaching 100% mortality by the end of the experiment, data were fit to sigmoidal models (Hill model or 5-parametric Weibull model) to derive lethal temperatures, lethal times (time until lethality) or lethal concentrations for certain percentages of the test groups ($LT_{emp}X\%$, $LT_{ime}X\%$ and LCX%, respectively).

2.4.2 ROUTINE METABOLIC RATE

Routine metabolic rate (RMR) was measured through oxygen consumption by optical sensors: Microx TX3 and OXY4, Oxygen Micro-Optode (NTH-PSt1-L5-TF-NS*35**x1,20-PC3,1-YOP) (Presens, Regensburg, Germany) in Teflon® flow-through chambers (cylindrical form) (Figure 8).

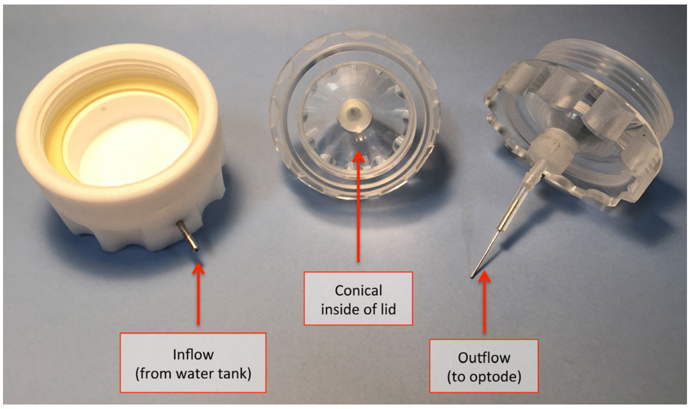


Figure 8:
Teflon flow-through chambers with plexiglass lids for oxygen consumption measurements. Chambers with individual volumes have been used for each species to account for their size differences; *E. verrucosus*: 15.5 mL; *E. cyaneus*: 3.0 mL and *G. lacustris*: 3.4 mL.

One hour prior to experimentation, the equipment was acclimated by placing the chambers into 2 L tanks containing oxygen-saturated Baikal water that was constantly aerated during experimentation. Tanks and tubing were put into a thermostatic bath to ensure temperature stability (± 0.2°C). Water was driven through the chambers by peristaltic pumps (Ismatec, Wertheim, Germany) connected to plasticizer-free tubing (Ismatec, MHSL 2001, Wertheim, Germany). Flow rates were adjusted according to species and temperature and recorded after each test series. Before transferring an animal to a chamber, the relative 0% oxygen saturation and 100% air saturation was calibrated. Over-saturated sodium sulfite (AppliChem, Darmstadt, Germany) solution was used to calibrate 0% oxygen. After cleaning the optode thoroughly with distilled water, the 100% air saturation was calibrated in the running flow-through system when the recorded value remained unchanged for about 20 minutes (Figure 9).

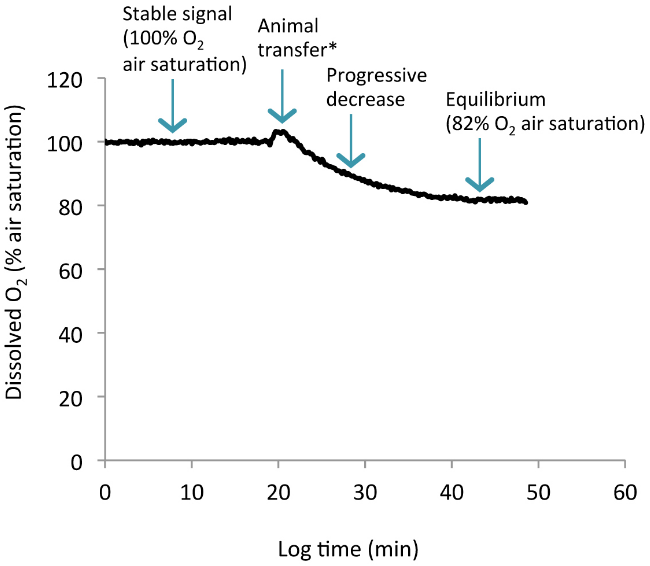


Figure 9:
Example of oxygen consumption measurement in the flow-through system.
*Opening the chamber might cause a peak in oxygen detection because the optode may contact air bubbles.

The optical sensor was connected with a 3-way T-piece in about 10 cm distance to the chamber. After calibration, the 100% value was logged for several minutes to visualize the stable baseline. Subsequently, an animal was carefully placed into the chamber that was relocked under water to exclude air bubbles. To prevent hypoxic conditions, flow rate was adjusted so that oxygen consumption was in steady state and the outflow drop in air saturation ranged between 6% and 23%. After about 20 - 40 minutes, depending on flow rate, oxygen inflow equaled oxygen consumption. When no further decrease of oxygen was recorded for at least 15 minutes the animal was removed from the chamber and was quickly and gently dried with lint-free paper tissue, weighed on a fine scale, and immediately placed back into the incubation system. Animals were allowed to recover from treatment stress for at least three days, after which time no differences between non-treated and previously treated animals were observed.

Between measurements, oxygen probes were stored in clean and aerated Baikal water to avoid initial signal drift in the following measurement. Oxygen consumption was calculated as follows:

Equation 2:

$$\mathbf{MO_2} = \frac{\mathbf{\Delta PO_2 \cdot \alpha O_2 \cdot VFR}}{\mathbf{FW}}$$

MO_2: Oxygen consumption (µmol O_2 h^{-1} g^{-1})
ΔPO_2: Difference of partial pressures of inflowing and outflowing water ((Barometric pressure (Torr) – Vapor pressure of H_2O (Torr)) 0.209)
αO_2: Oxygen capacity of water (µmol O_2 L^{-1} $Torr^{-1}$)
VFR: Volumetric flow rate (L h^{-1})
FW: Animal fresh weight (g)

Barometric pressure, vapor pressure and oxygen capacity of water are dependent on temperature and salinity, respectively, and values corrected according to Boutelier et al. (1984).

2.4.3 VENTILATION

Amphipods ventilate their gills by continuously moving their pleopods back and forth (Dahl 1977) (Figure 10). The gills consist of epipods on the coxa of peraeopods two to six and an expod on the basis of paraeopod seven (Steele and Steele 1991)

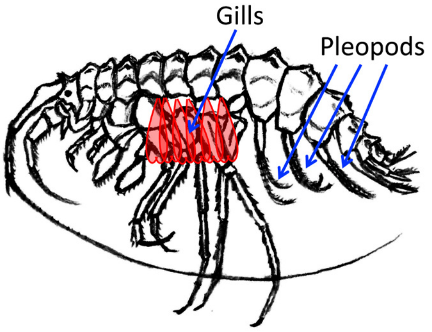


Figure 10:
Location of gills behind coxal plates in gammaridean amphipods. This artwork is a modified drawing of K. P. Vereshchagina (Irkutsk State University).

Ventilation (pleopod beat rate) was analyzed in periods of resting defined as the time spent in relaxed posture without locomotor or intense grooming activity.

Animals were transferred to Teflon® chambers with perforated depressions (Figure 11) to allow unhindered water circulation in temperature-controlled 2 L tanks containing aerated Baikal water.

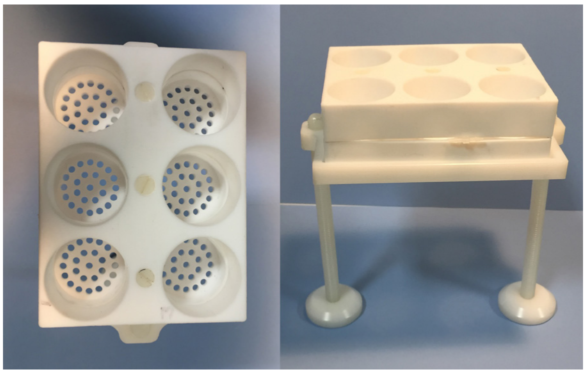


Figure 11:
Teflon chambers for the recording of ventilation rates. Chambers with a diameter of 3.5, 1.5 and 1 cm diameter were used for *E. verrucosus*, *G. lacustris* and *E. cyaneus*, respectively.

As the animals did not show any reaction to red light, a 625 nm LED light source was used for illumination during 3 min video recording sequences. Video-taped ventilation rates were analyzed manually in slow motion (0.5x or 0.25x). Further details of the methods are described in the material and methods chapters of Publication I and III.

2.4.4 HEMOLYMPH ION REGULATION

Extracellular concentrations of inorganic cations such as Na^+, K^+, Mg^{2+} and Ca^{2+} were determined by ion chromatography using a Dionex ICS 1500/2000 equipped with an Ion Pac CS16 column operated at 40°C. Methane sulfonic acid (30 mM) was used as an eluent at a flow rate of 0.36 mL min^{-1}. Hemolymph was extracted manually using ultra-fine capillaries, which were manufactured by treating Pasteur pipettes (230 mm; Brand, Wertheim Germany) with an electrode puller. Animals were dried with paper tissue and capillaries were dorsally introduced. Particular care was taken not to damage the guts. Samples (0.5 - 2.5 µL) were diluted with 50 µL of bidistilled H_2O and stored at -20°C prior to analysis. For peak identification the Dionex Six Cation II Standard (Dionex GmbH, Idstein, Germany) was used (Figure 12). Three-point calibrations of the cation standard were used for quantification; the dilution factors were adjusted according to the concentration of the respective ion in the diluted hemolymph samples. Calculations of the final concentrations were based on peak areas. The limit of determination was defined as 3x blank.

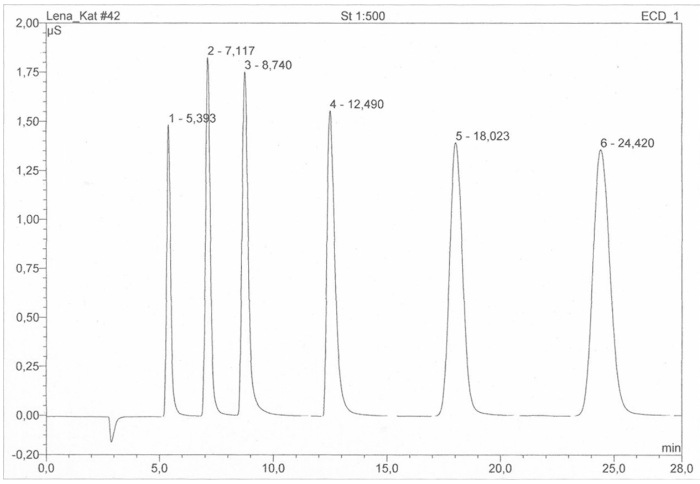


Figure 12: Chromatogram of ions contained in the Dionex Six Cation II Standard solution:
1. Lithium
2. Sodium
3. Ammonium
4. Potassium
5. Magnesium
6. Calcium

2.4.5 HEMOLYMPH PH

Extracellular pH was measured with a microelectrode (InLab® Ultra-Micro, Mettler Toledo, Giesen, Germany) in hemolymph immediately after extraction. Hemolymph was extracted as described under 2.4.4 and transferred to Eppendorf tubes. Measurements were performed at 6 ± 0.2°C in a water bath. Tubes and electrode tip were acclimated to 6°C prior to measurements.

2.4.6 ACTIVITIES OF KEY METABOLIC ENZYMES

Maximal enzyme activities of cytochrome-c-oxidase (COX), citrate synthase (CS), lactate dehydrogenase (LDH), pyruvate kinase (PK), 3-hydroxyacyl-CoA dehydrogenase (HADH), as well as glutamate dehydrogenase (GDH) were measured in crude extracts of *E. verrucosus*, *E. cyaneus* and *G. lacustris* after optimization of published enzyme assays. Procedures and assay parameters are compiled in Table 1 of Publication II. Table 2 summarizes the reaction equations and the references for the protocols adapted.

Table 2: Reaction equations for enzymes

Enzyme	Protocol after	Reaction mechanism
COX	Moyes et al. (1997)	4 Cytochrome-$c_{(red)}$ + 0_2 + $4H_+$ → 4 cytochrome-$c_{(ox)}$ + H_2O
CS	Sidell et al. (1987)	Acetyl-Co-A + Oxaloacetate + DNTB +H_2O → Citrate + DNTB-S-CoA
HADH	McClelland et al. (2005)	Acetyl-Co-A + NADH + H^+ → L-3-hydroxyl-Co-A + NAD^+
GDH	Sanchez-Muros et al. (1998)	α-Ketoglutarate + NH_4^+ + NAD(P)H + H^+ → Glutamate + NAD(P) +H_20
PK	Driedzic and De Almeida-Val (1996)	Phosphenolpyruvate + ADP + Mg^{2+} → Pyruvate + ATP + beta-NADH → Lactate + beta-NAD
LDH	Kornberg (1955)	Pyruvate + NADH +H^+ → Lactate + NAD^+

2.4.7 RNA TRANSCRIPT LEVELS

Total RNA was isolated from whole animals. Procedures and methods for homogenization of samples, isolation and purification of RNA, determination of RNA quantities and integrity, trancription into DNA, primer design and testing and calculation of changes in gene expression are reported in the material and methods chapter of Publication II.

2.5 Cadmium tissue compartmentalization and quantification

Cadmium was determined in different tissue fractions, i.e., heat stable proteins (HSP; fraction contains MTLPs), metal rich granules and undigested exoskeleton (MRG+exo), heat denaturable proteins (HDP), organelles and cell debris, which were obtained by a differential centrifugation procedure (Wallace et al. 2003). Here, the procedure described by Khan et al. (2010) was adapted, who fractionated tissue of *Gammarus pulex* (Amphipoda, Crustacea). For the first centrifugation step 1,800 x g instead of 800 x g because otherwise the supernatant (S1) was contaminated with small pieces of tissue (Figure 13).

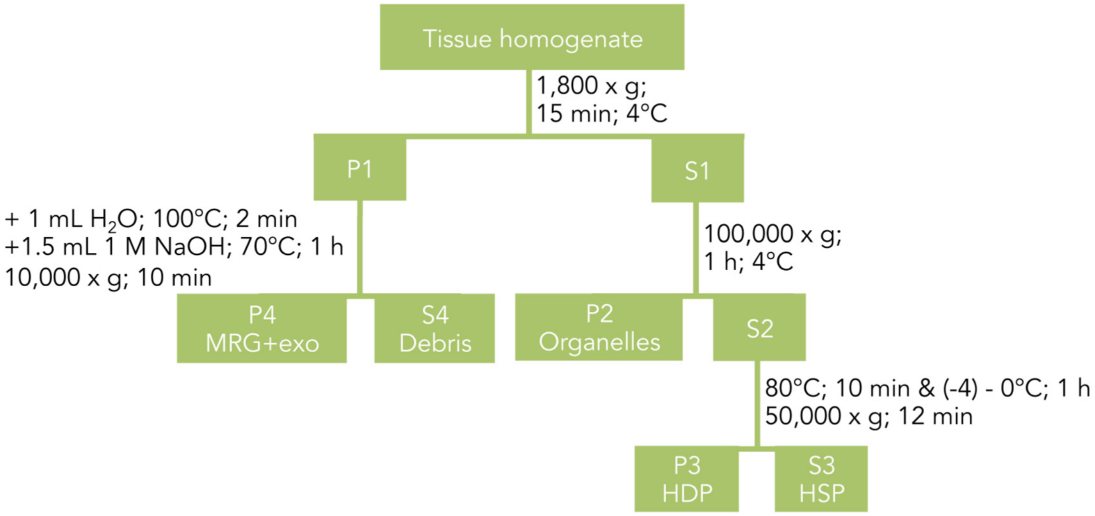


Figure 13: Subcellular compartmentalization adapted from Wallace et al. (2003) and Khan et al. (2010). "S" stands for supernatant and "P" for pellet. Subcellular compartments: MRG+exo = metal rich granules and undigested exoskeleton; Debris = cellular debris; Organelles = cell organelles like mitochondria, lysosomes and microsomes; HDP = heat denaturable proteins; HSP = heat stable proteins (metallothionein-like proteins).

Due to the small size of *E. cyaneus*, four animals were pooled for each sample whereas *E. verrucosus* samples consisted either of the anterior or the posterior half of an individual that were separated with a scalpel. The exoskeletons of amphipods mainly consist of chitin, which is a good adsorbent of heavy metals like cadmium. Chitin beads have successfully been used for the purification of metal-polluted water (Zhou et al. 2004). To remove weakly bound external cadmium, animals were washed with 10 mM EDTA solution with a physiological content of NaCl and KCl to prevent cellular water loss by osmosis and were then rinsed with deionized water prior to tissue fractionation. This cleaning procedure was repeated once. All further details of metal compartmentalization and quantification of cadmium in tissue and water samples are reported in the material and methods chapter of Publication III.

2.6 Data analysis and statistics

Standard statistical test are referred to in the material and methods chapters of the publications. For the derivation of breakpoint temperatures (BPTs), a nonlinear regression approach was used adapted from Marshall et al. (2013). This approach is in accordance with more general calls to fit functions to continuous data, thus proposing the use of nonlinear regression to fit various curvilinear functions to e.g. oxygen consumption data in order to estimate the partial pressure at which oxygen consumption begins to decrease (Figure 14). The authors compared the performance of their approach relative to the traditional broken stick regression approach for real world and simulated data and found that under realistic circumstances, nonlinear regression was more accurate and provided more powerful hypothesis tests.

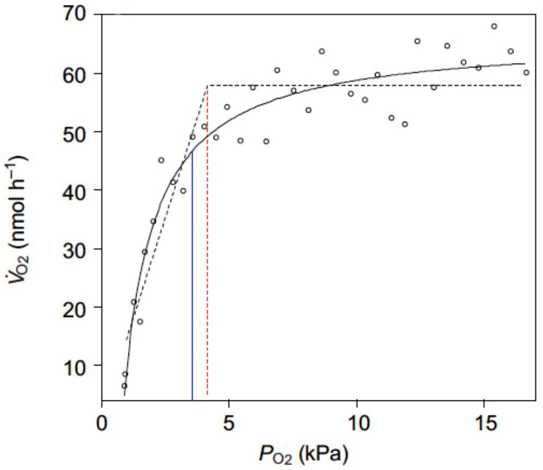


Figure 14:
Relationship between partial pressure of oxygen (PO2) and oxygen consumption (V_{O2}) for *Crinia georgiani* tadpoles. Black lines show predicted lines of best fit from nonlinear regression (NLR); dashed lines show lines of best fit from broken stick regression (BSR); red and blue lines show critical oxygen value (Pc) as estimated by BSR and NLR approaches, respectively. Figure and caption adopted from Marshall et. al (2013)

For data presented in this thesis, the "Dynamic Fit Wizard" in SigmaPlot 13 (Systat Software Inc, San Jose, California, USA) was used to find the best-fit model (those with lowest Akaike's information criterion) for the data sets of cumulative mortality, ventilation, and RMR. Candidate models were preselected by shape from a set of about 120 models provided by the Dynamic Fit Wizard. We aimed to find the upper BPT defined as the point where temperature stopped being the driver of ventilation or RMR, i.e., when the slope of the curve started to flatten out and approach zero. The upper BPT was defined according to Marshall et al. (2013), i.e., as the point where the model's slope equals 0.065. The 95% confidence intervals (CI) are given in parentheses for the modeled BPT values of RMR and ventilation. In case none of the selected parametric models fitted the data sets, which was most likely due to limited data resolution after the breakpoint, BPTs were estimated to correspond to the maximum values of the exponentially increasing rates that were followed by significant declines.

3 Results

List of publications and author contributions:

Publication I

Lena Jakob, Denis V. Axenov-Gribanov, Anton N. Gurkov, Michael Ginzburg, Daria S. Bedulina, Maxim A. Timofeyev, Till Luckenbach, Magnus Lucassen, Franz J. Sartoris and Hans-Otto Pörtner (2016).
Lake Baikal amphipods under climate change: thermal constraints and ecological consequences Ecosphere, 7, e01308.

The study was designed by myself with the help of HOP, FJS and ML. The experiments were performed by myself with technical help of DVAG, DSB, ANG and MG under the support of MAT. Data analyses and interpretation was done by myself and discussed with HOP and FJS. I wrote the manuscript, which was revised by HOP, FJS, ML, TL, DSB, MG, ANG and DVAG.

Publication II

Lena Jakob, Kseniya P. Vereshchagina, Anette Tillmann, Lorena Rivarola-Duarte, Denis V. Axenov-Gribanov, Anton N. Gurkov, Andreas Schüttler, Daria S. Bedulina, Maxim A. Timofeyev, Peter F. Stadler, Till Luckenbach, Hans-Otto Pörtner, Franz J. Sartoris and Magnus Lucassen (submitted to the Journal of Experimental Biology in September 2016).
Temperature-dependent capacities of key metabolic enzymes correlate with whole-animal responses in three boreal amphipod species.

The experimental setup was developed by myself with the help of ML. DVAG, DSB, AT, AS, KPV and myself performed the experiments and laboratory work with support of MAT. KPV and myself analyzed the data with technical help of DSB, LRD and ANG. I interpreted the data and discussed them with ML. The manuscript draft was prepared by myself and revised by ML, HOP, FJS, PFS, TL, DSB, ANG, DAVG, LRD and KPV.

Publication III

Lena Jakob, Daria S. Bedulina, Denis V. Axenov-Gribanov, Michael Ginzburg, Zhanna M. Shatilina, Yulia A. Lubyaga, Ekaterina V. Madyarova, Anton N. Gurkov, Maxim A. Timofeyev, Hans-Otto Pörtner, Franz J. Sartoris, Rolf Altenburger and Till Luckenbach (a previous version of this manuscript was submitted to Environmental Science & Technology in May 2016 and declined with encouragement to resubmit; the resubmission is planned for October 2016).
Uptake kinetics and subcellular compartmentalization explain lethal but not sublethal effects of cadmium in two closely related amphipod species

The concept of the study was elaborated by myself in cooperation with RA and TL. I performed the experiments with technical help of EVM, YAL, ZMS, DVAG, MG and ANG supported by MAT. Data analyses and interpretation was done by myself and discussed with TL, RA and DSB. I drafted the manuscript, which was revised by TL, RA, HOP, FJS, DSB, DVAG, ANG, MG and ZMS.

Publication I

Lake Baikal amphipods under climate change: thermal constraints and ecological consequences

L Jakob, DV Axenov-Gribanov, AN Gurkov, M Ginzburg, DS Bedulina, MA Timofeyev, T Luckenbach, M Lucassen, FJ Sartoris and H-O Pörtner

2016

Ecosphere, 7, e01308

submitted: 16th of June 2015

accepted: 6th of November 2015

published: 19th of March 2016

esa ECOSPHERE

Lake Baikal amphipods under climate change: thermal constraints and ecological consequences

Lena Jakob,[1,2,†] Denis V. Axenov-Gribanov,[3] Anton N. Gurkov,[3] Michael Ginzburg,[1]
Daria S. Bedulina,[3] Maxim A. Timofeyev,[3] Till Luckenbach,[4] Magnus Lucassen,[1]
Franz J. Sartoris,[1] and Hans-O. Pörtner[1,2]

[1]*Department of Integrative Ecophysiology, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570, Bremerhaven, Germany*
[2]*Department of Biology and Chemistry, University of Bremen, Leobener Straße, 28359, Bremen, Germany*
[3]*Institute of Biology, Irkutsk State University, Karl Marx str. 1, 664003, Irkutsk, Russia*
[4]*Department of Bioanalytical Ecotoxicology, UFZ – Helmholtz Centre for Environmental Research, Permoserstr. 15, 04318, Leipzig, Germany*

Citation: Jakob, L., D. V. Axenov-Gribanov, A. N. Gurkov, M. Ginzburg, D. S. Bedulina, M. A. Timofeyev, T. Luckenbach, M. Lucassen, F. J. Sartoris, and H.-O. Pörtner. 2016. Lake Baikal amphipods under climate change: thermal constraints and ecological consequences. Ecosphere 7(3):e01308. 10.1002/ecs2.1308

Abstract. Lake Baikal, the world's most voluminous freshwater lake, has experienced unprecedented warming during the last decades. A uniquely diverse amphipod fauna inhabits the littoral zone and can serve as a model system to identify the role of thermal tolerance under climate change. This study aimed to identify sublethal thermal constraints in two of the most abundant endemic Baikal amphipods, *Eulimnogammarus verrucosus* and *Eulimnogammarus cyaneus*, and *Gammarus lacustris*, a ubiquitous gammarid of the Holarctic. As the latter is only found in some shallow isolated bays of the lake, we further addressed the question whether rising temperatures could promote the widespread invasion of this non-endemic species into the littoral zone. Animals were exposed to gradual temperature increases (4 week, 0.8 °C/d; 24 h, 1 °C/h) starting from the reported annual mean temperature of the Baikal littoral (6 °C). Within the framework of oxygen- and capacity-limited thermal tolerance (OCLTT), we used a nonlinear regression approach to determine the points at which the changing temperature-dependence of relevant physiological processes indicates the onset of limitation. Limitations in ventilation representing the first limits of thermal tolerance (pejus (= "getting worse") temperatures (T_p)) were recorded at 10.6 (95% confidence interval; 9.5, 11.7), 19.1 (17.9, 20.2), and 21.1 (19.8, 22.4) °C in *E. verrucosus*, *E. cyaneus*, and *G. lacustris*, respectively. Field observations revealed that *E. verrucosus* retreated from the upper littoral to deeper and cooler waters once its T_p was surpassed, identifying T_p as the ecological thermal boundary. Constraints in oxygen consumption at higher than critical temperatures (T_c) led to an exponential increase in mortality in all species. Exposure to short-term warming resulted in higher threshold values, consistent with a time dependence of thermal tolerance. In conclusion, species-specific limits to oxygen supply capacity are likely key in the onset of constraining (beyond pejus) and then life-threatening (beyond critical) conditions. Ecological consequences of these limits are mediated through behavioral plasticity in *E. verrucosus*. However, similar upper thermal limits in *E. cyaneus* (endemic, Baikal) and *G. lacustris* (ubiquitous, Holarctic) indicate that the potential invader *G. lacustris* would not necessarily benefit from rising temperatures. Secondary effects of increasing temperatures remain to be investigated.

Key words: Amphipoda; breakpoint temperature; *Eulimnogammarus cyaneus*; *Eulimnogammarus verrucosus*; *Gammarus lacustris*; global warming; ion regulation; Lake Baikal; metabolic rate; thermal tolerance; ventilation.

Received 16 June 2015; revised 30 October 2015; accepted 6 November 2015. Corresponding Editor: J. Benstead.

† **E-mail:** Lena.Jakob@awi.de

Introduction

Lakes are sentinel ecosystems of climate change (Adrian et al. 2009). Lake Baikal, the world's largest freshwater body, has a volume of 23,615 km^3 (De Batist et al. 2006) and comprises 20% of global unfrozen surface freshwater resources. Since 1946 its mean surface water temperature increased by 1.21 °C (annual mean) and by 1.6 °C during summer (Hampton et al. 2008). Further, the ice-free season was prolonged by 18 d between 1869 and 2000 and the ice thickness in winter decreased by 12 cm in the southern basin between 1949 and 2000 (Shimaraev et al. 2002). By 2100 the surface water of Lake Baikal is projected to warm by more than 4.5 °C compared with present temperature (Moore et al. 2009).

Temperature is arguably the overarching abiotic factor for ectothermic organisms. Climate change will thus affect most aquatic ectotherms and challenge their physiological and ecological plasticity. This study focuses on the ecophysiological effects of rising temperature on amphipods, which comprise up to 90% of benthic biomass and species in Lake Baikal (Rusinek et al. 2012) and are key benthic decomposers in the complex nutrient cycling system of the lake. Lake Baikal is famous for its rich endemic amphipod fauna and is inhabited by more than 45% of the world's known gammaroidean species, all belonging to the suborder Gammaridea Latreille, 1802 (Takhteev et al. 2015). It is thus an ideal ecosystem to study ecosystem changes elicited through direct impacts of climate on the interaction of closely related species. Baikal endemics experienced stable abiotic conditions for millions of years with moderately fluctuating temperatures as contributing streams, currents, and winds are driving continuous mixing. Temperature fluctuates between 2.2 ° and 6.5 °C at 100 m depth (Kozhov 1963) and an annual mean temperature of 6 °C was measured in the littoral (Falkner et al. 1991, Weiss et al. 1991, Yoshioka et al. 2002). Hence, Baikal amphipods might be more vulnerable to a quickly changing environment than ubiquitous species like *Gammarus lacustris* Sars, 1863, a widespread amphipod of the Holarctic, including Siberia (Karaman and Pinkster 1977). The latter is a candidate species to widely invade Lake Baikal as it inhabits various shallow water bodies in proximity and even isolated bays of Lake Baikal characterized by a comparatively high content of benthic organic matter, high temperatures in summer and low wave impact. At present, it is not found in the typical Baikal littoral benthos (Kozhova and Izmest'eva 1998, Timoshkin 2001). It is unknown whether climate change will affect the distribution patterns of ubiquitous and endemic species; the biotic and abiotic factors constituting the "immiscibility barrier" separating the endemic Baikal and the ubiquitous Palearctic faunas remain to be identified (Mazepova 1990, Timoshkin 2001).

Here we compare *G. lacustris* with two Baikal amphipods, *Eulimnogammarus verrucosus* (Gerstfeldt, 1858) and *Eulimnogammarus cyaneus* (Dybowsky, 1874), which are two of the most abundant species of the littoral and thus highly important components of the unique benthic ecosystem. They inhabit rocky habitats at depths of 0–15 m and 0–1.5 m, respectively. As a sedentary species of the upper littoral, *E. cyaneus* is exposed to higher temperature fluctuations than *E. verrucosus*, which migrates to deeper waters in summer (Weinberg and Kamaltynov 1998). Previous short-term studies of preference temperatures (Timofeyev et al. 2001, Timofeyev and Kirichenko 2004, Timofeyev and Shatilina 2007), heat shock proteins (Shatilina et al. 2011, Bedulina et al. 2013, Protopopova et al. 2014), enzyme activities, and products of metabolism (Axenov-Gribanov et al. 2012) indicated that *E. verrucosus* is more sensitive to thermal stress than *E. cyaneus* and *G. lacustris*, but ecophysiological thermal limits and performances at the whole animal level have not been investigated.

Field observations of ecological consequences of climate change have been explained by the concept of oxygen- and capacity-limitation of thermal tolerance (OCLTT) (Pörtner 2010). Each species (and even life stage) has a limited thermal range of aerobic performance. Pejus (lat.) (= progressively deleterious) temperatures (T_p) characterize the first level of thermal limitations and the ecological thermal boundaries of a species. In the field, occurrence of temperatures beyond these thermal boundaries leads to decreasing abundance of a species (Pörtner and Knust 2007). Surpassing T_p implies decreased performance until reaching the critical temperature (T_c), which precludes long-term survival.

This study aimed to determine the points at which the changing temperature-dependence of relevant physiological processes indicates the onset of limitation in *E. verrucosus*, *E. cyaneus*, and *G. lacustris*. In light of the observations made, we discuss whether ecophysiological findings support an assessment of how increasing water temperatures will affect the three species and whether warming will be beneficial for *G. lacustris* in comparison with the Baikal endemics.

Material and methods

Sampling site

Eulimnogammarus verrucosus (550–885 mg) and *E. cyaneus* (19–49 mg) were caught with a hand net (kick sampling) in the Baikal littoral in the vicinity of Bolshie Koty (51°9137″ N, 105°0691″ E) at depths of 0–1.2 m. The sampling site represents the beach zone, according to underwater landscape zoning (Kravtsova et al. 2004) characterized by algae-covered boulders (mainly *Ulothrix zonata*), half-immersed in sand, and intensive hydrodynamics. *G. lacustris* (26–145 mg) was sampled in the same way in "Lake 14" (51° 55′ 14.39″ N, 105° 4′ 19.48″ E). This well-aerated eutrophic pond is supplied by groundwater and connected to a backwater of Bolshie Koty river. It is inhabited by fauna common in freshwaters across the Holarctic. The ground consists of boulders and gravel, submerged in a silty sediment and covered by detritus and macrophytes. See Protopopova et al. (2014) for a map with sampling locations. The investigated amphipod species are depicted in the Supporting Information in Fig. S1.

Experimental setup and animal maintenance

Experiments were performed between June and October (2011–2013) in the Institute of Biology at Irkutsk State University and its field station in Bolshie Koty. Adult animals were selected with a similar or larger size than those of previous studies in which body length was used to classify adult animals (*E. verrucosus*: 30 mm, *E. cyaneus*: 14 mm and *G. lacustris*: 14 mm) (Timofeyev et al. 2001). Immediately after sampling, amphipods were transported to the laboratory in insulated boxes and sorted under temperature-controlled conditions. Two hundred individuals of *E. cyaneus*, 75 of *G. lacustris*, or 25 of *E. verrucosus* were quickly placed into 2 L tanks (high-density polypropylene of food grade quality or glass) containing aerated 6 °C Baikal water and kept for at least 2 d with low illumination. About two-thirds of the tank bottoms were covered with pebbles from the littoral, precleaned with boiling Baikal water. Water was exchanged once every 1 to 3 d (increasing the exchange rate with rising temperatures). Commercial food for crustaceans (Tetra, Melle, Germany) was applied in 2011/2012 and animals were fed with a mix of amphipods, algae, water plants, and detritus collected in the Baikal littoral (frozen, air-dried at ≈ 30 °C and roughly mortared) in 2013. Food was supplied ad libitum and there was no visible difference in condition of animals fed with either food. Control animals were kept at a water temperature of 6 ± 0.8 °C, the reported annual mean temperature of the littoral. For *G. lacustris* an extra "Lake 14" control was set up containing water from "Lake 14" and animals were fed with alder, willow, and birch leaves collected in the pond. The cation composition (in μmol/L) was Na^+: 210, K^+: 10, Mg^{2+}: 240, Ca^{2+}: 300 in "Lake 14 and Na^+: 220, K^+: 40, Mg^{2+}: 140, Ca^{2+}: 450 in the Baikal littoral. In June 5.3–7.0 °C and 7.2–11 °C and in September 8.5–15 °C and 12.0–13.3 °C were monitored during sampling at depths of 0–1.2 m in Lake Baikal and "Lake 14," respectively. During long-term (4 week) experiments, water tanks were stored in an incubator (Sanyo MIR-254 (238 L), Osaka, Japan), and the water temperature was increased by 0.8 °C/d. In short-term (24 h) exposures, 2 L tanks were placed in water baths, increasing temperature by 1 °C/h by use of a thermostat (WiseCircu, Witeg GmbH, Wertheim, Germany). The experimental protocols were designed to mimic temperature fluctuations during a summer season and diurnal changes but went beyond natural habitat temperatures in order to clearly identify thermal limits. For each individual analysis, animals of a certain species were randomly taken out of 6–10 individual tanks.

Mortality

Dead animals were removed and counted daily to obtain the temperature-dependent cumulative mortality for each species excluding cannibalized individuals. The Weibull model (5-parametric; sigmoidal) was fitted to the data in order to calculate lethal temperatures (LT) for certain percentages of the test group (LTx%).

Routine metabolic rate

Routine metabolic rate (RMR), measured as oxygen consumption (MO_2) per gram of fresh weight (FW), was analyzed with optical sensors purchased from Presens, Regensburg, Germany: Microx TX3 and OXY4, Oxygen Micro-Optode (NTH-PSt1-L5-TF-NS*35**x1,20-PC3,1-YOP), with airtight connections to flow-through respiration chambers. Sensors were calibrated to 0% oxygen in oversaturated sodium sulfite (AppliChem, Darmstadt, Germany) and to 100% air saturation in the running system. After calibration, the 100% value was recorded for several minutes, and animals were carefully placed in airtight Teflon® chambers closed with plexiglass lids. Tanks and plasticizer-free tubing (MHSL 2001, Ismatec, Wertheim, Germany) were submerged in thermostatic baths to ensure temperature stability (±0.2 °C). Flow rate was adjusted so that oxygen consumption was in steady state and the outflow drop in air saturation ranged between 6% and 23%. When the values were stable for about 20 min, the animals were removed from the chambers, quickly and gently dried with lint-free paper towel, weighed on a fine scale, immediately placed back into the incubation system, and allowed to recover from manipulation for at least 3 d. No differences in RMR were found between nontreated and previously treated animals. The Q_{10} temperature coefficients were determined for each temperature increment to study irregular changes across temperatures.

Resting ventilation

Ventilation (pleopod beat rate) was analyzed in periods of resting defined as the time spent in relaxed posture without locomotor or intense grooming activity. Animals were transferred to Teflon® chambers with perforated depressions to allow unhindered water circulation in temperature-controlled 2 L tanks containing aerated Baikal water. As the animals did not show any reaction to red light, a 625 nm LED light source was used for illumination during 3 min video recording sequences. In the 4-week study in June/July 2013, *E. verrucosus* was video-recorded in the flow-through systems during simultaneous measurements of RMR. As pleopods of *E. cyaneus* and *G. lacustris* are small and transparent, simultaneous recordings of ventilation rate and RMR were impossible for these species. After recordings individuals were treated in the same way as after measuring RMR. Video-taped ventilation rates were analyzed manually in slow motion (0.5× or 0.25×).

If irregular changes were noted in RMR or ventilation, movies recorded for studying ventilation rates were examined by eye for any changes in movement activity by determining the ratio of time spent in motion in comparison to resting periods.

Ion regulation

Extracellular concentrations of inorganic cations (Na^+, K^+, Mg^{2+}, and Ca^{2+}) were determined chromatographically using a Dionex ICS 1500/2000 equipped with an Ion Pac CS16 column operated at 40 °C. Methane sulfonic acid (30 mM) was used as an eluent at a flow rate of 0.36 mL/min. Hemolymph was extracted manually using capillaries with ultrafine tips. Animals were dried with paper tissue and capillaries were introduced dorsally preventing damage to the guts. Samples (0.5–2.5 μL) were diluted with 50 μL of H_2O and stored at −20 °C prior to analysis. For peak identification and quantification (based on peak areas) three-point calibrations of the Dionex Six Cation II Standard were used. All products for ion analyses were obtained from Dionex GmbH, Idstein, Germany.

Tissue characteristics

To study percentages of water and inorganic content, frozen animals were thawed, weighed, dried for 24 h at 40 °C in a drying cabinet and reweighed. We checked whether all water had evaporated after another 3 h in the drying cabinet. Eventually, animals were incinerated in a muffle furnace at 560 °C and the ash-weights were determined.

Abundance

Abundance was measured in the field on a relative scale by calculating the personal catching success per unit time at different ambient temperatures (5.3 to 15.0 °C). Animals were sampled at ten sampling occasions in the period from the beginning of June to the beginning of September 2013. Only adult animals of the respective weight classes described in "Materials and methods: Sampling site" were taken into account. The amphipods were sampled at depths of 0–1.2 m.

Data analysis and statistics

Data were tested for normal distribution and homoscedasticity with the Ryan-Joiner and Levene's test, respectively, using Minitab 17 (Minitab Inc, State College, Pennsylvania, USA). They are presented as means ± standard error of the mean (SE) unless otherwise indicated. Standard statistical tests were applied to reveal significant differences ($P < 0.05$) and are mentioned in the results. If data showed no normal distribution, respective nonparametric tests were applied (e.g., ANOVA on ranks). The "Dynamic Fit Wizard" in SigmaPlot 13 (Systat Software Inc, San Jose, California, USA) was used to find the best-fit model (those with lowest Akaike's information criterion) for the data sets of cumulative mortality, ventilation, and RMR. Candidate models were preselected by shape. Weibull and lognormal models were fitted to all data sets of mortality, ventilation and to RMR data sets of *E. verrucosus* and *G. lacustris* (6–22 °C). We aimed to find the upper breakpoint temperature (BPT) defined as the point where temperature stopped being the driver of ventilation (= $BPT_{Vent.}$) or RMR (= $BPT_{Ox.}$), i.e., when the slope of the curve started to flatten out and approach zero. The upper BPT was defined according to Marshall et al. (2013), i.e., as the point where the model's slope equals 0.065 (nonlinear regression approach). The 95% confidence intervals (CI) are given in parentheses for the modeled BPT values of RMR and ventilation. In case none of the selected parametric models fitted the data sets, BPTs were estimated to correspond to the maximum values of the exponentially increasing rates that were followed by significant declines. These maximum values were similar to those derived by the nonlinear regression approach in this study (for comparison see Fig. 1). Only measurements at which survival was > 75% were taken into account to distinguish physiological phenomena that may appear right before death (e.g., a sudden increase in heart rate) from physiological responses to rising temperatures. However, we emphasize that these values are only rough estimates. All model equations are available in the Supporting Information in Table S1.

RESULTS

Mortality

LT50 values of 21.1, 28.4, and 28.3 °C were derived from Weibull models fitted to the data sets of *E. verrucosus*, *E. cyaneus*, and *G. lacustris* (Fig. 1a–c; 4-week study, June/July 2013). In comparison to June/July, the LT50 values determined in September/October were similar in *E. verrucosus* (21.5 °C) and slightly higher in *E. cyaneus* and *G. lacustris* (30.4 and 30.3 °C) (Table 1).

Routine metabolic rate

At control temperature (6 °C), oxygen consumption (μmol g $(FW)^{-1}$ h^{-1}) was lowest in *E. verrucosus* (3.3 ± 0.2) (Fig. 1a) and similar in *E. cyaneus* und *G. lacustris* (5.3 ± 0.4 and 5.7 ± 0.3, respectively) (Figs. 1b and c; 4-week study, June/July 2013). RMR increased up to 15.0 (14.0, 16.1) °C in *E. verrucosus* determined as $BPT_{Ox.}$. At 20.4 °C RMRs decreased with increasing weights of *E. verrucosus* specimens (Pearson correlation, $P = 0.0029$) and beyond 20.4 °C only relatively small animals were still alive (ANOM, $P < 0.05$). *E. cyaneus* and *G. lacustris* increased RMRs exponentially up to 25.2 ° and 23.6 °C, respectively. As parametric models (e.g., Weibull or lognormal) failed to fit these data sets, $BPT_{Ox.}$-values for *E. cyaneus* and *G. lacustris* could only be estimated and were considered to approximately correspond to the measured maximum values of the exponential increases, which were followed by significant declines in respiration rate (Figs. 1b and c).

In all species, the highest thermal sensitivity of RMR, deduced from the largest Q_{10}-values, occurred close to the upper $BPT_{Vent.}$. This leap in RMR was most pronounced in *G. lacustris* between 22.0 ° and 23.6 °C coinciding with a significant increase in movement activity from 52 ± 20% to 93 ± 13% (*t* test, $P = 0.002$). For the other species no such sudden elevations of movement activity were statistically detectable. Passing the $BPT_{Ox.}$

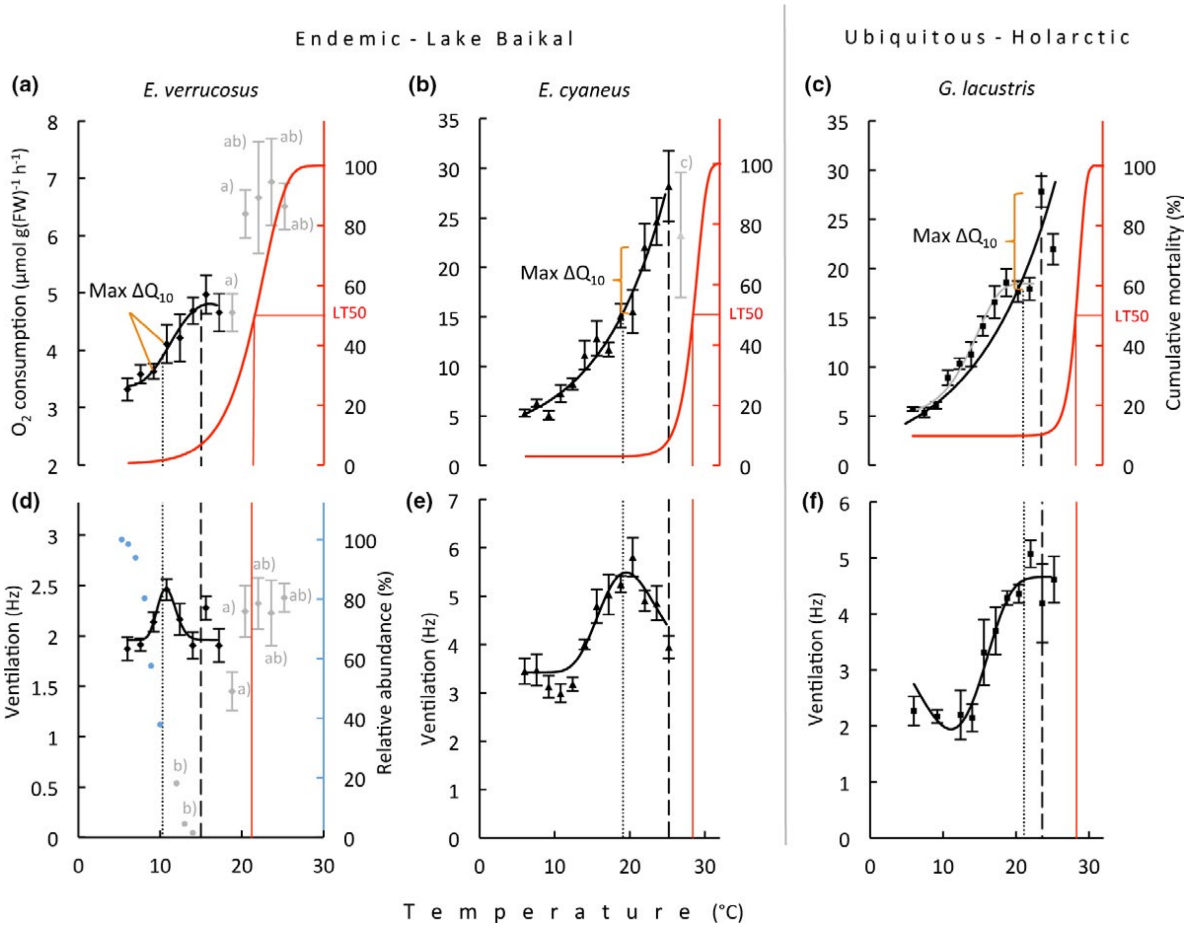


Fig. 1. Routine oxygen consumption (black symbols, a–c), cumulative mortality (red lines, a–c) and resting ventilation (black symbols, d–f) of *E. verrucosus* (left panels), *E. cyaneus* (central panels), and *G. lacustris* (right panels) exposed to gradual temperature increase (0.8 °C/d, 6–30 °C) (experimental period: June/July 2013). Model fits are represented by solid lines and model equations are summarized in the Supporting Information in Table S1. Field abundance (0–1.2 m depth) at different ambient temperatures is only depicted for *E. verrucosus* (blue dots, d). On each panel, vertical lines indicate the upper BPT$_{\text{Vent.}}$ (black dotted lines) and the upper BPT$_{\text{Ox.}}$ (black dashed lines) and the LT50 values (red solid lines), respectively. Gray symbols mark data subsets in which a) > 25% of the investigated population had died, b) negative deviations from the overall mean animal weight were stated (ANOM, $P < 0.05$) or c) only three replicates could be analyzed and were excluded from further analyses. Data are displayed as means ± SE (n = 5–18).

coincided with the onset of exponentially increasing mortality in all species.

Resting ventilation

The results of the 4-week study (June/July 2013) are depicted in Fig. 1d–f. A BPT$_{\text{Vent.}}$ of 10.6 (9.5, 11.7) °C was determined for *E. verrucosus* (Fig. 1d). Ventilation in *E. cyaneus* (Fig. 1e) and *G. lacustris* (Fig. 1f) was insensitive to temperatures up to 12.4 ° and 14.0 °C, respectively. Beyond those temperatures it increased exponentially, reaching an upper BPT$_{\text{Vent.}}$ of 19.1 (17.9, 20.2) °C in *E. cyaneus* and 21.1 (19.8, 22.4) °C in *G. lacustris*. Individuals of *G. lacustris* incubated in "Lake 14"-water showed a BPT$_{\text{Vent.}}$ of 21.8 (19.2, 24.4) °C (data not depicted).

Table 1 compiles BPT$_{\text{Vent.}}$ values from experiments differing with regard to duration and season. The BPT$_{\text{Vent.}}$ values of the two Baikal species from a 24-h study slightly exceeded those determined in 4-week investigations from the same

Table 1. LT50 and $BPT_{Vent.}$ (95% CI) of *E. verrucosus*, *E. cyaneus*, and *G. lacustris*. Values are displayed for long-term (4 week; 0.8 °C/d) studies conducted in different seasons (start of experiment in June or September) and for long- and short-term (24 h; 1 °C/h) studies performed in the same season (September), (n.a. = not analyzed).

	Endemic – Lake Baikal						Ubiquitous – Holarctic		
Parameter	*E. verrucosus*			*E. cyaneus*			*G. lacustris*		
Season	June	September	September	June	September	September	June	September	September
Duration	4 week	4 week	24 h	4 week	4 week	24 h	4 week	4 week	24 h
LT50 (°C)	21.1	21.5	29–29.5†	28.4	30.4	n.a.	28.3	30.3	n.a.
BPT_{Vent} (95% CI) (°C)	10.6 (9.5, 11.7)	9.6 (8.7, 10.5)	(11.4)‡	19.1 (17.9, 20.2)	20.8 (20.0, 21.6)	21.3 (20.5, 22.1)	21.1 (19.8, 22.4)	21.8 (20.5, 23.1)	≥ 27

† Axenov-Gribanov et al. (2012).

‡ Value could be derived from the model but with low confidentiality.

experimental season (September/October). They were determined as 11.4 °C (24 h) and 9.6 (8.7, 10.5) °C (4 week) in *E. verrucosus* and 21.3 (20.5, 22.1) °C (24 h) and 20.8 (20.0, 21.6) °C (4 week) in *E. cyaneus*. We were unable to determine the $BPT_{Vent.}$ for short-term-incubated *G. lacustris* as they tended to increase ventilation rates until reaching lethal temperatures ($BPT_{Vent.} \geq 27$ °C). In the 4-week study performed in September/October, *G. lacustris* showed a $BPT_{Vent.}$ of 21.8 (20.5, 23.1) °C.

Ion regulation

Extracellular ion concentrations are displayed in Fig. 2 (4-week study, June/July 2013). Potassium concentrations tended to be higher at 25.2 °C in all species and were positively correlated with temperature (6–25.2 °C) in *E. cyaneus* and *G. lacustris* (Pearson correlation, $P < 0.001$) (Fig. 2h–i). Sodium and calcium concentrations showed no clear trends in the investigated species. In the hemolymph of *E. verrucosus* and *G. lacustris* [Mg^{2+}] was reduced by 16–31% and 28–45%, respectively, at temperatures above 14.0 °C and negatively correlated with temperature (Pearson correlation, $P < 0.05$). *E. cyaneus* showed large interindividual variation and no clear temperature-dependent trend in [Mg^{2+}] (Fig. 2b). Only *G. lacustris* showed progressively decreasing [Mg^{2+}] over time at 6 °C (control). Likewise, in temperature treatment and control incubations with water from "Lake 14" [Mg^{2+}] also decreased in *G. lacustris* (Pearson correlation, $P = 0.033$) suggesting no effect from water from different sources on extracellular ion concentrations. No differences were seen between the effects of short- and long-term gradual temperature increases on ion levels except for [K^+] that in contrast to long-term experiments were not correlated with temperature (6–24 °C) in short-term studies. Data for controls (6 °C), 24-h studies and data recorded in different seasons in *E. verrucosus* are available in Supporting Information (Tables S2 & S3; Fig. S2). They are consistent with the patterns of regulation described here.

Tissue characteristics

The percentage of ash-weight based on the animal's wet weight was not significantly different between *E. verrucosus* (7.8 ± 0.4%) and *G. lacustris* (6.7 ± 0.3%), but higher in *E. cyaneus* (9.4 ± 0.3%) (ANOVA; $P < 0.001$; Holm-Sidak (post hoc test)); *E. verrucosus* vs. *E. cyaneus*, $P = 0.015$ and *E. cyaneus* vs. *G. lacustris*, $P < 0.001$). The water content of *G. lacustris* tissue was higher than found for the Baikal amphipods (ANOVA; $P < 0.001$; Holm-Sidak (post hoc test)); *E. cyaneus* vs. *G. lacustris*, $P < 0.001$ and *E. verrucosus* vs. *G. lacustris*, $P = 0.014$) (Table 2).

Abundance

Temperature and field abundance of *E. verrucosus* (550–885 mg) were negatively correlated (Spearman rank correlation, $P < 0.001$; Fig. 1d). The highest relative sampling success was recorded in June at water temperatures of 5–6 °C. At 14 °C, only a few small adults and juveniles of *E. verrucosus* were found. At temperatures ≥ 12 °C individuals showed lower weights than the overall mean (ANOM, $P < 0.05$). For

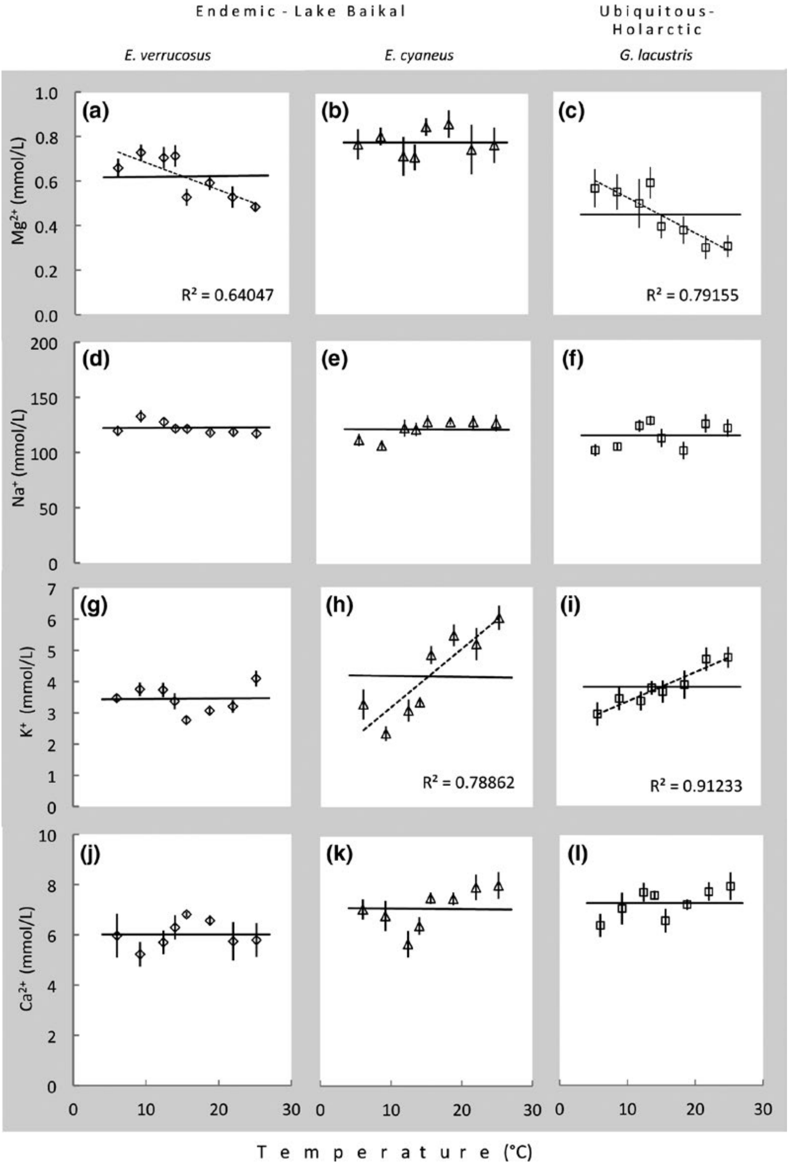


Fig. 2. Concentrations of Mg^{2+}, Na^{+}, K^{+}, Ca^{2+} in hemolymph of *E. verrucosus* (right panels), *E. cyaneus* (central panels), and *G. lacustris* (left panels) exposed to gradual temperature increase (0.8 °C/d) (experimental period: June/July 2013). Horizontal black lines (a–l) represent mean ion concentrations and dashed lines are linear fits (h and i). Data are presented as means ± SE, (*n* = 5–14).

Table 2. Wet weight, water content and inorganic content of *E. verrucosus*, *E. cyaneus*, and *G. lacustris*. Statistically significant differences (ANOVA, $P < 0.05$) are indicated by different superscripted letters in each column. Values represent means ± SE, n = 5–6.

Species	Wet weight (mg)	Water content (%)	Inorganic content (percentage of wet weight)
E. verrucosus	$693.9^{A} \pm 94.4$	$73.6^{A} \pm 0.5$	$7.8^{A} \pm 0.4$
E. cyaneus	$41.8^{B} \pm 4.1$	$71.0^{A} \pm 1.2$	$9.4^{B} \pm 0.3$
G. lacustris	$65.6^{C} \pm 9.8$	$77.4^{B} \pm 0.8$	$6.7^{A} \pm 0.3$

E. cyaneus sampling success was independent of ambient temperatures (5.3–14 °C; $R^2 = 0.024$) and highly variable over time (data not displayed). Field abundance was not quantitatively evaluated for *G. lacustris* as the small pond only provided few sampling sites and some of them were not accessible due to the funnel-shape of this small waterbody. However, we observed that adult individuals were far less frequently found in regions close to the water's edge when water temperatures reached up to 22 °C in July and August.

Discussion

Our discussion mainly focuses on the results for long-term studies (4 week; 0.8 °C/d) conducted in June/July (Figs 1 and 2). These are considered to be most ecologically relevant as animals were sampled at water temperatures close to control values (6 °C) and the rate of temperature change in the experiments is considered to be similar to field conditions. Thus, at 1–1.2 m depth water temperatures of around 6 °C were measured in the beginning of July, whereas by the end of the month up to 20 °C can be reached. Inter-experimental comparisons regarding duration and season (Table 1) are discussed in a separate section. Experiments performed in September/October addressed the question whether the animals had adapted to higher temperature during summer whereas short-term studies (24 h, 1 °C/h) reflect the responses to quick, e.g., diurnal, changes in the thermal regime.

Mortality, routine metabolic rate, and resting ventilation

The limit of oxygen supply capacity is likely key in the onset of constraining (beyond $BPT_{Vent.}$) and then life-threatening (beyond $BPT_{Ox.}$) conditions. Hypoxemic conditions (beyond $BPT_{Ox.}$) correlate with the start of exponentially increasing mortality in all species. The observed leaps in RMR reflect the highest thermal responsiveness and indicate a metabolic switch. As they occurred close to the first limit of thermal limitation represented by the $BPT_{Vent.}$, they might reflect a startling or escape response supported by the corresponding loss of field abundance in *E. verrucosus* and significant increase in movement activity in *G. lacustris*. Metabolic rate "sets the pace of life" and changes are thus fundamental to any animal (Brown et al. 2004). Evidently, beyond the limitations in ventilatory capacity the situation was progressively turning deleterious for *E. verrucosus* as a slight increase in the mortality rate (lag phase) was visible. The upper limit of the thermal optimum of *E. verrucosus* found in this study matches previous findings of a threefold increase in heat shock protein levels (Hsp70) and a doubling of catalase activity at 11 °C in a 24-h study (1 °C/h) (Axenov-Gribanov et al. 2012) indicating that physiological and biochemical transition phases go hand in hand (Pörtner 2010).

In all species studied here, resting ventilation failed to continually follow the increasing oxygen consumption rate, indicating an extended pejus range, which in a study investigating spider crab *Maja squinado* was also paralleled by heart rate leveling off (Frederich and Pörtner 2000). Ventilation comprises a significant fraction of metabolic costs (Glass and Wood 2009) and can thus reach capacity limits before the aerobic metabolic capacity becomes exploited. In addition, heart rate, oxygen affinity, and oxygen transport capacity of hemocyanin are likely to play a crucial role for maintaining aerobic metabolism at elevated temperatures or hypoxic conditions (Booth et al. 1982, Wilkes and McMahon 1982, Giomi and Pörtner 2013). Factors involved in modulating hemocyanin oxygen transport include changes in pH and lactate levels (independent of pH), in [Mg^{2+}] as well as in hormonal determinants (Mangum 1997, McMahon 2001). In the optimum range, hemocyanin might remain oxygen-saturated and might not be involved in tissue oxygen supply. Diffusion processes at low oxygen demand might play a large role for oxygen supply in highly oxygenated Baikal water, contributing to energy savings and low costs of oxygen supply in the cold. At low temperatures, RMR was even independent of ventilation in the similar-sized *E. cyaneus* and *G. lacustris* but not in the much larger *E. verrucosus*.

Eulimnogammarus verrucosus is considerably more thermally sensitive than *E. cyaneus* and *G. lacustris*, which may partly be due to its large body size, consistent with the allometry of thermal tolerance seen, e.g., in fishes (Pörtner et al. 2008). Warming exploits the capacity of the system to increase metabolic rate, reaching levels similar to those after maximum exercise before it collapses. Aerobic metabolic scope depends on three parameters, namely oxygen uptake, distribution and cellular exploitation. Oxygen uptake is a function of gill surface, which was shown to scale to body mass by an allometric coefficient of 0.792–0.843 in different species of the genus *Gammarus* (Moore and Taylor 1984). Thus, small animals have a relatively enlarged gill surface area, which is beneficial if oxygen is limited, likely contributing to higher thermal tolerance.

It has been stated as a unifying principle that climate warming benefits small organisms (Daufresne et al. 2009, Storch et al. 2014). Consistent with these studies and field observations in fish (Pörtner and Knust 2007), mortality occurred earliest in the largest individuals of *E. verrucosus* during the experimental temperature increase. Furthermore, at 20.4 °C RMR decreased with increasing animal weight. This indicates that smaller animals have a higher physiological capability to deal with elevated temperatures. However, other aspects also need to be considered when assessing the overall consequences of elevated temperatures for the species such as behavioral adaptations as discussed below. As smaller individuals have a smaller number of offspring the absence of large individuals will result in a declining population. Since food web structures and dynamics are mainly determined by body size (Woodward et al. 2005, 2010a,b, Barnes 2008, Yvon-Durocher et al. 2010), ecosystems would be severely affected by any temperature-mediated changes in organism sizes.

Ion regulation

The decrease in [Mg^{2+}] seen in *E. verrucosus* and *G. lacustris* at > 14 °C but not in *E. cyaneus* might be linked to increased activity as low extracellular [Mg^{2+}] in crustaceans go together with increased heart rate and support muscular activity. This might trigger behavioral changes, which in turn might influence field abundance. Moreover, a decrease in [Mg^{2+}] can counteract a temperature-dependent decrease in oxygen affinity of hemocyanin and support enhanced oxygen demand.

Magnesium is a modulator for numerous enzyme systems (Morritt and Spicer 1993) and important for regulatory processes concerning metabolism and growth. Decreasing intracellular [Mg^{2+}] correlate with lowered protein synthesis rates (Terasaki and Rubin 1985). However, as our findings are not uniform across the species, we suggest that the adjustment and regulation of Mg^{2+} is species-specific and possibly reflects its involvement in the control of hemocyanin or locomotor activity level more than other aspects.

The facultative hibernator *G. lacustris* might depend on elevated temperatures in summer to increase activity levels, forage and fill up energy stores. Animals caught at 6–7 °C in June were mostly found inactive in leaf litter suggesting that these individuals were still in their hibernation retreats. When kept at 6 °C after removing them from wintering substrates, extracellular [Mg^{2+}] progressively decreased. Moreover, individuals of *G. lacustris* that were caught in June showed a higher mortality than those sampled in September when they were kept at 6 °C for 4 weeks.

The positive correlation of extracellular [K^+] and temperature in *E. cyaneus* and *G. lacustris*, which are much smaller than *E. verrucosus*, is possibly due to the fact that small specimens were prone to membrane damage when extracting hemolymph with a glass capillary as the effect was absent in *E. verrucosus*. We suggest that the stability of membranes decreased during long-term warming as this effect was only visible in the 4-week studies. There is, however, no close relationship between sublethal thermal limits and [K^+].

In summary, ion regulation is a rather insensitive parameter when investigating sublethal thermal constraints in Baikal amphipods. This might be an indication of their excellent ion regulation capacities. Baikal amphipods need to efficiently take up essential ions as Baikal water is of extremely low ionic content (Na^+ = 155; Ca^{2+} = 402; Mg^{2+} = 126; Cl^- = 12; SO_4^{2-} = 57 μmol/kg; Zerbst-Boroffka et al. (2000)).

Abundance

Decreasing abundance of adult *E. verrucosus* at the field sampling sites paralleled rising water temperatures and can be explained through a decline in physiological performance and associated aerobic scope. Consistent with the allometry of thermal tolerance the largest animals reacted most sensitively. Similar to the field situation, *E. verrucosus* preferred temperatures of 5–6 °C in a previous behavioral laboratory study, which showed progressive decreases in abundance with rising temperatures in the experimental temperature gradient (Timofeyev and Shatilina 2007). It has previously been hypothesized that the migration patterns of *E. verrucosus* are temperature-dependent (Weinberg and Kamaltynov 1998). The present data suggest that migration behavior is initiated when temperatures exceed pejus limits. Considering that ecological niches are densely occupied in the Baikal littoral by 60–70% of the highly diverse macroinvertebrate fauna (Takhteev et al. 2003) the seasonal and diurnal migration behavior of *E. verrucosus* indicates a high ecological competitiveness of this species. This dominance of *E. verrucosus* is possibly due to the large size of adult specimens and their low metabolic rate; the latter may become advantageous as food availability decreases further with increasing depth in the oligotrophic lake. *E. verrucosus* is clearly dependent on the ability to escape to deeper zones, as in the upper littoral its T_c of 15.0 (14.0, 16.1) °C can easily be surpassed in summer.

Eulimnogammarus cyaneus is found in the upper littoral throughout the year and experiences temperatures from close to the freezing point up to 20 °C. It rarely faces its preferred temperature of 11–12 °C determined in a behavioral study (Timofeyev and Kirichenko 2004). A broad thermal tolerance range has been suggested as the most important trait for a species successful under climate change (Tepolt and Somero 2014), yet eurythermal species living at the edge of their thermal window are likely to be affected by global warming (Somero 2010). *E. cyaneus* was probably able to occupy its current niche due to its wide thermal window but summer temperatures in the littoral can reach the pejus range (> 19.1 °C). If temperatures exceed 19.1 °C in summer for extended periods of time, *E. cyaneus* may suffer as it, due to its small size and lower competitiveness, might not be able to conquer deeper habitats as niches are densely occupied. At any site this will depend on the community responses given that ecological interactions in the Baikal benthos are profoundly complex. In the bay of Bolshie Koty, 93 species of amphipods were found at depths of 0–20 m (Kravtsova et al. 2003). While *E. verrucosus* is less thermally tolerant than *E. cyaneus,* its temperature-related migration indicates behavioral plasticity.

The abundance of *G. lacustris* was difficult to quantify but it was noticeable that only few small adult individuals and large numbers of juveniles were found close to the water's edge in August and September when up to 22 °C were reached in "Lake 14". These findings suggest that *G. lacustris* also escaped rising surface temperatures in summer to stay below T_p.

Inter-experimental comparison—exposure duration and season

Long-term experimental warming led to falling thermal thresholds, which was most striking when comparing the LT50 values of *E. verrucosus*. These findings are likely due to the time dependence of lethal limits (Peck et al. 2009). Exploiting the passive range of tolerance during longer-term protocols possibly results in lower LTs. Considering ventilation, the divergence of limits between long- and short-term warming was highest in *G. lacustris*. Hence, we conclude that it is the most tolerant to diurnal temperature increases among the studied species, consistent with its shallow water habitat characterized by restricted hydrodynamics.

In comparison to June/July, the $BPT_{Vent.}$ of *E. cyaneus* and *G. lacustris* were marginally higher in September/October possibly due to acclimatization to higher temperatures during summer. In support of this, their LT50 values also exceeded those determined in June/July, which is likely mirrored in slightly right-shifted T_c following thermal acclimation. In contrast, the $BPT_{Vent.}$ of *E. verrucosus* was slightly lower in late summer and the LT50 remained unchanged probably because this species escapes rising temperatures and might thus not acclimatize.

$[Mg^{2+}]$ decreased similarly in 24-h and 4-week experiments, indicating its early decrease and then stabilization. $[K^+]$ was not positively correlated with temperature in *E. cyaneus* and *G. lacustris* in 24-h, but in 4-week studies. Maintaining a high stability might be energetically costly and effects thus only discernible in the long-term studies.

Gammarus lacustris—a potential invader to Lake Baikal?

To date *G. lacustris* is outcompeted by Baikal endemics in most Baikal habitats; low food availability, lack of hibernation retreats, and/

or intensive hydrodynamics might limit its distribution. Further, we observed that at low temperature (6 °C) Baikal amphipods were active whereas *G. lacustris* individuals were more rigid (sampling time: June). Animals sampled in September were more active than those caught in June indicating seasonal differences in physiological status causing different reactions to cold temperature. However, the observations suggest that the *Eulimnogammarus* species are more cold-tolerant than *G. lacustris* and should benefit from this advantage during cold seasons. Our results also show that the upper thermal limits of *G. lacustris* and *E. cyaneus* are similar and from the perspective of tolerance to high temperature *G. lacustris* should not be more competitive at elevated temperatures, but the competitive advantage from cold adaptation of Baikal endemics may be diminished.

Low metabolic rates likely improve a specie's fitness in the oligotrophic Lake Baikal. Even though mass-specific metabolic rates generally decrease with increasing body size, *E. cyaneus* (endemic, Baikal) has a lower mass-specific metabolic rate than the larger *G. lacustris*, (ubiquitous, Holarctic), which could be due to higher activity of the latter. Assuming that energy efficiency and savings are optimized in food-limited Baikal amphipods, it is likely that *G. lacustris* would be affected by low food availability. However, increasing temperatures will possibly lead to higher amounts of benthic organic matter, which could benefit *G. lacustris*.

Considering the intensive hydrodynamics in the Baikal littoral, we hypothesize that resistance to mechanical stress is another key parameter that prevents *G. lacustris* from invading the Baikal littoral. *G. lacustris* is generally found in lakes and slow-moving rivers (Matafonov 2007). A preliminary experiment revealed that in comparison to *E. verrucosus* and *E. cyaneus*, *G. lacustris* was less resistant to mechanical stress when gradually increasing pressure was applied to their exoskeleton by use of a glass capillary (diameter: 100 μm). Exoskeletons of *G. lacustris* were highly elastic and compressed completely in 45% of the tested animals. In contrast, Baikal species resisted equal pressure with little deformation. In addition, the inorganic content of whole animals was lowest in *G. lacustris*. This might indicate a lower resistance to mechanical disturbance than in Baikal endemics. It remains to be shown whether the exoskeleton of *G. lacustris* can evolve to resist the enormous physical disturbances in the Baikal littoral. Furthermore, a soft exoskeleton increases predation risk as we observed predation pressure to be highest on freshly molted amphipods. The abundance of *G. lacustris* correlates negatively with that of fish but positively with the occurrence of submerged aquatic vegetation (Anteau et al. 2011). Bullhead fish prey on benthic amphipods in Lake Baikal and submerged vegetation is absent around Bolshie Koty. This may also prevent the settlement of *G. lacustris* along the rocky shores of Lake Baikal but this situation may change due to ongoing coastal eutrophication (Silow 2014, Timoshkin et al. 2014).

CONCLUSIONS

Our data show the complex interaction of physiological constraints and habitat characteristics in shaping ecological patterns. In all investigated amphipod species, oxygen supply constraints became limiting at high temperatures. Comparing the two endemic species inhabiting the littoral of Lake Baikal, *E. verrucosus* (T_p = 10.6 (9.5, 11.7) °C) is significantly less thermally tolerant than *E. cyaneus* (T_p = 19.1 (17.9, 20.2) °C). By determining the upper (pejus) threshold temperature of physiological performance we could explain the warming induced retreat and loss of abundance of *E. verrucosus* in its natural habitat.

At first sight *E. verrucosus* may be more adversely affected by climate change than *E. cyaneus*, but as the former is able to escape from unfavorable conditions it might be the latter more sedentary species which is more vulnerable to increasing water temperatures despite its higher thermal tolerance. Temperatures of 20 °C can be reached in the upper littoral of Bolshie Koty bay, slightly exceeding the T_p of *E. cyaneus*. Following recent climate models for the Baikal region, this temperature is likely to be reached or even surpassed more often in future summer seasons and could negatively impact the fitness of *E. cyaneus*. If it is unable to escape from the rising temperatures to colder, deeper waters this species might be affected, e.g., suffer from reduced body size and, consequently, lower numbers of offspring.

Gammarus lacustris (T_p = 21.1 (19.8, 22.4) °C) showed similarities in thermal tolerance to *E. cyaneus* and displayed no distinct characteristics of thermal physiology that would benefit *G. lacustris* at higher temperatures. A combination of various other ecophysiological traits might prevent *G. lacustris* from becoming established in Lake Baikal. The importance of the variables food limitation, degree of cold adaptation, and sturdiness of exoskeletons to biomechanical stress remain to be investigated in future studies as well as further secondary factors of global warming and eutrophication.

Acknowledgments

We thank the students of Irkutsk State University who assisted field and laboratory work. Lena Perkuhn, Lukas Ehlers, Jan Arriens, and Alexander Massing are acknowledged for technical assistance. Moreover, we thank Christian Hamm and Gisela Lannig for scientific advice. This work was performed within the LabEglo project HRJRG-221, financed by the bilateral funding program "Helmholtz-Russia Joint Research Groups" (HRJRG) from the Helmholtz Association and the Russian Foundation for Basic Research (RFBR). Russian participants received further funding by the RFBR (grants: 15-29-01003; 14-04-00501; 15-04-06685), the Civilian Research and Development Foundation (CRDF) (grant: FSCX-15-61168-0) and were partially supported by the Russian Science Foundation (grant: 14-14-00400).

Data accessibility

Data presented here are available at: http://dx.doi.org/10.1594/PANGAEA.845942

Literature Cited

Adrian, R., et al. 2009. Lakes as sentinels of climate change. Limnology and Oceanography 54:2283–2297.

Anteau, M. J., A. D. Afton, A. C. E. Anteau, and E. B. Moser. 2011. Fish and land use influence *Gammarus lacustris* and *Hyalella azteca* (Amphipoda) densities in large wetlands across the upper Midwest. Hydrobiologia 664:69–80.

Axenov-Gribanov, D. V., J. A. Lubyaga, N. S. Shakhtanova, A. N. Gurkov, D. S. Bedulina, Z. M. Shatilina, E. M. Kondratyeva, K. P. Vereshchagina, and M. A. Timofeyev. 2012. Determination of Lake Baikal endemic amphipod *Eulimnogammarus verrucosus* (Gerstf., 1858) thermal optima limits by changes in its metabolic markers. Journal of Stress Physiology & Biochemistry 8:289–301.

Barnes, C. 2008. Predator and prey body sizes in marine food webs. Ecology 89:881.

Bedulina, D. S., et al. 2013. Expression patterns and organization of the hsp70 genes correlate with thermotolerance in two congener endemic amphipod species (*Eulimnogammarus cyaneus* and *E. verrucosus*) from Lake Baikal. Molecular Ecology 22:1416–1430.

Booth, C. E., B. R. McMahon, and A. W. Pinder. 1982. Oxygen uptake and the potentiating effects of increased hemolymph lactate on oxygen transport during exercise in the blue crab, *Callinectes sapidus*. Journal of Comparative Physiology - B 148:111–121.

Brown, J. H., J. F. Gillooly, A. P. Allen, V. M. Savage, and G. B. West. 2004. Toward a metabolic theory of ecology. Ecology 85:1771–1789.

Daufresne, M., K. Lengfellner, and U. Sommer. 2009. Global warming benefits the small in aquatic ecosystems. Proceedings of the National Academy of Sciences of the United States of America 106:12788–12793.

De Batist, M., M. Canals, P. Sherstyankin, and S. Alekseev; INTAS Project 99-1669 Team. 2006. A new bathymetric map of Lake Baikal. GeoForschungsZentrum GFZ, Potsdam, Germany. http://dx.doi.org/10.1594/GFZ.SDDB.1100.

Falkner, K. K., C. I. Measures, S. E. Herbelin, J. M. Edmond, and R. F. Weiss. 1991. The major and minor element geochemistry of Lake Baikal. Limnology and Oceanography 36:413–423.

Frederich, M., and H. O. Pörtner. 2000. Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, *Maja squinado*. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 279:1531–1538.

Giomi, F., and H. O. Pörtner. 2013. A role for haemolymph oxygen capacity in heat tolerance of eurythermal crabs. Frontiers in Physiology 4:110.

Glass, M. L., and S. C. Wood. 2009. Cardio-respiratory control in vertebrates, Twelfth edition. Springer, Berlin Heidelberg, Germany.

Hampton, S. E., L. R. Izmest'eva, M. V. Moore, S. L. Katz, B. Dennis, and E. A. Silow. 2008. Sixty years of environmental change in the world's largest freshwater lake - Lake Baikal, Siberia. Global Change Biology 14:1947–1958.

Karaman, G. S., and S. Pinkster. 1977. Freshwater *Gammarus* species from Europe, North-Africa and adjacent regions of Asia (Crustacea-Amphipoda):

Part I. *Gammarus pulex*-group and related species. Bijdragen tot de Dierkunde 47:1–97.

Kozhov, M. 1963. Lake Baikal and its life. Dr. W. Junk Publishers, The Hague, The Netherlands.

Kozhova, O. M., and L. R. Izmest'eva. 1998. Lake Baikal - evolution and biodiversity. Backhuys Publishers, Leiden, The Netherlands.

Kravtsova, L. S., E. B. Karabanov, R. M. Kamaltynov, S. K. Krivonogov, and D. Y. Shcherbakov. 2003. Macrozoobenthos of subaqueous landscapes in shoal of southern Lake Baikal. 1. Local diversity of bottom population and peculiarities of its spatial distribution. Zoologicheskii Zhurnal 82:307–317.

Kravtsova, L. S., et al. 2004. Macrozoobenthic communities of underwater landscapes in the shallow-water zone of southern Lake Baikal. Hydrobiologia 522:193–205.

Mangum, C. P. 1997. Adaptation of the oxygen transport system to hypoxia in the blue crab *Callinectes sapidus*. American Zoologist 37:604–611.

Marshall, D. J., M. Bode, and C. R. White. 2013. Estimating physiological tolerances – a comparison of traditional approaches to nonlinear regression techniques. Journal of Experimental Biology 216:2176–2182.

Matafonov, D. V. 2007. Ecology of *Gammarus lacustris* Sars (Crustacea: Amphipoda) in Transbaikalian water bodies. Biology Bulletin 34:148–155.

Mazepova, G. F. 1990. Baikal ostracoda. Nauka, Novosibirsk, Russia.

McMahon, B. R. 2001. Respiratory and circulatory compensation to hypoxia in crustaceans. Respiration Physiology 128:349–364.

Moore, P., and A. Taylor. 1984. Gill area relationships in an ecological series of gammaridean amphipods (Crustacea). Journal of Experimental Marine Biology and Ecology 74:179–186.

Moore, M. V., S. E. Hampton, L. R. Izmest'eva, E. A. Silow, E. V. Peshkova, and B. K. Pavlov. 2009. Climate change and the world's "Sacred Sea"-Lake Baikal, Siberia. BioScience 59:405–417.

Morritt, D., and J. I. Spicer. 1993. Mini review: a brief reexamination of the function and regulation of extra-cellular magnesium and its relationship to activity in crustacean arthropods. Comparative Biochemistry and Physiology - Part A 106:19–23.

Peck, L. S., M. S. Clark, S. A. Morley, A. Massey, and H. Rossetti. 2009. Animal temperature limits and ecological relevance: effects of size, activity and rates of change. Functional Ecology 23:248–256.

Pörtner, H. O. 2010. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. Journal of Experimental Biology 213:881–893.

Pörtner, H. O., and R. Knust. 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science 315:95–97.

Pörtner, H. O., C. Bock, R. Knust, G. Lannig, M. Lucassen, F. C. Mark, and F. J. Sartoris. 2008. Cod and climate in a latitudinal cline: physiological analyses of climate effects in marine fishes. Climate Research 37:253–270.

Protopopova, M. V., V. V. Pavlichenko, R. Menzel, A. Putschew, T. Luckenbach, and C. E. W. Steinberg. 2014. Contrasting cellular stress responses of Baikalian and Palearctic amphipods upon exposure to humic substances: environmental implications. Environmental Science and Pollution Research 21:14124–14137.

Rusinek, O. T., V. V. Takhteev, D. P. Gladkochub, T. V. Khodzher, and N. M. Budnev. 2012. Baikalovedenie (Baikalogy). Nauka, Novosibirsk, Russia.

Shatilina, Z. M., H. W. Riss, M. V. Protopopova, M. Trippe, E. I. Meyer, V. V. Pavlichenko, D. S. Bedulina, D. V. Axenov-Gribanov, and M. A. Timofeyev. 2011. The role of the heat shock proteins (HSP70 and sHSP) in the thermotolerance of freshwater amphipods from contrasting habitats. Journal of Thermal Biology 36:142–149.

Shimaraev, M. N., L. N. Kuimova, V. N. Sinyukovich, and V. V. Tsekhanovskii. 2002. Manifestation of global climatic changes in Lake Baikal during the 20th century. Doklady Earth Sciences 383:288–291.

Silow, E. A. 2014. Lake Baikal: current environmental problems. Pages 1–9 *in* S. E. Jørgensen, editor. Encyclopedia of environmental management. Taylor and Francis, New York, New York, USA.

Somero, G. N. 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine "winners" and "losers". Journal of Experimental Biology 213:912–920.

Storch, D., L. Menzel, S. Frickenhaus, and H.-O. Pörtner. 2014. Climate sensitivity across marine domains of life: limits to evolutionary adaptation shape species interactions. Global Change Biology 20:3059–3067.

Takhteev, V. V., I. V. Mechanikova, and E. B. Govoruchina. 2003. Ecological mechanisms of endemic evolution of amphipods (Crustacea Amphipoda) from Lake Baikal. Sibirskij Jecologicheskij Zhurnal 3:305–310.

Takhteev, V., N. A. Berezina, and D. A. Sidorov. 2015. Checklist of the Amphipoda (Crustacea) from continental waters of Russia, with data on alien species. Arthropoda Selecta 24:335–370.

Tepolt, C. K., and G. N. Somero. 2014. Master of all trades: thermal acclimation and adaptation of cardiac function in a broadly distributed marine invasive species, the European green crab, *Carcinus* *maenas*. Journal of Experimental Biology 217:1129–1138.

Terasaki, M., and H. Rubin. 1985. Evidence that intracellular magnesium is present in cells at a regulatory concentration for protein synthesis. Proceedings of the National Academy of Sciences of the United States of America 82:7324–7326.

Timofeyev, M. A., and K. A. Kirichenko. 2004. Experimental estimation of the role of abiotic factors in containment of endemics beyond the bounds of Lake Baikal. Sibirskij Jecologicheskij Zhurnal 1:41–50.

Timofeyev, M., and Z. Shatilina. 2007. Different preference reactions of three Lake Baikal endemic amphipods to temperature and oxygen are correlated with symbiotic life. Crustaceana 80:129–138.

Timofeyev, M. A., J. M. Shatilina, and D. I. Stom. 2001. Attitude to temperature factor of some endemic amphipods from Lake Baikal and Holarctic *Gammarus lacustris* Sars, 1863: a comparative experimental study. Arthropoda Selecta 10:93–101.

Timoshkin, O. A. 2001. Lake Baikal: diversity of fauna, problems of its immiscibility and origin, ecology and "exotic" communities. Pages 74–117 *in* O. A. Timoshkin, editor. Index of animal species inhabiting Lake Baikal and its catchment area. Nauka, Novosibirsk, Russia.

Timoshkin, O. A., N. A. Bondarenko, E. A. Volkova, I. V. Tomberg, V. S. Vishnyakov, and V. V. Maljnik. 2014. Mass development of filamentous algae of genera *Spirogyra* and *Stigeoclonium* (Chlorophyta) in the coastal zone of southern Baikal. Hydrobiological Journal 50:15–26.

Weinberg, I. V., and R. M. Kamaltynov. 1998. Zoobenthos communities on stony beach of Lake Baikal. Zoologichesky Zhurnal 77:259–265.

Weiss, R. F., E. C. Carmarck, and V. M. Koropalov. 1991. Deep water renewal and biological production in Lake Baikal. Nature 349:665–669.

Wilkes, P. R. H., and B. R. McMahon. 1982. Effect of maintained hypoxic exposure on the crayfish Oronectes Rusticus. 1. Ventilatory, acid-base and cardiovascular adjustments. Journal of Experimental Biology 98:119–137.

Woodward, G., B. Ebenman, M. Emmerson, J. M. Montoya, J. M. Olesen, A. Valido, and P. H. Warren. 2005. Body size in ecological networks. Trends in Ecology & Evolution 20:402–409.

Woodward, G., J. Blanchard, R. B. Lauridsen, F. K. Edwards, J. I. Jones, D. Figueroa, P. H. Warren, and O. L. Petchey. 2010a. Individual-based food webs: species identity, body size and sampling effects. Pages 211–266 *in* G. Woodward, editor. Advances in ecological research, Vol 43: integrative ecology: from molecules to ecosystems. Academic Press, Waltham, Massachusetts, USA.

Woodward, G., D. M. Perkins, and L. E. Brown. 2010b. Climate change and freshwaterecosystems: impacts across multiple levels of organization. Philosophical Transactions of the Royal Society B-Biological Sciences 365:2093–2106.

Yoshioka, T., S. Ueda, T. Khodzher, I. Bashenkhaeva, L. Korovyakova, L. Sorokovikova, and L. Gorbunova. 2002. Distribution of dissolved organic carbon in Lake Baikal and its watershed. Limnology 3:159–168.

Yvon-Durocher, G., J. I. Jones, M. Trimmer, G. Woodward, and J. M. Montoya. 2010. Warming alters the metabolic balance of ecosystems. Philosophical Transactions of the Royal Society B-Biological Sciences 365:2117–2126.

Zerbst-Boroffka, I., T. Grospietsch, I. Mekhanikova, and V. Takhteev. 2000. Osmotic and ionic hemolymph concentrations of bathyal and abyssal amphipods of Lake Baikal (Siberia) in relation to water depth. Journal of Comparative Physiology - B 170:615–625.

SUPPORTING INFORMATION

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.1308/supinfo

Publication II

Temperature-dependent capacities of key metabolic enzymes correlate with whole-animal responses in three boreal amphipod species

L Jakob, KP Vereshchagina, A Tillmann, L Rivarola-Duarte, DV Axenov-Gribanov, AN Gurkov, A Schüttler, DS Bedulina, MA Timofeyev, PF Stadler, T Luckenbach, H-O Pörtner, FJ Sartoris and M Lucassen

Submitted to the Journal of Experimental Biology in September 2016

Temperature-dependent capacities of key metabolic enzymes correlate with whole-animal responses in three boreal amphipod species

Lena Jakob*[ab], Kseniya P. Vereshchagina[c], Anette Tillmann[a], Lorena Rivarola-Duarte[de], Denis V. Axenov-Gribanov[c], Anton N. Gurkov[c], Andreas Schüttler[d], Daria S. Bedulina[c], Maxim A. Timofeyev[c], Peter F. Stadler[efghijkl], Till Luckenbach[d], Hans-Otto Pörtner[ab], Franz J. Sartoris[a] and Magnus Lucassen[a]

[a] Department of Integrative Ecophysiology, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570, Bremerhaven, Germany

[b] Faculty of Biology and Chemistry, University of Bremen, Leobener Straße, 28359 Bremen, Germany

[c] Institute of Biology, Irkutsk State University, Karl Marx str.1, 664003 Irkutsk, Russia

[d] Department of Bioanalytical Ecotoxicology, UFZ – Helmholtz Centre for Environmental Research, Permoserstr. 15, 04318 Leipzig, Germany

[e] Interdisciplinary Center for Bioinformatics, University Leipzig, Härtelstraße 16-18, 04107 Leipzig, Germany

[f] LIFE, Leipzig Research Center for Civilization Diseases, University Leipzig, Leipzig, Germany

[g] Bioinformatics Group, Department of Computer Science, University Leipzig, Leipzig, Germany

[h] RNomics Group, Fraunhofer Institut für Zelltherapie und Immunologie, Leipzig, Germany

[I] Max Planck Institute for Mathematics in the Sciences, Leipzig, Germany

[j] Department of Theoretical Chemistry, University of Vienna, Wien, Austria

[k] Center for Non-Coding RNA in Technology and Health, University of Copenhagen, Fredriksberg, Denmark

[l] Santa Fe Institute, Santa Fe, New Mexico

[*] Corresponding author: Lena.Jakob@awi.de

Abstract

Global warming may affect aquatic ectotherms as temperature exerts pervasive effects on molecular dynamics and biochemical reactions. Here, we investigated the thermal plasticity of key metabolic enzymes of two amphipod species endemic to Lake Baikal and a ubiquitous amphipod of the Holarctic in relation to whole animal responses (ventilation and oxygen consumption) determined in a parallel study. Animals were exposed to gradual temperature increments (0.8°C d^{-1}; 6 - 23.6°C); maximal activities of cytochrome-c-oxidase, citrate synthase, 3-hydroxyacyl-CoA dehydrogenase, glutamate dehydrogenase, pyruvate kinase, and lactate dehydrogenase and their RNA transcript levels were determined at incubation temperatures in freshly prepared tissue extracts. Except for lactate dehydrogenase, temperature-dependent enzyme activities followed peak-shaped or hyperbolic functions in *Eulimnogammarus verrucosus* (Baikal; endemic), indicating thermal compensation or constraints at high temperatures. This pattern could not be explained by changes in RNA expression levels, but correlated with limited oxygen consumption and a shift in transcript levels of metabolic enzymes indicating hypoxemic conditions and support of the glycogenic pathway. In *Eulimnogammarus cyaneus* (Baikal; endemic) and *Gammarus lacustris* (Holarctic, ubiquitous), enzyme activities rather increased with temperature over the entire thermal range (Q_{10} values: 1.6 - 4.9), again in accordance with oxygen consumption patterns. Ventilation was limited at lower temperatures than enzyme capacities in all species indicating constraints of the oxygen supply system that preceded metabolic changes. Besides, mRNA levels of most investigated genes were found warm-compensated in *E. cyaneus*, but uncompensated in *G. lacustris*. The different regulation patterns mirror the distinct thermal behavior and distribution of the three species within their natural thermal niches.

Key words: amphipoda, temperature, climate change, Lake Baikal, thermal limitation, cytochrome-c-oxidase (COX), citrate synthase (CS), lactate dehydrogenase (LDH), pyruvate kinase (PK), 3-hydroxyacyl-CoA dehydrogenase (HADH), glutamate dehydrogenase (GDH)

Introduction

Temperature is the most pervasive abiotic factor for animal life as it affects molecular dynamics and biochemical reaction rates (Hochachka and Somero 2002). As climate change is suggested to alter the thermal regime of many aquatic environments, it may affect the fitness or induce shifts in the distribution of aquatic organisms (Pörtner and Knust 2007). Eurythermal organisms are able to adapt to a broad thermal range and do consequently show a high degree in phenotypic plasticity in contrast to stenothermal species, which lack the physiological capacity to perform optimally over wide thermal ranges. Global warming is suggested to particularly affect animals that are adapted to a narrow temperature range or those that live at the edge of their thermal window (Somero 2010). The thermal plasticity of enzymes is a key factor to study when assessing the potential effects of global warming on aquatic organisms as activities of key metabolic enzymes may shift in response to temperature changes (Pörtner et al. 2007). Protein conformation may be altered in response to thermal changes, which may affect ligand and substrate binding events of enzymes (Somero 1995). Moreover, regulatory changes in the expression of genes that influence metabolic fuel use and oxygen utilisation were shown to occur in response to rising temperatures in aquatic organisms (Windisch et al. 2011, 2014).

Lake Baikal, the largest and most ancient freshwater body on earth, has experienced unprecedented warming during the last decades, which may affect its inhabitants (Hampton et al. 2008, Moore et al. 2009). The reported annual mean temperature for the littoral of Lake Baikal is 6°C (Weiss et al. 1991, Falkner et al. 1991, Yoshioka et al. 2002). Significant changes have been recorded since 1946 as the mean surface water temperature of Lake Baikal increased by 1.21°C (Hampton et al. 2008). Moreover, the ice-free season expanded by 18 days between 1869 and 2000 and the ice thickness in winter decreased by 12 cm in the southern basin between 1949 and 2000 (Shimaraev et al. 2002). Warming of surface temperature in summer exceeded the annual mean; by 2100 the surface water of the lake might warm by more than 4.5°C in summer and autumn compared to present temperatures (Moore et al. 2009).

Here, we studied the thermal plasticity of key metabolic enzymes of two closely related amphipod species endemic to Lake Baikal, (*Eulimnogammarus verrucosus* (Gerstfeldt, 1858) and *Eulimnogammarus cyaneus* (Dybowski, 1874)), and the related *Gammarus lacustris* Sars, 1863, which is ubiquitous across the Holarctic but not a part of the typical Baikal amphipod fauna. Amphipods of Lake Baikal are dominant members of the benthic invertebrate fauna in Lake

Baikal (Rusinek et al. 2012). However, *G. lacustris* was found in isolated bays of Lake Baikal termed sors, which are characterized by a comparatively high content of benthic organic matter, high temperatures in summer as well as little hydrodynamic mixing (Kozhova and Izmest'eva 1998, Timoshkin 2001). A comparative approach provides a powerful means to identify the physiological mechanisms causing variability in thermal tolerances among related species with different thermal niches (Somero 2010).

Studies of whole animal parameters such as oxygen consumption and ventilation rates (pleopod beat rates) within the framework of the conceptual model of oxygen- and capacity-limited thermal tolerance (OCLTT) (Pörtner 2010) revealed only slight differences in thermal tolerance between *G. lacustris* and the endemic Baikal amphipod *E. cyaneus* (Jakob et al. 2016), which mainly inhabits the littoral zone at depth of 0 - 1.5 m. The upper littoral shows the highest temperature fluctuations in Lake Baikal (Timoshkin 2009). The Baikal endemic *E. verrucosus* was shown to be significantly less thermally tolerant than the former two species (Timofeyev and Shatilina 2007, Timofeyev et al. 2010, Jakob et al. 2016). It migrates to deeper and cooler waters when temperatures rise in summer in the littoral of Lake Baikal (Weinberg and Kamaltynov 1998, Jakob et al. 2016) indicating a higher level of stenothermy, and consequently inhabits a greater depth range (0 - 25m) than *E. cyaneus*. Accordingly, *E. verrucosus* was shown to display earlier constraints in oxygen supply and consumption (Jakob et al. 2016), and to have lower constitutive levels of heat shock proteins (Hsp70) in comparison with *E. cyaneus* and *G. lacustris* (Bedulina et al. 2013, Protopopova et al. 2014).

In order to analyse the metabolic background of the oxygen supply system in the three amphipod species we quantified maximal enzyme activities of cytochrome-c-oxidase (COX), citrate synthase (CS), lactate dehydrogenase (LDH), pyruvate kinase (PK), 3-hydroxyacyl-CoA dehydrogenase (HADH), as well as glutamate dehydrogenase (GDH) were determined and their RNA transcripts were quantified. CS is a marker for intact mitochondria and together with COX, the final enzyme of the respiratory electron transport chain, mirrors the aerobic capacity of an organism. PK and LDH are key enzymes of the glycolytic metabolic pathway. LDH activity indicates the capacity for anaerobic glycolysis. HADH is essential for fatty acid metabolic processes, while GDH represents a key link between citric acid cycle and protein metabolism. By exposing the animals to an experimental setup with gradually increasing temperature (0.8°C per day), mimicking the thermal increase during a summer season in the upper littoral of Lake Baikal, but slightly exceeding natural temperatures, this study intended to reveal whether temperature-dependent capacities of key metabolic enzymes correlate with whole-animal responses (ventilation and respiration) investigated in a parallel study. Furthermore, we aimed to determine whether usage of metabolic fuel changes with rising temperatures.

Material and Methods

Sampling site

Sampling of the endemic amphipod species *E. verrucosus* and *E. cyaneus* was conducted with a hand net in the Baikal littoral (0 - 1.2 m depth) in the vicinity of Bolshie Koty (51°9137” N, 105°0691” E) by applying the kick sampling technique (Hynes 1961). The sampling site is part of the beach zone according to underwater landscape zoning (Kravtsova et al. 2004), which is characterized by algae-covered boulders and intensive hydrodynamics. *G. lacustris* was sampled in “Lake 14” (51° 55' 14.39" N, 105° 4' 19.48" E), a eutrophic pond in about 2 km distance to Lake Baikal. This artificial water body is inhabited by species, which are widespread across the Holarctic. It is connected to a backwater of Bolshie Koty river and supplied by groundwater. A map with the sampling locations is shown by Protopopova et al. (2014).

Experimental setup and animal maintenance

Animal incubations were performed in September 2013 in the Institute of Biology at Irkutsk State University. Only adult individuals were chosen using body size as selection criterion (for comparison see Timofeyev, Shatilina & Stom (2001) and Jakob *et al.* (2016)). The amphipods were transported to the laboratory in insulated boxes, sorted under temperature-controlled conditions and kept in 2 L tanks (high-density polypropylene of food grade quality or glass) filled with continuously aerated 6°C Baikal water. Numbers of individuals per container were 200, 75 and 25 for *E. cyaneus*, *G. lacustris* and *E. verrucosus*, respectively. The animals were kept for at least three days to acclimate to the test conditions and to check whether all animals were intact. Small clean pebbles on the tank bottoms served to provide shelter to the animals. Direct illumination was prevented. Water was exchanged once every one to three days increasing the rate of water exchanges with increasing temperature. A mix of amphipods, algae, water plants, and detritus collected in the Baikal littoral (frozen, air-dried at ≈ 30°C and roughly mortared) was applied as feed (*ad libitum*). Control groups were kept at 6 ± 0.8°C in a laboratory refrigerator during the entire experimental period. All tanks were stored in an incubator (Sanyo MIR-254 (238 L), Osaka, Japan) and the water temperature was increased by 0.8°C d^{-1}. The rate of increase was intended to resemble natural conditions as water temperature elevations of up to 15°C were monitored within about one month (July/August) close to the shoreline. Maximal temperatures in the littoral close to the shoreline may reach about 20°C in summer (Timoshkin 2009). The experimental temperature increase slightly exceeded temperature changes in the natural habitat (regarding the thermal increments and the maximum temperature) in order to account for a climate warming scenario. At each sampling time point individuals of each species were randomly taken out of 6 - 10 individual tanks, immediately frozen in liquid nitrogen and stored at -80°C prior to analysis. Control animals were sampled at four different time points during the experiment.

Enzyme assays

Related to the size differences of the here investigated species, numbers of individuals sampled at each time point were seven for *E. cyaneus*, two to three for *G. lacustris* and one for *E. verrucosus*. Animals were mortared upon addition of liquid nitrogen and aliquots of about 60 - 100 mg of tissue were prepared. Subsequently, the tissue was homogenized with an Ultra-Turrax (16,500 rpm) in ice-cold 20 mM Tris HCl buffer (pH 8.0) containing 1 mM EDTA, 0.1% Triton X 100 and 100 mM NaCl. Protease Inhibitor Cocktail powder P2714 (Sigma-Aldrich, Steinheim, Germany) was applied as recommended by the manufacturer. Ten µL of extraction buffer were added to 1 mg of tissue. Three intervals of 10 sec of homogenisation intermitted by 10-sec breaks were run under continuous cooling. Subsequently, cell debris was precipitated by a 10-min centrifugation step (4°C at 1,000 x g). The supernatant was used as a crude extract. Maximal enzyme activities of COX, CS, LDH, PK, HADH and GDH under optimized assay conditions (therefore also referred to as enzyme capacities) were determined in a temperature-controlled spectrophotometer system (SPECORD S600, Jena, Germany). Two aliquots of each samples were analysed (single amount and double amount of the extract) at their respective sampling temperature as well as at temperatures of 6, 18.8 and 23.6°C in order to evaluate Q_{10} relationships and the consequences of differential thermal sensitivities of the enzymes. UV-micro cuvettes for volumes of 70 - 550 µL (Brand, Wertheim, Germany) were used as reaction vessels (final volume = 200 µL). Table 1 compiles the components and parameters of the performed enzyme assays, which were modified according to assays of (Moyes et al. 1997) (COX), Sidell *et al.* (1987) (CS), (Kornberg 1955) (LDH), (Driedzic and De Almeida-Val 1996) (PK), McClelland *et al.* (2005) (HADH) and Sanchez-Muros *et al.* (1998) (GDH), respectively. The reactions were initiated by adding the start reagents after thermal equilibration of all components ensuring a stable absorbance signal. Maximal activities were calculated referring to animal fresh weight. Protein concentrations of the samples were determined according to (Bradford 1976) using bovine serum albumin as protein standard (Sigma-Aldrich, Steinheim, Germany). Enzyme activities presented in Figures 1 and 2 relate to fresh weight as whole animal parameters are also based on fresh weight. The protein content of samples taken along the warming treatment did not vary significantly in the studied species (ANOVA; $p > 0.05$).

Table 1: Summary of enzyme assays.

The given amounts relate to the final volume of 200 µL. Assays were conducted at the respective sampling temperature and at temperatures of 6, 18.8 and 23.6°C.

Enzyme	Physiological function (localization)	Components of enzyme assays	Start reagent	WL (nm)
Cytochrome-c-oxidase (COX)	Final enzyme in the respiratory electron transport chain (inner mitochondrial membrane)	- Tris HCl (20 mM, pH 8) - Tween (0.5%)	Cytochrome c (reduced) (50 µM)	550
Citrate synthase (CS)	Pace-making enzyme in the first step of the citric acid cycle, often used as a marker for intact mitochondria and aerobic capacity (mitochondrial matrix)	- Tris HCl (75 mM pH7.6) - Acetyl-CoA (0.4 mM) - 5,5'-Dithiobis-(2-nitrobenzoicacid) (0.25 mM)	Oxaloacetate (0.5 mM)	412
Lactate dehydrogenase (LDH)	Catalyzes the final step in anaerobic glycolysis (cytosol)	- Phosphate buffer (80mM, pH 7) - NADH (0.2 mM)	Sodium pyruvate (0.5 mM)	340
Pyruvate kinase (PK)	Catalyzes the final step in the glycolytic pathway (cytosol)	- Tris HCl (128 mM, pH 7.5) - MgSO4 (4.5 mM) - KCl (72 mM) - ADP (5mM) - LDH (1 µg) - NADH (0.2 mM)	Phosphoenol pyruvate (1mM)	340
3-Hydroxyacyl-CoA dehydrogenase (HADH)	Catalyzes the second oxidation step of fatty acid catabolism (mitochondrial matrix)	- Imidazole HCl (50mM, pH 6.6) - Antimycin (0.5 µg) - NADH (0.15 mM)	Aceto acetyl-CoA (0.1 mM)	340
Glutamate dehydrogenase (GDH)	Represents a key link between catabolic and metabolic pathways (mitochondrial matrix)	- Phosphate buffer (80mM, pH 8) - NH4 acetate (100 mM) - NADH (0.2 mM)	α-Ketoglutarate (5mM)	340

Quantification of transcript levels

The coding sequences of the genes of interest used for primer design and consecutive mRNA expression quantification were derived from the assembled transcriptomes of *E. verrucosus*, *E cyaneus* and *G. lacustris* (Rivarola-Duarte et al. unpublished). Briefly, mRNA from whole animals was extracted and cDNA was sequenced on an Illumina HiSeq2000 (Illumina, San Diego, USA). The trimmed 101 bp paired-end reads were assembled with Trinity (Grabherr et al. 2011). A small database with the sequences from the closest taxa available (*Amphipoda*, *Crustacea*, *Arthropoda*, *Insecta*, among others) for each gene in this study was constructed. This database was used as query against the transcriptome assemblies using tblastx (NCBI). The best ten to 15 hits for each gene were assembled with CAP3 (Huang and Madan 1999). Assembled contigs were validated using blast against the complete NCBI database to reduce spurious gene assemblies. Finally, one or a few (isoforms) consensus sequences were obtained for each gene of interest and used for primer design. These sequences have been deposited in GenBank (see Table 2 for accession numbers). Primers were designed using the PrimerExpress software (version 3.0) (Applied Biosystems, Darmstadt, Germany) (Table 2). For quantification of transcript levels total RNA was isolated from pool of animals. Forty to sixty mg of amphipod tissue were homogenized in 1 ml of Qiazol Reagent (Qiagen, USA) using a Precellys 24 Lysis and Homogenizer (Bertin Technologies, USA). A volume of 200 µl of chloroform was added to obtain a biphasic mixture. After phase separation the water phase containing the RNA was purified by columns using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A volume of 60 µl of RNase free water was used to elute RNA from the column. RNA quantities were determined by a NanoDrop 2000c spectrometer (PeqLab, Erlangen, Germany) and purity was estimated using ratios of 260/280 nm and 260/230 nm. RNA integrity was analysed by microfluidic electrophoresis in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Five µg of total RNA of each sample were treated with DNAse for DNA removal using the Turbo DNA-free kit (Ambion, USA) according to the manufacturer's protocol. The High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) was used to transcribe 0.4 µg DNA free RNA into cDNA, which was diluted 20-fold for PCR. Real-time PCR was performed on a ViiA™ 7 Real-Time PCR System (Applied Biosystems, USA) with SYBR® Green PCR master mix (Applied Biosystems, USA) and 300 nM of primer. All primer pairs were tested for performance and efficiency across a series of cDNA dilutions (1:20; 1:200; 1:2,000; 1:20,000; 1:200,000) in all three species. The selected primers displayed a suitable per cycle amplification rate, with an efficiency (E) of 2.0 ± 0.1 and $R_2 > 0.98$. To verify the amplification specificity of fragments a melting curve analysis was performed for each reaction. Only primer pairs and cDNA concentrations resulting in one specific fragment were used in the later analyses of the treatment groups (Table 2). NormFinder software (version 0.953) (MOMA, Aarhus University Hospital, Denmark) was used to identify the most stable genes within in a larger set of candidate genes. Accordingly, actin and *gapdh* were found to be similarly stable in all three species and the arithmetic mean of the CT values of both genes was used for normalisation in all three species, to ensure valuable interspecies comparisons of the expression data. Gene expression results of the qRT-PCR were calculated as log2 fold change (log2FC) of individual expressions of treatment and control normalized to the mean of the control.

Table 2: Genes and primer sequences used for real-time PCR

The primer pairs were derived from the genes of interest from the respective species and used after validation (see material and methods) in the respective organism: *E. verrrucosus* (Ev), *E. cyaneus* (Ec) and *G. lacustris* (Gl).

Organism	Gene ID	Annotation (Swissprot)	Accession No.	Forward-Primer	Backward primer	Used for:
E. verrucosus	*CISY*	Citrate synthase	KX831868	CACCTCCCTGATGACAACCTTT GGAGTGCCAACTTCACCAACA	GCCGGGCACCACAGTATAA GCCTCATGAGCTCGGTAAACTG	Ev, Ec Gl
	COX4	Cytochrome c oxidase subunit IV	KX831869	GAAAAGGAAAAGGGTGACTGGAA	AGATGCTCGGTATAAGGCCTTCT	Ev, Ec, Gl
	COX2	Cytochrome c oxidase subunit II	YP_008964129	GTAGCCCTACCAATTAACACTCAAATT	CCGCCCATGAGTGAATAACG	Ev
	GLUD1	Glutamate dehydrogenase 1, mitochondrial	KX831872	GTGGTGTCACCGTTTCCTACTTT	GGTGAGCCTGCCGTAAGAGA	Ev, Ec, Gl
	ECHA	Trifunctional enzyme subunit alpha, mitochondrial, includes: 3-hydroxyacyl-CoA dehydrogenase	KX831870	AAGTGGTGATTGTGGTGAAGGA	AACATGGGTGCCAGGATACG	Ev, Ec
	LDH-A	Lactate dehydrogenase A chain, muscle isoform	KX831873	TCGGCCTGTCCGTAGCA	GCAACGTCTCTGGTTCTTCATG	Ev, Ec
	PKM	Pyruvate kinase, muscle isoform	KX831875	CGCCATCATCGTCATTACCA	AACGAGGCCGGTACTTGGA	Ev
E. cyaneus	*ACT*	Actin	JN860427	CCGCCGAGCGAGAAATC	GGGCCACGTAGCAAAGCTT	Ec, Ev, Gl
	G3P	Glyceraldehyde-3-phosphate dehydrogenase	KF293381	GGCAAAGGTCCACTTCAACAA	GGCGGAGGGAGCAGAGAT	Ec, Ev, Gl
	PKM	Pyruvate kinase, muscle isoform	KX844831	CTGCGGTGGAGGCTTCTTT	GTGGTCGTGATGACGATGATG	Ec
G. lacustris	*ECHA*	Trifunctional enzyme subunit alpha, mitochondrial, includes: 3-hydroxyacyl-CoA dehydrogenase	KX831871	GGCCTATGGAGCAGAGTTTGA	TTCCAGATTTGGCGTAGTCTTG	Gl
	LDH-A	Lactate dehydrogenase A chain, muscle isoform	KX831874	GCCTGCGCGACCTCAA	CGTTGTACTTGTCAGGGTCATTG	Gl
	PKM	Pyruvate kinase, muscle isoform	KX831876	CTGCCGCAACATAGATTCCA	TCACCGCGAGCGATCAT	Gl

Data analysis and statistics

Data of maximal enzyme activities were fitted to non-linear models preselecting candidate models by shape using the Dynamic Fit Wizard in SigmaPlot (version 13, Systat Software Inc., USA/Canada) providing a selection of 120 different models. The Akaike's information criterion (AIC) was used to define the best-fit models; those with the lowest AIC were chosen. In peak-shaped curves we aimed to determine the point where the curve started to flatten out and approach zero, which consequently is close to the apex of the curve. According to the non-linear regression approach of Marshall, Bode & White (2013), this so-called breakpoint temperature (BPT) is reached when the model's slope equals 0.065.

Significant changes in transcript levels were determined by one-way ANOVA followed by Student–Newman–Keuls post hoc test ($\alpha < 0.05$) using SigmaPlot (version 12, Systat Software Inc., USA/Canada). Nalimov's test (Zanker 1984) was used to identify technical outliers.

Results

If not otherwise indicated, the results refer to samples, which were measured at the respective sampling temperatures (Figure 1). Results for measurements performed at 23.6°C are presented in Figure 2, they are described below in the section "Enzyme capacities determined at 23.6°C".

Aerobic capacity

Temperature affected CS and COX activities in species-specific ways: Fitting by non-linear regressions resulted in peak-shaped curves for *E. verrucosus* (BPT of CS = 13.5°C; BPT of COX = 17.1°C), whereas exponential curves were monitored for *E. cyaneus* and *G. lacustris* with Q_{10} values (6 - 23.6°C) ranging from 1.6 to 1.9 (Figures 1 A and B). CS and COX activities of *E. cyaneus* and *G. lacustris* were similar and differed only at 23.6°C for COX indicated by non-overlapping 95% confidence intervals (CI). Here, *G. lacustris* showed a higher mean COX activity but large inter-individual variation. Significantly higher CS and COX activities were determined for *E. cyaneus* compared to *E. verrucosus* at all sampling temperatures. Similar differences were observed comparing *E. verrucosus* and *G. lacustris*, however, they were not always statistically significant as indicated by overlapping of 95% CI at 6°C for COX and at 6, 9.2 and 12.4°C for CS. During the time series, gene expression of CS and COX remained more or less stable for *E. verrucosus* and *G. lacustris* (Figures 3A - F), whereas the expression of both genes decreased significantly in *E. cyaneus* (ANOVA; $p < 0.001$ for CS and $p = 0.007$ for COX4).

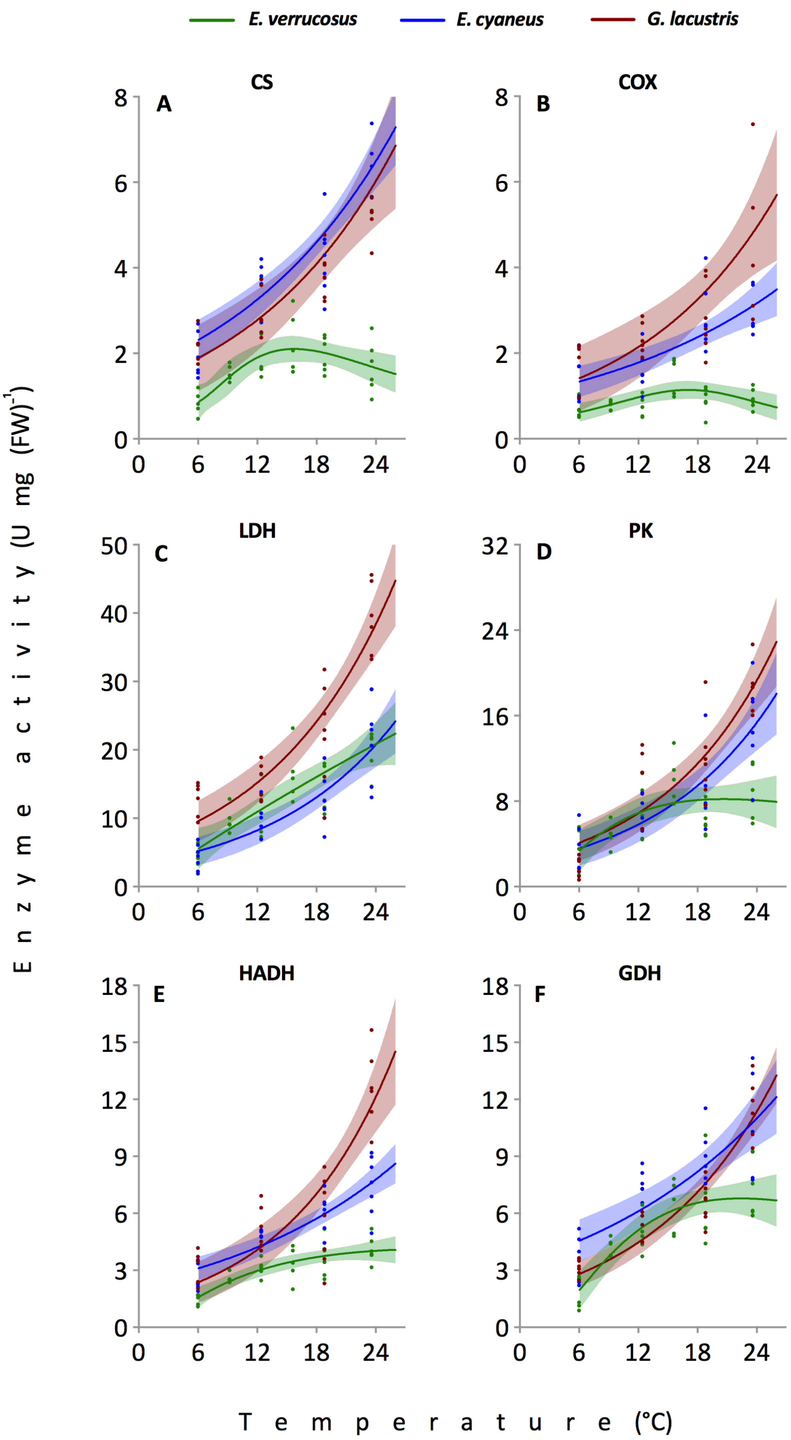


Figure 1: Maximal enzyme activities of CS, COX, LDH, PK, HADH and GDH extracted from tissues of *E. verrucosus*, *E. cyaneus* and *G. lacustris* exposed to gradual temperature increase (0.8°C d^{-1}). Samples of all three species were taken at 6, 12.4, 18.8, and 23.6°C; in addition, samples of *E. verrucosus* were taken at 9.2 and 15.6°C. Measurements were performed at the respective sampling temperatures. Dots represent raw data. Solid lines represent values calculated by nonlinear models (equations are summarized in Table 3) and shaded areas represent 95% confidence intervals (n = 5 - 7).

Glycolytic capacity

LDH activities (Figure 1C) were exponentially increasing with temperature in all species investigated (Q_{10} (6-23.6°C) = 1.89 - 2.43) and displayed identical capacities in the two endemic Baikal species. LDH was the only enzyme in *E. verrucosus* that showed an exponential increase over the entire thermal range (6 - 23.6°C). *G. lacustris* possessed the highest LDH capacities among the investigated species, and the 95% CI did not overlap with that of the Baikal species at sampling temperatures exceeding 12.4°C.

PK capacities (Figure 1D) were similar in all studied species up to a temperature of 15.6°C, whereupon the curve levelled off for *E. verrucosus*. *E. cyaneus* and *G. lacustris* rather showed exponential increases (Q_{10} (6 - 23.6°C): *E. cyaneus* = 2.4 and *G. lacustris* = 3.7). Significant differences in PK capacities between *E. verrucosus* and both *E. cyaneus* and *G. lacustris* became evident at 23.6°C. Regarding the transcript levels of the studied genes all three species showed distinct patterns: In *E. verrucosus* transcript levels of LDH and PK showed only reasonable variation but no specific trend. In *E. cyaneus* (Figure 3N) LDH showed a significant down-regulation ($p < 0.001$), but a tendency of increase in PK, which was non-significant due to high inter-individual variation. In *G. lacustris* (Figure 3R), LDH decreased at the first two temperatures, but turned back two control levels at the end of the trial (ANOVA; $p = 0.002$) (Figure 3O). Besides, PK transcript levels decreased slightly over time, which became significant at 23.6°C (ANOVA; $p = 0.016$) (Figure 3R).

Capacity of lipid and protein metabolism

HADH activities (Figure 1E) showed similar exponential increases in *E. cyaneus* and *G. lacustris*, which differed only at 23.6°C (no overlap of 95% CI). For *G. lacustris* a large activity increase was seen between 18.8 and 23.6°C (Q_{10} (18.8 - 23.6°C) = 5.5). Significantly lower HADH capacities were found in *E. verrucosus* compared to *E. cyaneus* at all sampling temperatures (no overlap of 95% CI). Comparing *E. verrucosus* and *G. lacustris*, HADH capacities were higher in the latter species at all sampling temperatures except at 9.2 and 12.4°C. Temperature-dependent HADH activities for *E. verrucosus* showed a hyperbolic-shaped curve.

GDH capacities (Figure 1F) were increasing over the entire thermal range in *E. cyaneus* and *G. lacustris* (Q_{10} (6 - 23.6°C): *E. cyaneus* = 1.9 and *G. lacustris* = 2.1), whereas they levelled off for *E. verrucosus* at temperatures surpassing 15.6°C.

HADH gene expression was found down-regulated with increasing temperature in *E. cyaneus* (Figures 3K; p < 0,001), but not in the other two species (Figure. 3J, L). For GDH a down-regulation was monitored in *E. verrucosus* at 15.6°C and beyond (ANOVA; p < 0.001) (Figure 3G). In *E. cyaneus* the downward trend in GDH expression remained non-significant due to high inter-individual variation. In *G. lacustris*, GDH gene expression was stable over the entire thermal range (Figure 3I).

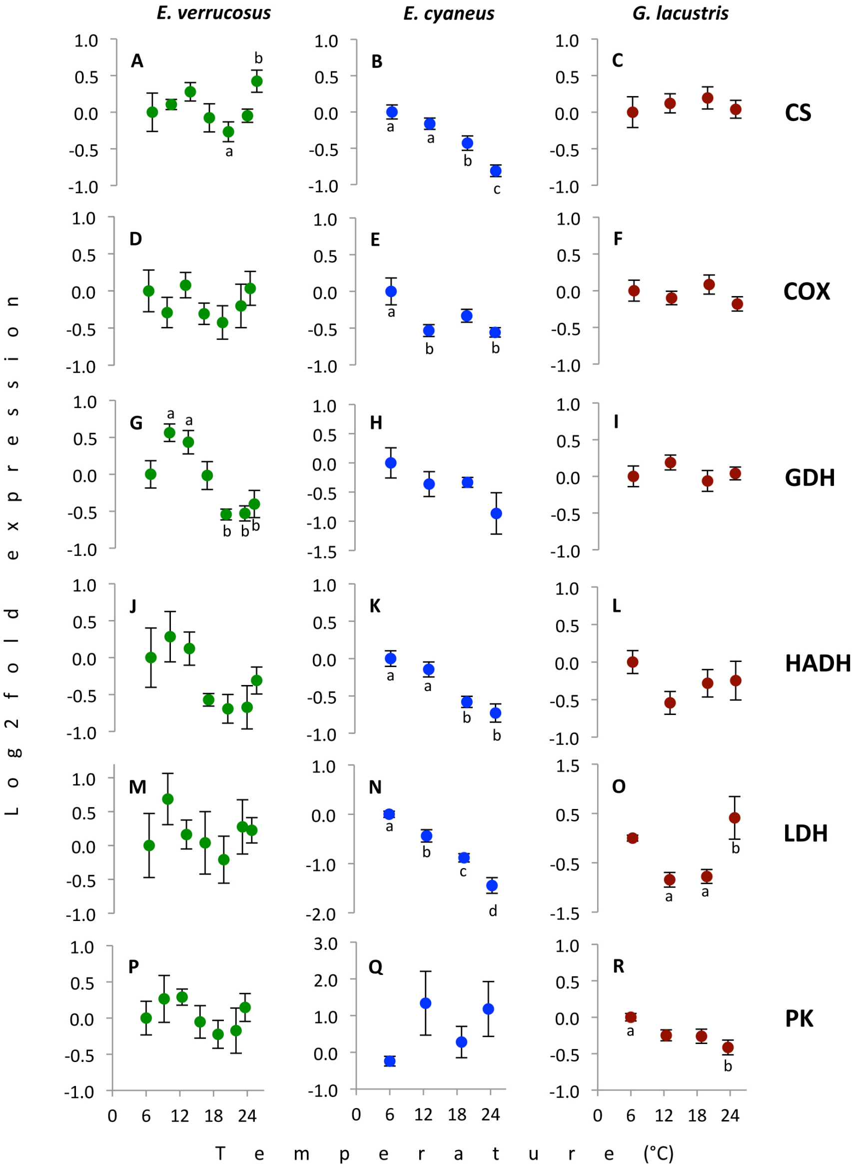


Figure 3: Expression of CS, COX, LDH, PK, HADH and GDH in *E. verrucosus*, *E. cyaneus* and *G. lacustris* exposed to gradual temperature increase (0.8°C d^{-1}). Samples of all three species were taken at 6, 12.4, 18.8, and 23.6°C; in addition, samples of *E. verrucosus* were taken at 9.2, 15.6, and 22°C. Letters indicate data that are statistically different (ANOVA; p < 0.05) Data are presented as means ±SE (n = 5 - 7).

Aerobic *vs.* anaerobic metabolism

With rising temperature a progressive shift to a higher disposition for anaerobic metabolism in comparison to aerobic capacity was monitored for *E. verrucosus* and *E. cyaneus* indicated by progressively increasing LDH/CS ratios (Pearson correlation; p = 0.0002 and p = 0.00183, respectively (Figures S1A - B, supporting information). However, *E. cyaneus* showed constant PK/LDH ratios over the entire thermal range, but in *E. verrucosus* the involvement of LDH increased with increasing temperature in comparison to PK. The opposite was observed for *G. lacustris* (Pearson correlation; p = 0.187) (Figure S2, supporting information).

Shifts in metabolic fuel use

In relation to aerobic capacity, protein catabolic capacity increased with rising temperatures indicated by increasing GDH/CS ratios for *E. verrucosus* (Pearson correlation; p = 0.00002) but not in *E. cyaneus* and only slightly in *G. lacustris* (Figure S1, supporting information). The ratio of capacities in fatty acid to carbohydrate metabolism indicated by HADH/PK decreased with increasing temperature in *G. lacustris* (Pearson correlation; p = 0.0099) but the HADH/LDH ratio remained stable. In *E. cyaneus* both HADH/PK and HADH/LDH ratios decreased with increasing temperature (Pearson correlation; p = 0.0336 and p = 0.0186, respectively). In *E. verrucosus* only HADH/LDH ratio decreased with increasing temperature (Pearson correlation; p = 0.0032) but no changes in the HADH/PK ratio were monitored (Figure S3, supporting information).

Enzyme capacities determined at 23.6°C

Maximal enzyme activities determined at 23.6°C are presented in Figure 2. Here, uniform thermal patterns for all investigated enzymes became visible for both, *E. verrucosus* and *G. lacustris*, albeit in opposite directions in the two species. Peak-shaped curves were monitored for *E. verrucosus* with BPTs ranging from 9.9°C (PK) to 13.5°C (COX), whereas *G. lacustris* showed more or less u-shaped curves. Consequently, highest enzyme capacities were observed at temperatures around 12.4°C in *E. verrucosus* while *G. lacustris* showed highest capacities of COX and LDH at 6 and 23.6°C, respectively. For *E. cyaneus* enzyme activities showed only marginal changes over the entire thermal range.

Significantly higher Q_{10} values of COX were measured in *E. verrucosus* samples taken at 12.4°C compared to those of samples taken at all other temperatures (ANOVA; $p \leq 0.003$). For PK Q_{10}

values of samples taken at 12.4°C were also significantly higher than those of samples taken at 6, 18.8 and 23.6°C (ANOVA; p ≤ 0.004) (data not shown).

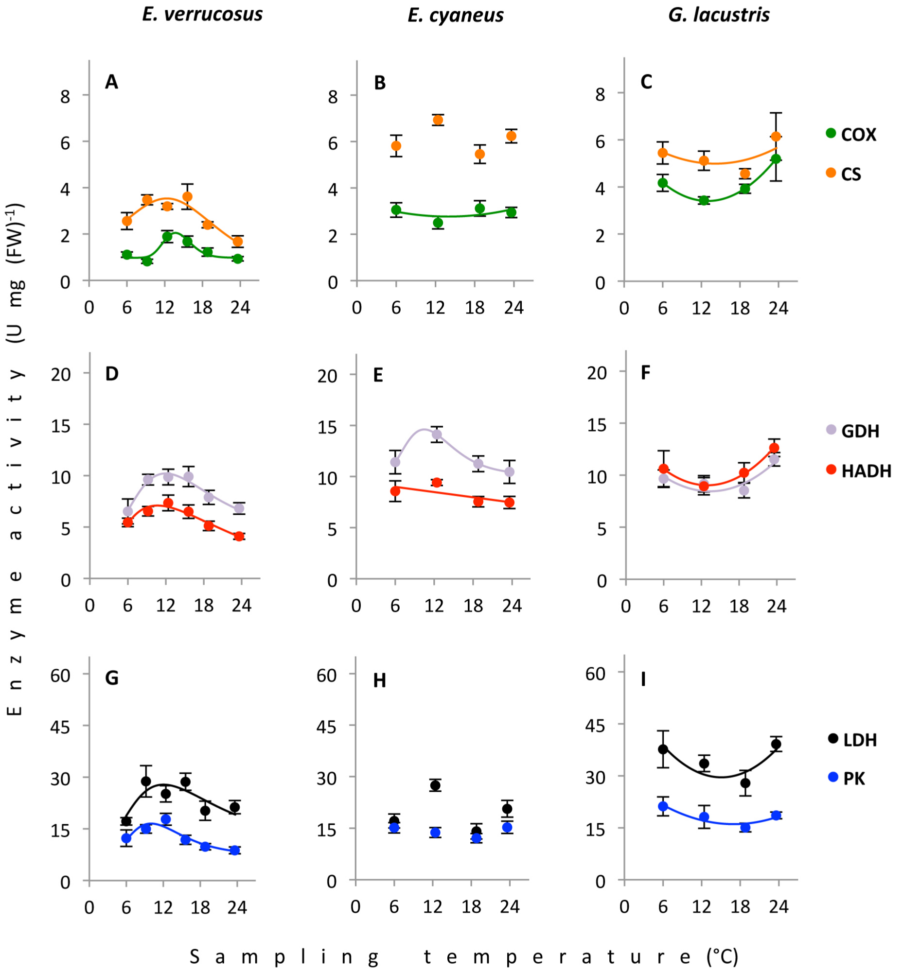


Figure 2: Maximal enzyme activities of CS, COX, LDH, PK, HADH and GDH extracted from tissues of *E. verrucosus*, *E. cyaneus* and *G. lacustris* exposed to gradual temperature increase (0.8°C d^{-1}). Samples of all three species were taken at 6, 12.4, 18.8, and 23.6°C; in addition, samples of *E. verrucosus* were taken at 9.2 and 15.6°C. All measurements were performed at 23.6°C. Data are presented as means ± SE (n = 5 - 7) and model equations are summarized in Table 3.

Table 3: Equations of models fitted to data presented in Figure 1 and 2 (Dynamic Fitting; Sigma Plot 13).

Data location	Equation category	Equation
Fig.1A (*E. verrucosus*, green) Fig.2A (CS), D (HADH, GDH), E (GDH), G (LDH, PK)	Lognormal; peak; 4 parameter	$y = y0 + \frac{a}{x}\exp\left[-0.5\left(\frac{\ln\left(\frac{x}{x0}\right)}{b}\right)^2\right]$
Fig.1B (*E. verrucosus*, green)	Lorentzian; peak; 3 parameter	$y = \frac{a}{1+\left(\frac{x-x_0}{b}\right)^2}$
Fig.1C (*E. verrucosus*, green)	Exponential growth; 3 parameter	$y = y0 + a\exp^{bx}$
Fig.1D-F (*E. verrucosus*, green)	Lognormal; peak; 3 parameter	$y = \frac{a}{x}\exp\left[-0.5\left(\frac{\ln\left(\frac{x}{x0}\right)}{b}\right)^2\right]$
Fig.1A-F (*E. cyaneus*, blue) Fig.1A-F (*G. lacustris*, red)	Exponential growth; 2 parameter	$y = a\exp^{bx}$
Fig.2A (COX)	Lorentzian; peak; 4 parameter	$y = y_0 + \frac{a}{1+\left(\frac{x-x_0}{b}\right)^2}$
Fig.2B (COX), C (CS, COX), F (HADH, GDH), I (PK, LDH)	Polynomial; quadratic	$y = y0 + ax + bx^2$
Fig.2E (HADH)	Linear	$y = mx + b$

Discussion

Thermal habitat and capacities of key metabolic enzymes

Most of the studied maximal enzyme capacities of *E. verrucosus* showed hyperbolic increases in capacity or peak-shaped curves, which flattened out at around 15°C (Figure 1). Adult individuals of *E. verrucosus* retreated completely from the upper littoral (0 – 1.2 m depth) to deeper waters, once this BPT was surpassed (Jakob et al. 2016). Temperatures of around 15°C are regularly reached close to the shoreline in Lake Baikal in summer as temperatures may rise to about 20°C (Timoshkin 2009) but *E. verrucosus* escapes deleterious thermal conditions by migration as has previously been shown (Weinberg & Kamaltynov 1998 Jakob *et al.* 2016). Enzyme activities determined at 23.6°C (Figure 2) and not at the respective sampling temperature (Figure 1) showed peak-shaped sampling temperature-dependent curves for all enzymes investigated in *E. verrucosus*. Kinetic stimulation of enzymatic processes in the thermal range of 6 to about 11°C in *E. verrucosus* might support a higher activity level and thus temperature-dependent migration activity. *E. verrucosus* represents the middle littoral and sublittoral fauna of amphipods of the rocky beaches of Lake Baikal, where it dominates by number and biomass (Kravtsova et al. 2004). This faunistic complex comprises many thermally sensitive species which are predominantly reproducing in winter (Takhteev 2000). These species likely evolved during the decreasing temperatures of the Pleistocene (Bazikalova 1941).

By contrast, enzyme capacities increased exponentially in *E. cyaneus* and *G. lacustris* and neither species showed noteworthy thermal compensation of enzyme activities during the thermal trial, meaning that after thermal acclimation, enzyme activities were similar. Consequently, enzyme activities simply followed the law of Arrhenius. However, when enzymes were measured at 23.6°C and not at the respective sampling temperature, there was a slight trend of *E. cyaneus* showing highest enzyme capacities when sampled at 12.4°C. By contrast, *G. lacustris* showed highest enzyme activities (measured at 23.6°C) when sampled at 6 and 23.6°C. This might be responsible for the generally lower enzyme activities in *E. cyaneus* than in *G. lacustris* at sampling temperatures of 23.6°C in combination with the thermally compensated transcription in *E. cyaneus*, which may help to stabilize the energy demand.

E. cyaneus mainly inhabits the upper littoral in a depth range of 0 - 1.5 m and represents a summer-reproducing complex of Baikal amphipods, which likely evolved under comparatively warm climate conditions during the Tertiary (Mats et al. 2011). *G. lacustris* inhabits shallow water habitats across the Holarctic and is thus exposed to large temperature fluctuations in the course of the seasons; like *E. cyaneus* it reproduces in summer. Food availability is highest in summer and could fuel the increasing energy demand caused by uncompensated activities. Thus, it might be beneficial to simply increase physiological rates and locomotor activity with increasing temperature during short warming periods. In summer, *E. cyaneus* and *G. lacustris* likely need to refill energy stores (especially *G. lacustris*, which hibernates during winter), which is likely supported by higher locomotor activity as indicated for other aquatic organisms (Marshall et al. 2011).

Oxygen availability and capacities of key metabolic enzymes

In *E. verrucosus*, oxygen consumption, analysed in a parallel study (Jakob et al. 2016), and enzymatic activity response to changing temperatures determined in the present study were represented by hyperbolic or peak-shaped curves with parallel breakpoints (around 15°C), beyond which both variables showed a clear decrease in magnitude. Only LDH enzyme activity, representing the anaerobic capacity of metabolism, increased beyond this BPT. Phosphofructokinase 2 (PFK2) was determined in a parallel gene expression study of the same samples (supporting information, Figure S4). PFK2 expression was up-regulated from 15.6°C in *E. verrucosus*. PFK2 genes are responsive to hypoxia in vivo (Minchenko et al. 2003), indicating a physiological role in the acclimation of the organism to environmental or systemic hypoxia.

In contrast to *E. verrucosus*, *E. cyaneus* and *G. lacustris* showed exponentially increasing maximal enzyme capacities and oxygen consumption over the entire investigated thermal range reflected in typical Q_{10} values. However, ventilation showed an earlier onset of limitation than oxygen consumption in all species investigated (Jakob et al. 2016). It has been hypothesized that the physiological thermal limits at different levels show a hierarchy of tolerance from the whole animal to the molecular level, with the tightest limits seen at the whole animal level, e.g. a progressive reduction in aerobic scope during warming (Pörtner 2002; Pörtner 2012). Our data support this hypothesis and suggest that the oxygen supply system likely contributes to setting the thermal frame to metabolic enzymes that in turn may respond to the availability of oxygen.

COX as the terminal enzyme of the respiratory chain transfers the electrons to oxygen and may thus represent aerobic capacities. Accordingly, COX capacities in this study resemble oxygen consumption curves determined in the parallel study (Jakob et al. 2016) (Figure 4). CS capacities (indicator for intact mitochondria) followed the same pattern. Specific differences in the temperature-dependent ratio of CS to COX have been demonstrated in other organisms, which may indicate functional differences of the tissues and the specific uses of fuels (Lucassen et al. 2003, 2006, Windisch et al. 2011). A relationship between changes in whole organism aerobic capacity and changes in CS activity has often been assumed (Vigelsø et al. 2014). The parallel temperature dependencies of CS and COX favor the view that CS is used mainly in aerobic pathways and that at the whole animal level, muscle mitochondria mostly contribute to the observed capacities.

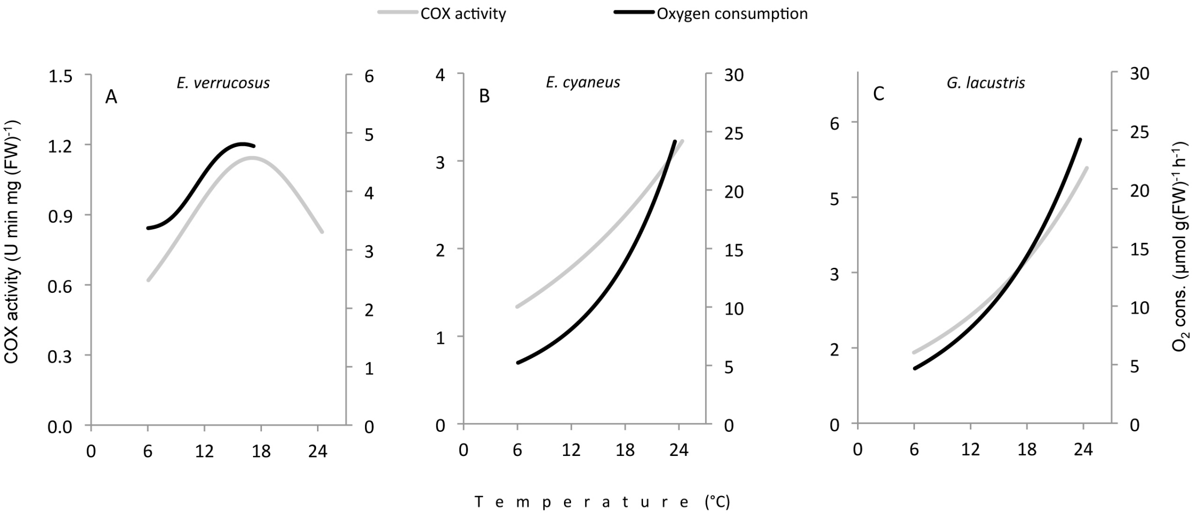


Figure 4: Non-linear models fitted to data of oxygen consumption and maximal enzyme activity of cytochrome-c-oxidase (COX) in *E. verrucosus* (A), *E. cyaneus* (B) and *G. lacustris* (C) exposed to a gradual temperature increase (0.8°C d^{-1}; 6 – 23.6°C). Measurements were performed at the respective sampling temperatures (n = 5 - 13).

Mitochondrial densities and capacities, and consequently aerobic capacities are generally higher for eurythermal species than for stenothermal species (Pörtner and Gutt 2016), which is in line with our observation of low maximum aerobic capacities in *E. verrucosus*, inhabiting the lowest temperature range in comparison to both, *E. cyaneus* and *G. lacustris*. Thus, for eurythermal species life is more costly at low temperatures (Pörtner, Hardewig & Peck 1999). However, cold-adapted species were shown to possess high mitochondrial densities, at low capacities of individual mitochondria (Johnston et al. 1998, O'Brien 2011), which is in accordance with the suggestion that the enhancements of mitochondrial capacity is negligible in stenothermal species (Pörtner et al. 1999).

Limitation and regulation of enzyme activity

The observed partial physiological inactivation of enzymes in *E. verrucosus* but neither in *E. cyaneus* nor in *G. lacustris* may be due to whole organism feedbacks, causing depression or down-regulation, or to differences in sensitive alterations of the 3-dimensional structure of individual proteins, which may affect molecular recognition events that are important for substrate binding by enzymes and oxygen binding by respiratory proteins (Pörtner et al. 2007). Mutations that affect the conformational stability of enzymes are favored by natural selection in dependence of the thermal environment (Hochachka and Somero 2002, Marx et al. 2007, Windisch et al. 2012). Flexibility mostly comes at the price of reduced substrate affinity (Angiletta 2009) and generally enzymes with less conformational stability function better at low temperatures (Somero 1995, Fields 2001).

Moreover, post-translational modifications of proteins during or after protein biosynthesis may also affect enzyme function and need to be accounted for before establishing a causal link between changes in amino acid sequences and observed temperature-adaptive differences in enzyme stability and function. Indeed, post-translational changes such as phosphorylation and temperature-dependent folding have been shown to occur in response to hypoxia (here possibly indicated in *E. verrucosus* by up-regulation of PFK2 gene expression), which may affect enzyme function and consequently regulate energy metabolism (Kumar and Klein 2003).

Stabilisation of the milieu to support optimal enzyme function is an adaptive trait of eurythermal organisms. Accordingly, *E. verrucosus* showed lower constitutive levels of heat shock proteins (Hsp70) than *E. cyaneus* and *G. lacustris* (Bedulina et al. 2013, Protopopova et al. 2014). High levels of Hsp70 and its chaperone function (Feder and Hofmann 1999), may foster eurythermy.

Furthermore, enhanced or decreased transcription of the genes encoding for the respective enzymes may be necessary during the acclimation process. During the time course of warm acclimation transient shifts in gene expression frequently occur and diminish when reaching a new steady state (Lucassen et al. 2003, Windisch et al. 2011). A complete acclimation of the amphipods can be excluded due to the applied warming scenario, however, in case of thermal compensation at least some responses should have become visible. All three species differed largely when comparing the expression of the investigated genes. Whereas *E. verrucosus* and *G. lacustris* showed only marginal shifts in expression with no clear pattern and only limited compensatory capacities at the transcription level, the gene expression of four out of six genes decreased upon the warming event in *E. cyaneus*. Although this classical warm-compensation took place for some genes quite early in the trial, it did not become manifested at functional levels, as enzyme capacities rose exponentially with temperature despite acclimation. However, enzyme activities showed less steep exponential increases in *E. cyaneus* in comparison with *G. lacustris*, which did not show any warm compensation. The question remains whether the reduced transcript levels failed to have measurable functional consequences because of the limited exposure time or whether it simply reflects higher efficiency of the transcription/translation machinery at higher temperature being able to support the same functional capacities at reduced transcript levels. In contrast, the exponential increments in enzyme capacities in *G. lacustris* at unchanged RNA levels indicate the absence of compensation and simple Q_{10} effects. The predominantly stable mRNA expression in *E. verrucosus* supports the view of post-translational modification of existing proteins (here: inactivation), which may have occurred in response to decreasing systemic oxygen availability.

Metabolic fuel use

Both elevated temperature and high locomotor activity may favor the use of the glycolytic metabolic pathway as energy is readily available and (initially) independent from oxygen supply whereas fatty acid oxidation relies on molecular oxygen. Accordingly, the capacity of anaerobic glycolysis rose with increasing temperature in *E. verrucosus*. This shows that there is a higher disposition to meet hypoxic conditions by anaerobic metabolism at elevated temperatures. Transcript levels of PFK2 were progressively increasing in *E. verrucosus* beyond 15.6°C (supporting information, Figure S4) indicating a low energy state at temperatures exceeding the breakpoint temperature of oxygen consumption (15.0°C) and enhancement of the glycolytic metabolic pathway. This metabolic rearrangement may be mediated by the hypoxia inducible factor HIF-1 pathway, as HIF-1 activates the transcription of genes, whose proteins mediate a switch from oxidative to glycolytic metabolism, when oxygen delivery is reduced (Semenza 2011).

The level of HADH and GDH transcripts decreased while PK and LDH transcripts remained stable and PFK2 transcripts increased in *E. verrucosus*, which indicates a higher involvement of the glycolytic pathway in response to rising energy demand at elevated temperatures. Similarly, a shift from lipid-based to carbohydrate-based metabolism in Antarctic fish (*Pachycara brachycephalum*) in the warmth was interpreted as `warm-hardiness´ (Windisch et al. 2011).

In *E. cyaneus* the capacity of the glycolytic pathway also increased with temperature but in contrast to *E. verrucosus* at stable LDH/PK ratios. *G. lacustris* showed the highest LDH/CS ratios at the highest incubation temperature also indicating a higher involvement of the glycolytic pathway. A high glycolytic capacity can be recruited short-term and may be an adaptive trait supporting sudden energy demand. The need for a high (anaerobic) glycolytic capacity of *G. lacustris* is possibly due to its ecology, which in some aspects differs from that of the two Baikal endemic species. *G. lacustris* lives in habitats, which may show higher and faster fluctuation in oxygen content and temperature (Luckenbach et al. 2015). The universally large Q_{10} values of enzyme activities and oxygen consumption between 18.8 and 23.6°C and the observation of an increase in locomotor activity in *G. lacustris* may indicate a startle response. This hypothesis is supported by the observation that only very few adult individuals of *G. lacustris* were found at the water edge, when temperatures in "Lake 14" reached about 22°C in summer (Jakob et al. 2016). By contrast, continuous hydrodynamics in Lake Baikal prevent rapid temperature fluctuations.

Moreover, species with high locomotor activity rates may show increased cristae surface areas per unit of mitochondrial volume (Leary and Moyes 2000). Comparing *E. cyaneus* and *G. lacustris*, CS capacities are fairly similar, indicating that both species may have similar mitochondrial densities. However, slightly higher COX capacities were found in *G. lacustris* than in *E. cyaneus*. This is likely due to a higher mitochondrial cristae density, which supports faster production of ATP.

Conclusions

This study indicates that temperature-dependent changes in activities of key metabolic (aerobic) enzymes were correlated with those in oxygen consumption rates in all three studied amphipod species (Figure 4). Moreover, thermal responses of key metabolic enzymes extracted from tissues of *E. verrucosus*, *E. cyaneus* and *G. lacustris* and analysed at the respective incubation temperatures of the animals mirror the thermal niche to which the organisms are adapted. Parallel constraints or regulation (around 15.0°C) on the whole animal and biochemical/molecular level in the stenothermal *E. verrucosus* (Baikal, endemic) suggest a narrowly regulated feedback system in response to hypoxemic conditions. By contrast, no breakpoints were monitored in the more eurythermal species *E. cyaneus* (Baikal, endemic) and *G. lacustris* (Holarctic, ubiquitous) within the studied thermal range (6 – 23.6°C).

Accordingly, the universal curve shapes of maximal enzyme capacities in *E. verrucosus* with an onset of thermal limitation at temperatures around 15°C indicate the strong degree of cold adaptation classifying *E. verrucosus* as a cold-stenothermal species. By contrast, *E. cyaneus* and *G. lacustris* did not show any capacity limitation in the investigated thermal range (6 – 23.6°C), which characterize them as more eurythermal species. The aerobic capacity of *E. verrucosus* indicated by COX and CS capacities was significantly lower than that of both *E. cyaneus* and *G. lacustris*, which is in line with the hypothesis that eurythermal species possess higher densities and capacities of mitochondria than stenothermal species. Besides, the species differed with respect to compensation mechanisms at the underlying mRNA expression level.

Increasing temperatures led to shifts in metabolic pathways in all three species, mostly directed to support the glycolytic metabolic pathway in the warmth. This was most striking for the cold-stenothermal *E. verrucosus*, where LDH followed the same Q_{10} pattern as in the more eurythermal species. Thus, evolutionary constraints may exist to preserve thermal plasticity for the anaerobic glycolytic pathway in cold-adapted species, when there is a loss of plasticity in the aerobic part. A higher involvement of the glycolytic metabolic pathway may be interpreted to prepare for hypoxemic conditions, which more likely occur at elevated temperatures (Windisch et al. 2011). It is yet unclear whether a higher involvement of glycolytic energy recruitment supports the Darwinian fitness of the here studied amphipod species. The ecological background of these metabolic alterations remains to be investigated.

Acknowledgements

Ekaterina Madyarova and Elizaveta Kondrateva are highly acknowledged for helping with animal maintenance and sampling during experimental exposures. We cordially thank Cornelia Kreiss for technical advice on the enzyme assays, and Jutta Jürgens, Alek Bolte, Marius Kemper and Michael Meyer for assistance. Marina Protopopova and Vasiliy Pavlichenko are thanked for their assistance in the field, and Jörg Hackermüller and Stephan Schreiber for their involvement in the transcriptome project, the genes of interest of the present study were extracted from. This study was financed by the bilateral funding program "Helmholtz-Russia Joint Research Groups" (HRJRG) from the Helmholtz Association and the Russian Foundation for Basic Research (RFBR). It was embedded in the LabEglo project HRJRG-221. Russian participants received further funding by the RFBR (grants: 15-29-01003; 14-04-00501; 15-04-06685), the Civilian Research and Development Foundation (CRDF) (grant: FSCX-15-61168-0) and were partially supported by the Russian Science Foundation (grant: 14-14-00400).

References

Angiletta, M. 2009. Thermal Adaptation - A theoretical and Empirical Synthesis. Oxford University Press, Oxford.

Bazikalova, A. J. 1941. Materials on the study of the amphipods of Baikal. II. Reproduction. Izvestya Acad. Sci. USSR:407–426.

Bedulina, D. S., M. B. Evgen'ev, M. A. Timofeyev, M. V. Protopopova, D. G. Garbuz, V. V. Pavlichenko, T. Luckenbach, Z. M. Shatilina, D. V. Axenov-Gribanov, A. N. Gurkov, I. M. Sokolova,and O. G. Zatsepina. 2013. Expression patterns and organization of the hsp70 genes correlatewith thermotolerance in two congener endemic amphipod species (*Eulimnogammarus cyaneus* and *E. verrucosus*) from Lake Baikal. Molecular Ecology 22:1416–1430.

Bradford, M. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry 72:248–254.

Driedzic, W. R., and V. M. F. De Almeida-Val. 1996. Enzymes of cardiac energy metabolism in Amazonian teleosts and the fresh-water stingray (*Potamotrygon hystrix*). Journal of Experimental Zoology 274:327–333.

Falkner, K. K., C. I. Measures, S. E. Herbelin, J. M. Edmond, and R. F. Weiss. 1991. The major and minor element geochemistry of Lake Baikal. Limnology and Oceanography 36:413–423.

Feder, M. E., and G. E. Hofmann. 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. Annual Review of Physiology 61:243 282.

Fields, P. A. 2001. Review: Protein function at thermal extremes: balancing stability and flexibility.Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology 129:417–431.

Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F. di Palma, B. W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman, and A. Regev. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29:644-U130.

Hampton, S. E., L. R. Izmest'eva, M. V. Moore, S. L. Katz, B. Dennis, and E. A. Silow. 2008. Sixty years of environmental change in the world's largest freshwater lake - Lake Baikal, Siberia. Global Change Biology 14:1947–1958.

Hochachka, P. W., and G. N. Somero. 2002. Biochemical adaptation: mechanism and process in physiological evolution. Oxford University Press, New York.

Huang, X. Q., and A. Madan. 1999. CAP3: A DNA sequence assembly program. Genome Research 9:868–877.

Hynes, H. B. N. 1961. The invertebrate fauna of a Welsh mountain stream. Archives of Hydrobiology 57:344–388.

Jakob, L., D. V. Axenov-Gribanov, A. N. Gurkov, M. Ginzburg, D. S. Bedulina, M. A. Timofeyev, T. Luckenbach, M. Lucassen, F. J. Sartoris, and H. O. Pörtner. 2016. Lake Baikal amphipods under climate change: Thermal constraints and ecological consequences. Ecosphere 7:e01308.

Johnston, I. A., J. Calvo, H. Guderley, D. Fernandez, and L. Palmer. 1998. Latitudinal variation in the abundance and oxidative capacities of muscle mitochondria in perciform fishes. Journal of Experimental Biology 201:1–12.

Kornberg, A. 1955. Lactic dehydrogenase of muscle: Pyruvate + DPNH↔Lactate + DPN. Methods in Enzymology 1:441–443.

Kozhova, O. M., and L. R. Izmest'eva. 1998. Lake Baikal - Evolution and Biodiversity. Backhuys Publishers, Leiden.

Kravtsova, L. S., E. B. Karabanov, I. V. Mekhanikova, T. Y. Sitnikova, N. A. Rozhkova, Z. V. Slugina, L. A. Izhboldina, I. V. Weinberg, T. V. Akinshina, and D. Y. Sherbakov. 2004. Macrozoobenthic communities of underwater landscapes in the shallow-water zone of southern Lake Baikal. Hydrobiologia 522:193–205.

Kumar, G. K., and J. B. Klein. 2003. Analysis of expression and posttranslational modification of proteins during hypoxia. Journal of Applied Physiology 96:1178–1186.

Leary, S. C., and C. D. Moyes. 2000. The effects of bioenergetic stress and redox balance on the expression of genes. Page 324. Environmental stressors and gene responses. Elsevier Sciene B.V.,Amsterdam, The Netherlands.

Lucassen, M., N. Koschnick, L. G. Eckerle, and H.-O. Pörtner. 2006. Mitochondrial mechanisms of cold adaptation in cod (*Gadus morhua* L.) populations from different climatic zones. Journal of Experimental Biology 209:2462–2471.

Lucassen, M., A. Schmidt, L. G. Eckerle, and H. O. Pörtner. 2003. Mitochondrial proliferation in the permanent vs. temporary cold: enzyme activities and mRNA levels in Antarctic and temperate zoarcid fish. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 285:R1410–R1420.

Luckenbach, T., D. S. Bedulina, and M. A. Timofeev. 2015. Is the Endemic Fauna of Lake Baikal Affected by Global Change? Pages 219–235. Water and Environment in the Selenga- Baikal Basin: International Research Cooperation for an Ecoregion of Global Relevance. ibidem Press.

Marshall, D. J., M. Bode, and C. R. White. 2013. Estimating physiological tolerances - a comparison of traditional approaches to nonlinear regression techniques. Journal of Experimental Biology 216:2176–2182.

Marshall, D. J., Y. Dong, C. D. McQuaid, and G. A. Williams. 2011. Thermal adaptation in the intertidal snail Echinolittorina malaccana contradicts current theory by revealing the crucial roles of resting metabolism. Journal of Experimental Biology 214:3649–3657.

Marx, J.-C., T. Collins, S. D'Amico, G. Feller, and C. Gerday. 2007. Cold-adapted enzymes from marine antarctic microorganisms. Marine Biotechnology 9:293–304.

Mats, V. D., D. Yu. Shcherbakov, and I. M. Efimova. 2011. Late Cretaceous-Cenozoic history of the Lake Baikal depression and formation of its unique biodiversity. Stratigr. Geol. Correl. 19:404–423.

McClelland, G. B., A. C. Dalziel, N. M. Fragoso, and C. D. Moyes. 2005. Muscle remodeling in relation to blood supply: implications for seasonal changes in mitochondrial enzymes. Journal of Experimental Biology 208:515–522.

Minchenko, O., I. Opentanova, and J. Caro. 2003. Hypoxic regulation of the 6-phosphofructo-2 kinase/fructose-2,6-bisphosphatase gene family (PFKFB-1-4) expression in vivo. Febs Letters 554:264–270.

Moore, M. V., S. E. Hampton, L. R. Izmest'eva, E. A. Silow, E. V. Peshkova, and B. K. Pavlov. 2009. Climate Change and the World's "Sacred Sea"-Lake Baikal, Siberia. Bioscience 59:405 417.

Moyes, C. D., O. A. MathieuCostello, N. Tsuchiya, C. Filburn, and R. G. Hansford. 1997. Mitochondrial biogenesis during cellular differentiation. American Journal of Physiology-Cell Physiology 272:C1345–C1351.

O'Brien, K. M. 2011. Mitochondrial biogenesis in cold-bodied fishes. Journal of Experimental Biology 214:275–285.

Pörtner, H. O. 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology 132:739–761.

Pörtner, H. O. 2010. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. Journal of Experimental Biology 213:881–893.

Pörtner, H. O., and J. Gutt. 2016. Impacts of climate variability and change on (marine) animals: Physiological underpinnings and evolutionary consequences. Integrative and Comparative Biology 56:31–44.

Pörtner, H. O., I. Hardewig, and L. S. Peck. 1999. Mitochondrial function and critical temperature in the Antarctic bivalve, *Laternula elliptica*. Comparative Biochemistry and Physiology a Molecular & Integrative Physiology 124:179–189.

Pörtner, H. O., and R. Knust. 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science 315:95–97.

Pörtner, H. O., L. Peck, and G. Somero. 2007. Thermal limits and adaptation in marine Antarctic ectotherms: an integrative view. Philosophical Transactions of the Royal Society B-Biological Sciences 362:2233–2258.

Pörtner, H.-O. 2012. Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes. Marine Ecology Progress Series 470:273–290.

Protopopova, M. V., V. V. Pavlichenko, R. Menzel, A. Putschew, T. Luckenbach, and C. E. W. Steinberg. 2014. Contrasting cellular stress responses of Baikalian and Palearctic amphipods upon exposure to humic substances: environmental implications. Environmental Science and Pollution Research 21:14124–14137.

Rusinek, O. T., V. V. Takhteev, D. P. Gladkochub, T. V. Khodzher, and N. M. Budnev. 2012. Baikalovedenie (Baikalogy). Nauka, Novosibirsk.

Sanchez-Muros, M. J., G. Rejon, G. Salguero, M. de la Higuera, and J. A. Lupianez. 1998. Long term nutritional effects on the primary liver and kidney metabolism in rainbow trout. Adaptive response to nutritional effects on the primary liver and kidney metabolism in rainbow trout. Adaptive response to starvation and a high-protein, carbohydrate-free diets on glutamate dehydrogenase and alanine amino transferade kinetics.
Biochemistry and Cellular Biology 30:55–63.

Semenza, G. L. 2011. Regulation of metabolism by Hypoxia-inducible Factor 1. Cold Spring Harbor Symposium on Quantitative Biology 76:347–353.

Shimaraev, M. N., L. N. Kuimova, V. N. Sinyukovich, and V. V. Tsekhanovskii. 2002. Manifestation of global climatic changes in Lake Baikal during the 20th century. Doklady Earth Sciences 383:288–291.

Sidell, B. D., W. R. Driedzic, D. B. Stowe, and I. A. Johnston. 1987. Biochemical correlations of power development and metabolic fuel preferenda in fish hearts. Physiological Zoology 60:221 232.

Somero, G. N. 1995. Proteins and temperature. Annual Review of Physiology 57:43–68.

Somero, G. N. 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine “winners” and “losers.” Journal of Experimental Biology 213:912–920.

Takhteev, V. V. 2000. Trends in the evolution of Baikal amphipods and evolutionary parallels with some marine Malacostracan fauna. Advances in Ecological Research 31:197–220.

Timofeyev, M. A., K. A. Kirichenko, Z. M. Shatilina, A. V. Rokhin, and T. P. Pobezhimova. 2010. Attitude of Baikal and Palearctic amphipods to oxygen as a factor of environment and mechanism of adaptation to its lowered level. Contemporary Problems of Ecology 3:522–527.

Timofeyev, M. A., J. M. Shatilina, and D. I. Stom. 2001. Attitude to temperature factor of some endemic amphipods from Lake Baikal and Holarctic *Gammarus lacustris* Sars, 1863: A comparative experimental study. Arthropoda selecta 10:93–101.

Timofeyev, M., and Z. Shatilina. 2007. Different preference reactions of three Lake Baikal endemic amphipods to temperature and oxygen are correlated with symbiotic life. Crustaceana 80:129–138.

Timoshkin, O. A. 2001. Lake Baikal: fauna diversity, problems of its "immiscibility" and origin, ecology and "exotic" communities. Pages 16–73Index of animal species inhabiting Lake Baikal and its catchment area. Nauka, Novosibirsk.

Timoshkin, O. A. 2009. Index of Animal Species Inhabiting Lake Baikal and its Catchment Area. Nauka, Novosibirsk.

Vigelsø, A., N. B. Andersen, and F. Dela. 2014. The relationship between skeletal muscle mitochondrial citrate synthase activity and whole body oxygen uptake adaptations in response to exercise training. International Journal of Physiology, Pathophysiology and Pharmacology 6:84–101.

Weinberg, I. V., and R. M. Kamaltynov. 1998. Zoobenthos communities on stony beach of Lake Baikal. Zoologichesky Zhurnal 77:259–265.

Weiss, R. F., E. C. Carmarck, and V. M. Koropalov. 1991. Deep water renewal and biological production in Lake Baikal. Nature 349:665–669.

Windisch, H. S., S. Frickenhaus, U. John, R. Knust, H.-O. Pörtner, and M. Lucassen. 2014. Stress response or beneficial temperature acclimation: transcriptomic signatures in Antarctic fish (*Pachycara brachycephalum*). Molecular Ecology 23:3469–3482.

Windisch, H. S., R. Kathoever, H.-O. Pörtner, S. Frickenhaus, and M. Lucassen. 2011. Thermal acclimation in Antarctic fish: transcriptomic profiling of metabolic pathways. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 301:R1453–R1466.

Windisch, H. S., M. Lucassen, and S. Frickenhaus. 2012. Evolutionary force in confamiliar marine vertebrates of different temperature realms: adaptive trends in zoarcid fish transcriptomes. Bmc Genomics 13:549.

Yoshioka, T., S. Ueda, T. Khodzher, I. Bashenkhaeva, L. Korovyakova, L. Sorokovikova, and L. Gorbunova. 2002. Distribution of dissolved organic carbon in Lake Baikal and its watershed. Limnology 3:159–168.

Zanker, A. 1984. Detection of outliers by means of Nalimov's test. Chemical Engineering 91:74.

Supporting information

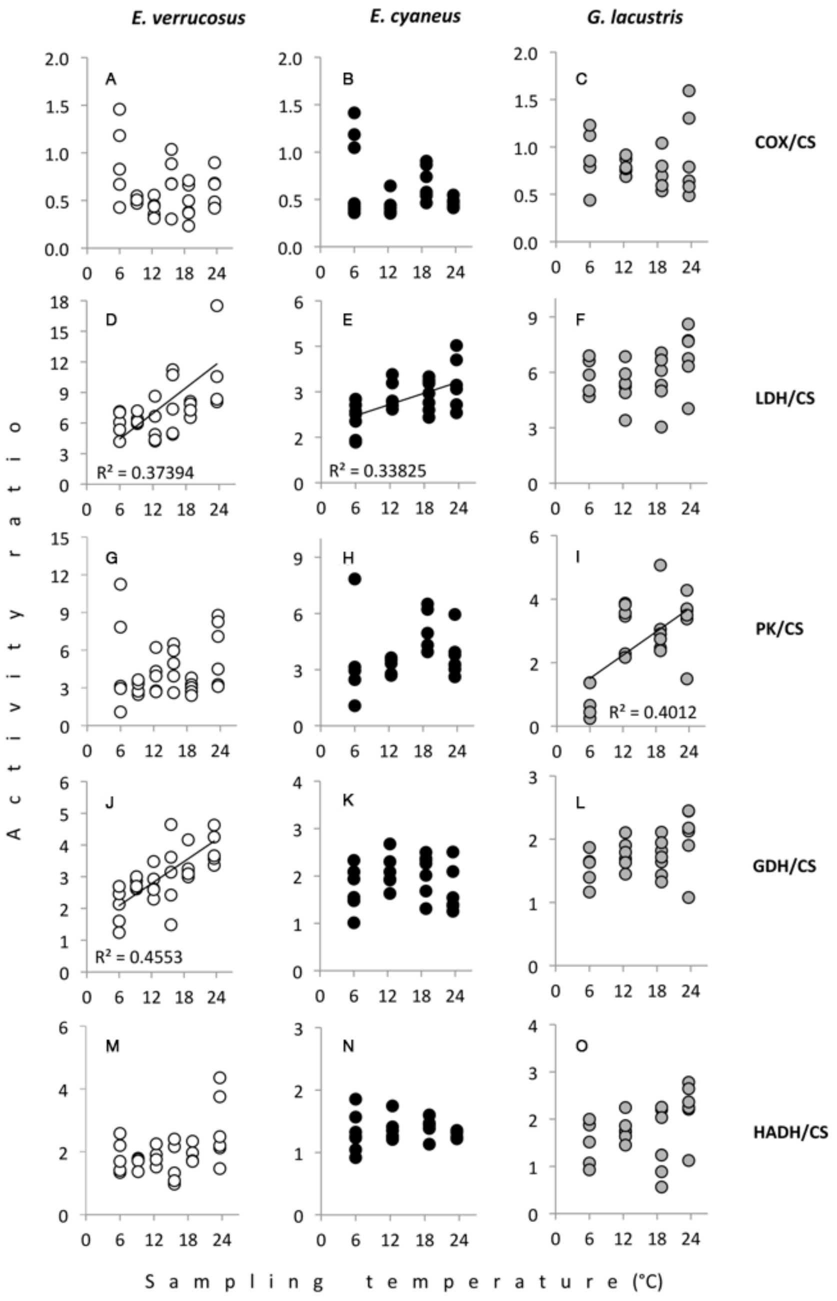


Figure S1: Maximal activities of COX, LDH, PK, HADH and GDH in relation to CS activity in *E. verrucosus*, *E. cyaneus* and *G. lacustris* exposed to a gradual temperature increase (0.8°C d^{-1}). Samples of all three species were taken at 6, 12.4, 18.8, and 23.6°C; in addition, samples of *E. verrucosus* were taken at 9.2 and 15.6°C. Measurements were performed at the respective sampling temperatures. Linear regressions and R^2 values are indicated when significant correlations were found. Data are presented as means ±SE (n = 5 - 7).

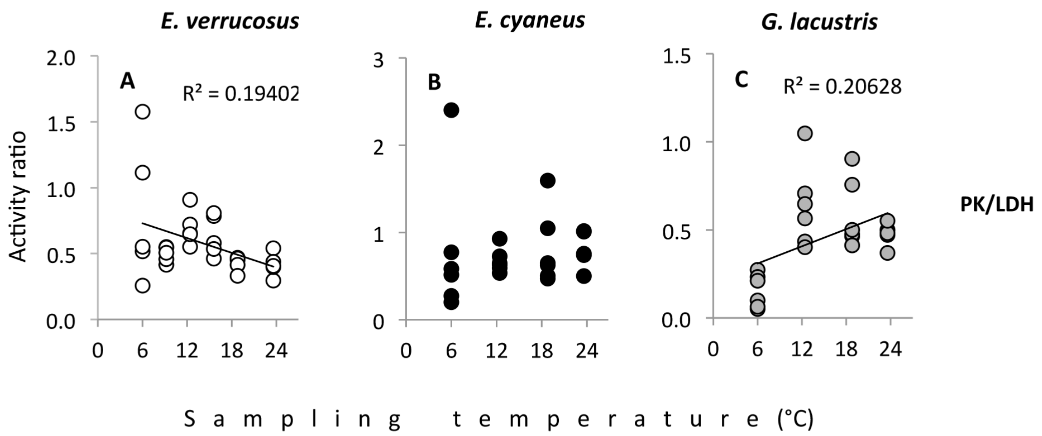


Figure S2: Maximal activity of PK in relation to LDH activity in *E. verrucosus* (A), *E. cyaneus* (B) and *G. lacustris* (C) exposed to a gradual temperature increase (0.8°C d^{-1}). Measurements were performed at the respective sampling temperatures. Data are presented as means ±SE (n = 5 - 7).

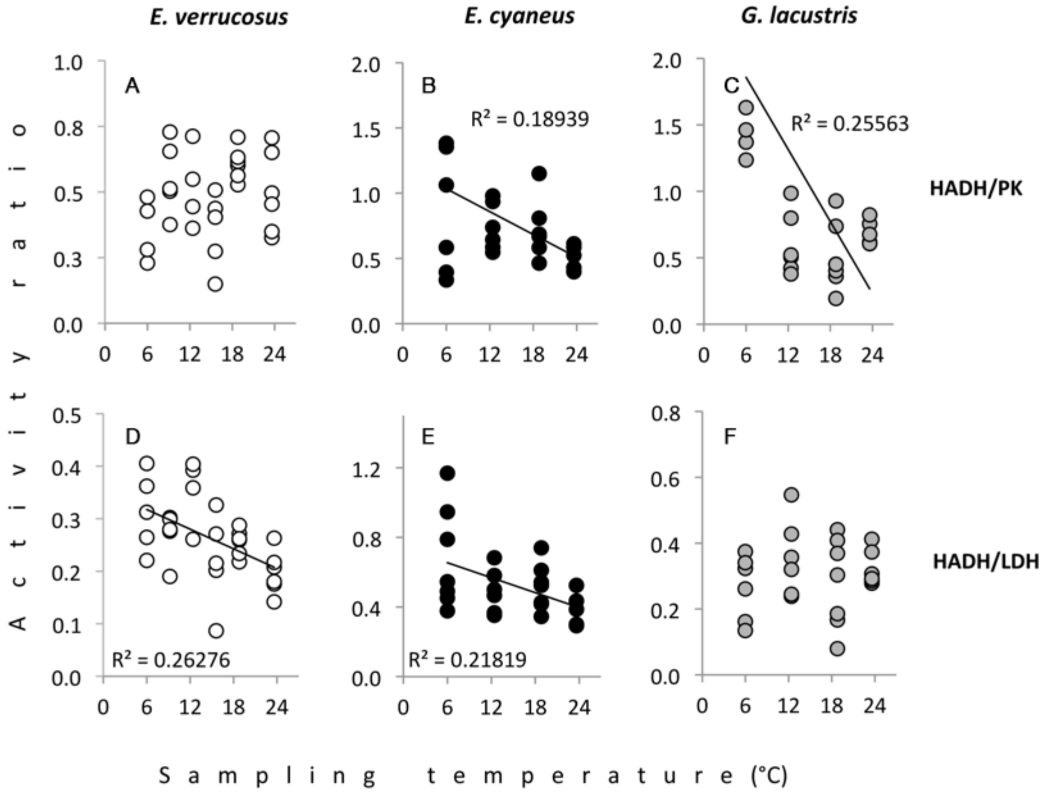


Figure S3: Maximal activity of HADH in relation to PK and LDH activity in *E. verrucosus*, *E. cyaneus* and *G. lacustris* exposed to gradual temperature increase (0.8°C d^{-1}). Measurements were performed at the respective sampling temperatures. Data are presented as means ± SE (n = 5 - 7).

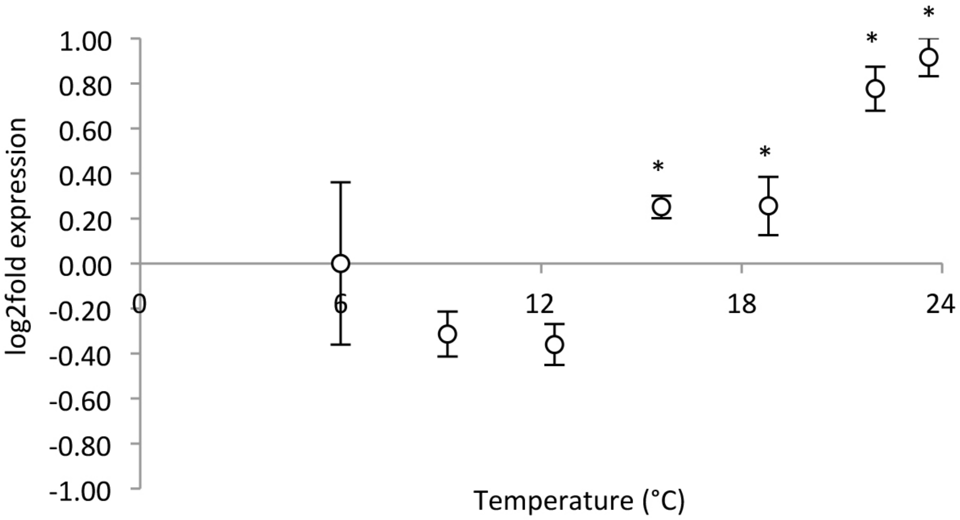


Figure S4: Expression of Phosphofructokinase-2 (PFK2) in *E. verrucosus* exposed to gradual temperature increase (0.8°C d^{-1}). Asterisks indicate data different from control values (ANOVA; $p > 0.05$). Data are presented as means ±SE (n = 5 - 6).

Publication III

Uptake kinetics and subcellular compartmentalization explain lethal but not sublethal effects of cadmium in two closely related amphipod species

L Jakob, DS Bedulina, DV Axenov-Gribanov, M Ginzburg, ZM Shatilina, YA Lubyaga, EV Madyarova, AN Gurkov, MA Timofeyev, H-O Pörtner, FJ Sartoris, R Altenburger and T Luckenbach

A previous version of this manuscript was submitted to Environmental Science & Technology in May 2016 and declined with encouragement to resubmit, the resubmission is planned for October 2016

Uptake kinetics and subcellular compartmentalization explain lethal but not sublethal effects of cadmium in two closely related amphipod species

Lena Jakob*[ab], Daria S. Bedulina[c], Denis V. Axenov-Gribanov[c], Michael Ginzburg[a], Zhanna M. Shatilina[c], Yulia A. Lubyaga[c], Ekaterina V. Madyarova[c], Anton N. Gurkov[c], Maxim A. Timofeyev[c], Hans-O. Pörtner[ab], Franz J. Sartoris[a], Rolf Altenburger[d] and Till Luckenbach[d]

[a] Department of Integrative Ecophysiology, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570, Bremerhaven, Germany

[b] Faculty of Biology and Chemistry, University of Bremen, Leobener Straße, 28359 Bremen, Germany

[c] Institute of Biology, Irkutsk State University, Karl Marx str.1, 664003 Irkutsk, Russia

[d] Department of Bioanalytical Ecotoxicology, UFZ – Helmholtz Centre for Environmental Research, Permoserstr. 15, 04318 Leipzig, Germany

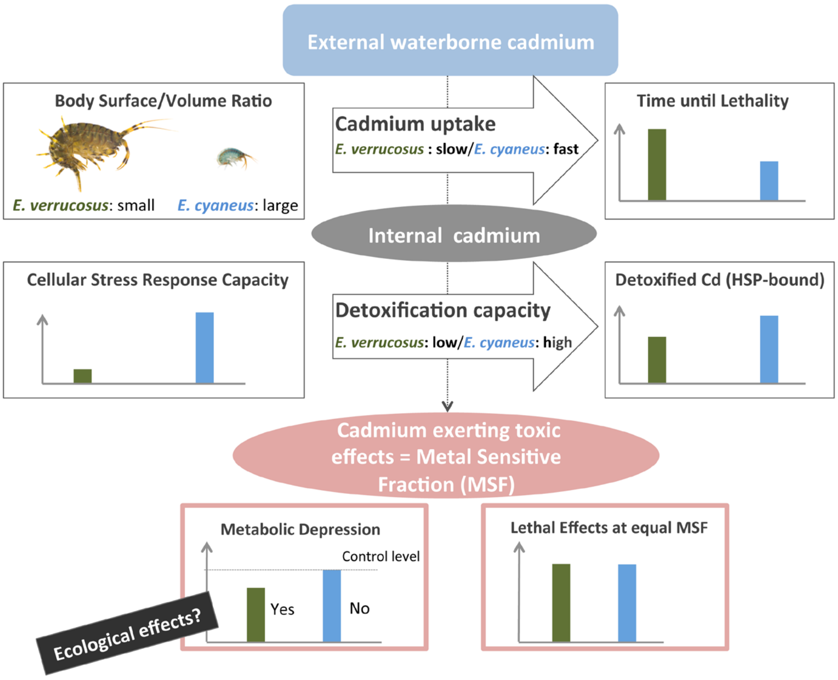
* Corresponding author: Lena.Jakob@awi.de

Abstract

This study aimed to compare toxic sensitivities to waterborne cadmium (Cd^{2+}) of two closely related amphipod species endemic to Lake Baikal with different body sizes and concentrations of cellular stress response (CSR) proteins. Higher Cd uptake rates of *Eulimnogammarus cyaneus* (fresh weight: 20 - 44 mg) than in *Eulimnogammarus verrucosus* (fresh weight: 418 - 942 mg) in concentration-mortality-studies (4 week; 6°C) explained that lethal concentrations of *E. cyaneus* were lower than in *E. verrucosus*. When exposed to the respective LC1 (Cd concentration inducing 1% of mortality; *E. cyaneus*: 18 nM and *E. verrucosus*: 115 nM; 4 weeks at 6°C), the metal sensitive tissue fractions of Cd (MSF) were similar in *E. verrucosus* (0.26 ± 0.07 µg g (fresh weight)$^{-1}$) and *E. cyaneus* (0.25 ± 0.06 µg g (fresh weight)$^{-1}$) (6°C, 4 wk). In accordance with the higher constitutive concentration of CSR proteins of *E. cyaneus* than in *E. verrucosus*, more Cd was biologically detoxified in the former species (*E. verrucosus* = 0.66 ± 0.11 and *E. cyaneus* = 0.83 ± 0.13 µg g (fresh weight)$^{-1}$). LC1 exposure induced decreases in respiration and ventilation (15 – 38% in comparison to parallel controls) at all sampling time points only in *E. verrucosus* but not in *E. cyaneus*. The physiological trait of decreasing metabolism, which has evolved to endure adverse conditions in the short-term, might turn detrimental in the presence of persisting toxicant pressure in the long-term.

Key words: Crustacea, cadmium, metabolic rate, ventilation, sublethal effects, metabolic depression, metal sensitive fraction, aquatic toxicology

Graphical Abstract



Introduction

Susceptibility of aquatic organisms to dissolved metal varies largely due to differences in bioavailability, bioaccumulation kinetics and internal distribution of metals (Rainbow 2002). Waterborne cadmium enters the body of aquatic organisms mainly through permeable body surface such as gill epithelia (Marsden and Rainbow 2004). According to the biotic ligand model (BLM) only the free ion is available for uptake (Campbell et al. 2002, Luoma and Rainbow 2008, Lavoie et al. 2012). This is due to the intrinsically low permeability of epithelia to dissolved hydrophilic trace metal ions; uptake is thus thought to be facilitated by diffusion through calcium channels as cadmium and calcium ions have the same charge and similar radii (Simkiss and Taylor 1995). Moreover, uptake is mediated by specific calcium transport mechanisms such as Ca^{2+}-ATPases and Na^{+}/Ca^{2+}-exchangers (McGeer et al. 2011, Tan and Wang 2011). Aquatic organisms show highly variable calcium (cadmium) uptake rates because of their species-specific calcium demands (Philips and Rainbow 1994). Thus, both metal speciation in the exposure medium and physiological regulation of (cation) transport systems are important factors for metal toxicity (Campbell et al. 2002, Luoma and Rainbow 2008, Lavoie et al. 2012). Consequently, cadmium uptake was explained by phylogenetic factors, however, only when accounting for body size (Buchwalter et al. 2008) because (permeable) body surface increases with body size to a higher degree than body volume. Gill surface area in amphipods, for instance, was shown to be relatively large in small amphipods compared to larger ones (Moore and Taylor 1984).

There is no known critical threshold of total body concentration of accumulated metal determining the onset of toxic effects (Rainbow 2002, Rainbow and Luoma 2011). Rather, toxicity is related to a critical concentration of metabolically available accumulated metal that builds up when the rate of metal influx exceeds the combined rates of excretion and detoxification (Rainbow 2002). Bioaccumulation kinetics can be deduced from the biodynamic metal bioaccumulation model, which combines targeted, geochemical data from a site of interest with parameterization of key physiological constants for a species from that site (Luoma and Rainbow 2005). Metabolically available accumulated metal can pragmatically be determined by subcellular compartmentalization (Wallace et al. 2003, Vijver et al. 2004). The underlying principle of this approach is that metal toxicity is predominantly explained by the amount of metal associated with enzymes and cell organelles contained within the "metal sensitive fraction" (MSF) post tissue fractionation. By contrast, metal that is bound to heat stable proteins or metal rich granules is considered as biologically detoxified (Wallace et al. 2003, Vijver et al. 2004, Wang and Rainbow 2006). However, behavioral and sublethal effects of aquatic organisms to the same concentrations of metabolically available metal have yet not been studied across a set of different species; effects may vary depending on the stress response strategy.

Animals may adopt different strategies to cope with environmental stress such as elevated trace metal concentrations that elicit various cellular, physiological and behavioral responses (Sulmon et al. 2015). Cellular stress response systems are important mediators of resistance against adverse conditions such as exposure to chemical or thermal stressors (Kültz 2005). The universal stress response system that cells of animals are generally equipped with is activated as a reaction to damage of biological macromolecules regardless of the type of stress (Kültz 2005). Components of the cellular stress response system mitigate or repair the damage of macromolecules and mediate transformation or sequestration of stress causing agents (Kültz 2005). Behavioral and physiological strategies can be directed to reduce stress impact and may include down-scaling of metabolic activity (Storey and Storey 2004).

Crustaceans are particularly sensitive to toxic metals like cadmium (Von Der Ohe and Liess 2004) and frequently included in risk assessments. Lake Baikal endemic amphipods (Amphipoda, Crustacea) constitute key components of benthic communities from littoral to deepwater zones of the lake (Rusinek et al. 2012). Cadmium concentrations of Baikal water and biota are presently very low (Ciesielski et al. 2016) but industrialization and further sources of pollution (e.g., thawing of permafrost, agriculture and tourism) of the region have increased significantly and progressively during the last decades (Moore et al. 2009, Thorslund et al. 2012). As the Baikal fauna has experienced stable abiotic conditions for millions of years (Kozhova and Izmest'eva 1998) it is unclear whether Baikal endemics are able to adapt to a rapidly changing environment. Pollutants that enter the Lake nowadays could build up for centuries to come because water prevails in Lake Baikal for about 377 - 400 years (Gronskaya and Litova 1991). The extremely low mineralization and low calcium ion concentration in Baikal water (Ca^{2+} = 402 µmol L^{-1} Na^{+} = 155, Mg^{2+} = 126, Cl^{-} = 12 and SO_4^{2-} = 57 µmol L^{-1}) (Zerbst-Boroffka et al. 2000) can increase the toxicity of cadmium to aquatic organisms (Hollis et al. 2000). Similar observations have been made under reduced salinity conditions (Bjerregaard and Depledge 1994, Barbieri and Paes 2011).

The objectives of this study were to study cadmium uptake of waterborne cadmium, subcellular compartmentalization and sublethal physiological effects (ventilation and metabolic rate) of endemic amphipods to cadmium exposure in a comparative approach. The species chosen for this study are closely related but differ with regard to parameters that may determine chemical uptake and toxic sensitivity. *Eulimnogammarus cyaneus* (Dybowski, 1874) is smaller than *Eulimnogammarus verrucosus* (Gerstfeldt, 1858) and thus, likely to possess higher cadmium uptake rates. *E. cyaneus*, however, may possess a higher potential to detoxify or mitigate the effects of internal cadmium than *E. verrucosus*, owing to its higher constitutive of cellular stress response related proteins (Bedulina et al. 2013).

Concentration-mortality relationships were determined to derive low biologically effective doses, which were then applied to investigate physiological responses on an effect-scaled basis. Uptake kinetics and metal compartmentalization were studied to discriminate between the influences of these two factors on effect propagation. The extent to which sublethal concentrations elicit adverse functional effects was investigated through the quantification of physiological parameters. Experimental exposures were performed over four weeks to simulate long-term exposure conditions in-situ.

Material and Methods

Animal sampling

Adult (identified by size (Timofeyev et al. 2001)) *E. cyaneus* and *E. verrucosus* specimens were collected by kick-sampling (Hynes 1961) in the littoral zone of Lake Baikal at depths of 0 - 1.2 m in proximity to the Bolshie Koty settlement area (51°9137″ N, 105°0691″ E). Egg-carrying females and individuals with visible parasites (i.e., leeches) were excluded. Immediately after sampling, amphipods were transported to the laboratory in insulated boxes filled with Baikal water. Specimens of *E. cyaneus* (n = 150 - 200, fresh weight: 20 - 44 mg) and *E. verrucosus* (n = 25, fresh weight: 418 - 942 mg) were quickly transferred into separate 2 L polypropylene (PP) tanks (high density PP of food grade quality) containing aerated Baikal water at 6°C corresponding to the reported annual mean water temperature for the Baikal littoral (Weiss et al. 1991, Falkner et al. 1991, Yoshioka et al. 2002). About two thirds of the tank bottoms were covered with small pebbles from the littoral, which were previously cleaned with boiling Baikal water. Animals were kept in acclimation tanks for at least two days to assure that all animals were intact. Experiments were performed in 2013 (July - October) at the field station of Irkutsk State University in Bolshie Koty.

Long-term concentration-mortality relationships

Prior to experiments, tanks were prepared by keeping them filled with Baikal water for two weeks exchanging the water once every other day. Tanks were then pre-soaked with water containing Cd at the respective exposure concentrations for two days. Individuals of *E. cyaneus (n = 15)* and *E. verrucosus (n =10)* were placed in each plastic container with 0.4 and 1.2 L of well-aerated $CdCl_2$-contaminated 6°C Baikal water, respectively. In parallel to a water control devoid of additional Cd, treatments were set up with 8.7, 35, 105, 210, 419, 559, 1117 and 2235 nM Cd (nominal; three tank replicates per concentration) following the OECD guideline for the testing of chemicals that requires at least five geometric concentrations (OECD 2012). The applied concentrations were based on previously conducted range-finding tests. Tanks were randomly distributed in a laboratory refrigerator. Water with respective Cd concentrations was exchanged every fourth day. During the 4-weeks exposure, the omnivorous animals were fed *ad libitum* with a mix of amphipods, algae and water plants collected in the Baikal littoral (frozen, air-dried at ≈30°C and roughly mortared). Low background concentrations of Cd were measured in amphipods and phytoplankton of the sampling area (range: 0.0375 – 0.804 µg g (dry weight)$^{-1}$) (Ciesielski et al. 2016). Thus, a noteworthy contribution of the applied food to metal uptake in the studied amphipods can be excluded. Mortality was monitored daily and dead animals were removed.

Physiological experiments

EXPERIMENTAL DESIGN AND ANIMAL MAINTENANCE

Animals were exposed for four weeks at 6 ± 0.8°C to their species-specific LC1 (lethal concentrations for 1% of the test groups; LC1 for *E. verrucosus* = 115 nM, LC1 for *E. cyaneus* = 18 nM) as sublethal concentration. Water was exchanged every fourth day. Oxygen consumption and ventilation rates were determined weekly as well as hemolymph pH (measured only in *E. verrucosus*; small size of individuals precluded measurements in *E. cyaneus*). Hemolymph samples for analysis of major cations (Na^+, K^+, Mg^{2+} and Ca^{2+}) and whole animal samples for Cd analysis were taken at the same time points, frozen in liquid nitrogen and stored at -80°C. All parameters were analyzed in parallel also in controls.

ROUTINE METABOLIC RATE AND RESTING VENTILATION

Experimental set up and measurement procedures are described in detail elsewhere (Jakob et al. 2016). In brief, routine metabolic rate was measured as oxygen consumption in flow-through respiration chambers equipped with optical sensors Microx TX3 and OXY4 and Oxygen Micro-Optode (NTH-PSt1-L5-TF-NS*35**x1,20-PC3,1-YOP) (Presens, Regensburg, Germany). Teflon® chambers were closed with plexi-glass lids and submerged in 2 L temperature-controlled water tanks (6.0 ± 0.2°C). For analysis of ventilation rates (= pleopod beat rates) animals were placed in Teflon® chambers with perforated depressions to allow unhindered water circulation and pleopod movements were video-recorded. Video sequences (if necessary in slow-motion) were analyzed by eye at the computer screen to quantify pleopod beat rates (Hz).

HEMOLYMPH ION REGULATION AND PH

Concentrations of the major hemolymph cations Na^+, K^+, Mg^{2+} and Ca^{2+} were determined in diluted hemolymph samples. For hemolymph extraction, amphipods were carefully patted dry with lint-free paper towel and glass capillaries with ultrafine tips were introduced dorsally without damaging the intestines. Samples were stored at -20 – (-25)°C prior to analysis. Ion concentrations were determined chromatographically with a Dionex ICS 1500 equipped with an Ion Pac CS 16 column operated at 40°C. Methane sulfonic acid (30 mM) was used as eluent at a flow rate of 0.36 mL min^{-1}. Three point calibrations of the Dionex™ Combined Six Cation Standard-I (Dionex GmbH, Idstein, Germany) were used for peak identification and peak area based quantification.

Hemolymph pH was measured with a microelectrode (InLab® Ultra-Micro, Mettler Toledo, Giesen, Germany) in hemolymph immediately after extraction. Measurements were performed at 6 ± 0.2°C in a water bath.

Cadmium analysis

SUBCELLULAR CADMIUM COMPARTMENTALIZATION

Cadmium was quantified in five subcellular compartments: heat stable proteins (HSP; fraction contains MTLPs), metal rich granules and undigested exoskeleton (MRG+exo), heat denaturable proteins (HDP), organelles and cell debris. For quantification of Cd in these compartments, amphipods were subjected to fractionation with differential centrifugation (Wallace et al. 2003) and adapted the protocol of Khan et al. (2010) who investigated subcellular fractionation in

Gammarus pulex. Due to the small size of *E. cyaneus* four animals were pooled for each sample; *E. verrucosus* samples consisted either of the anterior or the posterior half of an individual that were separated with a scalpel. Prior to tissue extraction weakly bound external Cd was removed by thorough washing of animals in 10 mM EDTA solution with a physiological content of NaCl and KCl to prevent cellular water loss by osmosis and subsequent quick rinsing with deionized water. This cleaning procedure was repeated once. Tissues were then homogenized with an Ultra-Turrax (20 - 30 sec at 17,000 rpm) in 1.5 mL ice-cold Tris-HCl buffer (pH 8.0). Homogenates were centrifuged at 1,800 x g (15 min, 4°C). The supernatant was ultracentrifuged at 100,000 x g (1 h, 4°C) in order to produce a pellet containing mitochondria, lysosomes and microsomes (= organelle fraction). The ultracentrifuge supernatant was incubated at 80°C for 10 min and kept on ice for 1 h to precipitate HDP. HSP and HDP fractions were separated by ultracentrifugation at 50,000 x g (12 min). The pellet of the first centrifugation step was resuspended in 1 mL deionized water and heated to 100°C for 2 min. Subsequently, it was digested with 1.5 mL 1M NaOH (1h, 70°C) and centrifuged at 10,000 x g (10 min) for separating the MRG + exo fraction which was pelleted and digested exoskeleton, nuclei and cell debris (= debris fraction) contained in the supernatant. All fractions were transferred to microwave Teflon® tubes; 5 mL of 65% HNO_3 (p.a. degree; Merck, Darmstadt, Germany) were added and incubated for 4 h. Later, 0.5 mL of 30% H_2O_2 (p.a. degree; Merck Darmstadt, Germany) were added and the samples were subjected to complete digestion in a microwave-system (CEM MARSXpress, Matthews, USA). Power was gradually increased from 100 to 500 W within 5 min and held at 800 W for 15 min. After cooling, the samples were diluted with deionized water in volumetric flasks (25 mL). Rhodium Standard for ICP, (TraceCERT®, Sigma-Aldrich, Darmstadt, Germany) was used as internal standard at a concentration of 10 µg L^{-1}. Blanks were randomly included and treated like samples.

CADMIUM QUANTIFICATION

Cadmium was determined by inductively coupled plasma mass spectrometry (ICP-MS; Elan 6000 (PerkinElmer, Waltham, USA)) using Argon as nebulizer gas at a flow rate of 0.9 L min^{-1} (plasma power: 1000 W) in water samples taken at day 1 (day of water exchange) and four (shortly prior to water exchange) from aquaria as well as in tissue samples. Each sample was analyzed in triplicate. Cadmium concentrations of external standard solutions were 0.1, 1 and 10 µg L^{-1} (Multi-element Calibration Standard 3, PerkinElmer, Waltham, USA). Nominal and measured water concentrations of Cd in concentration-mortality experiments and LC1 exposures are summarized in the supporting information in Table S1 and S2, respectively.

Data analysis and statistics

The modified non-linear HILL model (Equation 1, supporting information) was fitted to concentration-mortality data in order to derive effective lethal concentrations for certain percentages of the test groups (LCx%). Data were tested for normal distribution and homoscedasticity with the Shapiro-Wilk-test and Levene's test, respectively, using SigmaPlot 12 (Systat Software GmbH, Germany). For the physiological parameters, depending on the assumptions, the t-test or the Mann-Whitney rank sum test was applied to identify significant differences ($p < 0.05$) between treatments and their parallel control groups and between the two investigated species, respectively. They are presented as means ± standard error of the mean (SE) unless otherwise indicated.

Results

Cadmium water concentrations

Measured concentrations deviated from nominal concentrations by 24 and 11% at the lowest concentration applied (8.7 nM) in *E. verrucosus* and *E. cyaneus*, respectively. Deviations from nominal concentrations increased progressively with increasing test concentrations reaching 40% (*E. verrucosus*) and 38% (*E. cyaneus*) at the highest exposure concentration (2235 nM). Between water exchanges (every fourth day) Cd concentrations decreased by 26 and 56% in the lowest exposure concentration and by 36 and 26% in the highest test concentration in treatments with *E. verrucosus* and *E. cyaneus*, respectively (Table S1, supporting information).

In LC1-exposures, measured concentrations deviated from nominal ones by 0 - 17% and decreased by 27 - 54% within four days (Table S2, supporting information). All nominal concentrations are based on the measured concentration of a Cd stock solution (4.4 mM) applied in all experiments presented here.

Deviations of measured from nominal concentrations are likely due to adsorption of Cd to tank walls, food particles and stones needed to provide shelter for the amphipods as it is known that the free Cd ion can be removed from aqueous solutions by chelation, electrostatic interaction, such as ion exchange or the formation of ionic pairs (Dzul Erosa et al. 2001).

Storage capacities for contaminated water are very limited in the field station at Lake Baikal where the experiments were carried out; more frequent water changes in the semi-static system or use of a flow through system were therefore not possible. However, as Cd concentrations decreased similarly in the experimental setups for both examined species, the deviations of measured from nominal concentrations do not affect the overall conclusions regarding species-specific differences in Cd uptake and sequestration and Cd effects found in this study.

Mortality

Time periods until lethality (lethal times; LT) were significantly shorter for *E. cyaneus* than for *E. verrucosus* in treatments with identical $CdCl_2$ concentrations as shown for the 1117 nM $CdCl_2$ treatments in Figure 1B (LT50 *E. verrucosus* = 7.6 d (95% CI = 6.8, 8.4) and LT50 *E. cyaneus* 3.1 d (95% CI = 2.8, 3.4)). Higher Cd sensitivity of *E. cyaneus* compared to *E. verrucosus* with respect to mortality is also reflected by LC50 values derived from nonlinear regressions for the concentration-mortality relationships, which were significantly lower for *E. cyaneus* than for *E. verrucosus* (159 nM (95% CI = 141, 179) vs. 366 nM (95% CI = 316, 406) (Figure 1A). No mortality was observed in controls.

In LC1 experiments, mortality in treatments and controls was < 1.5% during the entire experimental period.

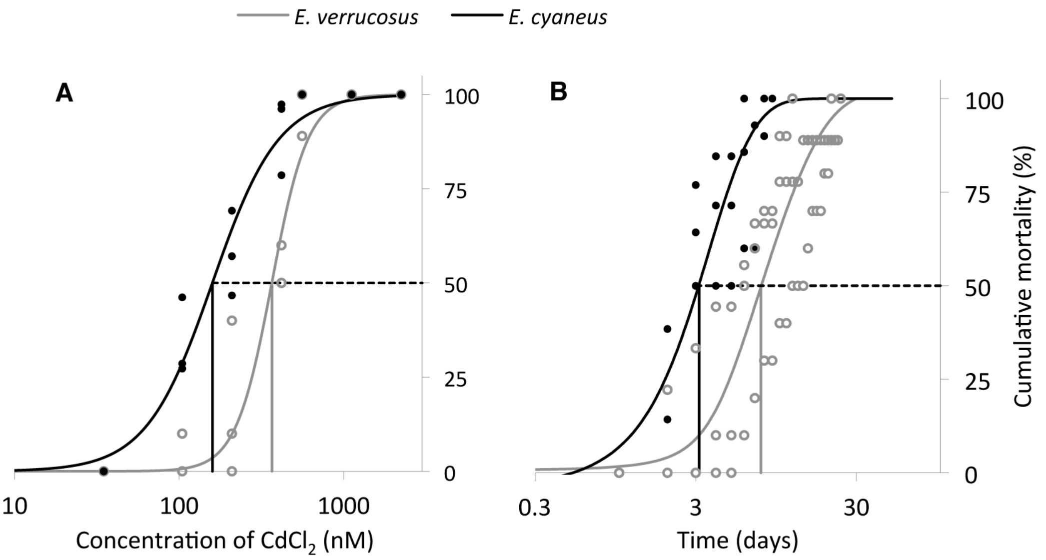


Figure 1: Cumulative mortality of *E. verrucosus* (open circles, grey curve) and *E. cyaneus* (closed circles, black curve) exposed to different nominal concentrations of $CdCl_2$ (0, 8.7, 35, 105, 210, 419, 559, 1117 and 2235 nM) **(A)** and to a nominal concentration of 1117 nM $CdCl_2$ **(B)** for 4 weeks (6°C) (n = 3). Vertical lines mark the LC50 **(A)** and the LT50 values **(B)**, respectively.

Routine metabolic rate and resting ventilation

In *E. verrucosus* exposed to Cd at LC1 oxygen consumption and ventilation rates were reduced by 15 - 36% and 18 - 38%, respectively, in comparison to parallel controls; these differences were found in all measurements performed weekly within the four-week exposure time; the difference magnitudes were independent of exposure time (Figure 2A,C). By contrast, no differences between LC1 treatments and controls were seen in *E. cyaneus* (Figure 2B,D). When both species were exposed to the same Cd concentration (35 nM) for four weeks, *E. verrucosus* showed a decrease in oxygen consumption by 26% compared to controls (control: 2.1±0.2 *vs.* treatment: 1.6±0.2; $p = 0.045$) whereas no differences from controls were found for *E. cyaneus* (control: 6.0±0.4 *vs.* treatment: 5.7±0.4; $p = 0.507$).

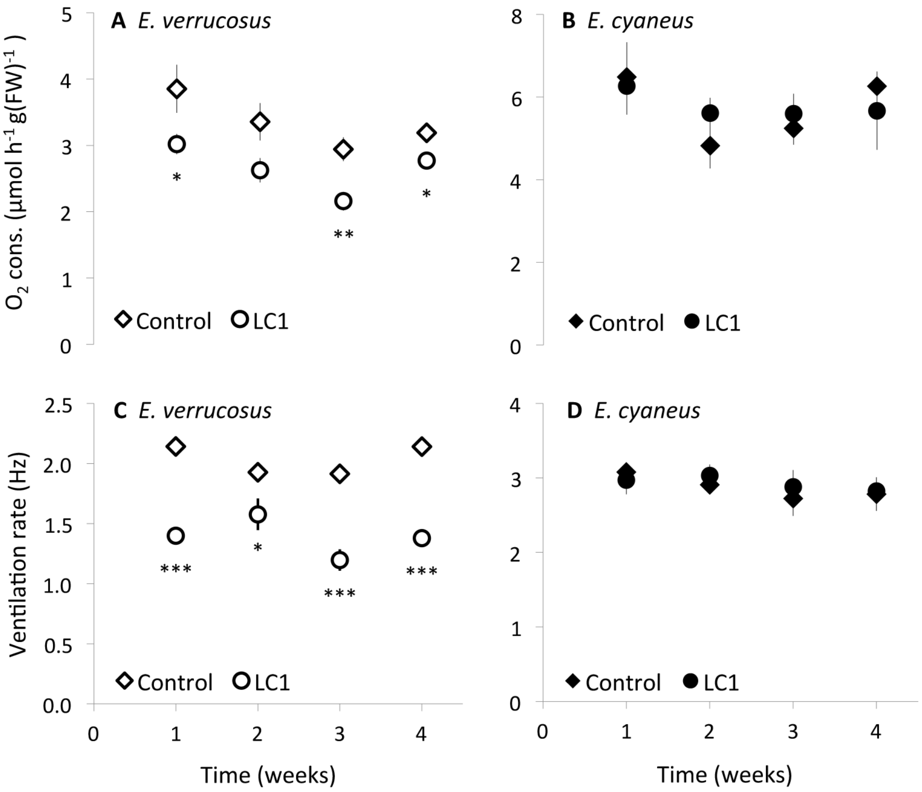


Figure 2: Oxygen consumption and resting ventilation (pleopod beating) of *E. verrucosus* **(A, C)** and *E. cyaneus* **(B, D)** exposed to their species-specific LC1 ($CdCl_2$); LC1 *E. verrucosus* = 115 nM, LC1 *E. cyaneus* = 18 nM for 4 weeks at 6°C. Circles represent LC1 treatments and rhombs parallel controls. Data are displayed as means ± SE (n = 7 - 13). Statistically significant differences between treatments and parallel controls are indicated by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Hemolymph ion concentration and pH

In *E. verrucosus* and *E. cyaneus* specimens exposed to Cd at the respective LC1 for four weeks, hemolymph levels of the major cations Ca^{2+}, Na^{+}, K^{+} and Mg^{2+} were not significantly altered from control values.

For hemolymph pH (only determined in *E. verrucosus*) no significant differences were seen between Cd treatments and controls after one week of exposure, but from week two on the pH of the hemolymph of treated animals was marginally but statistically significantly decreased in comparison to control samples (difference in pH: 0.062; $p = 0.037$) (Figure S1, supporting information).

Cadmium body burdens and compartmentalization

Total Cd body burdens (i.e., sums of Cd amounts in the different tissue fractions, which are displayed in Figure 3) progressively increased in *E. verrucosus* and did not reach equilibrium within the entire exposure time (Figure 4).

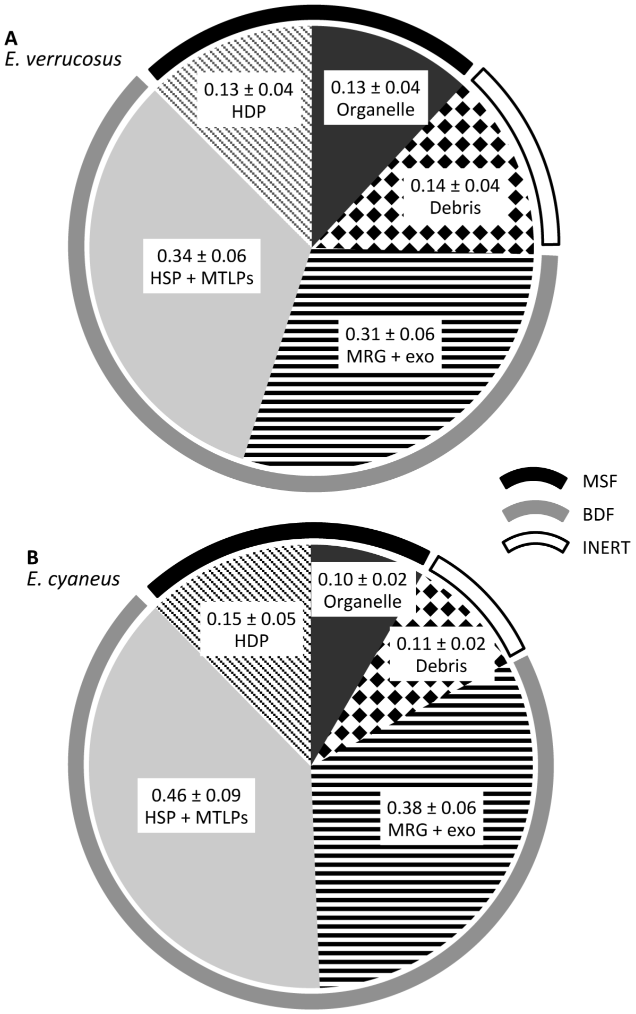


Figure 1: Cadmium concentration in different subcellular fractions of *E. verrucosus* **(A)** and *E. cyaneus* **(B)** exposed to their species-specific LC1 ($CdCl_2$), LC1 *E. verrucosus* = 115 nM, LC1 *E. cyaneus* = 18 nM for 4 weeks at 6°C. Metal sensitive fraction (MSF) = Organelle (mitochondria, lysosomes and microsomes) + HDP (heat denaturable proteins); *E. verrucosus* = 0.26±0.07 and *E. cyaneus* = 0.25 ± 0.06, Biologically detoxified fraction (BDF) = MRG+exo (metal rich granules+exoskeleton) + HSP + MTLPs (heat stable proteins including metallothionein-like proteins); *E. verrucosus* = 0.66 ± 0.11 and *E. cyaneus* = 0.83 ± 0.13 and the toxicologically inert fraction = Debris (cell debris). Data are presented as means ± SE in µg g (fresh weight)$^{-1}$ (n = 5-10).

In *E. cyaneus*, total Cd tissue levels increased until week 2 of exposure and remained more or less unchanged thereafter (Figure 4). After four weeks, bioconcentration factors (BCF) of total accumulated Cd were 79 and 500 for *E. verrucosus* and *E. cyaneus*, respectively. Major increases of Cd concentrations of the different fractions were seen between weeks 1 and 2 in *E. cyaneus* and between weeks 2 and 3 in *E. verrucosus* (Figure S2, supporting information). Cadmium concentrations of the MSF, representing the accumulated bioavailable Cd, were more than two-fold higher in *E. cyaneus* compared to *E. verrucosus* in week 3 but almost equal in weeks 2 and 4 (Figure 4). BDF-Cd was generally higher in *E. cyaneus* than in *E. verrucosus*, however, differences were only significant after two weeks of exposure ($p = 0.048$). Cadmium that is associated with cell debris (debris fractions) can be considered as toxicologically inert and makes a contribution of 9 and 14% to the total Cd body burden after four weeks in *E. verrucosus* and *E. cyaneus*, respectively. After 4 weeks, the MSF-Cd accounted for 24 and 20% and the BDF-Cd for 63 and 70% of the total Cd in *E. verrucosus* and *E. cyaneus*, respectively. The distribution of Cd among the different cellular compartments (percentages of the fractions in relation to total body concentration) was very similar to that found in *Gammarus pulex* (Khan et al. 2010).

When exposed to the same external Cd concentration (105 nM) for four weeks, the amount of BDF-Cd and MSF-Cd determined in *E. cyaneus* exceeded that found in *E. verrucosus* by 3.7 and 7.2-fold, respectively (Figure S3, supporting information).

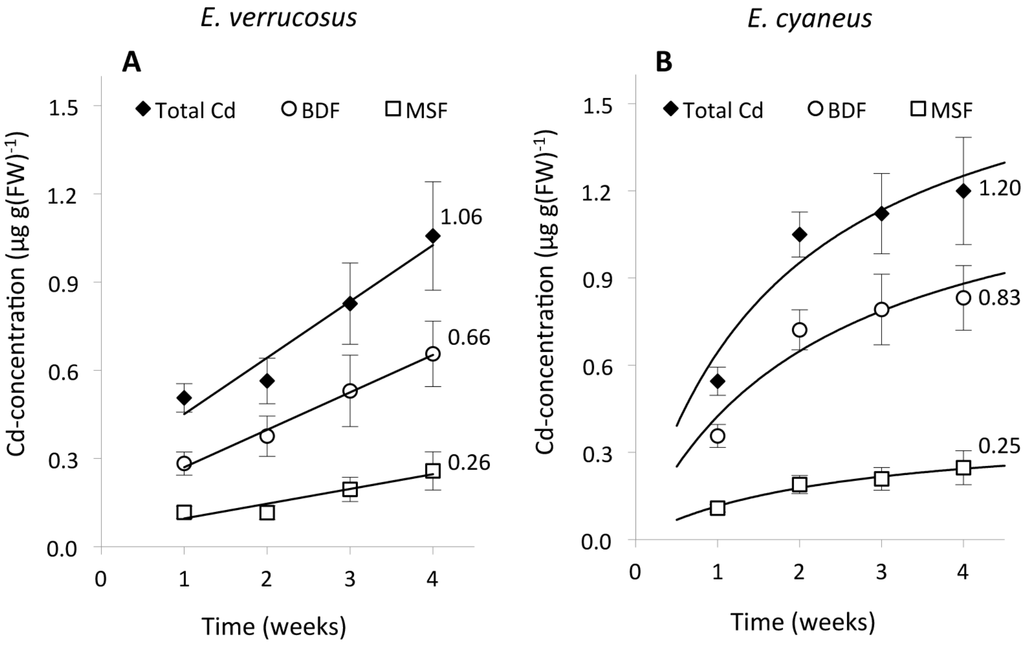


Figure 4: Amounts of total, biologically detoxified (BDF) and metal sensitive (MSF) Cd in *E. verrucosus* **(A)** and *E. cyaneus* **(B)** upon exposure to their species-specific LC1 (LC1 of *E. verrucosus* = 115 nM, LC1 of *E. cyaneus* = 18 nM) for up to four weeks. The MSF represents the biologically available Cd fraction associated with toxic effects; the BDF comprises sequestered Cd. Linear models were fit to data of *E. verrucosus* and a ligand binding (+ nonspecific binding) model was fitted to the data sets of *E. cyaneus* (equation below). Data are presented as means ± SE (n = 5 - 10).

$$y = \frac{B_{\max} x}{K_d + x} + N_s x$$

Discussion

In the present study we examined the toxic impact of Cd on two phylogenetically closely related amphipod species, *E. verrucosus* and *E. cyaneus*, that differ with regard to parameters that may determine metal uptake and toxic sensitivity, namely body size and titer of cellular stress-response related heat shock proteins. The physiological effects of exposure to the same lethal concentration of cadmium (1117 nm $CdCl_2$) were observed at shorter incubation times in *E. cyaneus* than in *E. verrocosus*. LC50 values (based on nominal concentrations) were 159 and 366 nM for *E. verrucosus* and *E. cyaneus*, respectively. The lower sensitivity of *E. verrucosus* to Cd is likely associated with lower uptake rates and may be due to its larger body size. Whereas during the 4-week exposure to species-specific LC1 ($CdCl_2$) accumulation showed saturation in *E. cyaneus* this was not the case for *E. verrucosus*. The relative internal Cd amount found in the BDF was higher in *E. cyaneus* than in *E. verrucosus*, which shows that biological detoxification of Cd is more effective in *E. cyaneus* and may mitigate toxic Cd effects to a higher degree. *E. verrucosus*, by contrast, displayed reduced metabolism in response to sublethal Cd concentrations, which may have added to the relatively low Cd uptake in specimens of this species. The finding of metabolic depression following Cd exposure exclusively in *E. verrucosus and not E. cyaneus* is remarkable considering (1) that it occurred at Cd concentrations that were substantially below lethal levels and (2) that the metabolic depression reaction did not show concentration or time dependency and paralleled decreases in ventilation. Thus, the decreases may not be directly related to metabolically available cadmium and may rather be the result of specific molecular cascades stimulated by cadmium exposure.

Uptake kinetics and compartmentalization of total cadmium body burden

Cadmium uptake proceeded slower in the larger *E. verrucosus* than in *E. cyaneus,* which is likely associated with differences in the surface/body volume ratios of the two species (Figure 4 and Figure S3) as observed in previous studies on aquatic insects (Buchwalter et al. 2008). Dependencies of Cd accumulation kinetics in relation to body size have been earlier reported for other crustaceans (mysid shrimps) (Lasenby and Vanduyn 1992) and aquatic insects (Smock 1983). Although those studies focused on surface bound metal while we studied internal metal here, similar scaling coefficients can be expected across studies as they are due to the same body surface/volume dependencies. However, similar scaling coefficients can be expected as they are due to the same body surface/body volume dependencies (Buchwalter et al. 2008). Observed levels of total body concentrations of cadmium in control animals (kept in clean Baikal water) further support the uptake/body size dependency described above as concentrations of 0.03 (range: 0.03 - 0.04) and 0.09 (range: 0.08 – 0.10) µg g (FW)$^{-1}$ were determined in *E. verrucosus* and *E. cyaneus*, respectively (n = 4) (data not shown).

Whether bioaccumulation of Cd reaches steady state within the experimental exposure period depends on external concentration, lifetime, uptake and elimination rates, as well as on internal storage capacity, which is often related to body size. Amphipods are net accumulators of trace metals and typically their steady state body concentrations increase with local metal availability (Rainbow 1998, 2002). In *E. cyaneus* exposed to its LC1 ($CdCl_2$), our data indicate that equilibrium is approached after approximately two weeks. A similar time period until reaching equilibrium has been observed in the small freshwater amphipod *Hyalella azteca* when exposed to 0.8 nM of cadmium (Stephenson and Turner 1992). However, Cd concentrations in *E. verrucosus* increased progressively over the entire experimental period and did not reach a steady state. This comparison shows that it is important to consider uptake kinetics when estimating metal toxicity as otherwise the sensitivity of species with slow Cd accumulation (e.g. *E. verrucosus)* might be misleadingly interpreted to be comparatively low and field effects may consequently be underestimated.

Previously, it has been stated that Cd toxicity occurs when excretion and detoxification rates are exceeded by Cd uptake leading to metabolically available excess Cd (Rainbow 2002). Our results emphasize that compartmentalization of the total accumulated amount of a metal contributes to explain differences in toxicity because only a portion of the total body burden exerts toxic effects (Vijver et al. 2004). Similar susceptibility of closely related species due to similar accumulation into potentially sensitive compartments has been previously shown for aquatic insects (Buchwalter et al. 2008). In accordance with the results of Buchwalter et al. (2008), derivation of species-specific effective doses (LC1) resulted in the same amounts of MSF-Cd in the species studied here (*E. verrucosus* = 0.26 ± 0.07 µg g (fresh weight)$^{-1}$; *E. cyaneus* = 0.25 ± 0.06 µg g (fresh weight)$^{-1}$ (6°C, 4 wk)). Based on these observations we suggest that MSF-Cd is a good predictor for lethal effects in *E. verrucosus* and *E. cyaneus*. By contrast, the total Cd body burden was higher in *E. cyaneus* mainly due to a higher Cd content in the HSP fraction (including MTLPs). Although induction of small heat shock proteins by Cd was found in both species in a 24 h-study (Timofeyev et al. 2008), *E. cyaneus* started from a 5-fold higher constitutive level of Hsp70 than *E. verrucosus* under normal physiological conditions (Bedulina et al. 2013).

Animals exposed to high bioavailable metal concentrations may show higher amounts of metal detoxified in lysosomes contained in the organelle fraction (Rainbow et al. 2015). Pre-exposure to high levels of bioavailable cadmium can be excluded as a confounding factor for our study since the cadmium concentration at our sampling site was low (Ciesielski et al. 2016). However, for general applicability, lysosomes/microsomes can be isolated upon subcellular metal partitioning (Rosabal et al. 2014).

In conclusion, differences in uptake kinetics and Cd compartmentalization likely explain the observed differences in mortality. Metal accumulation and sequestration are species-specific and only explicit consideration of the uptake kinetics as well as determining the amount of Cd associated with the MSF can resolve the relevant metrics for effective lethal doses, which were similar across the two species. In turn, considering both uptake kinetics and compartmentalization of metals will likely improve the quality of models aiming to predict biologically effective lethal effects.

Mechanisms of stress response

Uptake kinetics and compartmentalization of Cd provide explanations for the different species-specific concentration-mortality relationships but not for the different physiological responses (i.e. reduction of ventilation and metabolic rates) to low Cd doses. In contrast to *E. cyaneus*, *E. verrucosus* showed metabolic depression when exposed to its LC1 ($CdCl_2$) (115 nM) and at 35 nM (4 wk; 6°C); the degree of metabolic depression was comparable at these two concentrations, i.e. it showed no concentration dependence. Further, under LC1 exposure there was no time dependence although metabolically available cadmium increased over time. Metabolic depression is a strategy that has been recorded in all major animal phyla and is reported to maximize survival time under adverse environmental conditions such as hyperthermia, hypoxia, desiccation, hypersalinity and food deprivation (Guppy and Withers 1999, Storey and Storey 2004). The strategy of decreasing metabolic rate involves a reduction in energy utilization and implies extended use of stored energy reserves, and usually involves reduced activity and physiological rates (e.g., heart rate and ventilation) (Guppy and Withers 1999, Storey and Storey 2004).

Studies on the estuarine crab *Chasmagnathus granulata* or the white shrimp *Litopenaeus vannamei* showed that Cd causes gill impairment (e.g. necrotic tissue, gill thickening) and consequently hinders oxygen diffusion across the gill surface at higher Cd concentrations than applied here (Rodriguez et al. 2001, Wu and Chen 2004). However, gill impairment at sublethal Cd concentrations might not necessarily be the reason for decreased oxygen consumption in *E. verrucosus* since we neither found a progressive decrease of these rates over time nor dose-dependence of the effect as seen in other studies (Gaudy et al. 1991, Barbieri and Paes 2011). Moreover, ventilation rates in *E. verrucosus* decreased in concert with decreased oxygen consumption rates, which suggests a mechanistic link between the two. The finding of only a negligible affect on the pH of the hemolymph in *E. verrucosus* and the unchanged concentrations of major hemolymph cations in *E. verrucosus* and *E. cyaneus* in LC1 exposures, support the assumption that hemolymph homeostasis was only negligibly affected and, by extension, that severe toxic effects were absent. Thus, it is unlikely that damaged gill tissue was the reason for the decreased oxygen consumption. The negligible pH effects might be due to the fact that hemocyanin binds Cd, which might reduce its buffering capacity as Cd^{2+} replaces Ca^{2+} at its binding sites (Brouwer et al. 1983). Hemocyanin, the oxygen transporting protein, is the major hemolymph protein in crustaceans (90 - 95%) (De Fur et al. 1985) and buffers hemolymph pH. Further, the altered metabolism may have caused the slightly changed hemolymph pH.

Perspectives

The ecophysiology of the two amphipod species studied here may contribute to explain their different reactions to low doses of Cd. *E. verrucosus* is thermally sensitive and has a comparably low upper thermal limit in comparison to *E. cyaneus* (Jakob et al. 2016). Hypoxemia, which develops in the animal toward both ends of the thermal window likely contributes to the pattern of oxidative stress (Heise et al. 2007). As cadmium was also shown to induce oxidative stress (Nemmiche et al. 2011) and causes depletion of oxygen availability in cells, which causes hypoxia (Simmons et al. 2009), we suggest that an at least partly similar mode of action of the two stressors in accordance with previous propositions (Lushchak 2011). *E. verrucosus* exhibits behavioral plasticity to cope with adverse environmental conditions. In summer, it escapes rising temperatures in the upper littoral by migration to deeper and cooler waters (Weinberg and Kamaltynov 1998, Jakob et al. 2016) and in experimental setups it was shown to avoid reduced oxygen concentrations (Timofeyev and Shatilina 2007). Although competitive pressure in the littoral benthos of Lake Baikal is probably high as it is densely populated by 93 amphipod species at the sampling site close to Bolshie Koty (Kravtsova et al. 2003), the relatively large size of adult *E. verrucosus* individuals enables their migration to different zones withstanding competition with other species. However, escaping from environmental pollution might not be possible.

We highlight the fact that metabolic depression in *E. verrucosus* occurred at relatively low stress levels (LC1 = 115 nM; 35 nM) and was permanent during the exposure time. Metabolic depression is typically paralleled by reduced activity, food uptake and reproduction (Guppy and Withers 1999, Storey and Storey 2004). In the short term, this strategy may help to avoid adverse impacts of chemicals. However, in the long run, metabolic depression may have negative consequences on the population level. Less active organisms are more vulnerable to predation, reduced food uptake may result in depletion of internal energy resources and reduced reproduction will lead to lower recruitment. Thus, even low levels of a chemical stressor may have dramatic consequences on *E. verrucosus* populations in the wild. Amphipod diversity in the Baikal littoral is extremely high but *E. verrucosus* as also *E. cyaneus* are among species with large population sizes and impacts on their population structure is likely to have dramatic consequences on the ecosystem.

In contrast to *E. verrucosus, E. cyaneus is* a sedentary species of the upper littoral and does not migrate to escape environmental changes. The species is exposed to large changes in environmental conditions such as seasonal temperature fluctuations from close to freezing in winter to up to 20°C in summer (Timoshkin 2009). *E. cyaneus* did not show metabolic depression in our experiments. It might rather respond to adverse conditions by increasing cellular stress defense mechanisms, e.g., by increasing catalase activity at low Cd concentrations as shown in other freshwater crustaceans (Lei et al. 2011). This may lead to increased energy demand to support these processes and might alter the energy budget of *E. cyaneus.* Shifts in energy budget at unchanged respiration rates have been observed e.g. in *Porcelio scaber* when exposed to Cd (Khalil et al. 1995).

In conclusion, further sublethal effects such as on somatic growth, reproduction, enzyme activities and energy state caused by low doses of Cd remain to be studied in the here investigated species to identify possible further processes that could ultimately lead to reduced Darwinian fitness of a species. However, the extreme sensitivity of an important Lake Baikal endemic amphipod to low levels of a chemical stressor underscores the necessity of water management strategies required to strictly avoid the chemical contamination of Lake Baikal waters.

Acknowledgements

We highly acknowledge Ilsetraut Stölting for performing ICP-MS analyses and Birgit Daus for technical advice. Moreover, our thanks go to Sabine Kasten and Ingrid Stimac who provided their laboratory and microwave equipment. The students of Irkutsk State University and Michael Meyer are acknowledged for laboratory assistance. This study was performed within the LabEglo project HRJRG-221, financed by the bilateral funding program “Helmholtz-Russia Joint Research Groups” (HRJRG) from the Helmholtz Association and the Russian Foundation for Basic Research (RFBR). Russian participants received further funding by the Russian Science Foundation (grant: 14-14-00400), the RFBR (grants: 14-04-00501 and 15-04-06685), and were partially supported by the Civilian Research and Development Foundation (grant: FSCX-15-61168-0).

References

Barbieri, E., and E. T. Paes. 2011. The use of oxygen consumption and ammonium excretion to evaluate the toxicity of cadmium on *Farfantepenaeus paulensis* with respect to salinity. Chemosphere 84:9–16.

Bedulina, D. S., M. B. Evgen'ev, M. A. Timofeyev, M. V. Protopopova, D. G. Garbuz, V. V. Pavlichenko, T. Luckenbach, Z. M. Shatilina, D. V. Axenov-Gribanov, A. N. Gurkov, I. M. Sokolova, and O. G. Zatsepina. 2013. Expression patterns and organization of the hsp70 genes correlate with thermotolerance in two congener endemic amphipod species (*Eulimnogammarus cyaneus* and *E. verrucosus*) from Lake Baikal. Molecular Ecology 22:1416–1430.

Bjerregaard, P., and M. H. Depledge. 1994. Cadmium accumulation in Littorina littorea, *Mytilus edulis* and *Carcinus maenas*: the influence of salinity and calcium ion concentrations. Marine Biology 119:385–395.

Brouwer, M., C. Bonaventura, and J. Bonaventura. 1983. Metal-ion interactions with *Limulus polyphemus* and *Callinectes sapidus* hemocyanins - Stochiometry and structural and functional consequences. Biochemistry 22:4713–4723.

Buchwalter, D. B., D. J. Cain, C. A. Martin, L. Xie, S. N. Luoma, and T. Garland. 2008. Aquatic insect ecophysiological traits reveal phylogenetically based differences in dissolved cadmium susceptibility. Proceedings of the National Academy of Sciences of the United States of America 105:8321–8326.

Campbell, P. G. C., O. Errecalde, C. Fortin, W. R. Hiriart-Baer, and B. Vigneault. 2002. Metal bioavailability to phytoplankton - applicability of the biotic ligand model. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 133:189–206.

Ciesielski, T. M., M. V. Pastukhov, S. A. Leeves, J. Farkas, S. Lierhagen, V. I. Poletaeva, and B. M. Jenssen. 2016. Differential bioaccumulation of potentially toxic elements in benthic and pelagic food chains in Lake Baikal. Environmental Science and Pollution Research 23:15593–15604.

De Fur, P. L., C. P. Mangum, and B. R. McMahon. 1985. Cardivascular and ventilatory changes during ecdysis in the blue crab *Callinectes sapidus* Rathbun. Journal of Crustacean Biology 5:207–215.

Dzul Erosa, M. S., T. I. S. Medina, R. N. Mendoza, M. A. Rodriguez, and E. Guibal. 2001. Cadmium sorption on chitosan sorbents: kinetic and equilibrium studies. Hydrometallurgy 61:157–167.

Falkner, K. K., C. I. Measures, S. E. Herbelin, J. M. Edmond, and R. F. Weiss. 1991. The major and minor element geochemistry of Lake Baikal. Limnology and Oceanography 36:413–423.

Gaudy, R., J. P. Guerin, and P. Kerambrun. 1991. Sublethal effects of cadmium on respiratory metabolism, nutrition, excretion and hydrolase activity in *Leptomysis lingvura* (Crustacea, Mysidacea). Marine Biology 109:493–501.

Gronskaya, T. P., and T. E. Litova. 1991. Short characteristics of the water balance of Lake Baikal during 1962–1988. Pages 153–158. Monitoring of Lake Baikal Environment. Hydrometeoizdat, Leningrad.

Guppy, M., and P. Withers. 1999. Metabolic depression in animals: physiological perspectives and biochemical generalizations. Biological Reviews of the Cambridge Philosophical Society 74:1–40.

Heise, K., M. S. Estevez, S. Puntarulo, M. Galleano, M. Nikinmaa, H. O. Pörtner, and D. Abele. 2007. Effects of seasonal and latitudinal cold on oxidative stress parameters and activation of hypoxia inducible factor (HIF-1) in zoarcid fish. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 177:765–777.

Hollis, L., J. C. McGeer, D. G. McDonald, and C. M. Wood. 2000. Protective effects of calcium against chronic waterborne cadmium exposure to juvenile rainbow trout. Environmental Toxicology and Chemistry 19:2725–2734.

Hynes, H. B. N. 1961. The invertebrate fauna of a Welsh mountain stream. Archives of Hydrobiology 57:344–388.

Jakob, L., D. V. Axenov-Gribanov, A. N. Gurkov, M. Ginzburg, D. S. Bedulina, M. A. Timofeyev, T. Luckenbach, M. Lucassen, F. J. Sartoris, and H. O. Pörtner. 2016. Lake Baikal amphipods under climate change: Thermal constraints and ecological consequences. Ecosphere 7:e01308.

Khalil, M. A., M. H. Donker, and N. M. van Straalen. 1995. Long-term and short-term changes in the energy budget of *Porcellio scaber* Latreille (Crustacea) exposed to cadmium polluted food. European Journal of Soil Biology 31:163–172.

Khan, F. R., N. R. Bury, and C. Hogstrand. 2010. Cadmium bound to metal rich granules and exoskeleton from *Gammarus pulex* causes increased gut lipid peroxidation in zebrafish following single dietary exposure. Aquatic Toxicology 96:124–129.

Kozhova, O. M., and L. R. Izmest'eva. 1998. Lake Baikal - Evolution and Biodiversity. Backhuys Publishers, Leiden.

Kravtsova, L. S., E. B. Karabanov, R. M. Kamaltynov, S. K. Krivonogov, and D. Y. Shcherbakov. 2003. Macrozoobenthos of subaqueous landscapes in shoal of southern Baikal. 1. Local diversity of bottom population and peculiarities of its spatial distribution. Zoologicheskii Zhurnal 82:307–317.

Kültz, D. 2005. Molecular and evolutionary basis of the cellular stress response. Annual Review of Physiology 67:225–257.

Lasenby, D. C., and J. Vanduyn. 1992. Zinc and cadmium accumulation by the opossum shrimp *Mysis relicta*. Archives of Environmental Contamination and Toxicology 23:179–183.

Lavoie, M., P. G. C. Campbell, and C. Fortin. 2012. Extending the Biotic Ligand Model to Account for Positive and Negative Feedback Interactions between Cadmium and Zinc in a Freshwater Alga. Environmental Science & Technology 46:12129–12136.

Lei, W., L. Wang, D. Liu, T. Xu, and J. Luo. 2011. Histopathological and biochemical alternations of the heart induced by acute cadmium exposure in the freshwater crab *Sinopotamon yangtsekiense*. Chemosphere 84:689–694.

Luoma, S. N., and P. S. Rainbow. 2005. Why is metal bioaccumulation so variable? Biodynamics as a unifying concept. Environmental Science & Technology 39:1921–1931.

Luoma, S. N., and P. S. Rainbow. 2008. Metal Contamination in Aquatic Environments - Science and Lateral Management. Camebridge University Press, Camebridge.

Lushchak, V. I. 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology 101:13–30.

Marsden, I. D., and P. S. Rainbow. 2004. Does the accumulation of trace metals in crustaceans affect their ecology - the amphipod example? Journal of Experimental Marine Biology and Ecology 300:373–408.

McGeer, J. C., S. Niyogi, and D. Scott Smith. 2011. Homeostasis and Toxicology of non-essential metals-3 - Cadmium. Fish Physiology 31:125–184.

Moore, M. V., S. E. Hampton, L. R. Izmest'eva, E. A. Silow, E. V. Peshkova, and B. K. Pavlov. 2009. Climate Change and the World's "Sacred Sea"-Lake Baikal, Siberia. Bioscience 59:405–417.

Moore, P., and A. Taylor. 1984. Gill area relationships in an ecological series of gammaridean amphipods (Crustacea). Journal of Experimental Marine Biology and Ecology 74:179–186.

Nemmiche, S., D. Chabane-Sari, M. Kadri, and P. Guiraud. 2011. Cadmium chloride-induced oxidative stress and DNA damage in the human Jurkat T cell line is not linked to intracellular trace elements depletion. Toxicology in Vitro 25:191–198.

OECD. 2012. OECD guideline for the testing of chemicals - Section 2: Effects on biotic systems; Test No. 211: *Daphnia magna* reproduction test.

Philips, D. J. H., and P. S. Rainbow. 1994. Biomonitoring of trace aquatic contaminants. Springer-Science + Business Media, Dordrecht.

Protopopova, M. V., V. V. Pavlichenko, R. Menzel, A. Putschew, T. Luckenbach, and C. E. W. Steinberg. 2014. Contrasting cellular stress responses of Baikalian and Palearctic amphipods upon exposure to humic substances: environmental implications. Environmental Science and Pollution Research 21:14124–14137.

Rainbow, P. S. 1998. Phylogeny of trace metal accumulation in crustaceans. Pages 285–319. Metal Metabolism in Aquatic Environments. Chapman & Hall, London.
Rainbow, P. S. 2002. Trace metal concentrations in aquatic invertebrates: why and so what? Environmental Pollution 120:497–507.

Rainbow, P. S., F. Liu, and W.-X. Wang. 2015. Metal accumulation and toxicity: The critical accumulated concentration of metabolically available zinc in an oyster model. Aquatic Toxicology 162:102–108.

Rainbow, P. S., and S. N. Luoma. 2011. Metal toxicity, uptake and bioaccumulation in aquatic invertebrates-Modelling zinc in crustaceans. Aquatic Toxicology 105:455–465.

Rodriguez, E. M., R. Bigi, D. A. Medesani, V. S. Stella, L. S. L. Greco, P. A. R. Moreno, J. M. Monserrat, G. N. Pellerano, and M. Ansaldo. 2001. Acute and chronic effects of cadmium on blood homeostasis of an estuarine crab, *Chasmagnathus granulata*, and the modifying effect of salinity. Brazilian Journal of Medical and Biological Research 34:509–518.

Rosabal, M., L. Hare, and P. G. C. Campbell. 2014. Assessment of a subcellular metal partitioning protocol for aquatic invertebrates: preservation, homogenization, and subcellular fractionation. Limnology and Oceanography-Methods 12:507–518.

Rusinek, O. T., V. V. Takhteev, D. P. Gladkochub, T. V. Khodzher, and N. M. Budnev. 2012. Baikalovedenie (Baikalogy). Nauka, Novosibirsk.

Simkiss, K., and M. G. Taylor. 1995. Simkiss, K., Taylor, M.G., 1995. Transport of metals across membranes. In: Tessier, A., Turner, D.R. (Eds.), Wiley, New York, pp. 1–44. Metal Speciation and Bioavailability in Aquatic Systems. Wiley, New York.

Simmons, S. O., C.-Y. Fan, and R. Ramabhadran. 2009. Cellular Stress Response Pathway System as a Sentinel Ensemble in Toxicological Screening. Toxicological Sciences 111:202–225.

Smock, L. 1983. Relationships between metal concentrations and organism size in aquatic insects. Freshwater Biology 13:313–321.

Stephenson, M., and M. A. Turner. 1992. A field study of cadmium dynamics in periphyton and in *Hyalella azteca* (Crustacea: Amphipoda). Water, Air, and Soil Pollution 68:341–361.

Storey, K. B., and J. M. Storey. 2004. Metabolic rate depression in animals: transcriptional and translational controls. Biological Reviews 79:207–233.

Sulmon, C., J. van Baaren, F. Cabello-Hurtado, G. Gouesbet, F. Hennion, C. Mony, D. Renault, M. Bormans, A. El Amrani, C. Wiegand, and C. Gerard. 2015. Abiotic stressors and stress responses: What commonalities appear between species across biological organization levels? Environmental Pollution 202:66–77.

Tan, Q.-G., and W.-X. Wang. 2011. Acute Toxicity of Cadmium in *Daphnia magna* under Different Calcium and pH Conditions: Importance of Influx Rate. Environmental Science & Technology 45:1970–1976.

Thorslund, J., J. Jarsjo, S. R. Chalov, and E. V. Belozerova. 2012. Gold mining impact on riverine heavy metal transport in a sparsely monitored region: the upper Lake Baikal Basin case. Journal of Environmental Monitoring 14:2780–2792.

Timofeyev, M. A., J. M. Shatilina, and D. I. Stom. 2001. Attitude to temperature factor of some endemic amphipods from Lake Baikal and Holarctic *Gammarus lacustris* Sars, 1863: A comparative experimental study. Arthropoda selecta 10:93–101.

Timofeyev, M. A., Z. M. Shatilina, D. S. Bedulina, M. V. Protopopova, V. V. Pavlichenko, O. I. Grabelnych, and A. V. Kolesnichenko. 2008. Evaluation of biochemical responses in Palearctic and Lake Baikal endemic amphipod species exposed to $CdCl_2$. Ecotoxicology and Environmental Safety 70:99–105.

Timofeyev, M., and Z. Shatilina. 2007. Different preference reactions of three Lake Baikal endemic amphipods to temperature and oxygen are correlated with symbiotic life. Crustaceana 80:129–138.

Timoshkin, O. A. 2009. Index of Animal Species Inhabiting Lake Baikal and its Catchment Area. Nauka, Novosibirsk.

Vijver, M. G., C. A. M. Van Gestel, R. P. Lanno, N. M. Van Straalen, and W. Peijnenburg. 2004. Internal metal sequestration and its ecotoxicological relevance: A review. Environmental Science & Technology 38:4705–4712.

Von Der Ohe, P. C., and M. Liess. 2004. Relative sensitivity distribution of aquatic invertebrates to organic and metal compounds. Environmental Toxicology and Chemistry 23:150–156.

Wallace, W. G., B. G. Lee, and S. N. Luoma. 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). Marine Ecology Progress Series 249:183–197.

Wang, W.-X., and P. S. Rainbow. 2006. Subcellular partitioning and the prediction of cadmium toxicity to aquatic organisms. Environmental Chemistry 3:395–399.

Weinberg, I. V., and R. M. Kamaltynov. 1998. Zoobenthos communities on stony beach of Lake Baikal. Zoologichesky Zhurnal 77:259–265.

Weiss, R. F., E. C. Carmarck, and V. M. Koropalov. 1991. Deep water renewal and biological production in Lake Baikal. Nature 349:665–669.

Wu, J. P., and H. C. Chen. 2004. Effects of cadmium and zinc on oxygen consumption, ammonium excretion, and osmoregulation of white shrimp (*Litopenaeus vannamei*). Chemosphere 57:1591–1598.

Yoshioka, T., S. Ueda, T. Khodzher, I. Bashenkhaeva, L. Korovyakova, L. Sorokovikova, and L. Gorbunova. 2002. Distribution of dissolved organic carbon in Lake Baikal and its watershed. Limnology 3:159–168.

Zerbst-Boroffka, I., T. Grospietsch, I. Mekhanikova, and V. Takhteev. 2000. Osmotic and ionic hemolymph concentrations of bathyal and abyssal amphipods of Lake Baikal (Siberia) in relation to water depth. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 170:615–625.

Supporting information

Table S1: Nominal and measured water concentrations of Cd in dose-mortality experiments at the day of water exchange (start) and before water exchange (after 4 days) in randomly selected replicates of each concentration.

Species	Measured conc. at 0 d (nM)	Measured conc. at 4 d (nM)	Nominal conc. (nM)	% loss (0-4 d)	% deviation from nominal (0 d)	% deviation from nominal (4 d)	
E. cyaneus	0	0	0	0	0	0	
	7.7	3.4	8.7	55.8	11.5	60.9	
	33	11	35	66.7	5.7	68.6	
	66	38	105	42.4	37.1	63.8	
	144	112	210	22.2	31.4	46.7	
	407	208	419	48.9	2.9	50.4	
	290		559		48.1		
	673	578	1117	14.1	39.7	48.3	
	1385	1019	2235	26.4	38.0	54.4	
				14.1	**2.9**	**46.7**	**min**
				66.7	**48.1**	**68.6**	**max**
				39.5	**26.8**	**56.1**	**average deviation**
				19.2	**17.4**	**8.4**	**SD**
E. verrucosus	0	0	0	0	0	0	
	6.6	4.9	8.7	25.8	24.1	43.7	
	22	9.8	35	55.5	37.1	72.0	
	62	65	105	-4.8	41.0	38.1	
	130	95	210	26.9	38.1	54.8	
	284	206	419	27.5	32.2	50.8	
	371		559		33.6		
	883	546	1117	38.2	20.9	51.1	
	1333	853	2235	36.0	40.4	61.8	
				-4.8	**20.9**	**38.1**	**min**
				55.5	**41.0**	**72.0**	**max**
				29.3	**33.4**	**53.2**	**average deviation**
				18.2	**7.4**	**11.2**	**SD**

Table S2: Nominal and measured water concentrations of Cd in experimental LC1-exposures at the day of water exchange (start) and before water exchange (after 4 days) (n = 3).

Species	Replicate No.	Measured conc. at 0 d (nM)	Measured conc. at 4 d (nM)	Nominal conc. (nM)	% loss (0-4 d)	% deviation from nominal (0 d)	% deviation from nominal (4 d)
E. cyaneus	1	18	9.6	18	53.3	0,0	46.7
	2	17	13	18	76.5	5.6	27.8
	3	21	14	18	66.7	-16.7	22.2
E. verrucosus	1	114	55	115	48.2	0.9	52.2
	2	114	53	115	46.5	0.9	53.9
	3	111	81	115	73.0	3.5	29.6

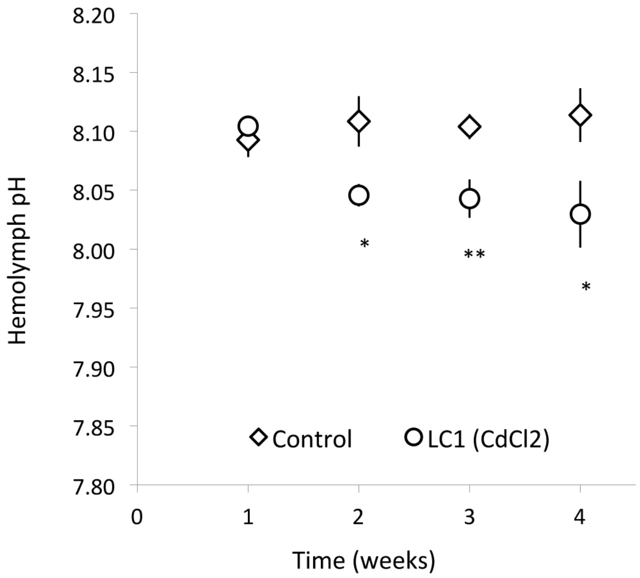


Figure S1: Hemolymph pH in *E. verrucosus* exposed to its species-specific LC1 ($CdCl_2$) (115 nM) for 4 weeks at 6°C. Data are represented as means ± SE (n = 10). Statistically significant differences between treatments (circles) and parallel controls (rhombs) are indicated by asterisks (*$p < 0.05$; **$p < 0.01$)

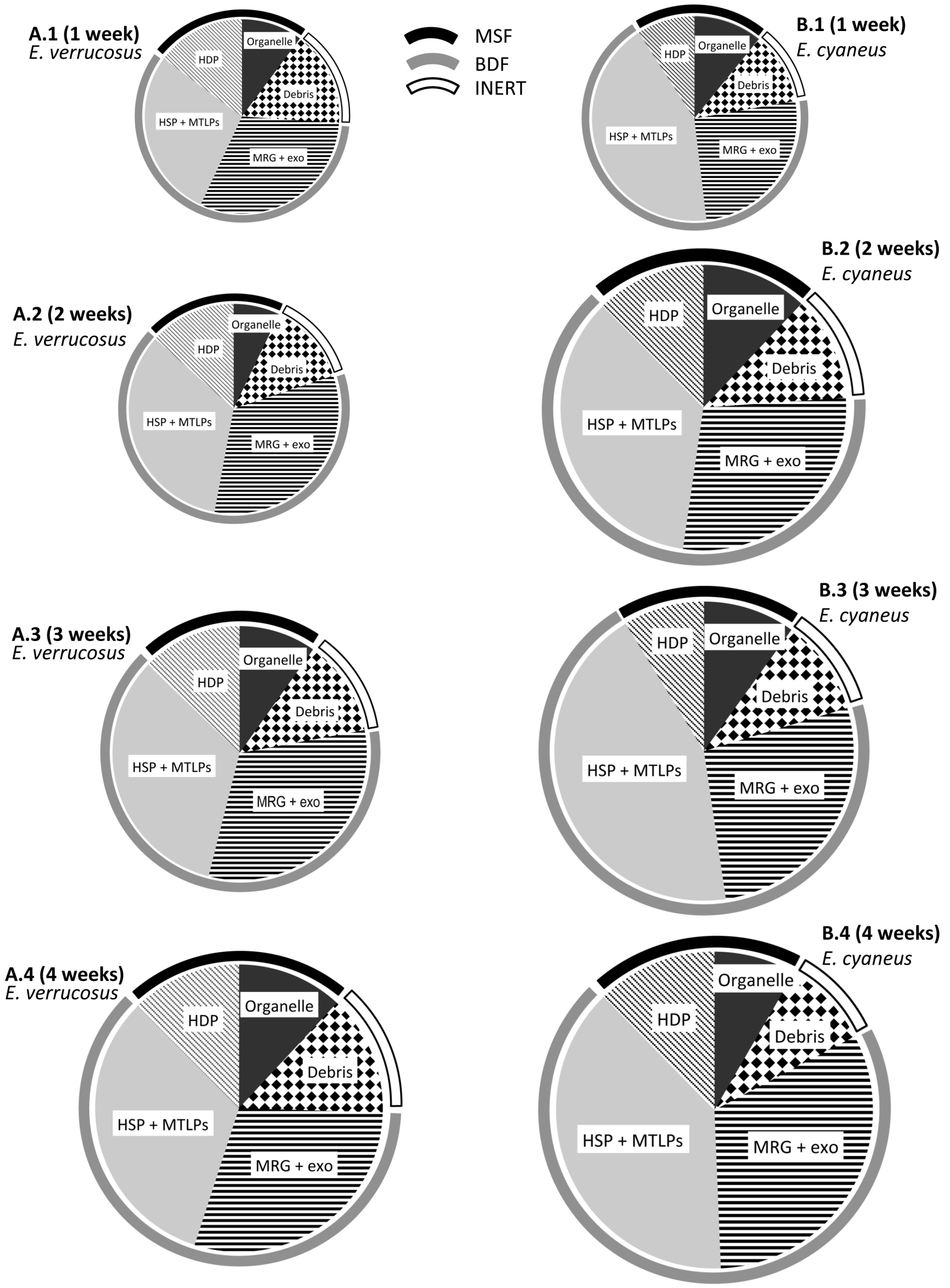


Figure S2: Cadmium concentrations in different subcellular fractions of *E. verrucosus* **(A)** and *E. cyaneus* **(B)** exposed to their species-specific LC1 ($CdCl_2$), LC1 *E. verrucosus* = 115 nM, LC1 *E. cyaneus* = 18 nM for 4 weeks at 6°C. Metal sensitive fraction (MSF) = Organelle (mitochondria, lysosomes and microsomes) + HDP (heat denaturable proteins), Biologically detoxified fraction (BDF) = MRG+exo (metal rich granules+exoskeleton) + HSP + MTLPs (heat stable proteins including metallothionein-like proteins) and the toxicologically inert fraction = Debris (cell debris). The areas of the circles relate to total Cd tissue concentrations; *E. verrucosus*: 0.51 ± 0.05 (1 wk), 0.56 ± 0.08 (2 wk), 0.83 ± 0.14 (3 wk), 1.06 ± 0.18 (4 wk) and *E. cyaneus*: 0.54 ± 0.03 (1 wk), 1.05 ± 0.19 (2 wk), 1.12 ± 0.16 (3 wk) and 1.20 ± 0.18 (4 wk), presented as means ± SE in µg g (fresh weight)$^{-1}$ (n = 5 - 10).

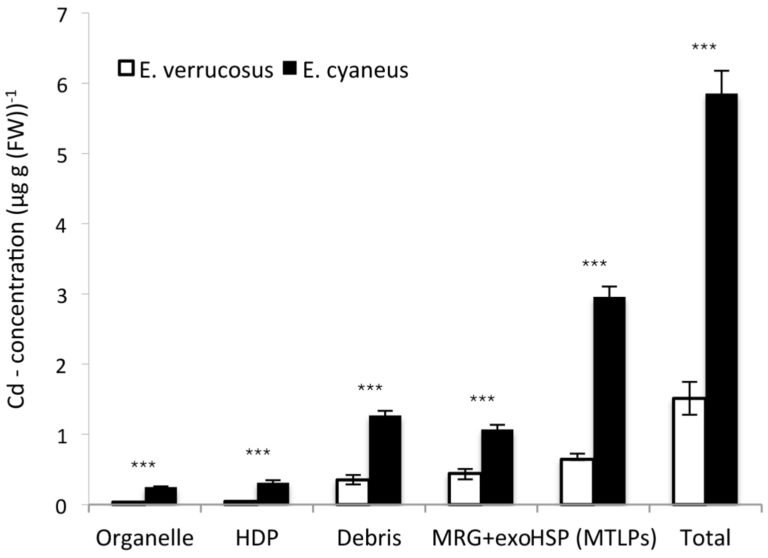


Figure S3: Cadmium concentration in different fractions of *E. verrucosus* (white columns) and *E. cyaneus* (black columns) exposed to 105 nM $CdCl_2$ for 4 weeks (6°C). Data are presented as means ± SE (n = 7). Statistically significant differences between the two species are indicated by asterisks (*** $p < 0.001$).

4 Additional Results

This section presents results on cadmium toxicity in *G. lacustris* (ubiquitous in the Holarctic) in comparison with the studied Baikal endemic amphipods. Due to a very high rate of cannibalism (up to 77%) in 4-week exposures to cadmium-containing water in some of the experimental setups with *G. lacustris*, no fully reliable derivation of LC values was possible. Consequently, this species was excluded from the follow-up study on inter-specific variation of cadmium toxicity; i.e. Publication III. However, despite the high predation LC50 values were derived for *G. lacustris*, which may give indication for toxicity values for this species. Moreover short-term effects (LC10, 24 h, 6°C) are reported. These data were not included in Publication III because here it was focused on long-term data, which have a higher environmental relevance. However, the short-term data may contribute to differentiate between physiological effects to short-term exposure to relatively high cadmium concentrations (LC10, 24 h, 6°C) and long-term exposure to relatively low cadmium concentrations (LC1, 4 wk, 6°C). Furthermore, data on the combined effects of temperature and cadmium are reported, which give first indication whether synergistic effects can be expected. These datasets may be extended in future research.

Cadmium concentrations are given as nominal concentration (upright letters) and wherever determined also as measured concentration (italic letters). As cadmium concentrations decreased significantly between water exchanges in the semi-static incubation system (water was exchanged every 4th day), the mean measured concentration is indicated for long-term exposures.

4.1 Cadmium toxicity in *E. verrucosus*, *E. cyaneus* and *G. lacustris*

While the LC50 value for *G. lacustris* should be regarded as an indication due to the high rate of cannibalism, lethal times for 50% of the animals ($LT_{ime}50$) exposed to 1117 nM (*626 nM*) could be determined as cannibalism was negligible in this treatment.

Lethal concentrations (LC50 values) were lowest in *E. cyaneus* (159 nM; 95% confidence interval (CI) = 141, 179) and slightly higher in *G. lacustris* (234 nM; 95% CI = 197, 281) (Figure 15A). A higher LC50 value was obtained for *E. verrucosus* (366 nM (95% CI = 316, 406) (Figure 15A). $LT_{ime}50$ values were 3.6 d (95% CI = 3.5, 3.6) for *G. lacustris* at this concentration, 7.6 d (95% CI = 6.8, 8.4) for *E. verrucosus* and 3.1 d (95% CI = 2.8, 3.4) for *E. cyaneus*, respectively (Figure

15B). Thus, 95% CI of the curve of *E. verrucosus* did not overlap with that of both *E. cyaneus* and *G. lacustris,* whereas they overlapped for the latter two species.

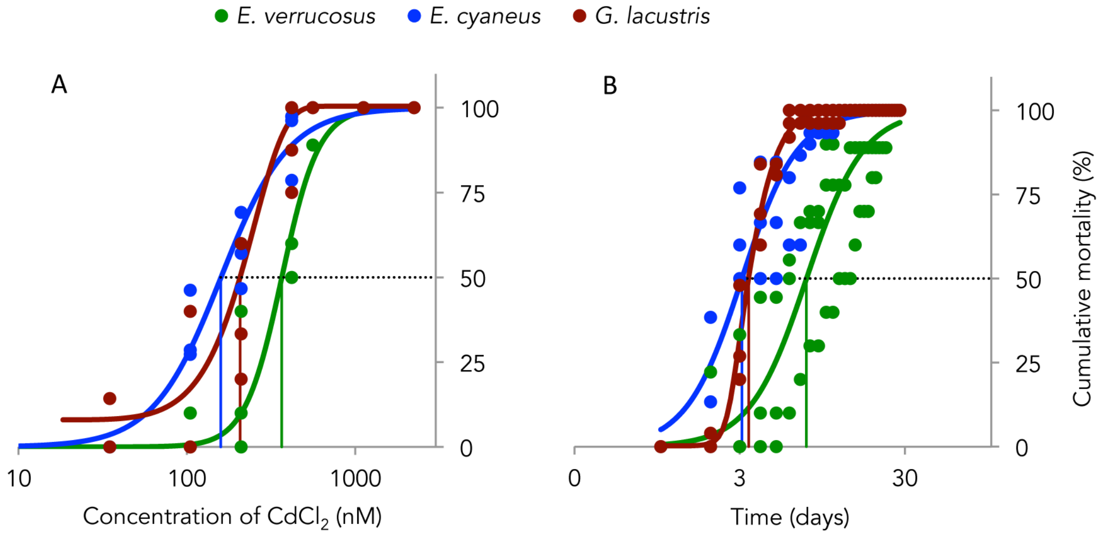


Figure 15: Concentration-mortality curves **(A)** and lethal times (at 1117 nM (*626 nM*) of CdCl$_2$) **(B)** in 4 week-exposures of *E. verrucosus*, *E. cyaneus* and *G. lacustris* (N = 3). The Hill model was fitted to all datasets (solid lines), the equation can be found in Publication III.

Tissue concentration in *G. lacustris*, *E. cyaneus* and *E. verrucosus* were determined after 4 week-exposures to 105 nM (*52 nM*) Cd^{2+} (Figure 16A). The results show that *E. cyaneus* and *G. lacustris* had similar body concentrations whereas *E. verrucosus* displayed significantly lower total body concentrations of cadmium (ANOVA; $p < 0.05$, Table 1). Accordingly, the concentration of cadmium associated with the MSF was significantly lower in *E. verrucosus* in comparison with both *E. cyaneus* and *G. lacustris* (Figure 16B) (ANOVA; $p < 0.05$, Table 1).

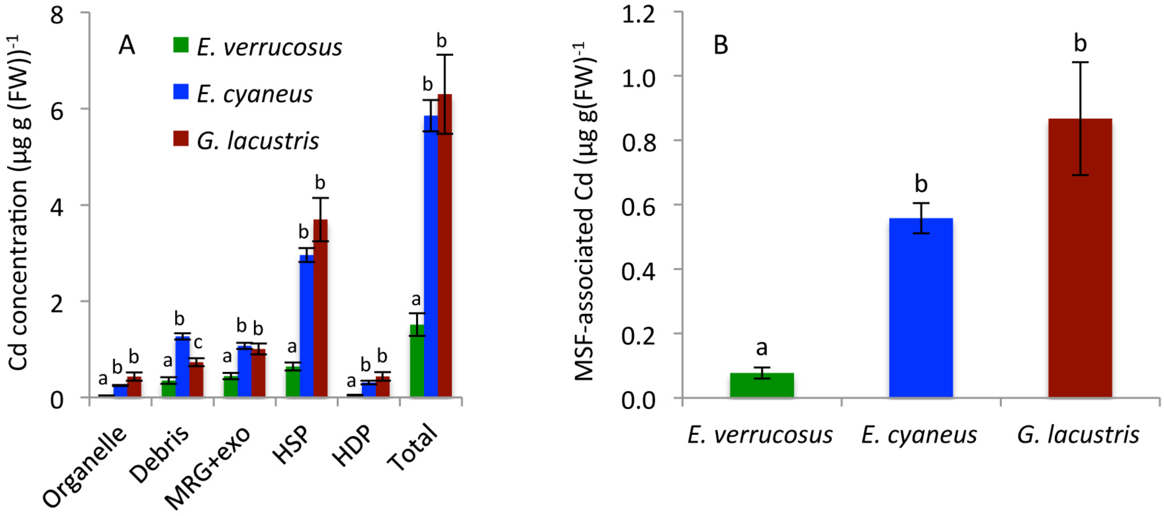


Figure 16: Cadmium concentration of different subcellular compartments **(A)** and of the metal sensitive fraction (MSF) **(B)** in *E. verrucosus*, *E. cyaneus* and *G. lacustris* exposed to 105 nM (*52 nM*) of Cd^{2+} for 4 weeks. Different letters mark statistical differences between groups ($p < 0.05$); see Table 1 for ANOVA results. Data is presented as means ± SE (n = 7).

Table 3: Summary of ANOVA results presented in chapter 4.

Data location	Parameter	Statistical test	F or H value	DF	p	Post hoc test
Figure 16A	Total	Kruskal-Wallis	13.455	2	0.001	Tukey
Figure 16B	MSF	Kruskal-Wallis	14.043	2	< 0.001	Tukey
Figure 16A	HDP	Kruskal-Wallis	13.799	2	0.001	Tukey
Figure 16B	Debris	One-way ANOVA	39.484	2	< 0.001	Holm-Sidak
Figure 16A	HSP + MTLPs	Kruskal-Wallis	13.900	2	< 0.001	Tukey
Figure 16A	MRG + exo	One-way ANOVA	16.666	2	< 0.001	Holm-Sidak
Figure 16A	Organelle	Kruskal-Wallis	15.216	2	< 0.001	Tukey
Figure 17A	Ventilation	Kruskal-Wallis	12.045	3	= 0.007	Dunn's test
Figure 20A	O_2 consumption	One-way ANOVA	3.094	2	0.066	

4.2 Long-term and short-term exposure to cadmium

Ventilation was increasing in *E. verrucosus* during a short-term (24 h) study at 6°C when animals were exposed to their LC10 (obtained in 24 h studies at 7°C; (Protopopova et al. unpublished)) (Figure 17A) whereas ventilation was neither increasing nor decreasing significantly in *E. cyaneus* and *G. lacustris* (Figure 17B and C). In 4 week-setups, ventilation was significantly decreased in *E. verrucosus* when animals were exposed to their LC1 (obtained in 4 week-studies at 6°C) in comparison to the parallel control group (Figure 2 of Publication III) but again, no deviations of treated animals from control animals were determined in *E. cyaneus* (Figure 2 of Publication III).

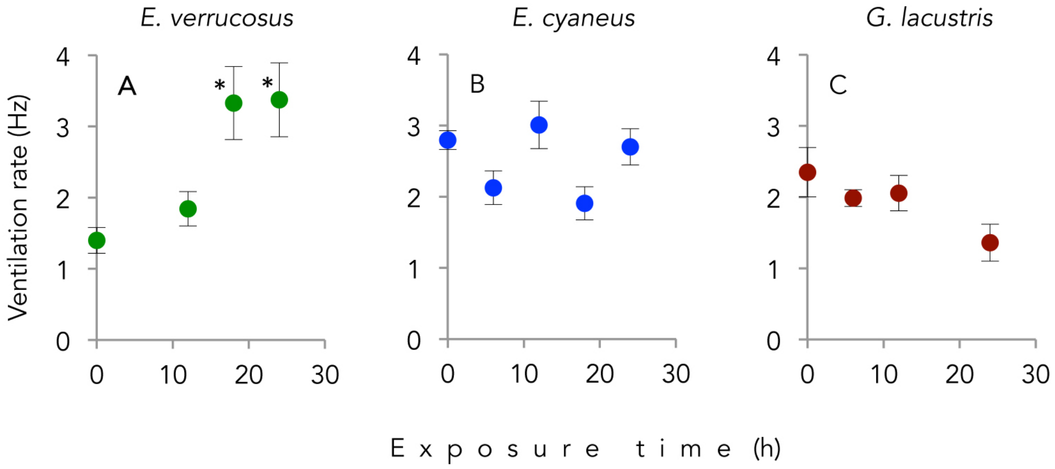


Figure 17: Exposure of *E. verrucosus* **(A)**, *E. cyaneus* **(B)** and *G. lacustris* **(C)** to their species-specific LC10 (24 h, 7°C; Protopopova et al. unpublished) for 24 h at 6°C. LC10 of *E. verrucosus*, *E. cyaneus* and *G. lacustris* = 2.9, 3.7 and 3.2 µM, respectively. Asterisks indicate statistical differences from the start control ($p < 0.05$); see Table 1 for ANOVA results. Data are represented as means ± SE (n = 3 - 12).

4.3 Combined effects of temperature and cadmium

Mortality was increased in both *E. verrucosus* and *E. cyaneus* when individuals were exposed to a combination of gradual temperature increase and cadmium in comparison to individuals that were exposed to temperature increase alone (Figure 18). Here, animals were exposed to their respective LC1 of $CdCl_2$ (LC1 of *E. verrucosus* = LC1-EV (115 nM, *88 nM*); LC1 of *E. cyaneus* LC1-EC (18 nM, *18 nM*), obtained in 4 week-exposures at 6°C) and gradual temperature increase (0.8°C d^{-1}). The $LT_{emp}50$ of *E. verrucosus* shifted from 21.2 to 19.0°C (Figure 18A); the $LT_{emp}50$ of *E. cyaneus* shifted from 27.1 to 24.0°C (Figure 18B). In a previous experiment, *E. verrucosus* was exposed to cadmium concentration of 139 nM (4 weeks, 6°C). This concentration was derived as sublethal concentration from a 24 h dose mortality experiment at 7°C (Protopopova et al. unpublished). Under exposure to 139 nM of $CdCl_2$, the $LT_{emp}50$ of the cadmium exposed group was decreased by 2.2°C in comparison to the parallel control (data not displayed).

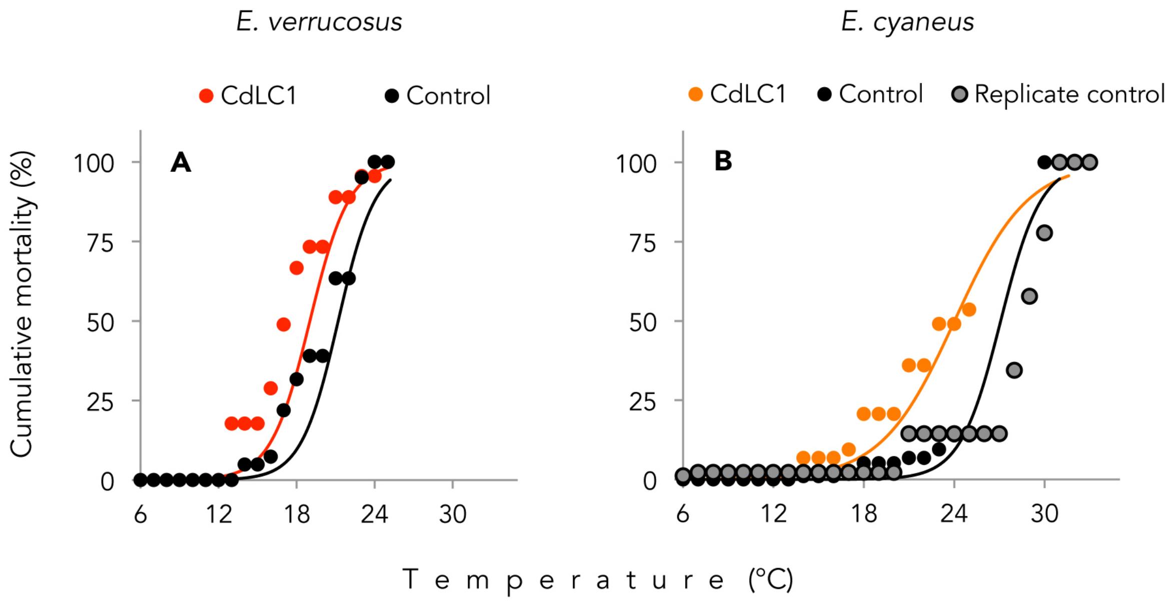


Figure 18: Cumulative mortality (in percentage) of *E. verrucosus* **(A)** and *E. cyaneus* **(B)** exposed to gradual temperature increase of 0.8°C d^{-1} in $CdCl_2$-containing Baikal water. Each species was exposed to its LC1 obtained in 4 week-experiments at 6°C (LC1 of *E. verrucosus*: 105 nM, *88 nM*; LC1 of *E. cyaneus*: 18 nM, *18 nM*); colored dots. Control animals were exposed to the same temperature increase in clean Baikal water (black dots). Gray dots **(B)** represent a replicate experiment (not taken in parallel) as the actual experiment was terminated at 25.2°C due to technical problems. The Hill model was fitted to all datasets (solid lines), the equation is found in Publication III.

Oxygen consumption was measured in *E. verrucosus* and *E. cyaneus* exposed to gradual temperature increase of 0.8°C d^{-1} in comparison with animals exposed to $CdCl_2$-containing water for 24 h during the incremental temperature increase (Figure 19). Each species was exposed to its LC1 obtained in 4 week-experiments at 6°C (LC1 of *E. verrucosus*: 105 nM, *88nM*; LC1 of *E. cyaneus*: 18 nM, *18 nM*). Oxygen consumption was lower at 10.8 and 12.4°C in cadmium-treated individuals than in control animals, which were exposed to temperature increase alone (T-Test; $p < 0.05$) (Figure 19A). However, otherwise no statistically significant differences were found between treatments and controls.

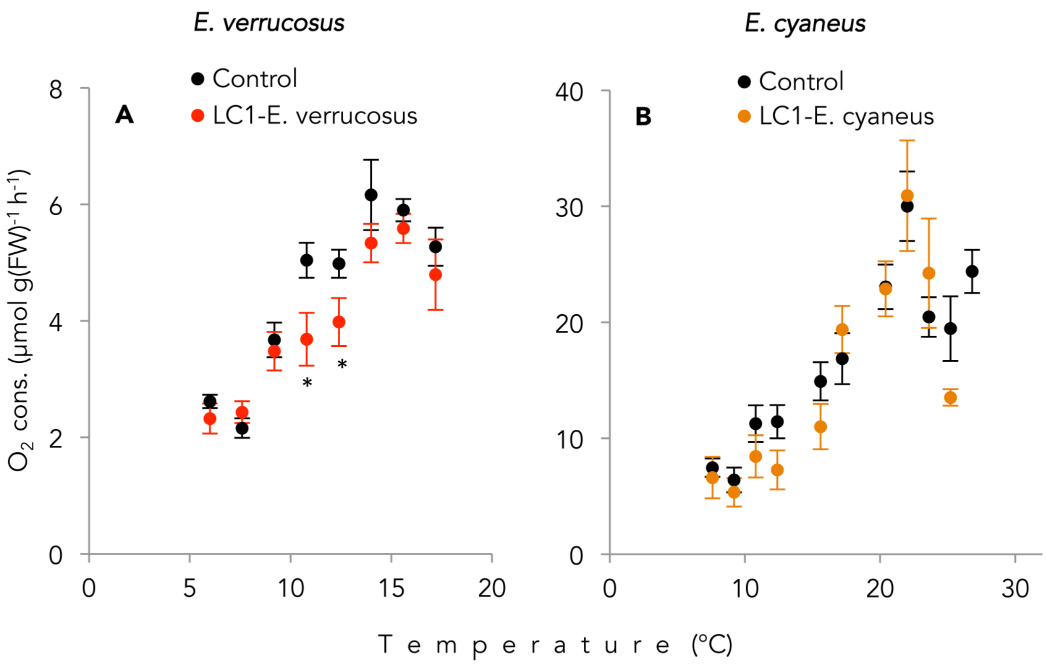


Figure 19: Oxygen consumption of *E. verrucosus* **(A)** and *E. cyaneus* **(B)** exposed to gradual temperature increase of 0.8°C d^{-1} (black dots: controls) in comparison with animals exposed to $CdCl_2$-containing water for 24 h at the respective temperatures (colored dots: cadmium treatments). Each species was exposed to its LC1 obtained in 4 week-experiments at 6°C (LC1 of *E. verrucosus*: 105 nM, *88nM*; LC1 of *E. cyaneus*: 18 nM, *18 nM*). Control animals, which were kept in clean Baikal water were also transferred to different tanks 24 h prior to measurements to keep potential handling stress equal in treatments and controls. The experiments were terminated when mortality exceeded 25%. At each sampling time point new individuals of both LC1-treatments and control groups were analyzed, which were randomly taken from the exposure tanks. Asterisks indicate results that are significantly different from parallel control samples (T-Test; $p < 0.05$); (n = 5 - 12).

In a second setup, individuals of *E. verrucosus* and *E. cyaneus* were exposed for 14 days to both the LC1 of *E. cyaneus* = LC1-EC (18 nM, *18 nM*) and the LC1 of *E. verrucosus* = LC1-EV (115 nM, *88 nM*) at 6°C and a temperature close to the respective T_p of the species (T_p of *E. verrucosus* = 10.6°C; T_p of *E. cyaneus* = 19.1°C) (Figure 20). In the 6°C group of *E. verrucosus* metabolism was decreased compared to the parallel control group kept in clean Baikal water as reported in Publication III. Even when exposed to LC1-EC, which is 6.4 (nominal) and *4.9 (measured*) times lower than LC1-EV, metabolism was similarly decreased as under LC1-EV exposure but differences marginally failed to be statistically significant (ANOVA, p = 0.066) (Figure 20A). At 10°C, however, metabolism of individuals kept in clean Baikal water was lower than at 6°C. In contrast to the 6°C-setup, metabolism of animals kept in cadmium containing water (LC1-EV and LC1-EC) was not decreased in comparison to the control (Figure 20A). Mortality reached 50% in the LC1-EV exposure at 10°C whereas no mortality was monitored in the other setups (Figure 20C). In *E. cyaneus* metabolic rates of cadmium-exposed groups were similar to control groups

(Figure 20B); oxygen consumption in the 18°C group exposed to LC1-EV was not measured since mortality reached 90% whereas mortality was 10 and 20% in the control and the LC1-EC exposure, respectively (Figure 20D). In the 6°C setup no mortality was determined in the control and the LC1-EC group whereas 20% mortality was determined in the LC1-EV exposure of *E. cyaneus* (Figure 20D).

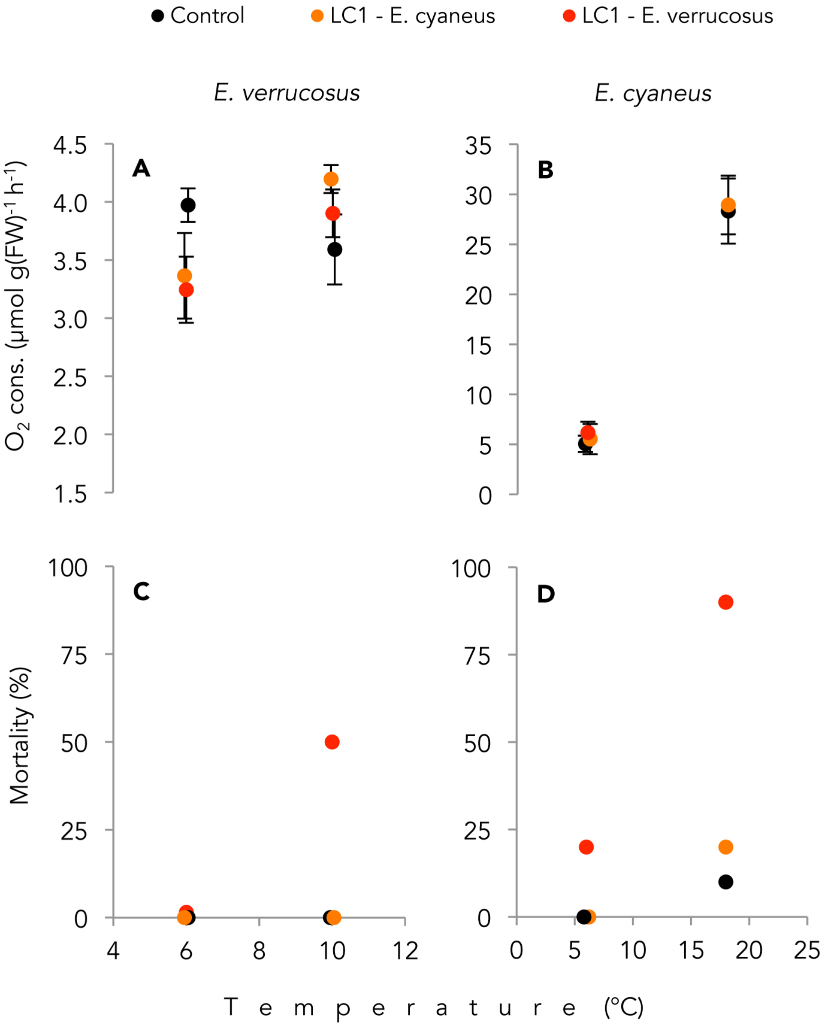


Figure 20: Oxygen consumption and mortality of *E. verrucosus* **(A, C)** and *E. cyaneus* **(B, D)** exposed to both the LC1 of *E. verrucosus* and *E. cyaneus* (obtained in 4 week-exposures at 6°C) for 14 days at 6°C and at temperatures close to the proposed pejus temperatures of the two species (T_p of *E. verrucosus*: 10.6°C; T_p of *E. cyaneus*: 19.1°C). Control animals were kept in parallel in clean Baikal water at the same temperatures as treated animals. Data on oxygen consumption is shown as means ± SE and mortality is presented as percentages (n = 8 - 10).

Hemolymph pH was determined in parallel (after 14 days) but only in *E. verrucosus* as the small size of *E. cyaneus* precluded sufficient hemolymph extraction for pH measurements. It was found that pH was slightly decreased in both the 6°C and the 10°C group; however, not always statistically significant (Table 3). A similar decrease of hemolymph pH in animals exposed to their LC1 for 14 days at 6°C was found before (Publication III); a summary of all results on hemolymph pH is given in Table 4.

Table 4: Hemolymph pH in *E. verrucosus*.
Animals were exposed to different concentrations of $CdCl_2$ for 14 days. Asterisks mark results that are statistically significantly different from controls; depending on the number of comparisons either the T-Test or One-way ANOVA has been performed ($p < 0.05$).

Year	Exposure concentration of $CdCl_2$	Temperature (°C)	Hemolymph pH	n
2013	Control (≤ 0.0066 nM)	6	8.11 ± 0.02	10
2013	LC1 – *E. verrucosus* (115 nM, 88 nM)	6	8.05 ± 0.01*	10
2014	Control (≤ 0.0066 nM)	6	8.14 ± 0.04	8
2014	LC1 – *E. verrucosus* (115 nM, 88 nM)	6	8.08 ± 0.04	8
2014	LC1 – *E. cyaneus* (18 nM, 18 nM)	6	8.02 ± 0.08*	8
2014	Control (≤ 0.0066 nM)	10 - 11	8.10 ± 0.02	8
2014	LC1 – *E. verrucosus* (115 nM, 88 nM)	10 - 11	8.04 ± 0.04	6
2014	LC1 – *E. cyaneus* (18 nM, 18 nM)	10 - 11	8.05 ± 0.04	7

5 Discussion

Lake Baikal endemic amphipods are exposed to quickly changing thermal and chemical conditions. This thesis seeks to provide insights to physiological processes determining thermal tolerance and plasticity as well as toxicant susceptibility in Lake Baikal endemic amphipods compared to *G. lacustris*, which is ubiquitous in the Holarctic. Publications I and II addressed the topic of thermal limitation and thermal plasticity on different organizational levels. Publication III elucidated, which factors including those affecting thermal tolerance, may influence cadmium susceptibility in Baikal amphipods. Discussions of the single results are part of the publications. The following chapter compiles the key findings of the publications, which are discussed in an integrated way and complemented with additional results and corresponding literature.

5.1 Temperature and thermal tolerance

The conceptual model of oxygen- and capacity-limitation of thermal tolerance (OCLTT) (Pörtner 2010) outlined in the introduction of this thesis states that temperature becomes constraining for an organism at increasing temperatures reflected in a progressive decrease of systemic oxygen availability, due to a mismatch in oxygen supply capacity and demand, which is important for the maintenance of fitness parameters such as growth and reproduction, maintenance of aerobic metabolism and ultimately survival. The following two sections (5.1.1 and 5.1.2) focus on linking the observed limitations of the oxygen supply system (i.e. ventilation rate and oxygen consumption) determined in the studied amphipods species to further physiological and biochemical parameters as well as to behavioral and ecological phenomena. Moreover, the theory of a hierarchy of thermal tolerance in the conceptual model of OCLTT is addressed, regarding the whole animal and biochemical levels investigated in Publication I and II, respectively.

5.1.1 WHOLE ANIMAL THERMAL LIMITATIONS

KEY QUESTION 1

At which points does the temperature-dependence of relevant physiological processes on the highest hierarchical level (whole animal) indicate the onset of limitation in the studied species?

The main findings are schematically displayed in Figure 21.

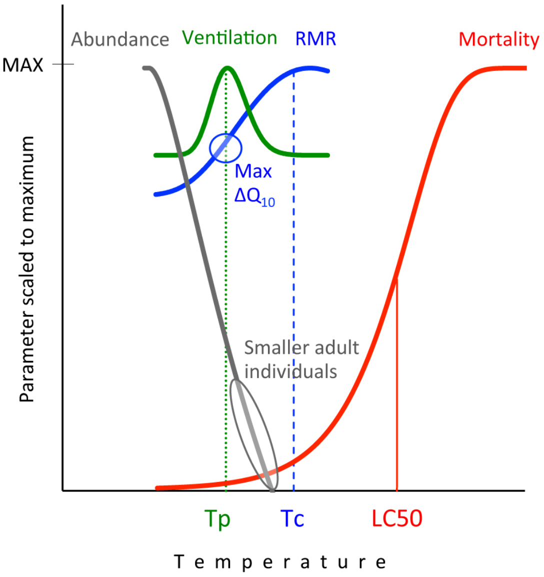


Figure 21: Schematic compilation of different parameters (field abundance, resting ventilation, routine metabolic rate and mortality) investigated under gradual thermal increase (0.8°C d^{-1}) (example: *E. verrucosus*). All factors are scaled to their respective maximum values. Measurements of routine metabolic rate (RMR) and ventilation were taken into account up to a cumulative mortality of 25%.

KEY FINDINGS AND SIGNIFICANCE

Lake Baikal provides a great opportunity to study physiological factors limiting thermal tolerance because it is home to a variety of closely related amphipod species inhabiting different thermal niches. Constraints in ventilation rates were seen earliest in E. verrucosus *(10.6°C) and much later in* E. cyaneus *(19.1°C) and* G. lacustris *(21.1°C). These so-called breakpoint temperatures (BPTs) were correlated with migration of* E. verrucosus *from the upper littoral to deeper and cooler areas. Moreover, there was strong indication that the BPTs of ventilation correspond to the maximal habitat temperature of* E. cyaneus *and likely also to that of* G. lacustris*. Thus, within the framework of oxygen- and capacity-limitation of thermal tolerance (OCLTT), it is suggested that the BPTs of ventilation reflect the first level of thermal limitation, i.e. the pejus ("getting worse") temperatures of the species. Oxygen consumption was as well as ventilation firstly constrained in* E. verrucosus *(15.0°C) and much later in* E. cyaneus *(25.2°C) and* G. lacustris *(23.6°C). Surpassing the BPTs of oxygen consumption was attended by exponentially increasing mortality. Consequently, the BPTs of oxygen consumption were proposed to correspond to the critical temperatures of the studied species. In conclusion, species-specific limits in oxygen supply capacity are likely key in the onset of constraining (beyond pejus) and then life-threatening (beyond critical) conditions. Ecological consequences of these limits are mediated through behavioral plasticity in* E. verrucosus.

The OCLTT model suggests that an organism's thermal tolerance is limited by oxygen supply capacity in relation to demand (Pörtner 2010). The results presented in this thesis support the hypothesis that the (upper) pejus temperature can be derived from limitations of the oxygen supply system. Resting ventilation was the most sensitive parameter and was clearly limited before reaching lethal ranges in all species studied here (Publication I). In replicate experiments similar BPTs for ventilation were found for all species investigated (Table 1 of Publication I). Also for oxygen consumption replicate experiments were well in accordance with previous results; BPTs for oxygen consumption were 15.0 (95% CI; 14.0, 16.1)°C and 13.9 (11.9, 16.1)°C for *E. verrucosus* in 4-week studies performed in 2013 and 2014, respectively. For *E. cyaneus* 25.2 and 22.9 (19.0, 26.8)°C have been determined in the respective experiments (see Figure 1 of Publication I and Figure 19).

One of the main purposes of ventilation is to supply the gills with oxygen-rich water. Although the ventilation current was described as the result of a constant beating action of the pleopods (Dahl 1977), ventilation rates can be changed in response to environmental changes. Indeed, ventilation rates of aquatic amphipods increase in response to decreasing dissolved oxygen concentration, while those of terrestrial amphipods respond readily to changes in levels of CO_2 in the environment (Vernberg and Vernberg 1972). In the present work, pleopod beating frequencies increased in parallel with oxygen consumption rates up to the species-specific BPTs. However, ventilation was found to be independent of oxygen consumption up to 12.4 and 14.0°C (experiment started at 6°C) in *E. cyaneus* and *G. lacustris*, respectively. Therefore, ventilation cannot always be used as a direct proxy for oxygen consumption despite it being the respiratory movement in an amphipod. In small organisms diffusion processes independent of ventilation might be sufficient for oxygen supply within a certain thermal range. Ventilation is a multiple-purpose physiological process, which besides from oxygen supply may serve to filter food particles in combination with setose pereiopods (Platvoet et al. 2006). In the species studied here it was observed that the amphipods regularly cleaned their pleopods and pereiopods and ingested filtered food particles (personal observation). Furthermore, the continuous pleopod movement possibly aid in preventing the settlement of epizoites and particles, which may cover and foul epithelia and consequently hinder gas exchange, locomotor abilities (impeded appendages and setae) and sensation (preventing receptor contact with stimuli) (Bauer 2013). A constant movement might also be beneficial for the maintenance of pleopod muscles; pleopods are the key propulsors for swimming amphipods (Boudrias 2013).

The earlier decease of ventilation as compared to oxygen consumption may be explained by the high energetic costs associated with the physical activity of ventilation (Glass and Wood 2009) and the inability of an organism to allocate the required energy sources in the pejus range. Similar cessation of ventilation and increased oxygen consumption in the putative pejus range has been shown in several species, e.g. polychaetes (Kristensen 1983). Oxygen extraction consequently must have increased beyond the BPT of ventilation. Oxygen consumption rates beyond the range that can be supported by increasing ventilation rates might be due to the activation of an “emergency system” which, however, can most likely not be sustained in the long term and was therefore proposed to correspond to the pejus temperature of the studied species. Elevated oxygen consumption beyond the BPT of ventilation was probably achieved through a drastic increase in heart rates and a higher involvement of hemocyanin in oxygen distribution potentially including changes in lactate content, decreased hemolymph pH as well as hormonal factors. In exercising Dungeness crabs, oxygen consumption was elevated 4-fold, which was achieved by a 2-fold increase of both ventilation and cardiac output in combination with a greater participation of hemocyanin in oxygen supply (McMahon et al. 1979), which indicates that there is a complex interplay between these physiological processes. In the amphipods studied, the observations of a decreasing ventilation at increasing oxygen consumption beyond the T_p implies that heart rate and the contribution of hemocyanin to oxygen delivery must have increased to a higher degree than described for the Dungeness crabs.

Given the higher efficiency of oxygen consumption after cessation of ventilation and the high energy cost of ventilation, it may be questioned why organisms do not further reduce their ventilation rates below their constitutive levels so as to minimize ventilation associated energy costs and still maintain high oxygen supply. In other words, why are the emergency mechanisms not used constitutively? Most probably, the permanent activation of such mechanisms would increase the risk of physiological breakdown, e.g. heart failure. If ventilation rates could have been optimized selection would have favored it.

The large Q_{10} of oxygen consumption rates at the BPT of ventilation (most apparent in *E. cyaneus* and *G. lacustris*, Publication I) might reflect a significant change of physiological functioning and most likely also a higher movement activity, which was, however, only significant in *G. lacustris*. Adult individuals of *E. verrucosus* retreated progressively with increasing ambient temperatures resulting in retreat of large adults at around 10 - 11°C in their natural environment. Furthermore, the investigated population of *E. cyaneus* experiences a maximum temperature of 20°C in summer in the upper littoral zone of Lake Baikal and almost no adult individuals of *G. lacustris* were found at the water edge of the studied pond, Lake-14, when temperatures had reached 22°C. The close correlation of the BPT of ventilation and the maximal habitat temperatures of the investigated populations, supports the suggestion that the BPT of ventilation reflects the T_p and underlines its ecological relevance.

The ranking of thermal sensitivity was in accord with previous studies on preference temperatures (Timofeyev et al. 2001, Timofeyev and Kirichenko 2004, Timofeyev and Shatilina 2007) and correlated with the constitutive levels of the molecular chaperone Hsp70 (Bedulina et al. 2013, Protopopova et al. 2014). Migration behavior in *E. verrucosus* has previously been hypothesized to be linked to thermal changes (Weinberg and Kamaltynov 1998) and the findings presented here provide the physiological underpinnings for this behavior. They further support the assumption of an allometry of thermal limitation and its correlation with oxygen uptake as seen before in cuttlefish (Melzner et al. 2007). Small sized animals have a higher oxygen uptake and extended thermal tolerance, owing to their higher gill surface area to body volume ratio and shorter diffusion path lengths for oxygen molecules. Accordingly, large adults of *E. verrucosus* were the first to escape from the upper littoral to cooler temperatures. The following chapter intends to link whole animal thermal limitations to observations for temperature-dependent activities of key metabolic enzymes.

5.1.2 THERMAL PLASTICITY OF KEY METABOLIC ENZYMES AND WHOLE ANIMAL RESPONSES

KEY QUESTION 2

Does the thermal plasticity of key metabolic enzymes correlate with organismic thermal tolerance and does metabolic fuel use change with temperature?

The main findings are schematically displayed in Figures 22, 23 and 24.

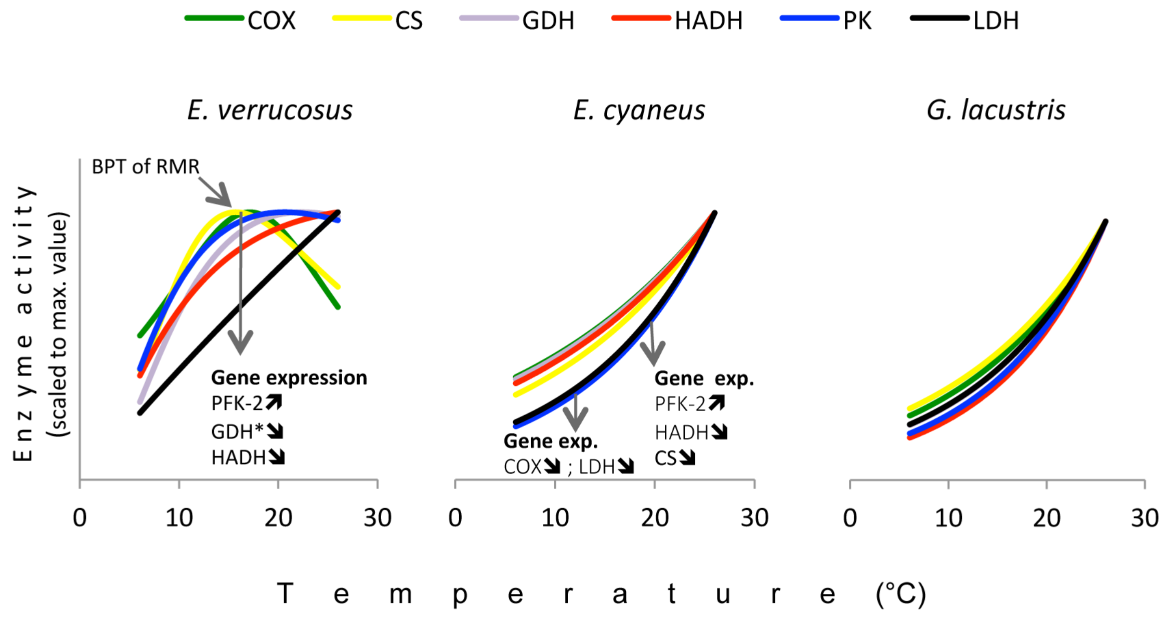


Figure 22: Activities of key metabolic enzymes scaled to their respective maximum values in order to visualize curve shapes. Arrows mark points at which significant physiological or molecular changes were noted, i.e. BPT breakpoint temperature of routine metabolism and changes in gene expression (temperature range: 6 - 23.6°C).

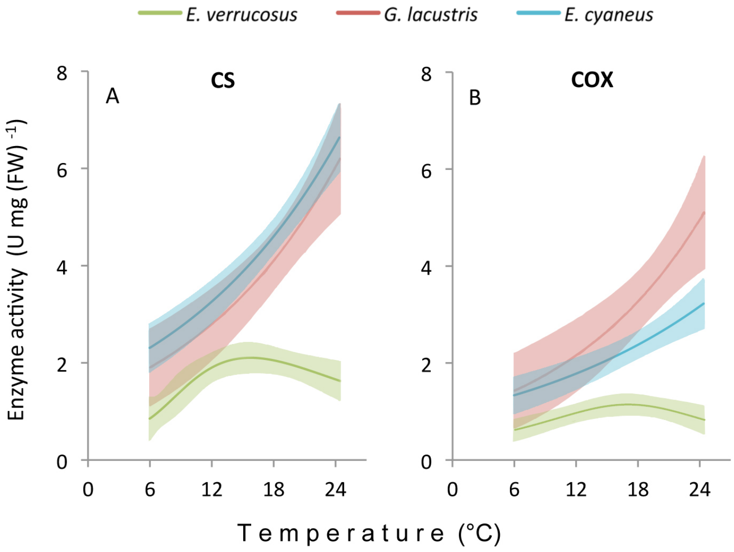


Figure 23: Enzyme activities of CS and COX. Modeled values (solid lines) and 95% confidence intervals (shaded areas); see Publication II for model equations.

KEY FINDINGS AND SIGNIFICANCE

Temperature dependent changes in activities of key enzymes involved in aerobic metabolism were correlated with those in oxygen consumption rates in all three amphipod species, however, the shapes of curves representing these changes differed between species. In accord with the OCLTT hypothesis, the higher thermal tolerance of the eurythermal E. cyaneus *and* G. lacustris*, as compared to* E. verrucosus*, can be attributed to their higher aerobic capacities (reflected by CS and COX capacities) and in the allover higher activities and incremental growth in both enzymatic activity and oxygen consumption during the progressive temperature increase. In* E. verrucosus*, oxygen consumption and enzymatic activity responses to changing temperatures were represented by hyperbolic or peak-shaped curves with isothermal breakpoints (around 15°C), beyond which both variables showed a clear decrease in magnitude. Furthermore, transcriptional levels of genes coding for enzymes involved in aerobic processes (HADH and GDH) were down-regulated and genes implicated in hypoxic conditions (PFK2) simultaneously up-regulated. Concomitant changes at cellular (enzymatic) and molecular (gene expression) levels, which correlated with changes in respiration and followed changes at the whole animal level (constraints in ventilation) suggests a tightly regulated feedback system in response to decreasing oxygen availability in* E. verrucosus. *In E. cyaneus and G. lacustris enzyme activities showed exponential increases.* E. cyaneus *showed slight thermal compensation as RNA transcript levels of many enzymes decreased progressively with temperature, whereas* G. lacustris *showed no thermal compensation. Consequently, Q_{10} - relationships at high temperatures were lower in* E. cyaneus *than in* G. lacustris*. The tightest constraints were always seen at the whole animal level supporting the theory of a systemic to molecular hierarchy of thermal tolerance.*

There was a constant increase in enzymatic activity in *E. cyaneus* and *G. lacustris* and a reduction in *E. verrucosus* beyond a temperature of about 15°C, which was not generally accompanied by a change in the transcription levels of the genes encoding the respective enzymes and, by extension, enzyme concentrations. This suggests that the enzymes of the three species may differ in enzymatic efficiencies at high temperature rather than absolute concentrations. The efficiency of an enzyme depends on, besides other factors, the structural conformation and stability of its tertiary structure, which can be disturbed at high temperatures. High temperatures increase the frequency of protein folding failures and can result in lower enzymatic activity and efficiency. Protein misfolding is prevented by chaperones in the cell and differences in the levels of Hsp70 between species correspond with those in their enzymatic efficiencies (Bettencourt et al. 2008). Indeed, higher constitutive levels of Hsp70 in limpets from the high-intertidal, as compared to those from the more low-and mid-intertidal limpets, has been termed as a protection mechanism against extreme temperature fluctuations (Dong et al. 2008). Similarly, constitutive levels of Hsp70 in *E. verrucosus* were shown to be lower than in the other two species (Bedulina et al. 2013, Protopopova et al. 2014). Differences in constitutive levels and not only inducible levels of genes between species is indicative of their adaptive strategies developed over evolutionary time scales.

Modifications of the genes encoding the respective enzymes may alter their thermal stability. Only a single change in amino acid sequence is needed to change the thermal stability and function of enzymes, as shown for the heart-type LDH (LDH-B) (Powers et al. 1993). Previous studies showed that even when active site sequences were fully conserved, closely related eurythermal and stenothermal species showed intraspecific differences in K_m values. No active site residues need to be affected because any changes affecting the conformational flexibility of an enzyme may lead to alterations in structure and function (Somero 1995). Slight temperature-dependent conformational changes may cause the decreased enzyme activities or, in other words, physiological inactivation. This may affect molecular recognition events, which are important for substrate binding by enzymes and oxygen binding by respiratory proteins (Pörtner et al. 2007). *E. verrucosus* escapes rising temperatures in the upper littoral by migrating to deeper areas and consequently experiences a relatively stable thermal habitat. The absence of fluctuating thermal conditions may result in a reduced thermal flexibility of enzymes because a high flexibility usually comes at the price of reduced substrate affinity (Somero 1995, Fields

2001). However, the fact that juveniles were observed to stay in the upper littoral during summer contradicts the above-described theory.

Further, the expression of different isoforms with specific thermal characteristics may support enzymatic functioning over a wide thermal range because the paralogous isozymes might vary in thermal stability and in kinetic properties. Isozymes with different thermal characteristics might be synthesized constitutively at all temperatures, or solely the isozyme appropriate for the current thermal range. However, the evolution of isozymes is probably less common than the evolution of allozyms (Fields 2001), which is due to the fact that sustaining a set of different isozymes is costly. Allozymes are alternative forms of an enzyme, which are encoded by different alleles at the same locus; isozymes are enzymes that have the same function but are encoded by different genes with different loci (Berg et al. 2002). Moreover, closely related species likely have the same isozymes as changes would imply a large step in evolution.

Furthermore, posttranslational modifications might be responsible for the close correlation between oxygen consumption and activities of key metabolic enzymes. Changes in phosphorylation and temperature-dependent folding have been shown to occur in response to hypoxia, which may affect enzyme functioning and consequently regulate e.g. energy metabolism (Kumar and Klein 2003, and references therein). In Publication II it was hypothesized that a long evolution time in the isolated Baikal system may have led to synchronization of thermal limitations on different organizational levels (i.e. oxygen-dependent metabolic enzymes = biochemical level and oxygen consumption rates = whole animal level) in the thermally sensitive *E. verrucosus*. A narrowly regulated feedback system affecting the functionality of enzymes might have evolved in response to hypoxemia in *E. verrucosus*. This narrow regulation is likely beneficial for preventing accumulation of metabolites that cannot be further processed at limited oxygen availability. Thus, adapting enzyme activities to oxygen availabilities is beneficial to maintain homeostasis (at a decreased metabolic state). Eventually this may lead to decreasing enzyme activities of oxygen-dependent key metabolic enzymes, which seem to parallel decreasing oxygen consumption rates. An indication that processes are regulated by systemic oxygen availability is the activation of PFK2 gene expression (support of the glyconeogenic metabolic pathway), which correlated with the BPTs of oxygen consumption and activities of key metabolic enzymes in *E. verrucosus*. PFK2 was shown to be regulated by the hypoxia inducible factor (HIF) (Kumar and Klein 2003) and is thus related to decreasing systemic oxygen availability. The theory of hypoxia inducing posttranslational modifications would be consistent with the observation that juveniles are more thermally tolerant than adult individuals (M. A. Timofeyev, personal communication) as this would be in accord with the theory of an allometry of thermal limitation. Furthermore, LDH was not constrained in the same manner as the other enzymes. As LDH is needed for anaerobic energy supply it is unlikely to be down-regulated in response to hypoxia.

In conclusion, sensitive responses to hypoxemia may explain the physiological and biochemical reactions of *E. verrucosus*. Long evolution time in the isolated Baikal system may have favored synchronization of thermal limitations on different organizational levels (i.e. oxygen-dependent metabolic enzymes and oxygen consumption rates) in the thermally and hypoxia sensitive *E. verrucosus*. The molecular underpinnings for the specific enzyme activity curves need to be studied in detail in future research.

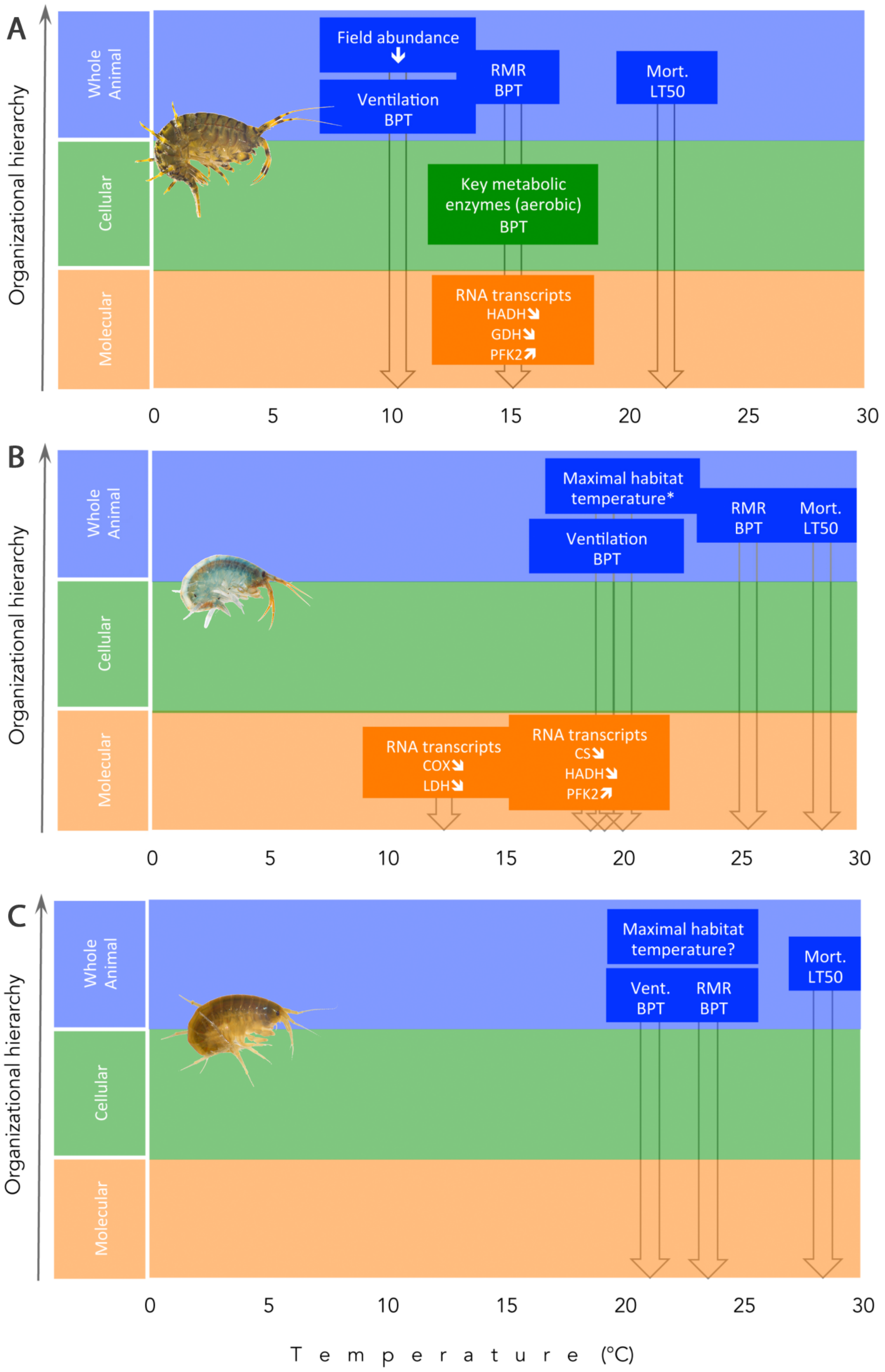


Figure 24: Summary of responses to thermal changes on different hierachical levels in *E. verrucosus* **(A)** *E. cyaneus* **(B)** and *G. lacustris* **(C)**. All physiological, biochemical and molecular parameters were determined upon gradual warming in laboratory experiments (0.8°C d^{-1})

↓ Significant loss of abundance of large adult individuals in the upper littoral (0 – 1.2 m depth)

↘ Onset of progressive decrease

↗ Onset of progressive increase

*A maximum temperature of about 20°C was reported for the upper littoral of Lake Baikal (Timoshkin et al. 2009), the habitat of *E. cyaneus*; the maximal habitat temperature of *G. lacustris* is likely more variable and thus not indicated with an arrow.

5.2 What makes an organism susceptible to waterborne cadmium?

KEY QUESTION 3

How do closely related species, which differ with respect to body surface to volume ratio and cellular stress response capacity, respond to cadmium exposure regarding uptake from aqueous solutions, internal metal distribution (cellular compartmentalization), concentration-mortality relationships and physiological reactions to low biologically effective doses?

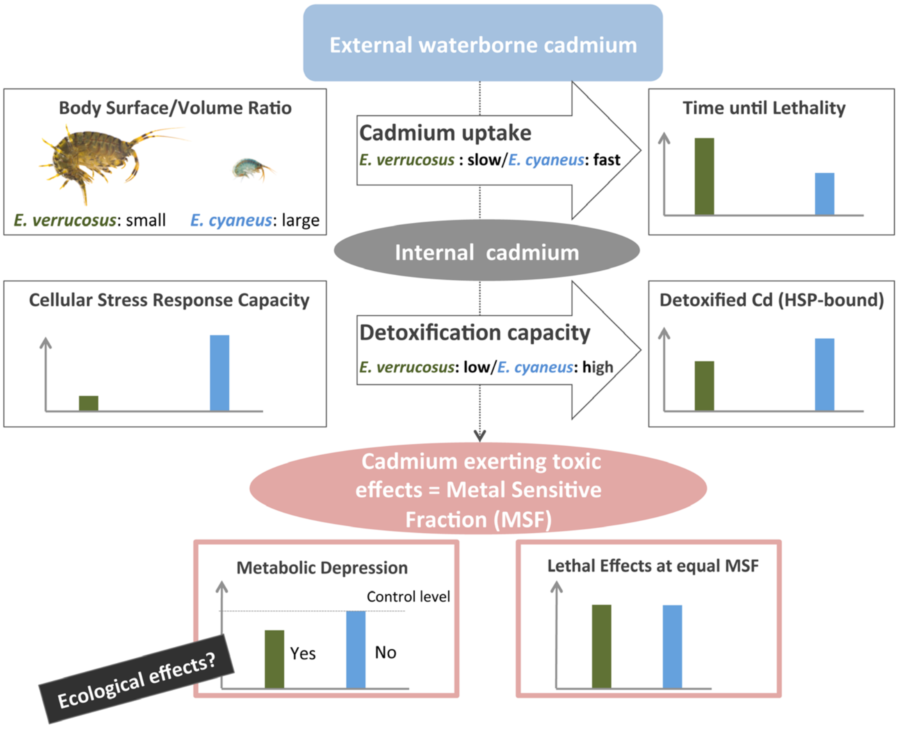


Figure 25: Graphical abstract for Publication III.

KEY FINDINGS AND SIGNIFICANCE

Lake Baikal offers the opportunity to study endemic species of the same genus but with different physiological characteristics, which inhabit pristine waters. This may enable us to elucidate physiological factors, which influence cadmium sensitivity as many confounding factors such as pre-exposure and phylogenetic effects can be excluded. Faster uptake rates of E. cyaneus *in comparison to* E. verrucosus *and thus lower lethal concentrations were likely determined by the body surface to body volume ratio. In 4 week-exposures (effect-scaled setup: species-specific LC1 of* $CdCl_2$*) it was found that more cadmium was bound to heat stable proteins in* E. cyaneus *than in* E. verrucosus*, which was in accordance with the higher CSR capacity of the former. In this setup similar concentrations of MSF-associated cadmium (*E. verrucosus *= 0.26 ± 0.07 µg g* $(FW)^{-1}$*;* E. cyaneus *= 0.25 ± 0.06 µg g* $(FW)^{-1}$*) were determined in the two species. Metabolic depression and reduced ventilation upon exposure to cadmium was only found in* E. verrucosus *for a range of sublethal concentrations but not in* E. cyaneus*. The finding of lower thermal tolerance and metabolic rate depression of* E. verrucosus *in response to low concentrations of cadmium might be due to the allover high sensitivity to hypoxemic stress of this species.*

5.2.1 PHYLOGENY AND ALLOMETRY OF CADMIUM UPTAKE FROM AQUEOUS SOLUTIONS

The data presented in this thesis support the assumption that within closely related species body size is a determining factor for cadmium uptake and toxicity (cf. Buchwalter et al. 2008). After correcting uptake rates of dissolved cadmium for body size, phylogeny is a further determining factor for cadmium uptake due to specific calcium demands (Philips and Rainbow 1994). Data on cadmium uptake from water in the studied species show similar body size dependencies as reported for other closely related species (Buchwalter et al. 2008). In turn, this would indicate a similar calcium demand of the studied species. This can be concluded because the studied species were kept in the same medium and were consequently exposed to the same calcium bioavailabilities, which is an important parameter for cadmium uptake (Wright 1980, Chang et al. 1997, Tan and Wang 2011). Owing to the similarities in size and equal charges of cadmium and calcium ions, the permeability of a body surface for cadmium ions is expected to be determined by calcium channels or by mediation of specific calcium transport mechanisms such as Ca^{2+}-ATPases. Thus, cadmium may substitute for calcium on a calcium regulatory mechanism (Wright 1980). Indeed, studies on fish (Wicklund and Runn 1988, Chang et al. 1997), molluscs (Wang and Evans 1993, Bjerregaard and Depledge 1994), and crustaceans (Bjerregaard and Depledge 1994, Tan and Wang 2011) provide evidence for a common route of uptake of both calcium and cadmium. Although this relationship is less clear for aquatic insects (Poteat and Buchwalter 2014), several patterns point towards the correlation between calcium and cadmium uptake (rates and routes) in aquatic organisms (Wright 1980, Wright and Frain 1981, Wang and Evans 1993, Bjerregaard and Depledge 1994, Tan and Wang 2011). In conclusion, only the free ion is considered as bioavailable. Thus, metal speciation in the medium and physiological regulation in response to the ionic composition of the exposure medium are important factor for metal toxicity (Campbell et al. 2002, Luoma and Rainbow 2008, Lavoie et al. 2012). However, this is less important when comparing metal toxicity of closely related species retained from the same site and kept in the same exposure medium as it was done in the present study.

The magnitude of trace metal uptake and body concentrations is highly variable and the same concentration may be considered as high in one species and low in another (Rainbow 2002). Organisms with high calcium demand for the formation of shells or calcification of the exoskeleton like molluscs and malacostracean crustaceans may exhibit high calcium pump activities, particularly in waters with low ion concentrations (Philips and Rainbow 1994). Thus, it is likely that organisms that efficiently pump calcium ions at low concentration in the medium will take up more cadmium than others. Reduced calcium concentrations or salinity may consequently enhance cadmium toxicity as shown for many aquatic organisms (Bjerregaard and Depledge 1994, Rodriguez et al. 2001, Ballatori 2002, Pellet et al. 2009, Barbieri and Paes 2011).

In conclusion, uptake rates are influenced by the calcium requirement of a species. Accordingly, phylogeny was a major factor explaining cadmium uptake from aqueous solutions by aquatic insects (Buchwalter et al. 2008), which has also been concluded for calcium uptake in the same taxa (Poteat and Buchwalter 2014). *E. verrucosus* and *E. cyaneus* are suggested to have similar calcium requirements due to the similarities in composition and habitus (body build and general appearance) of their exoskeletons but nevertheless show large differences in cadmium uptake. This is likely explained by their different body sizes because, besides calcium requirement, uptake is mainly determined by the ratio of permeable body surface to body volume, which is expected to decrease with increasing body size within species and possibly families of aquatic organisms. For example, gill surface area (considered as permeable surface area) was shown to decrease with increasing body size in various amphipods (Moore and Taylor 1984). These observations are further supported by the results presented in this thesis as the uptake rates of cadmium were higher in *E. cyaneus* than in the *E. verrucosus*. Moreover, the results for *G. lacustris* support this assumption as similar cadmium uptake and body concentrations (Figure 16A and B) and accordingly similar LC50 and LT$_{ime}$50 values (Figure 15) were found in the similar-sized *E. cyaneus* and *G. lacustris*. Similarly, allometry of cadmium influx has been previously shown in bivalves as metal uptake from the dissolved phase decreased with an increase of body sizes of scallops (Pan and Wang 2008), mytilids (Wang and Fisher 1997) and within families of aquatic insects (Buchwalter et al. 2008). In mysid shrimps, the inverse relationship between cadmium body burden and body size was described as the power of function: $Y = 0.20X^{0.60}$, where Y = total cadmium burden and X = body weight (Lasenby and Vanduyn 1992). Interestingly and despite the fact that Lasenby and Vanduyn (1992) studied surface-bound metal in contrast to metal influx, there is a similar ratio in the here studied species. As gill surface is a fraction of the entire body surface, uptake rates (among the same or closely related species) likely show similar allometric scaling. Similar allometric scaling was determined within families of aquatic insects for uptake from aqueous solutions (Buchwalter et al. 2008). Background concentrations of *E. verrucosus* and *E. cyaneus* sampled in Bolshie Koty were similar to those previously reported concentrations for Baikal gammarids (mean: 0.039; range: 0.0075 – 0.074 µg g (FW)$^{-}$) (Leeves 2011). We found 0.03 (range: 0.03 - 0.04) and 0.09

(range: 0.08 – 0.10) µg g $(FW)^{-1}$ in *E. verrucosus* and *E. cyaneus*, respectively (n = 4). Thus, also for the background concentrations it is evident that *E. cyaneus* shows higher cadmium concentrations than the larger *E. verrucosus*.

Our results emphasize the importance of taking allometric effects of cadmium uptake from water into account when modeling toxic effects of metal pollution. Furthermore, they provide initial evidence that metal uptake scales with body size within closely related Baikal amphipods. However, this must be studied in more detail in future research including a larger set of species. If exoskeletons are of very different habit (thickness and structure) it might be necessary to report uptake rates based on ash-free weight. Metals bound to the body surface should be excluded for the comparison of uptake rates because a highly structured body surface may diminish the scaling effect as cadmium was shown to efficiently bind to chitinous material (Robinson et al. 2003, Zhou et al. 2004). This might be important for many Baikal endemic amphipods such as species of the genera *Pallasea*, *Acanthogammarus* and *Brandtia*, which have highly structured exoskeletons in comparison to the species studied here. In the present work, metals bound to body surface were removed by chelating agents as the focus of this thesis was to study uptake and toxicity of cadmium. However, surface-bound metal is highly important for ecological implications such as predator-prey interactions regarding metal transfer along the food chain (Robinson et al. 2003), which was not addressed in the present thesis. Moreover, food chain transfer is controlled not only by the amount of the metal accumulated in the prey but also its physicochemical detoxification process favored by the prey species (Wallace and Lopez 1996, 1997, Wallace et al. 1998).

5.2.2 CELLULAR STRESS RESPONSE CAPACITY AND METAL DETOXIFICATION

Among the species studied here, *E. cyaneus* showed a higher fraction of cadmium associated with heat stable proteins in comparison to *E. verrucosus* when both species were exposed to their LC1 of $CdCl_2$ (Publication III). Higher constitutive levels of the heat shock protein Hsp70 were found in *E. cyaneus* and *G. lacustris* than in *E. verrucosus* (Bedulina et al. 2013, Protopopova et al. 2014), which suggests higher CSR capacities of the former two species and is congruent with the results presented here. When exposed to the same concentration of cadmium (105 nM, 4 wk, 6°C) *E. verrucosus* accumulated 42% of the total cadmium in the HSP fraction whereas in *E. cyaneus* and *G. lacustris* 52 and 58% of the total body concentration of cadmium were associated with the HSP compartment, respectively.

It has been suggested that heat stable proteins are most likely strictly MTs (Rainbow et al. 2015). However, others failed to demonstrate the existence of MTs in the heat stable protein fraction and suggested that it mainly contains glutathion (Buchwalter et al. 2008). Metallothionein and glutathione as well as heat shock protein and antioxidant enzymes like catalase are expressed upon various environmental stress factors (Kültz 2005). Cells sense stress stimuli on the basis of macromolecular damage and consequently respond to a variety of stressors independent of the type of stress causing the damage (Kültz 2005). Cadmium exerts a variety of toxic effects but many of them are the consequence of oxidative stress, which induces universal stress responses (CSR), e.g. induction of metallothioneins (MT), heat shock proteins, glutathion and catalase. MTs are low-molecular weight, cysteine-rich, metal-binding non-enzymatic proteins and highly conserved across species (Klaassen et al. 1999). They have been shown to function as a storage for zinc, act as free-radical scavengers and protect against cadmium toxicity (Klaassen et al. 1999). Heat-shock proteins play important roles in cellular homeostasis under normal physiological conditions and under stressful conditions (Feder and Hofmann 1999). Both molecules were shown to perform chaperone function (Georgopoulos and Welch 1993, Costello et al. 2004) and a correlation between their expression has been demonstrated in cadmium in aquatic animals like oysters (Ivanina et al. 2008) as well as in mammals (Bauman et al. 1993). Cadmium can be detoxified by metallothionein (MT) because of its high content of cysteine residues that efficiently bind cadmium and therefore reduce the amount of metabolically available cadmium in the organism (Kägi and Schäffer 1988, Klaassen et al. 1999). Thus, high levels of MT are beneficial to sequester cadmium and may result in a higher cadmium tolerance like seen in previous studies (Klaassen et al. 1999 and references therein). Though, the higher level of CSR related proteins is most likely the reason for the higher amounts of cadmium associated with the HSP fraction in *E. cyaneus* and *G. lacustris* in comparison with *E. verrucosus*. However, the contribution of metallothionein-like proteins was not determined in this study.

While the binding of metals to MT is reversible, metals are irreversibly detoxified when they have been included in metal rich granules (Vijver et al. 2004). No general localization of metal-containing granules in invertebrates were reported, although they were mostly associated with organs with digestive, storage and excretory functions (e.g. hepatopancreas, midgut, digestive gland, Malpighian tubules and kidney) are most likely prime sites for metal storage and detoxification in crustacean, molluscs and insects (Brown 1982). Mineral granules or concretions have been identified in intestinal cells of many invertebrates, including collemboles (Van Straalen et al. 1987) and crustaceans (Brown 1982). In earthworms, phosphate-rich structures termed chloragocytes, are metal-sequestrating organelles that respond to elevated metal exposure by increasing the volume of the debris vesicles and thereby decreasing the chloragosome volume (Morgan et al. 2002). Metal accumulation in inclusion bodies was previously monitored in isopods by X-ray microanalysis of different types of granules (Hopkin et al. 1989). The granule type accumulating cadmium contains mainly acid phosphatase and originates from the lysosomal system (Hopkin et al. 1989). In the present study the differences between detoxified and metabolically available metal were mainly due to the higher fraction of cadmium bound to heat stable proteins whereas the fractions containing metal rich granules were similar. Thus, it can be concluded that during the 4-week exposure to species-specific LC1 of $CdCl_2$, the different concentrations of detoxified metal were due to a higher level of heat stable proteins in *E. cyaneus* in comparison to *E. verrucosus* and not related to differences in long-term metal sequestration via metal rich granules. However, as the subcellular fraction containing metal rich granules also contained undigested exoskeleton in samples of both *E. verrucosus* and *E. cyaneus*, the conclusion is only valid, when similar amounts of exoskeleton are contained in the samples of both species. This needs to be determined in future research.

In conclusion, the different amounts of biodetoxified cadmium were in accord with previous findings on concentrations of CSR related proteins in the studied species. However, detoxification capacity seems to make only a small contribution to the differences in mortality, which are largely explained by the different uptake rates of the two species. However, a different capacity in the CSR system (e.g. antioxidant capacity) may contribute to explain the different physiological reactions of *E. verrucosus* and *E. cyaneus* to low doses of cadmium, which is discussed in chapter 5.4.

5.2.3 SUBCELLULAR METAL COMPARTMENTALIZATION: IS METAL BOUND TO ORGANELLES AND HEAT LABILE PROTEINS ALWAYS A GOOD PREDICTOR OF TOXIC EFFECTS?

In *E. verrucosus* and *E. cyaneus* the MSF fraction of cadmium was remarkably similar when the species were exposed to their species-specific LC1. Also the data for *G. lacustris* supports the assumption that the MSF is responsible for lethal effects as *E. cyaneus* and *G. lacustris* show similar MSF fractions when exposed to 105 nM $CdCl_2$ for 4 weeks (6°C) and *E. verrucosus* shows a much lower MSF fraction, which was in accordance with mortality data. However, effects of low cadmium concentrations were not similar as ventilation was reduced in *E. verrucosus* but not in

E. cyaneus in long-term exposures (4 wk, LC1 at 6°C). Thus, it can be questioned whether the MSF is a good predictor for sublethal toxic effects.

Metal speciation in aqueous solutions is highly important for the uptake of cadmium from the medium as only the free ion is considered to be bioavailable, i.e. ready for uptake by aquatic organisms (Campbell et al. 2002). However, metal speciation is not further discussed here since the primary focus of this subchapter is on internal bioavailable metal, which is termed as metabolically available metal, hereon, and is distinguished from the bioavailable metal.

It is generally agreed upon that only metabolically available metal causes toxic effects (Rainbow 2002, Vijver et al. 2004). Thus, there is no critical threshold of total body concentration of accumulated metal determining the onset of toxic effects. Rather, toxicity is related to a critical concentration of metabolically available accumulated metal that ensues when the rate of metal influx exceeds the combined rates of excretion and detoxification (Rainbow 2002). Buchwalter et al. (2008) proposed that the conceptual model of (Rainbow 2002) can be tested by combining bioaccumulation kinetics (Luoma and Rainbow 2005) and subcellular fractionation (Wallace et al. 2003, Wang and Rainbow 2006). Bioaccumulation kinetics can be deduced from the biodynamic metal bioaccumulation model, which combines targeted, geochemical data from a site of interest with parameterization of key physiological constants for a species from that site. The physiological factors cover metal influx from water, influx rates from food, rate constants of loss, and growth rates (when high) (Luoma and Rainbow 2005). Subcellular fractionation into heat stable proteins (HSP; fraction contains MTLPs), metal rich granules (MRG), heat denaturable proteins (HDP), organelles and cell debris is a pragmatic approach to determine metabolically available and thus potentially toxic metal, which has been applied on many aquatic organisms (Wallace et al. 2003, Martin et al. 2007, Buchwalter et al. 2008, Khan et al. 2011, Publication III). The hypothesis that toxicity relates to metal bound to organelles and non-detoxificatory soluble proteins contained in the HDP fraction was supported by the results presented in this thesis and has been previously found in studies on bivalves (Wallace et al. 2003) and diatoms (Wang and Wang 2008). Rainbow et al. (2015) investigated oysters from unpolluted sites and sites with raised zinc bioavailabilities. In oysters retained from the unpolluted site, the MSF approximated to the theoretical metabolically available zinc concentration, while in oysters previously exposed to elevated zinc concentrations, the MSF would include more zinc detoxified in the lysosome component of the organelle-bound zinc. Thus the MSF in such populations would deviate more from the theoretical MA metal concentration. In this thesis, both species were sampled at the same unpolluted site, which thus would render this factor irrelevant for the here studied species. For the sake of general applicability, it is helpful to separate the organelle fraction into two separate fractions containing mitochondria and lysosomes as it has been recently proposed in a common guideline for metal compartmentalization for aquatic invertebrates (Rosabal et al. 2014).

Criticisms of the subcellular partitioning approach may address the fact that fractions can be contaminated by other fractions; for instance the organelle fraction can contaminate the debris fraction. Moreover, the fractions need to be characterized in more detail and exoskeletons are often not digestible (Buchwalter et al. 2008, Khan et al. 2010, Publication III). Further, species may show a high variability in their repair capacities and thus show different sensitivities, which cannot be elucidated by the compartmentalization approach. In the present study, different physiological reactions of the two species in response to equal concentrations of MSF-associated cadmium were found, which emphasizes the need for studying sublethal effects in addition to metal uptake and internal distribution for the estimation of ecological effects.

5.2.4 SUBLETHAL PHYSIOLOGICAL AND BEHAVIORAL EFFECTS OF CADMIUM

In long-term exposures (4 wk, LC1 at 6°C) with low cadmium concentrations *E. verrucosus* showed decreased ventilation rates whereas it showed progressively increasing ventilation in a short-term study (24h, LC10 at 6°C). By contrast, *E. cyaneus* did not show any changes in ventilation rates in both setups.

The reported toxic effects of cadmium vary widely in aquatic ectotherms due to the numerous abiotic and biotic factors altering the reaction of an organism to waterborne cadmium and, moreover, different exposure scenarios (time and concentration). Consequently, published data on cadmium toxicity to aquatic organisms with a focus on metabolic and behavioral effects is highly discordant as discussed below. Moderate sublethal stress might be compensated by increasing metabolic costs for detoxification, excretion, and to fuel the repair machinery (Calow 1991). This may in turn decrease the scope for growth, reproduction, locomotor activity or other energy consuming processes (e.g. grooming). Decreased energy reserves and reduced sizes of elytrae, tibiae and rear femora were monitored in carabid beetles exposed to cadmium-contaminated food (Maryanski et al. 2002). In the short-term, an allover increase in metabolic rate may be observed (see e.g., Lannig et al. 2006) possibly due to mobilizing energy storages to cope with the toxicant. However, elevated metabolic rates at unchanged food availabilities are unlikely in the long-term (field conditions) and a redistribution of the energy budget seems more realistic. Behavioral reactions like locomotor activity are often more sensitive markers than growth rate as growth might not be affected due to unlimited feeding in laboratory setups (Khalil et al. 1995).

More extreme stress may result in significant down-scaling of metabolic rate in order to conserve energy and prevent damage (Storey and Storey 2004). Decreased metabolic rates have been observed in aquatic ectotherms when exposed to sublethal cadmium concentrations (Gaudy et al. 1991, Publication III). The results obtained for *E. verrucosus* in this thesis reflect the time and/or concentration dependence of physiological responses to cadmium. Figure 17A displays that *E. verrucosus* significantly increased ventilation rate when exposed to its LC10 for 24 h at 6°C. By contrast, ventilation rates of animals exposed to their LC1 for 4 weeks at 6°C were significantly reduced in *E. verrucosus* in comparison to parallel controls (Figure 2 of Publication III). Ventilation rate of *E. cyaneus* and *G. lacustris* (*G. lacustris* was only investigated in 24 h studies) did not change in the same experimental setups (Figure 17B and C). Since *E. verrucosus* and *E. cyaneus* were exposed to species-specific biologically effective concentrations it is interesting that the two species show different strategies to cope with cadmium, as the metabolically available fractions of cadmium are the same.

Except in cases of environmental accidents, low toxicant concentrations are of greater ecological relevance as environmental concentrations are usually low but persistent (Fent 2013). The presented results for *E. verrucosus* emphasize that the mechanisms are highly dependent on exposure time and concentration, which has been shown in many other species. For example, a decrease of ventilation rate was recorded in cadmium exposed *G. pulex* (Felten et al. 2008, Vellinger et al. 2012) whereas individuals of *Dikerogammarus villosus* increased mobility under short-term exposition to comparatively high cadmium water concentrations. The differences compared to the study with *G. pulex* were suggested to be due to the different exposure scenarios (Sornom et al. 2012). Consequently, a high cadmium concentration might induce an escape response due to a rapid change in water chemistry, which is further confirmed by data on temporal comparison for *E. verrucosus* in this thesis comparing short- and long-term exposures. However, *E. cyaneus* does not show any changes in ventilation over different temporal scales (24 h, 4 weeks). Why do the closely related species *E. verrucosus* and *E. cyaneus* show the same mortality when exposed to the same metabolically available concentrations of cadmium but not the same physiological and behavioral reaction? Is this reaction related to the higher thermal sensitivity of *E. verrucosus* in comparison to *E. cyaneus*? The limited sensitivity of *E. cyaneus* could be explained by its exposure to frequent environmental changes and inability to migrate to other densely occupied niches in the littoral of Lake Baikal. Thus, it might have evolved different strategies to react to a changing environment. The molecular basis for the sensitivity of *E. verrucosus* in contrast to *E. cyaneus*, which did not show alterations of metabolic rate or ventilation behavior, is focused on in chapter 5.4.

5.3 Combined effects of temperature and cadmium

KEY QUESTION 4
Does temperature enhance the deleterious effects of cadmium in Baikal amphipods?

KEY FINDINGS AND SIGNIFICANCE

In order to test whether temperature enhances the toxicity of cadmium concentrations that were sublethal at 6°C, animals were exposed to gradual temperature increase (0.8°C d^{-1}) and low biologically effective concentrations of cadmium (LC1). Lethal temperatures for animals exposed to a combination of gradual thermal increase and species-specific LC1 of $CdCl_2$ *were lower than those for animals exposed to temperature increase alone. Moreover, mortality increased when* E. verrucosus *was exposed for 14 days to a constant temperature of 10°C (close to* T_p*) in comparison to the 6°C exposure where no mortality occurred. In* E. cyaneus *mortality increased significantly at 18°C (close to* T_p*) only when exposed to the LC1 of* E. verrucosus *but not when exposed to its species-specific LC1. Metabolic rate depression was observed at 6°C in* E. verrucosus *when exposed to a range of sublethal concentrations. No metabolic depression was observed at 10°C (14 day-exposure), however, the mortality markedly increased when exposed to its species-specific LC1 at this temperature.*

Interestingly, mortality was increased in *E. verrucosus* in LC1 exposures at T_p in comparison to the temperature treatment alone. By contrast, mortality was not increased under the same setup in *E. cyaneus* (Figure 20D). This indicates that on an effect-scaled basis *E. verrucosus* reacts more sensitively to the combination of the two stressors. However, for ecological implications faster uptake rates in *E. cyaneus* need to be taken into account.

Less clear results are seen for the metabolic rate depression, which was observed at sublethal concentrations at 6°C in *E. verrucosus*. Metabolic rate depression is likely a protection mechanism; its induction might not be possible under already suboptimal conditions when cost is enhanced. Likewise, a sick animal would not likely enter a state of hibernation (Lyman 1982).

Temperature and cadmium have often been shown to act synergistically (Holmstrup et al. 2010). Synergism comes from the word "synergos" meaning working together and refers to the interaction between two or more factors when the combined effect is greater than the sum of the single effects. The mechanisms of synergism are variable but mostly appear to be determined by the effects on enzymes. Cadmium causes oxidative stress by inhibiting antioxidant enzymes (Ikediobi et al. 2004, Kim et al. 2005, Ognjanovic et al. 2008, Jiang and

Zhou 2012), which are also important for coping with oxidative stress caused by increased temperature (see Lesser 2006 for review). Cherkasov et al. (2007) found ROS levels in oysters to increase upon cadmium exposure and stated that elevated temperatures enhanced this effect. When chemicals have larger effects than each of the single factors alone as seen here for cadmium and temperature, the potential hazards of the chemicals should be re-evaluated, taking their synergistic properties into consideration (Holmstrup et al. 2010).

5.4 Sensitivity to temperature and cadmium – Physiological and behavioral plasticity

KEY QUESTION 5

Are thermally sensitive organisms more susceptible to cadmium?

Aquatic animals may show physiological and behavioral responses to changing environmental conditions (Figure 26).

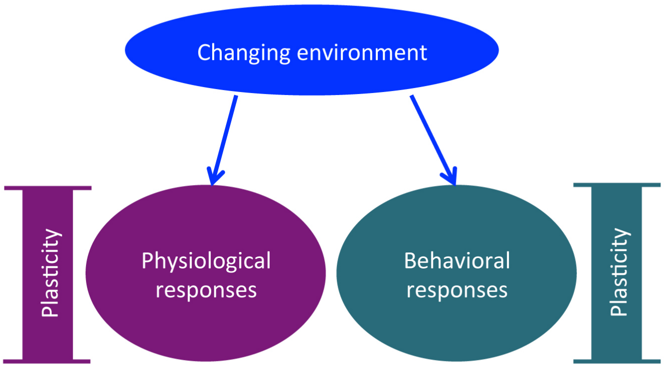


Figure 26: Coping with a changing environment: Physiological and behavioral plasticity.

KEY FINDINGS AND SIGNIFICANCE

E. verrucosus *showed sensitive reactions to both temperature (BPT of ventilation and oxygen consumption, 10.6 and 15.0°C, respectively); it actively avoids rising temperatures in the upper littoral in summer by migrating to sublittoral areas where colder temperatures prevail.* E. verrucosus *reacted sensitively to low biologically effective doses of cadmium (metabolic depression at LC1 exposure), whereas* E. cyaneus *was more tolerant (BPT of ventilation and oxygen consumption: 19.1 and 25.2°C, respectively and no metabolic depression at LC1 exposure).*

E. verrucosus has a high behavioral plasticity to cope with thermal stress mediated by migration to its preferred thermal range. Further, it shows metabolic depression in response to sublethal cadmium concentrations (LC1), which may prevent cellular damage. By contrast, *E. cyaneus* shows a high physiological plasticity as it tolerates high temperature fluctuations in the upper littoral and shows no metabolic depression under exposure to sublethal cadmium levels (LC1).

Is there a common mechanism explaining the species-specific reactions to both environmental stressors?

The higher physiological plasticity of *E. cyaneus* might be explained by its higher CSR capacity in comparison to *E. verrucosus*. Both cadmium and temperature may induce oxidative stress (increase in ROS) (Lushchak 2011), which can be tackled by different strategies, e.g. defense (universal cellular stress response) (Kültz 2005) or avoidance of stress (i.e. by physiological or behavioral responses such as metabolic depression or escape reactions) (Storey and Storey 2004, Lushchak 2011).

Reactive oxygen species are formed as a natural byproduct of aerobic metabolism and are key regulators of metabolic and defense pathways (Abele et al. 2011, Lushchak 2011). As heart rate and ventilation activity significantly contribute to standard metabolic rate, a reduction in physiological functions will reduce ROS production. Reduction in metabolic rate is a physiological strategy that relies on endogenous energy storages, and mostly involves a reduction of energy utilization (e.g. activity) and physiological functions (heart rate and ventilation) (Storey and Storey 2004). As cadmium impairs antioxidant enzymes, which are scavengers of ROS, metabolism may be down-regulated to prevent oxidative damage. Higher organisms are likely to share a universal oxygen-sensing apparatus that includes a haem oxidase, which produces ROS that in turn control the activity of transcription factors which control gene expression (Guppy and Withers 1999). The hypoxia-inducible factor (HIF-1α) is induced by ROS and controls the expression of various genes including those involved in metabolism (Giordano 2005). HIF-1 has also shown to be induced under normoxic conditions when ROS levels were increased. Thus, a critical level of ROS even induce a metabolic depression under oxidative stress. In *Caenorhabditis elegans* even a modest increase in ROS even induced longevity, but the underlying physiological mechanisms remain to be identified (Hwang et al. 2014). It can be hypothesized that these effects are also based on metabolic depression as the lifespan of an organism is dependent on its metabolic rate (Rate of living theory (Pearl 1928)). However, Hwang et al. (2014) did not report whether the investigated organisms showed, for example, reduced activity rates, foraging behavior or reproduction, which could have indicated metabolic depression in the nematodes.

In conclusion, the higher thermal sensitivity of *E. verrucosus* might be related to its sensitive reaction to sublethal cadmium concentrations under the premise that both stressors may cause oxidative stress (see Lushchak 2011 for review). As HIF-1α is activated in order to support cell survival, decreased respiration is paralleled by activation of the glycolytic metabolic pathway and mitochondrial autophagy (Semenza 2010), which remains to be studied in cadmium-exposed individuals of *E. verrucosus*. In thermally challenged *E. verrucosus*, cessation of respiration curves at 15.0°C (Publication I) was paralleled by an increase of PFK2, which indicates the activation of the glycolytic metabolic pathway (Publication II). However, the above stated possible molecular underpinnings of the observed effects remain to be co-determined.

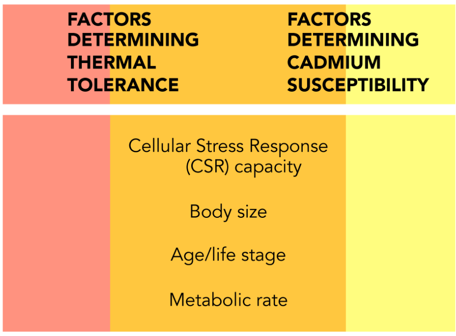


Figure 27: Physiological factors that may affect both thermal tolerance and cadmium tolerance. (The list is not complete).

Several physiological factors may affect both thermal and cadmium tolerance (Figure 27) such as CSR capacity (cf. Kültz 2005), body size (Buchwalter et al. 2008, Daufresne et al. 2009), age/life stage (Lopez Greco et al. 2001), metabolism and hypoxia sensitivity (Alonso et al. 2010, Anttila et al. 2013), however, sometimes in opposite directions. In contrast to high CSR capacity, which is beneficial for both heat and cadmium tolerance, small body size was shown to be beneficial for heat tolerance (Daufresne et al. 2009, Storch et al. 2014, Publication I and II) whereas smaller individuals (within closely related species) were more susceptible to cadmium (Buchwalter et al. 2008, Publication III).

Cadmium was investigated on an effect-scaled basis (i.e. biologically effective concentrations; LC1). Here, *E. cyaneus* tolerated a higher concentration of total internal cadmium because a larger fraction of cadmium was bound to heat stable proteins than in the less thermal tolerant species *E. verrucosus*. However, this effect was negligible when the species were exposed to the same external concentration because uptake rates were much faster in *E. cyaneus* in comparison to its close relative *E. verrucosus*. Consequently, lethal concentrations of waterborne cadmium for *E. cyaneus* were much lower than for *E. verrucosus*. Certainly, higher precision can be achieved when results would be based on at least three parameters, namely, 1) measured concentration of bioavailable cadmium of the external medium; 2) internal cadmium (excluding cadmium that is bound to the body surface), and; 3) the cadmium concentration of the metal sensitive tissue fraction. Furthermore, effects should, whenever possible, be based on concentrations at equilibrium. As shown for *E. verrucosus* cadmium uptake proceeded extremely slowly (Publication III), which might lead to underestimation of long-tern effects.

6 Conclusions

By investigating thermal tolerance on the whole animal level, it was revealed that species-specific limits in oxygen supply capacity are likely key for the onset of constraining (beyond pejus) and then life-threatening (beyond critical) conditions. *E. verrucosus* was far more sensitive to gradual temperature increase than *E. cyaneus* and *G. lacustris*, which both had similar whole animal thermal limits. The results support the theory that pejus temperatures represent the ecological thermal boundary of a species, i.e. they correlate with the maximal habitat temperatures, which was supported by field observations of maximum summer temperatures in the respective habitats. Moreover, this was mediated through behavioral plasticity (migration behavior) in *E. verrucosus*.

Studying thermal plasticity on the biochemical level by investigating temperature-dependent activities of key metabolic enzymes elucidated that oxygen consumption rates correlated with maximal activities of (oxygen-dependent) key metabolic enzymes. The close correlation of oxygen consumption and enzyme activities in *E. verrucosus*, both showing hyperbolic or peak-shaped curves with similar breakpoints is remarkable and may be due to a narrowly regulated feedback system in response to decreasing systemic oxygen availability. Limitations in oxygen consumption and enzyme activities were preceded by constraints on the ventilation system, which emphasizes the sensitivity of the oxygen supply system to temperature as suggested by the OCLTT model (Pörtner 2010) and, moreover, supports the theory of a hierarchy of thermal tolerance (Fry 1947, Fry and Hart 1948, Pörtner 2002). The findings clearly characterize the degree of eurythermy of these species, with *E. verrucosus* being stenothermal while *E. cyaneus* and *G. lacustris* are more eurythermal. *E. verrucosus* is well behaviorally adapted to stay in a certain thermal range and long evolution time in the isolated Baikal system may have led to synchronization of physiological processes on different organizational levels driven by the constraints of systemic oxygen supply.

Cadmium toxicity was mainly determined by different uptake rates whereas differences in detoxification capacity made a rather small contribution to the differences in mortality. Mortality data were best explained by the metal sensitive fraction (MSF), i.e. cadmium associated with cell organelles and heat labile proteins. However, physiological and behavioral responses to sublethal cadmium concentrations are highly variable and depend on the respective stress response strategy. Here, *E. verrucosus* and *E. cyaneus* showed different physiological reactions at equal concentrations of MSF-associated cadmium, which underlines that sublethal effects need to be studied in addition to metal uptake and internal distribution in order to estimate ecological effects. For example, reduced activity may severely affect the Darwinian fitness of a species.

Differences in strategies to cope with oxidative stress may explain the sensitive reaction of *E. verrucosus* to sublethal cadmium concentrations in comparison to *E. cyaneus* as only the former species showed metabolic depression under exposure to sublethal cadmium concentrations. Cadmium induces oxidative stress, which can be tackled by different strategies, such as down-regulation of metabolism or activation of the cellular stress response system (e.g. antioxidant system); both strategies are directed to prevent cellular damage. In accord, the higher thermal sensitivity of *E. verrucosus* might be related to its sensitive reaction to sublethal cadmium concentrations under the premise that both stressors cause oxidative stress and signaling pathways mediated by ROS may induce metabolic depression. The low sensitivity of *E. cyaneus* could be explained by its high physiological plasticity to deal with frequent environmental changes in the upper littoral of Lake Baikal. However, all adaptation and repairing mechanisms are energetically costly (Somero 2002). Increased energy for defense mechanisms might thus lead to reductions of the available energy for parameters such as growth and reproduction (Somero 2002).

Both physiological and behavioral plasticity may determine a species success in a changing environment (Hofmann and Todgham 2010). *E. verrucosus* and *E. cyaneus* are perfect examples that both factors need to be considered when aiming at estimating a species success under global change. *E. verrucosus* has a high behavioral plasticity (mediated by migration behavior) but low physiological plasticity to cope with rising temperatures. By contrast *E. cyaneus* tolerates thermal fluctuations in the upper littoral. However, present summer temperatures may already touch the pejus range of *E. cyaneus*. Consequently, despite the higher thermal tolerance of *E. cyaneus* it might be more severely affected by global change than *E. verrucosus*. Further, despite the fact that lethal concentrations of cadmium were much lower for *E. cyaneus*, *E. verrucosus* showed sensitive reactions (metabolic depression) to concentrations far below lethal ones. Consequently, the extremely sensitive physiological/behavioral reaction of *E. verrucosus* to low levels of a chemical stressor underlines that sublethal effects may not necessarily mirror sensitivity rankings based on mortality data. Despite the fact that metabolic depression is beneficial for short-term survival, it might turn detrimental in the long term because important parameters for growth and reproduction such as locomotor activity are likely reduced. Moreover, the findings underscore the necessity of water management strategies strictly avoiding chemical contamination of Lake Baikal waters. Rising temperatures are likely to enhance the deleterious effects of chemical stressors as shown here for cadmium.

7 Lake Baikal endemic amphipods under global change: Ecological perspectives

The results presented in this thesis suggest that changes in the thermal regime will likely negatively affect *E. verrucosus* and *E. cyaneus*, which are two of the most successful species of the littoral community. Present temperatures may reach a maximum of about 20°C close to the shoreline (Timoshkin 2009) and a temperature increase of more than 4.5°C may be expected considering surface temperatures in summer and fall (Moore et al. 2009). Thus the pejus temperature of *E. cyaneus* (19.1°C) is likely to be exceeded more often in future summers. Furthermore, *E. verrucosus* was shown to be thermally sensitive and would avoid the upper littoral in summer. Extended periods with elevated temperature might thus affect the migration behavior of *E. verrucosus*. Secondary effects of these changes need to be investigated but may include selection of smaller individuals based on the theory of an allometry of thermal limitation, which was supported in the present thesis. However, changes in organism size have been shown to have significant ecological implications, such as reductions in growth and reproductive success (Levitan 1991, Gillooly et al. 2002, Woodward et al. 2005, 2010).

The finding of metabolic depression in *E. verrucosus* in response to far below lethal cadmium concentrations (which was not found in *E. cyaneus*) is alarming because we did not yet determine a NOEC (= no observed effect level), and so further study is required to not only identify the NOEC, but also secondary effects of cadmium contamination. Metabolic depression usually implies a reduction of locomotor activity and reproduction; it is beneficial for surviving adverse conditions in the short-term. The results of this thesis support previous suggestions that the metal sensitive tissue fraction (MSF) is related to toxicological effects but only when considering mortality. A significant finding was that physiological effects were not directly related to the MSF, which raises concern regarding the derivation of ecological effects based on MSF-associated cadmium as sublethal effects, which may severely affect the Darwinian fitness of a species might not be indicated.

The combination of cadmium and temperature as stressors was shown to be more deleterious than each single factor alone and sublethal effects need to be studied in more detail. Not only increasing temperature as a single factor is important because regional increases in temperature variation may increase the sensitivity to cadmium (Kimberly and Salice 2014).

Whether global change would provide a competitive advantage for *G. lacustris* (ubiquitous, Holarctic) in comparison to Baikal endemics and, by extension, promote the widespread invasion of this non-endemic species, could not be elucidated. *G. lacustris* showed only slightly higher thermal tolerance in comparison to the Baikal endemic *E. cyaneus* and showed similar cadmium susceptibility as *E. cyaneus*. However, secondary effects of global change such as eutrophication, which has been monitored in the littoral of Lake Baikal (Timoshkin et al. 2016), are important factors that need to be considered in future studies. An increase of oxygen consuming processes in the littoral regions will almost certainly affect Baikal endemic amphipods, especially forms such as *E. verrucosus*, which are highly sensitive to hypoxic condition.

References

Abele, D., J. P. Vazquez-Medina, and T. Zenteno-Savin. 2011. Oxidative stress in aquatic organisms. Wiley-Blackwell.

Adrian, R., C. M. O'Reilly, H. Zagarese, S. B. Baines, D. O. Hessen, W. Keller, D. M. Livingstone, R. Sommaruga, D. Straile, E. Van Donk, G. A. Weyhenmeyer, and M. Winder. 2009. Lakes as sentinels of climate change. Limnology and Oceanography 54:2283–2297.

Alonso, A., H. J. De lange, and E. T. H. M. Peeters. 2010. Contrasting sensitivities to toxicants of the freshwater amphipods *Gammarus pulex* and *G. fossarum*. Ecotoxicology 19:133–140.

Anestis, A., H. O. Pörtner, A. Lazou, and B. Michaelidis. 2008. Metabolic and molecular stress responses of sublittoral bearded horse mussel *Modiolus barbatus* to warming sea water: implications for vertical zonation. Journal of Experimental Biology 211:2889–2898.

Anttila, K., R. S. Dhillon, E. G. Boulding, A. P. Farrell, B. D. Glebe, J. A. K. Elliott, W. R. Wolters, and P. M. Schulte. 2013. Variation in temperature tolerance among families of Atlantic salmon (*Salmo salar*) is associated with hypoxia tolerance, ventricle size and myoglobin level. Journal of Experimental Biology 216:1183–1190.

Arrhenius, S. 1889. Svante Arrhenius, Z. Phys. Chem. 1889, 4, S. 226–248. Zeitung für Physikalische Chemie 4:226–248.

Ballatori, N. 2002. Transport of toxic metals by molecular mimicry. Environmental Health Perspectives 110:689–694.

Barbieri, E., and E. T. Paes. 2011. The use of oxygen consumption and ammonium excretion to evaluate the toxicity of cadmium on *Farfantepenaeus paulensis* with respect to salinity. Chemosphere 84:9–16.

Bauer, R. T. 2013. Adaptive modification of appendages for grooming (cleaning, antifouling) and reproduction in the crustacea. Page Functional morphology and diversity. Oxford University Press, Oxford.

Bauman, J. W., J. Liu, and C. D. Klaassen. 1993. Production of metallothionein and heat-shock proteins in response to metals. Fundamental and Applied Toxicology 21:15–22.

Bedulina, D. S., M. B. Evgen'ev, M. A. Timofeyev, M. V. Protopopova, D. G. Garbuz, V. V. Pavlichenko, T. Luckenbach, Z. M. Shatilina, D. V. Axenov-Gribanov, A. N. Gurkov, I. M. Sokolova, and O. G. Zatsepina. 2013. Expression patterns and organization of the hsp70 genes correlate with thermotolerance in two congener endemic amphipod species (*Eulimnogammarus cyaneus* and *E. verrucosus*) from Lake Baikal. Molecular Ecology 22:1416–1430.

Bedulina, D. S., B. Köntopp, V. V. Pavlichenko, M. V. Protopopova, M. Schmidt, and K. Thiemann. 2007. Integration of the Ecology and Biodiversity of the Macrozoobenthos of Lake Baikal (Lake Baikal Summer School Report).

Berg, J. M., J. L. Tymoczko, and L. Stryer. 2002. Biochemistry. Fifth edition. W. H. Freeman and Company, New York.

Bettencourt, B. R., C. C. Hogan, M. Nimali, and B. W. Drohan. 2008. Inducible and constitutive heat shock gene expression responds to modification of Hsp70 copy number in *Drosophila melanogaster* but does not compensate for loss of thermotolerance in Hsp70 null flies. Bmc Biology 6:5.

Bjerregaard, P., and M. H. Depledge. 1994. Cadmium accumulation in *Littorina littorea*, *Mytilus edulis* and *Carcinus maenas*: the influence of salinity and calcium ion concentrations. Marine Biology 119:385–395.

Boltzmann, L. 1872. Weitere Studien über das Wärmegleichgewicht unter Gasmolekülen. Wiener Berichte 66:275–370.

Boudrias, M. A. 2013. Swimming fast and furious: body and limb propulsion at higher Reynolds numbers. Functional morphology and diversity. Oxford University Press, Oxford.

Boutelier, R. G., T. A. Heming, and G. K. Iwana. 1984. Anatomy, gas transfer, and acid-base regulation. Fish Physiology 10:403–430.

Braeckman, B., G. Smagghe, N. Brutsaert, R. Cornelis, and H. Raes. 1999. Cadmium uptake and defense mechanism in insect cells. Environmental Research 80:231–243.

Brown, B. E. 1982. The form and function of metal-containing “granules” in invertebrate tissues. Biological Reviews 57:621–667.

Buchwalter, D. B., D. J. Cain, C. A. Martin, L. Xie, S. N. Luoma, and T. Garland. 2008. Aquatic insect ecophysiological traits reveal phylogenetically based differences in dissolved cadmium susceptibility. Proceedings of the National Academy of Sciences of the United States of America 105:8321–8326.

Calow, P. 1991. Physiological costs of combating chemical toxicants: ecological implications. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology 100:3–6.

Campbell, P. G. C., O. Errecalde, C. Fortin, W. R. Hiriart-Baer, and B. Vigneault. 2002. Metal bioavailability to phytoplankton - applicability of the biotic ligand model. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 133:189–206.

Chang, M. H., H. C. Lin, and P. P. Hwang. 1997. Effects of cadmium on the kinetics of calcium uptake in developing tilapia larvae, *Oreochromis mossambicus*. Fish Physiology and Biochemistry 16:459–470.

Cherkasov, A. A., R. A. Overton, E. P. Sokolov, and I. M. Sokolova. 2007. Temperature-dependent effects of cadmium and purine nucleotides on mitochondrial aconitase from a marine ectotherm, *Crassostrea virginica*: a role of temperature in oxidative stress and allosteric enzyme regulation. Journal of Experimental Biology 210:46–55.

Ciesielski, T. M., M. V. Pastukhov, S. A. Leeves, J. Farkas, S. Lierhagen, V. I. Poletaeva, and B. M. Jenssen. 2016. Differential bioaccumulation of potentially toxic elements in benthic and pelagic food chains in Lake Baikal. Environmental Science and Pollution Research 23:15593–15604.

Costello, L. C., Z. X. Guan, R. B. Franklin, and P. Feng. 2004. Metallothionein can function as a chaperone for zinc uptake transport into prostate and liver mitochondria. Journal of Inorganic Biochemistry 98:664–666.

Currie, R. S., D. C. G. Muir, W. L. Fairchild, M. H. Holoka, and R. E. Hecky. 1998. Influence of nutrient additions on cadmium bioaccumulation by aquatic invertebrates in littoral enclosures. Environmental Science and Chemistry 17:2435–2443.

Dahl, E. 1977. Amphipod functional model and its bearing upon systematics and phylogeny. Zoologica Scripta 6:221–228.

Daufresne, M., K. Lengfellner, and U. Sommer. 2009. Global warming benefits the small in aquatic ecosystems. Proceedings of the National Academy of Sciences of the United States of America 106:12788–12793.

De Batist, M., M. Canals, P. Sherstyankin, and S. Alekseev. 2006. A new bathymetric map of Lake Baikal. INTAS Project 99-1669 Team.

Dong, Y., L. P. Miller, J. G. Sanders, and G. N. Somero. 2008. Heat-Shock Protein 70 (Hsp70) Expression in Four Limpets of the Genus *Lottia*: Interspecific Variation in Constitutive and Inducible Synthesis Correlates With in situ Exposure to Heat Stress. Biological Bulletin 215:173–181.

Driedzic, W. R., and V. M. F. De Almeida-Val. 1996. Enzymes of cardiac energy metabolism in Amazonian teleosts and the fresh-water stingray (*Potamotrygon hystrix*). Journal of Experimental Zoology 274:327–333.

Elliott, J. M. 1981. Some aspects of thermal stress on freshwater fish - water temperature and inland fisheries. Pages 209–245. Stress and Fish. Academic Press Inc., London.

EPA. 2014. Priority pollutant list.

EPA. 2016. Aquatic life ambient water quality criteria - update for cadmium.

Falkner, K. K., C. I. Measures, S. E. Herbelin, J. M. Edmond, and R. F. Weiss. 1991. The major and minor element geochemistry of Lake Baikal. Limnology and Oceanography 36:413–423.

Feder, M. E., and G. E. Hofmann. 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. Annual Review of Physiology 61:243–282.

Felten, V., G. Charmantier, R. Mons, A. Geffard, P. Rousselle, M. Coquery, J. Garric, and O. Geffard. 2008. Physiological and behavioural responses of *Gammarus pulex* (Crustacea : Amphipoda) exposed to cadmium. Aquatic Toxicology 86:413–425.

Fent, K. 2013. Ökotoxikologie. Fourth edition. Georg Thieme, Stuttgart, New York.

Fields, P. A. 2001. Review: Protein function at thermal extremes: balancing stability and flexibility. Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology 129:417–431.

Fry, F. E. J. 1947. Effects of the Environment on Animal Activity, by F. E. J. Fry.

Fry, F. E. J. 1971. The effect of environmental factors on the physiology of fish. Pages 1–98.Fish Physiology. Academic Press Inc., New York.

Fry, F. E. J., and J. S. Hart. 1948. Cruising speed of goldfish in relation to water temperature. Journal of Fisheries Research Board of Canada 7:169–175.

Gaudy, R., J. P. Guerin, and P. Kerambrun. 1991. Sublethal effects of cadmium on respiratory metabolism, nutrition, excretion and hydrolase activity in *Leptomysis lingvura* (Crustacea, Mysidacea). Marine Biology 109:493–501.

Gavrilov, G. B. 1949. On the problem of the time of reproduction in amphipods and isopods in Lake Baikal. Doklady Akademii Nauk SSSR 64:739–742.

Georgopoulos, C., and W. J. Welch. 1993. Role of the major heat-shock proteins as molecular chaperones. Annual Review of Cell Biology 9:601–634.

Gillooly, J. F., E. L. Charnov, G. B. West, V. M. Savage, and J. H. Brown. 2002. Effects of size and temperature on developmental time. Nature 417:70–73.

Giordano, F. J. 2005. Oxygen, oxidative stress, hypoxia, and heart failure. Journal of Clinical Investigation 115:500–508.

Glass, M. L., and S. C. Wood. 2009. Cardio-respiratory control in vertebrates. 12th edition. Springer, Berlin Heidelberg.

Goh, B. P. L., and C. H. Lai. 2014. Establishing the thermal threshold of the tropical mussel *Perna viridis* in the face of global warming. Marine Pollution Bulletin 85:325–331.

Grigorovich, I. A., T. R. Angradi, E. B. Emery, and M. S. Wooten. 2008. Invasion of the Upper Mississippi River system by saltwater amphipods. Fundamental and Applied Limnology 173:66–77.

Gronskaya, T. P., and T. E. Litova. 1991. Short characteristics of the water balance of Lake Baikal during 1962–1988. Pages 153–158. Monitoring of Lake Baikal Environment. Hydrometeoizdat, Leningrad.

Guderley, H. E., and J. S. St-Pierre. 2008. Phenotypic plasticity and evolutionary adaptations of mitochondria to temperature. Pages 127–152. Animals and Temperature: Phenotypic and Evolutionary Adaptation. Camebridge University Press, Camebridge.

Guppy, M., and P. Withers. 1999. Metabolic depression in animals: physiological perspectives and biochemical generalizations. Biological Reviews of the Cambridge Philosophical Society 74:1–40.

Hampton, S. E., D. K. Gray, L. R. Izmest'eva, M. V. Moore, and T. Ozersky. 2014. The Rise and Fall of Plankton: Long-Term Changes in the Vertical Distribution of Algae and Grazers in Lake Baikal, Siberia. Plos One 9:e88920.

Hampton, S. E., L. R. Izmest'eva, M. V. Moore, S. L. Katz, B. Dennis, and E. A. Silow. 2008. Sixty years of environmental change in the world's largest freshwater lake - Lake Baikal, Siberia. Global Change Biology 14:1947–1958.

Hartung, T., and C. Rovida. 2009. Chemical regulators have overreached. Nature 460:1080–1081.

Hofmann, G. E., and A. E. Todgham. 2010. Living in the Now: Physiological Mechanisms to Tolerate a Rapidly Changing Environment. Annual Review of Physiology 72:127–145.

Holmstrup, M., A.-M. Bindesbol, G. J. Oostingh, A. Duschl, V. Scheil, H.-R. Koehler, S. Loureiro, A. M. V. M. Soares, A. L. G. Ferreira, C. Kienle, A. Gerhardt, R. Laskowski, P. E. Kramarz, M. Bayley, C. Svendsen, and D. J. Spurgeon. 2010. Interactions between effects of environmental chemicals and natural stressors: A review. Science of the Total Environment 408:3746–3762.

Hopkin, S. P. 1989. Ecophysiology of Metals in Terrestrial Invertebrates. Elsevier Applied Science, London.

Hopkin, S. P., C. A. C. Hames, and A. Dray. 1989. X-ray microanalytical mapping of the intracellular distribution of pollutant metals. Microscopy and Analysis 14:23–27.

Hwang, A. B., E.-A. Ryu, M. Artan, H.-W. Chang, M. H. Kabir, H.-J. Nam, D. Lee, J.-S. Yang, S. Kim, W. B. Mair, C. Lee, S. S. Lee, and S.-J. Lee. 2014. Feedback regulation via AMPK and HIF-1 mediates ROS-dependent longevity in Caenorhabditis elegans. Proceedings of the National Academy of Sciences of the United States of America 111:E4458–E4467.

Hynes, H. B. N. 1961. The invertebrate fauna of a Welsh mountain stream. Archives of Hydrobiology 57:344–388.

Ikediobi, C., I. Christopher, B. Veera, A.-T. Lambert, L. Lekan, and W. John. 2004. Response of antioxidant enzymes and redox metabolites to cadmium-induced oxidative stress in CRL-1439 normal rat liver cells. Int. J. Mol. Med.

IPCC. 2014. Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Geneva, Switzerland.

IPCC Secretariat. 2016, April 14. IPCC agrees special report, AR6 workplan.

Ivanina, A. V., A. S. Cherkasov, and I. M. Sokolova. 2008. Effects of cadmium on cellular protein and glutathione synthesis and expression of stress proteins in eastern oysters, *Crassostrea virginica* Gmelin. Journal of Experimental Biology 211:577–586.

Jaishankar, M., T. Tseten, N. Anbalagan, B. B. Mathew, and K. N. Beeregowda. 2014. Toxicity, mechanism and health effects of some heavy metals. Interdisciplinary Toxicology 7:60–72.

Jiang, S. Y., and P. J. Zhou. 2012. Effects of Cadmium on the Expression of Antioxidant Enzymes, Oxidative Stress and Apoptosis in Primary Hepatocytes of *Carassius Auratus*. Adv. Mat. Res. 518–523:341–346.

Kägi, J. H. R., and A. Schäffer. 1988. Biochemistry of metallothionein. Biochemistry 27:8509–8515.

Khalil, M. A., M. H. Donker, and N. M. van Straalen. 1995. Long-term and short-term changes in the energy budget of *Porcellio scaber* Latreille (Crustacea) exposed to cadmium polluted food. European Journal of Soil Biology 31:163–172.

Khan, F. R., N. R. Bury, and C. Hogstrand. 2010. Cadmium bound to metal rich granules and exoskeleton from *Gammarus pulex* causes increased gut lipid peroxidation in zebrafish following single dietary exposure. Aquatic Toxicology 96:124–129.

Khan, F. R., J. R. Irving, N. R. Bury, and C. Hogstrand. 2011. Differential tolerance of two *Gammarus pulex* populations transplanted from different metallogenic regions to a polymetal gradient. Aquatic Toxicology 102:95–103.

Kim, Y. K., W. I. Yoo, S. H. Lee, and M. Y. Lee. 2005. Proteomic Analysis of Cadmium-Induced Protein Profile Alterations from Marine Alga Nannochloropsis oculata. Ecotoxicology 14:589–596.

Kimberly, D. A., and C. J. Salice. 2014. Complex interactions between climate change and toxicants: evidence that temperature variability increases sensitivity to cadmium. Ecotoxicology 23:809–817.

Klaassen, C. D., J. Liu, and S. Choudhuri. 1999. Metallothionein: An intracellular protein to protect against cadmium toxicity. Annual Review of Pharmacology and Toxicology 39:267–294.

Kornberg, A. 1955. Lactic dehydrogenase of muscle: Pyruvate + DPNH↔Lactate + DPN. Methods in Enzymology 1:441–443.

Kozhov, M. 1963. Lake Baikal and its life. Dr. W. Junk Publishers, The Hague, The Netherlands.

Kozhova, O. M., and L. R. Izmest'eva. 1998. Lake Baikal - Evolution and Biodiversity. Backhuys Publishers, Leiden.

Kravtsova, L. S., E. B. Karabanov, I. V. Mekhanikova, T. Y. Sitnikova, N. A. Rozhkova, Z. V. Slugina, L. A. Izhboldina, I. V. Weinberg, T. V. Akinshina, and D. Y. Sherbakov. 2004.

Macrozoobenthic communities of underwater landscapes in the shallow-water zone of southern Lake Baikal. Hydrobiologia 522:193–205.

Kristensen, E. 1983. Comparison of polychaete (*Nereis spp.*) ventilation in plastic tubes and natural sediment. Marine Ecology Progress Series 12:307–309.

Kültz, D. 2005. Molecular and evolutionary basis of the cellular stress response. Annual Review of Physiology 67:225–257.

Kumar, G. K., and J. B. Klein. 2003. Analysis of expression and posttranslational modification of proteins during hypoxia. Journal of Applied Physiology 96:1178–1186.

Lane, T. W., and F. M. M. Morel. 2000. A biological function for cadmium in marine diatoms. Proceedings of the National Academy of Sciences of the United States of America 97:4627–4631.

Lannig, G., J. F. Flores, and I. M. Sokolova. 2006. Temperature-dependent stress response in oysters, *Crassostrea virginica*: Pollution reduces temperature tolerance in oysters. Aquatic Toxicology 79:278–287.

Larison, J. R., G. E. Likens, J. W. Fitzpatrick, and J. G. Crock. 2000. Cadmium toxicity among wildlife in the Colorado Rocky Mountains. Nature 406:181–183.

Lasenby, D. C., and J. Vanduyn. 1992. Zinc and cadmium accumulation by the opossum shrimp *Mysis relicta*. Archives of Environmental Contamination and Toxicology 23:179–183.

Lavoie, M., P. G. C. Campbell, and C. Fortin. 2012. Extending the Biotic Ligand Model to Account for Positive and Negative Feedback Interactions between Cadmium and Zinc in a Freshwater Alga. Environmental Science & Technology 46:12129–12136.

Leeves, S. A. 2011. Bioaccumulation of arsenic, cadmium, mercury, lead and selenium in the benthic and pelagic food chain of Lake Baikal. Master thesis, NTU Trondheim Norway.

Lesser, M. P. 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. Annu. Rev. Physiol. 68:253–278.

Levitan, D. R. 1991. Influence of Body Size and Population Density on Fertilization Success and Reproductive Output in a Free-Spawning Invertebrate. Biol. Bull. 181:261.

Lopez Greco, L. S., M. V. Sanchez, G. L. Nicoloso, D. A. Medesani, and E. M. Rodriguez. 2001. Toxicity of cadmium and copper on larval and juvenile stages of the estuarine crab *Chasmagnathus granulata* (Brachyura, Grapsidae). Archives of Environmental Contamination and Toxicology 41:333–338.

Luoma, S. N., and P. S. Rainbow. 2005. Why is metal bioaccumulation so variable? Biodynamics as a unifying concept. Environmental Science & Technology 39:1921–1931.

Luoma, S. N., and P. S. Rainbow. 2008. Metal Contamination in Aquatic Environments - Science and Lateral Management. Camebridge University Press, Camebridge.

Lushchak, V. I. 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology 101:13–30.

Lyman, C. P. 1982. Hibernation: Responses to external challenges. Pages 196–205. Hibernation and torpor in mammals and birds. Academic Press, New York.

Macdonald, K. S., L. Yampolsky, and J. E. Duffy. 2005. Molecular and morphological evolution of the amphipod radiation of Lake Baikal. Molecular Phylogenetics and Evolution 35:323–343.

Marshall, D. J., M. Bode, and C. R. White. 2013. Estimating physiological tolerances - a comparison of traditional approaches to nonlinear regression techniques. Journal of Experimental Biology 216:2176–2182.

Martin, C. A., S. N. Luoma, D. J. Cain, and D. B. Buchwalter. 2007. Cadmium ecophysiology in seven stonefly (Plecoptera) species: Delineating sources and estimating susceptibility. Environmental Science & Technology 41:7171–7177.

Martin, M. H., P. J. Coughtrey, and E. W. Young. 1976. Observations on the availability of lead, zinc, cadmium and copper in woodland litter and the uptake of lead, zinc and cadmium by the woodlouse, *Oniscus asellus*. Chemosphere 5:313–318.

Martin, P., B. Goddeeris, and K. Martens. 1993. Oxygen concentration profiles in soft sediment of Lake Baikal (Russia) near the Selenga delta. Freshwater Biology 29:343–349.

Maryanski, M., P. Kramarz, R. Laskowski, and M. Niklinska. 2002. Decreased energetic reserves, morphological changes and accumulation of metals in carabid beetles (*Poecilus cupreus* L.) exposed to zinc- or cadmium-contaminated food. Ecotoxicology 11:127–139.

Mats, V. D., D. Yu. Shcherbakov, and I. M. Efimova. 2011. Late Cretaceous-Cenozoic history of the Lake Baikal depression and formation of its unique biodiversity. Stratigr. Geol. Correl. 19:404–423.

McClelland, G. B., A. C. Dalziel, N. M. Fragoso, and C. D. Moyes. 2005. Muscle remodeling in relation to blood supply: implications for seasonal changes in mitochondrial enzymes. Journal of Experimental Biology 208:515–522.

McMahon, B. R., D. G. McDonald, and C. M. Wood. 1979. Ventilation, oxygen-uptake and hemolymph oxygen-transport, following enforced exhausting activity in the Dungeness crab *Cancer magister*. Journal of Experimental Biology 80:271–285.

Melzner, F., C. Bock, and H. O. Pörtner. 2007. Allometry of thermal limitation in the cephalopod *Sepia officinalis*. Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology 146:149–154.

Moore, M. V., S. E. Hampton, L. R. Izmest'eva, E. A. Silow, E. V. Peshkova, and B. K. Pavlov. 2009. Climate Change and the World's "Sacred Sea"-Lake Baikal, Siberia. Bioscience 59:405–417.

Moore, P., and A. Taylor. 1984. Gill area relationships in an ecological series of gammaridean amphipods (Crustacea). Journal of Experimental Marine Biology and Ecology 74:179–186.

Morgan, A. J., M. P. Turner, and J. E. Morgan. 2002. Morphological plasticity in metalseque - stering earthworm chloragocytes: Morphometric electron microscopy provides a biomarker of exposure in field populations. Environmental Toxicology and Chemistry 21:610–618.

Mouneyrac, C., B. Berthet, and J.-C. Amiard. 1999. Cd distribution in the tissues of oysters (*Crassostrea gigas*) exposed chronically in situ. Water, Air and Soil Pollution 112:187–196.

Moyes, C. D., O. A. MathieuCostello, N. Tsuchiya, C. Filburn, and R. G. Hansford. 1997. Mitochondrial biogenesis during cellular differentiation. American Journal of Physiology-Cell Physiology 272:C1345–C1351.

Nemmiche, S., D. Chabane-Sari, M. Kadri, and P. Guiraud. 2011. Cadmium chloride-induced oxidative stress and DNA damage in the human Jurkat T cell line is not linked to intracellular trace elements depletion. Toxicology in Vitro 25:191–198.

OECD. 2012. OECD guideline for the testing of chemicals - Section 2: Effects on biotic systems; Test No. 211: *Daphnia magna* reproduction test.

Ognjanovic, B. I., S. D. Markovic, S. Z. Pavlovic, R. V. Zikic, A. S. Stajn, and Z. S. Saicic. 2008. Effect of chronic cadmium exposure on antioxidant defense system in some tissues of rats: protective effect of selenium. Physiol. Res. 57:403–411.

OSPAR. 2010. Review statement for the OSPAR background document on cadmium.

Pan, K., and W.-X. Wang. 2008. Allometry of cadmium and zinc concentrations and bioaccumulation in the scallop *Chlamys nobilis*. Marine Ecology Progress Series 365:115–126.

Pearl, R. 1928. The Rate of Living, by Raymond Pearl. Alfred A. Knopf, New York.

Pellet, B., O. Geffard, C. Lacour, T. Kermoal, C. Gourlay-France, and M.-H. Tusseau-Vuillemin. 2009. A model predicting waterborne cadmium bioaccumulation in *Gammarus pulex*: The effects of dissolved organic ligands, calcium, and temperature. Environmental Toxicology and Chemistry 28:2434–2442.

Philips, D. J. H., and P. S. Rainbow. 1994. Biomonitoring of trace aquatic contaminants. Springer-Science + Business Media, Dordrecht.

Platvoet, D., J. T. A. Dick, N. Konijnendijk, and G. van der Velde. 2006. Feeding on micro-algae in the invasive Ponto-Caspian amphipod *Dikerogammarus villosus* (Sowinsky, 1894). Aquatic Ecology 40:237–245.

Pörtner, H. O. 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology 132:739–761.

Pörtner, H. O. 2010. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. Journal of Experimental Biology 213:881–893.

Pörtner, H. O., I. Hardewig, and L. S. Peck. 1999. Mitochondrial function and critical temperature in the Antarctic bivalve, *Laternula elliptica*. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 124:179–189.

Pörtner, H. O., and R. Knust. 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science 315:95–97.

Pörtner, H. O., F. C. Mark, and C. Bock. 2004. Oxygen limited thermal tolerance in fish? Answers obtained by nuclear magnetic resonance techniques. Respiratory Physiology & Neurobiology 141:243–260.

Pörtner, H. O., L. Peck, and G. Somero. 2007. Thermal limits and adaptation in marine Antarctic ectotherms: an integrative view. Philosophical Transactions of the Royal Society B-Biological Sciences 362:2233–2258.

Pörtner, H.-O. 2012. Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes. Marine Ecology Progress Series 470:273–290.

Poteat, M. D., and D. B. Buchwalter. 2014. Calcium uptake in aquatic insects: influences of phylogeny and metals (Cd and Zn). Journal of Experimental Biology 217:1180–1186.

Powers, D. A., M. Smith, I. Gonzales-Villasenor, L. DiMichele, D. Crawford, G. Bernardi, and T. Lauerman. 1993. A multi-disciplinary approach to the selectionist/neutralist controversy using the model teleost, *Fundulus heteroclitus*. Pages 43–107Oxford surveys in evolutionary biology. Oxford University Press, Oxford.

Protopopova, M. V., V. V. Pavlichenko, R. Menzel, A. Putschew, T. Luckenbach, and C. E. W. Steinberg. 2014. Contrasting cellular stress responses of Baikalian and Palearctic amphipods upon exposure to humic substances: environmental implications. Environmental Science and Pollution Research 21:14124–14137.

Pure Earth (Blacksmith Institute). 2015. World's worst pollution problems - The new top six toxic threats: A priority list for remediation.

Rainbow, P. S. 1997. Ecophysiology of Trace Metal Uptake in Crustaceans. Estuar. Coast. Shelf Sci. 44:169–176.

Rainbow, P. S. 2002. Trace metal concentrations in aquatic invertebrates: why and so what? Environmental Pollution 120:497–507.

Rainbow, P. S., F. Liu, and W.-X. Wang. 2015. Metal accumulation and toxicity: The critical accumulated concentration of metabolically available zinc in an oyster model. Aquatic Toxicology 162:102–108.

Robinson, K. A., D. J. Baird, and F. J. Wrona. 2003. Surface metal adsorption on zooplankton carapaces: implications for exposure and effects in consumer organisms. Environmental Pollution 122:159–167.

Rodriguez, E. M., R. Bigi, D. A. Medesani, V. S. Stella, L. S. L. Greco, P. A. R. Moreno, J. M. Monserrat, G. N. Pellerano, and M. Ansaldo. 2001. Acute and chronic effects of cadmium on blood homeostasis of an estuarine crab, *Chasmagnathus granulata*, and the modifying effect of salinity. Brazilian Journal of Medical and Biological Research 34:509–518.

Rosabal, M., L. Hare, and P. G. C. Campbell. 2014. Assessment of a subcellular metal partitioning protocol for aquatic invertebrates: preservation, homogenization, and subcellular fractionation. Limnology and Oceanography-Methods 12:507–518.

Rusinek, O. T., V. V. Takhteev, D. P. Gladkochub, T. V. Khodzher, and N. M. Budnev. 2012. Baikalovedenie (Baikalogy). Nauka, Novosibirsk.

Sanchez-Muros, M. J., G. Rejon, G. Salguero, M. de la Higuera, and J. A. Lupianez. 1998. Long-term nutritional effects on the primary liver and kidney metabolism in rainbow trout. Adaptive response to nutritional effects on the primary liver and kidney metabolism in rainbow trout. Adaptive response to starvation and a high-protein, carbohydrate-free diets on glutamate dehydrogenase and alanine amino transferade kinetics. Biochemistry and Cellular Biology 30:55–63.

Schmidt-Nielsen, K. 1997. Animal Physiology - Adaptation and Environment. Fifth edition. Camebridge University Press.

Semenza, G. L. 2010. HIF-1: upstream and downstream of cancer metabolism. Current Opinion in Genetics & Development 20:51–56.

Shelford, V. E. 1931. Some concepts of bioecology. Ecology:455–467.

Shimaraev, M. N., L. N. Kuimova, V. N. Sinyukovich, and V. V. Tsekhanovskii. 2002. Manifestation of global climatic changes in Lake Baikal during the 20th century. Doklady Earth Sciences 383:288–291.

Sidell, B. D., W. R. Driedzic, D. B. Stowe, and I. A. Johnston. 1987. Biochemical correlations of power development and metabolic fuel preferenda in fish hearts. Physiological Zoology 60:221–232.

Simkiss, K., and M. G. Taylor. 1989. Metal fluxes across membranes of aquatic organisms. CRC Critical Reviews in Aquatic Sciences 1:137–188.

Sinensky, M. 1974. Homeoviscous adaptation - a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America 71:522–525.

Somero, G. N. 1995. Proteins and temperature. Annual Review of Physiology 57:43–68.

Somero, G. N. 2002. Thermal physiology and vertical zonation in intertidal animals: optima, limits, and costs of living. Integrative and Comparative Biology 42:780–789.

Somero, G. N. 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine “winners” and “losers.” Journal of Experimental Biology 213:912–920.

Somero, G. N. 2012. The Physiology of Global Change: Linking Patterns to Mechanisms. Pages 39–61*in* C. A. Carlson and S. J. Giovannoni, editors. Annual Review of Marine Science.

Sornom, P., E. Gismondi, C. Vellinger, S. Devin, J.-F. Ferard, and J.-N. Beisel. 2012. Effects of Sublethal Cadmium Exposure on Antipredator Behavioural and Antitoxic Responses in the Invasive Amphipod Dikerogammarus villosus. Plos One 7:e42435.

Stachowicz, J. T., J. R. Terwin, R. B. Whitlatch, and R. W. Osman. 2002. Linking climate change and biological invasions: Ocean warming facilitates nonindigenous species invasions. Proceedings of the National Academy of Sciences of the United States of America 99:15497–15500.

Steele, D. H., and V. J. Steele. 1991. The structure and organization of the gills of gammaridean Amphipoda. Journal of Natural History 25:1247–1258.

Storch, D., L. Menzel, S. Frickenhaus, and H.-O. Pörtner. 2014. Climate sensitivity across marine domains of life: limits to evolutionary adaptation shape species interactions. Global Change Biology 20:3059–3067.

Storey, K. B., and J. M. Storey. 2004. Metabolic rate depression in animals: transcriptional and translational controls. Biological Reviews 79:207–233.

Takhteev, V., N. A. Berezina, and D. A. Sidorov. 2015. Checklist of the Amphipoda (Crustacea) from continental waters of Russia, with data on alien species. Arthropoda selecta 24:335–370.

Tan, Q.-G., and W.-X. Wang. 2011. Acute Toxicity of Cadmium in *Daphnia magna* under Different Calcium and pH Conditions: Importance of Influx Rate. Environmental Science & Technology 45:1970–1976.

Tepolt, C. K., and G. N. Somero. 2014. Master of all trades: thermal acclimation and adaptation of cardiac function in a broadly distributed marine invasive species, the European green crab, *Carcinus maenas*. Journal of Experimental Biology 217:1129–1138.

Timofeyev, M. A., and K. A. Kirichenko. 2004. Experimental estimation of the role of abiotic factors in containment of endemics beyond the bounds of Lake Baikal. Sibirskij Jecologicheskij Zhurnal 1:41–50.

Timofeyev, M. A., J. M. Shatilina, and D. I. Stom. 2001. Attitude to temperature factor of some endemic amphipods from Lake Baikal and Holarctic *Gammarus lacustris* Sars, 1863: A comparative experimental study. Arthropoda selecta 10:93–101.

Timofeyev, M., and Z. Shatilina. 2007. Different preference reactions of three Lake Baikal endemic amphipods to temperature and oxygen are correlated with symbiotic life. Crustaceana 80:129–138.

Timoshkin, O. A. 2001. Lake Baikal: fauna diversity, problems of its "immiscibility" and origin, ecology and "exotic" communities. Pages 16–73. Index of animal species inhabiting Lake Baikal and its catchment area. Nauka, Novosibirsk.

Timoshkin, O. A. 2009. Index of Animal Species Inhabiting Lake Baikal and its Catchment Area. Nauka, Novosibirsk.

Timoshkin, O. A., N. A. Bondarenko, E. A. Volkova, I. V. Tomberg, V. S. Vishnyakov, and V. V. Maljnik. 2014. Mass development of filamentous algae of genera *Spirogyra* and *Stigeoclonium* (Chlorophyta) in the coastal zone of southern Baikal. Hydrobiological Journal 50:15–26.

Timoshkin, O. A., D. P. Samsonov, M. Yamamuro, M. V. Moore, O. I. Belykh, V. V. Malnik, M. V. Sakirko, A. A. Shirokaya, N. A. Bondarenko, V. M. Domysheva, G. A. Fedorova, A. I. Kochetkov, A. V. Kuzmin, A. G. Lukhnev, O. V. Medvezhonkova, A. V. Nepokrytykh, E. M. Pasynkova, A. E. Poberezhnaya, N. V. Potapskaya, N. A. Rozhkova, N. G. Sheveleva, I. V. Tikhonova, E. M. Timoshkina, I. V. Tomberg, E. A. Volkova, E. P. Zaitseva, Y. M. Zvereva, A. B. Kupchinsky, and N. A. Bukshuk. 2016. Rapid ecological change in the coastal zone of Lake Baikal (East Siberia): Is the site of the world's greatest freshwater biodiversity in danger? Journal of Great Lakes Research 42:487–497.

Väinölä, R., J. D. S. Witt, M. Grabowski, J. H. Bradbury, K. Jazdzewski, and B. Sket. 2008. Global diversity of amphipods (Amphipoda; Crustacea) in freshwater. Hydrobiologia 595:241–255.

Van Straalen, N. M., T. B. A. Burghouts, M. J. Doornhof, G. M. Groot, M. P. M. Janssen, E. N. G. Joosse, J. H. Van Meerendonk, J. P. J. J. Theeuwen, H. A. Verhoef, and H. R. Zoomer. 1987. Efficiency of lead and cadmium excretion in populations of *Orchesella cincta* (Collembola) from various contaminated forest soils. Journal of Applied Ecology 24:953–968.

Vellinger, C., V. Felten, P. Sornom, P. Rousselle, J.-N. Beisel, and P. Usseglio-Polatera. 2012. Behavioural and Physiological Responses of *Gammarus pulex* Exposed to Cadmium and Arsenate at Three Temperatures: Individual and Combined Effects. Plos One 7:e39153.

Vernberg, W. B., and F. J. Vernberg. 1972. Environmental physiology of marine animals. Springer, New York.

Vijver, M. G., C. A. M. Van Gestel, R. P. Lanno, N. M. Van Straalen, and W. Peijnenburg. 2004. Internal metal sequestration and its ecotoxicological relevance: A review. Environmental Science & Technology 38:4705–4712.

Votintsev, K. K. 1961. The hydrochemistry of Lake Baikal. Izd. AN SSSR, Moscow.

Wallace, W. G., B. G. Lee, and S. N. Luoma. 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). Marine Ecology Progress Series 249:183–197.

Wallace, W. G., and G. R. Lopez. 1996. Relationship between subcellular cadmium distribution in prey and cadmium transfer to a predator. Estuaries 19:923–930.

Wallace, W. G., and G. R. Lopez. 1997. Bioavailability of biologically sequestered cadmium and the implifications for metal detoxification. Marine Ecology Progress Series 147:149–157.

Wallace, W. G., G. R. Lopez, and J. S. Levinton. 1998. Cadmium resistance in an oligochaete and its effect on cadmium trophic transfer. Marine Ecology Progress Series 172:225–237.

Wang, M., and W.-X. Wang. 2008. Cadmium toxicity in a marine diatom as predicted by the cellular metal sensitive fraction. Environmental Science & Technology 42:940–946.

Wang, W. X., and N. S. Fisher. 1997. Modeling the influence of body size on trace element accumulation in the mussel *Mytilus edulis*. Marine Ecology Progress Series 161:103–115.

Wang, W.-X., and P. S. Rainbow. 2006. Subcellular partitioning and the prediction of cadmium toxicity to aquatic organisms. Environmental Chemistry 3:395–399.

Wang, Y., and R. D. Evans. 1993. Influence of calcium concentrations on cadmium uptake by the freshwater mussel *Elliptio complanata*. Canadian Journal of Fisheries and Aquatic Sciences 50:2591–2596.

Weinberg, I. V., and R. M. Kamaltynov. 1998. Zoobenthos communities on stony beach of Lake Baikal. Zoologichesky Zhurnal 77:259–265.

Wicklund, A., and P. Runn. 1988. Calcium effects on cadmium uptake, redistribution, and elimination in minnows, *Phoxinus phoxinus*, acclimated to different calcium concentrations. Aquatic Toxicology 13:109–121.

Wilson, M. P., and M. R. Schwarzman. 2009. Toward a new U.S. chemicals policy: Rebuilding the foundation to advance new sience, green chemistry, and environmental health. Environmental Health Perspectives 117:1202–1209.

Woodward, G., J. Blanchard, R. B. Lauridsen, F. K. Edwards, J. I. Jones, D. Figueroa, P. H. Warren, and O. L. Petchey. 2010. Individual-based food webs: species identity, body size and sampling effects. Pages 211–266 *in* G. Woodward, editor.Advances in Ecological Research, Vol 43: Integrative Ecology: From Molecules to Ecosystems.

Woodward, G., B. Ebenman, M. Emmerson, J. M. Montoya, J. M. Olesen, A. Valido, and P. H. Warren. 2005. Body size in ecological networks. Trends in Ecology & Evolution 20:402–409.

Wright, D. A. 1980. Cadmium and calcium interactions in the freshwater amphipod *Gammarus pulex*. Freshwater Biology 10:123–133.

Wright, D. A., and J. W. Frain. 1981. The effect of calcium on cadmium toxicity in the freshwater amphipod, Gammarus pulex (L.). Arch. Environ. Contam. Toxicol. 10:321–328.

Yoshioka, T., S. Ueda, T. Khodzher, I. Bashenkhaeva, L. Korovyakova, L. Sorokovikova, and L. Gorbunova. 2002. Distribution of dissolved organic carbon in Lake Baikal and its watershed. Limnology 3:159–168.

Yu, T. L., and J. B. Chen. 2013. Mating patterns of *Gammarus lacustris* (Amphipoda: Gammaridae) from three populations across a gradient on the Tibetan Plateau. Italian Journal of Zoology 80:608–613.

Zadereev, E. S., A. P. Tolomeyev, A. V. Drobotov, A. Y. Emeliyanova, and M. V. Gubanov. 2010. The vertical distribution and abundance of *Gammarus lacustris* in the pelagic zone of the meromictic lakes Shira and Shunet (Khakassia, Russia). Aquatic Ecology 44:531–539.

Zerbst-Boroffka, I., T. Grospietsch, I. Mekhanikova, and V. Takhteev. 2000. Osmotic and ionic hemolymph concentrations of bathyal and abyssal amphipods of Lake Baikal (Siberia) in relation to water depth. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 170:615–625.

Zhou, D., L. N. Zhang, J. P. Zhou, and S. L. Guo. 2004. Cellulose/chitin beads for adsorption of heavy metals in aqueous solution. Water Research 38:2643–2650.

Acknowledgements

First of all I would like to thank Hans-Otto Pörtner who supported my work in the Integrative Ecophysiology section. Thank you for leaving me free reign to develop my thesis, your professional advice, and prompt responses.

Franz Josef Sartoris and Magnus Lucassen are sincerely acknowledged for their guidance, scientific discussions and the effort to find further financial support.

I would like to thank Till Luckenbach and Maxim Timofeyev for launching this project. Maxim Timofeyev supported my work in Siberia; thank you a lot for your confidence. Till Luckenbach helped me a lot with the "toxic" of this thesis. Thank you so much for scientific discussions, your help with overcoming doubts and the good times at Lake Baikal. Your interpretation of "Schifoan" was hilarious but you might want to work on your talent of telling (German) jokes to foreigners.
;-)

Rolf Altenburger, thank you a lot for taking the time to evaluate my dissertation and for your contributions to this thesis.

Furthermore, I would like to thank Martin Zimmer for completing the dissertation committee.

I am very grateful for the hard work, patience and dedication of Denis Axenov-Gribanov, Kseniya Vereshchagina, Daria Bedulina, Anton Gurkov, Zhanna Shatilina, Yulia Lubyaga and Ekaterina Madyarova in helping me with the experimental work and the research progress. Thank you for your great company in the field, adventurous trips and for sharing room(s) ☺. Moreover, I would like to thank all the friendly students of Irkutsk State University who assisted field and lab work. Michael Meyer is kindly acknowledged for his help with catching amphipods and lab work, his contagious cheerfulness, belief in the "impossible", and the best cinematic art ever.

Kuzma Kirichenko, I sincerely thank you for your kindness, hospitality, tea times, unlimited supply with buckwheat and chicken (still on the list of my favorite foods) and aid with everyday life in Irkutsk.

The Integrative Ecophysiologists are gratefully thanked for their integration-friendliness. I would like to thank Anette Tillmann, Timo Hirse, Jutta Jürgens, and Nils Koschnik for keeping up the spirits and for their great support in the lab. Thank you Timo for so many "Ioni"-fixing-hours. The biotechnician trainees are cordially thanked for their assistance.

Ilsetraut Stölting is highly acknowledged for running ICP-MS analysis. Furthermore, I thank Sabine Kasten and Ingrid Stimac for providing their lab and microwave digestion system.

Laura, Corni, Kristina, Burgel, Kadda, Lars and Mela, I thank you for having been my companions in fortune and misfortune, fun activities and the many useful and useless (but funny) discussions and for your help with my thesis.

Thank you very much Kyle and Laura for the language check, and commenting on this thesis.

Shobhit, I thank you a lot for caring so much about the many fractions of the whole (thesis), your patience in trying to cheer me up and your support during the final phase of this work.

I thank my family for always being there for me, encouraging words and for reminding me of things that count in life besides amphipods.

Misha, the list of things I want to thank you for goes beyond the scope of this thesis; I thank you wholeheartedly for your unshakeable belief in me.

Erklärung

Lena Jakob
Fehrfeld 25
28203 Bremen

Bremen 15.09.2016

Erklärung gemäß § 6(5) der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Hiermit erkläre ich, dass ich die Arbeit mit dem Titel:

"Thermal Tolerance and Cadmium Susceptibility of Amphipods Endemic to Lake Baikal"

1. ohne unerlaubte fremde Hilfe angefertigt habe,
2. keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und
3. die den benutzen Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe

Lena Jakob