Physiological indicators of fitness in freshwater ecological studies: fundamentals and application examples

by

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Chapter 5

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Chapter 1

General Introduction

Freshwater ecosystems may be considered the most endangered ecosystems in the world, which particularly relates to the decline in biodiversity that is greater than in the most affected terrestrial ecosystems (Dudgeon *et al.*, 2006; Sala, 2000). The loss of biodiversity is mainly caused by factors such as overexploitation of water and organisms, water pollution, flow modification, and habitat degradation as well as the invasion of non-native species (Dudgeon *et al.*, 2006). Further, climate change contributes to these effects either directly by changing temperatures and run-off patterns or indirectly leading to altered structures and function of these ecosystems (Allan and Castillo, 2007; Wrona *et al.*, 2006). Owing to the already evident and further expected crisis to biodiversity, the protection of aquatic biodiversity, as well as the documentation of species loss and the identification of its causes, has become a major part of contemporary freshwater ecology, and particularly led to growing efforts to restore degraded habitats or control water pollution within conservation biology (Strayer and Dudgeon, 2010). To effectively avoid and prevent further damage to lotic environments and their biota, understanding the interaction between organisms and the environment as well as their response to changing environmental conditions is mandatory. The related analysis is a fundamental objective of freshwater ecological studies and the gained knowledge is applied for the management of populations, communities and ecosystems, as well as their services (Sutherland *et al.*, 2013).

Within their natural habitats organisms are affected by multiple environmental factors that vary in relevance depending on the particular system. With respect to lotic systems, water current velocity, water temperature, and oxygen concentration can be considered the most important abiotic factors, whereas species interactions such as competition and predation represent most relevant biotic factors. Moreover, because nowadays almost all ecosystems are increasingly affected by impacts caused by humans or their activities, anthropogenic factors are considered to represent major factors influencing natural habitats and the inhabiting organisms as well (Sokolova, 2013).

To evaluate the effects of natural environmental and anthropogenic factors, freshwater ecological studies mostly focus on the higher levels of biological organization, frequently the community level, and various indicators are applied, e.g. to assess environmental conditions or to monitor temporal trends in those conditions and to provide an early warning signal of environmental degradation (Dale and Beyeler, 2001; Niemi and McDonald, 2004). In this context the monitoring and subsequent evaluation of the benthic community structure is a widely applied method (Culp and Baird, 2007) and commonly focuses on structural characteristics, such as species abundance, richness, diversity, and composition. Beside the observation of these general aspects, the presence of specific taxa within the benthic community, so-called bioindicator species, is regularly used as an ecological indicator within environmental health assessment. One of the most traditional examples of this bioindicator approach is the ‘saprobic system’ (Kolkwitz and Marsson, 1909, 1908), which classifies the degree of organic pollution, i.e. saprobity, in rivers based on the presence of benthic invertebrates and water plants (Rosenberg and Resh, 1993). Nowadays the use of ecological indicators is implemented in the environmental assessment within the framework of the European Water Framework Directive (2000/60/EC; European Commission, 2000). It stipulates the evaluation of ‘biological quality elements’, such as benthic invertebrates, fish, phytoplankton and macro-algae in order to determine the status of water bodies based on comparison of the species community with type-specific undisturbed reference conditions (Borja *et al.*, 2009).

The response of organisms to changing environmental conditions, however, is not always directly observable at the community level. While mortality caused by abiotic conditions exceeding the tolerance limits of organisms or the consumption of a prey species by its predator, represent a lethal response that directly manifests in the benthic community via changes in species abundances, sub-lethal effects of moderate environmental stress, e.g. the presence of toxicants at non-lethal concentrations or non-consumptive predation effects, are often less obvious. Sub-lethal effects can include various cellular, physiological and behavioural mechanisms to reduce the negative effects of stress (Kassahn *et al.*, 2009; Sokolova *et al.*, 2012), which, in turn, can cause a reduction in growth rates, reproductive outputs and survival probability of organisms (Calow, 1989). Even though sub-lethal effects may ultimately cause the death of organisms in the long term, most often less drastic effects, such as reduced fitness, are observed. The concept of ‘fitness’ is a central element in biological sciences and in ecology it is used to characterize how well organisms are adapted to their environment and how well they perform under the prevailing environmental conditions. The individuals producing the highest number of descendants that are able to reproduce are considered to have the highest ‘ecological fitness’ (Begon *et al.*, 2006). Therefore, in practice, parameters either directly describing reproductive success, e.g. the total number of offspring, or fitness-related parameters such as fecundity (i.e. potential reproductive output), fertility (i.e. actual reproductive performance) or the individual growth rate are considered as adequate measures. While the observation and determination of fitness-related parameters of individual organisms is quite feasible in laboratory or mesocosm experiments, this is often impractical in field-studies. The limited accessibility of organisms within their natural habitats often does not allow the repeated sampling of specific individuals or their continuous observation and thus impedes the determination of individual growth rates or reproductive success under field conditions. This might have led to the frequent determination of population characteristics, such as its density, biomass, or growth rate, in order to estimate the condition of aquatic organisms in ecological field studies, which appears to be reasonable because an increase in these measures often results from a high reproductive activity of the individuals within a population.

Another field of application of indicators is their use in experimental ecology. Within an experimental context they are mostly used as a measure of performance while studying organisms or populations under intentionally altered environmental conditions. Experimental ecology focuses on studies conducted under various experimental settings, ranging from controlled laboratory conditions to field experiments under near natural conditions, and hence the spectrum of applied measures or indicators varies according to the specific experimental context. Studies in experimental ecology can include the monitoring of the benthic community, but can also extend down to the analyses at the organism level, such as observation of individual behaviour, the direct measurement of reproduction and growth rate or food consumption (e.g. Gergs and Rothhaupt, 2008; Peckarsky and McIntosh, 1998; Welton and Clarke, 1980). These methods often involve continuous investigation of individual organisms and consequently are mostly less appropriate to be applied under field conditions.

Even though the effects of environmental stress and the quality of aquatic ecosystems is traditionally assessed based on measures of biodiversity and benthic community characteristics (Vasseur and Cossu-Leguille, 2003), and ecological indicators can provide an early warning of occurring changes (Dale and Beyeler, 2001), these common approaches are limited in their ability to detect slow and gradual changes such as climate effects, non-point impacts or sub-lethal impacts affecting population dynamics at an early stage. This relates to the fact that community-based measures generally reflect the response of organisms to the prevailing environmental conditions and, consequently, changing conditions can only be recognised *a posteriori* when certain taxa become extinct or at least drastically less abundant. Such a rather slow response often impairs the detection of potential effects within the timeframe of common monitoring programs (Cooke *et al.*, 2013) and, hence, increases the time until counteractive measures could be initiated (Adams *et al.*, 2005).

To overcome these potential limits of traditional ecological approaches, physiological or biochemical indicators have been suggested as an appropriate tool for the investigation and assessment of ecological impacts within aquatic systems, as well as their management (Adams and Ham, 2011; Koop *et al.*, 2008). In this context, the physiological perspective focuses on the sub-individual effects of environmental stress rather than on the community response in terms of changes of benthic community structure. Based on various physiological studies, the sub-individual response of aquatic invertebrates to important environmental factors (e.g. water temperature, oxygen concertation) has been investigated in detail and it has been shown that benthic organisms employ various physiological strategies such as the expression of heat shock proteins, antioxidative defence mechanisms, or adjustments of the metabolism to cope with changing and harsh environmental conditions (for review see e.g. Grieshaber *et al.*, 1994; Hochachka and Somero, 2002; Hofmann and Todgham, 2010) and are generally associated with increased energetic costs (Calow, 1991; Koop and Grieshaber, 2000). To analyse the physiological mechanisms of stress response, most studies have been conducted under highly controlled laboratory conditions and often focused on species well suited to laboratory rearing. However, such laboratory studies can hardly simulate the complex conditions of natural environments, where organisms are affected by multiple, interacting factors simultaneously. As a consequence, laboratory studies often only provide a mechanistic understanding of the underlying processes in response to single or few environmental factors.

Nevertheless, the general conclusion drawn from these studies that stress response is energetically costly for organisms is most important from an ecological perspective and provides the foundation for an integrative assessment of the effects of multiple stressors in natural environments as it is for example applied within the ‘Concept of energy-limited tolerance to stress’ (cf. Sokolova, 2013). Energy unarguably plays a central role in the function of organisms. From a thermodynamically point of view living organisms can be characterized as open systems (Lamprecht, 2003). Consequently, the balance between energy acquisition from resources and expenditure for basal maintenance or fitness-related functions, like ontogenetic development, growth and reproduction, fundamentally affects the survival of organisms. It appears that ecological fitness might be reduced in favour of basal maintenance, if increased energetic costs of stress response or reduced energy acquisition cannot be compensated from stored energy resources (Sokolova, 2013). Hence, the amount of energy available from stored resources as well as their metabolic conversion, which enable organisms to sustain vital functions over a broad range of environmental conditions, can be regarded as the foundation of the ecological success of organisms. For this reason the determination of sub-individual energetic measures, such as the amount of energy reserves, e.g. glycogen or lipid, as well as measures providing insight into the current cellular energy status, e.g. the adenylate energy charge (i.e. the ratio of the cellular concentration of the adenosine phosphates ATP, ADP and AMP; cf. Atkinson, 1977, 1968), have been suggested as suitable indicators to detect exposure to stressful environmental conditions and to estimate effects of toxic substances related to environmental pollution (Amiard-Triquet *et al.*, 2012; Sokolova, 2013; Sokolova *et al.*, 2012). Furthermore, the actual energy consumption is regarded as a good indicator of an animal's performance and adaptation (Normant et al., 2007; Willmer et al., 2005).

Although the use of physiological indicators appears to be well established in marine ecology and in ecotoxicology, they are still rarely used in freshwater ecology. While physiological indicators were initially introduced to marine biology to evaluate the condition of commercially used wild fish stocks and their population dynamics (Buckley, 1984; Chícharo and Chícharo, 2008), they are meanwhile applied to assess the quality of marine environments as well as to explain and predict species' distribution limits due to environmental change (Sokolova *et al.*, 2012). In ecotoxicology, their application mainly focuses on providing evidence for the exposure to and effects of environmental pollutants. In this context, some recent ecotoxicological studies applied physiological indicators to investigate the effects of common environmental pollutants such as pesticides, wastewater, diet-related and waterborne heavy metals (e.g. Cu, Cd) on the behaviour and physiology of common amphipod species (e.g. *Hyalella* spp., *Gammarus* spp. and *Dikerogammarus villosus;* Bundschuh *et al.*, 2011; Dutra *et al.*, 2011, 2008; Sroda and Cossu-Leguille, 2011a; Zubrod *et al.*, 2015). Moreover, the effects of ambient ammonia have been studied in *Asellus aquaticus*, as well as *Gammarus pulex* and *Gammarus fossarum* by Dehedin *et al.* (2013). Even though these studies provide important information on the effects of specific environmental stressors, the related knowledge is of limited relevance for the application of physiological indicators in freshwater ecological field studies, because the experimental conditions do not reflect the complex environmental conditions of natural habitats. Some ecotoxicological studies, however, aimed at investigating physiological indicators in unstressed organisms under field conditions rather than assessing the effects of specific stressors in the laboratory. These studies provide information e.g. on the inter-sex differences as well as temporal variation within the energy storage compounds of *Gammarus roeselii* and *Hyalella* spp. (Braghirolli *et al.*, 2016; Dutra *et al.*, 2008, 2007; Gismondi *et al.*, 2012; Sroda and Cossu-Leguille, 2011b).

Even though to date only few studies intentionally assessed physiological indicators in freshwater ecosystems within an ecological context, these rare studies indicate the high potential for the assessment of sub-lethal effects of biotic interactions as well as environmental stress in field-collected invertebrates. Winkelmann *et al.* (2007), for example, assessed sub-lethal effects of benthivorous fish on a mayfly (*Rhithrogena semicolorata*) and the amphipod *G. pulex* by analysing their energetic condition. Further, Koop *et al.* (2008) reported that the energetic condition of several aquatic invertebrate taxa (*Heptagenia flava, Glossiphonia complanata, D. villosus*) correlates with their spatial distribution and abundance within a natural environmental gradient of a large river, and Cavaletto *et al.* (2003) demonstrated the adaptive use of seasonal varying food resources in the nymphs of two *Hexagenia* spp. populations based on the different seasonal patterns in energy storage compounds.

Compiling the data on specific physiological indicators in aquatic invertebrates from published ecotoxicological and ecological studies, there appears to be considerable evidence for large interspecific, spatial as well as seasonal variability in these measures. However, these data have mostly been acquired under differing experimental conditions and the applied analytical methods, as well as data analysis often vary between studies. Comparative studies investigating closely related taxa or taxa occurring in the same habitat within the same study are very rare (but see Sroda and Cossu-Leguille, 2011a). This apparent lack of comparative studies as well as the scare knowledge on the natural variability in common freshwater invertebrates stimulated my research within this thesis. It focuses on selected amphipod species belonging to the genera *Gammarus* and *Dikerogammarus*. Due to their important role in freshwater ecosystems and widespread distribution in Central European riverine ecosystems, *Gammarus* species (i.e. gammarids) may be suitable candidates for the determination of physiological indicators in ecological studies. In fact, these omnivorous invertebrates are considered keystone species in freshwater habitats because they contribute to detritus processing, by breaking down allochthonous, course organic matter (mainly leaves) to fine particulate organic matter and through this activity provide large amounts of faeces (Glazier, 2009). Moreover, gammarids provide an important trophic link by constituting an abundant prey for fish, vertebrates, and other macroinvertebrates, but can also act as predator for other invertebrates themselves (Macneil *et al.*, 1999, 1997).

In central Europe, both *Gammarus fossarum* and *G. pulex* are common native *Gammarus* species which exhibit a characteristic distribution within different stream reaches (*G. fossarum*: upper reaches, *G. pulex*: middle and lower reaches; Karaman and Pinkster, 1977a). The lower reaches of streams and small rivers are often inhabited by *G. roeselii* (Siegismund, 1988), which, although formerly originating from the south-eastern part of Europe or Asia minor (Karaman and Pinkster, 1977b), is considered a naturalized species in Central Europe (Hesselschwerdt *et al.*, 2008; Josens *et al.*, 2005). While the amphipod taxa occurring in streams and small rivers are mostly characterized by native amphipod species, the benthic community of large rivers is nowadays dominated by non-native amphipods, such as the invasive *D. villosus*. Originating from the Ponto-Caspian region this species established high population densities in many European rivers, such as the Moselle, Rhine or Elbe rivers (Bij de Vaate *et al.*, 2002; Devin *et al.*, 2005; Tittizer *et al.*, 2000). Considering the invasion success of *D. villosus*, that is supported by beneficial life-history traits like an early maturity or high reproductive rate (for review see Grabowski *et al.*, 2007; Rewicz *et al.*, 2014) it represents an ideal species to compare its physiological species traits to the native gammarids which are often characterised by less beneficial traits.

Objectives and outline of the thesis

The aims of the present thesis are twofold. One objective is to highlight the potential of assessing the fitness of organisms in freshwater ecological studies by means of physiological indicators. To this end **Chapter 2** provides the basic assumptions and the relevant theoretical as well as methodological fundamentals necessary for the application of physiological indicators within an ecological context and, furthermore, points out their applicability by several case studies.

The second aim of the thesis is to provide ecophysiological background knowledge that is crucial for a successful application of physiological indicators in ecological studies. In particular the following fundamental questions are addressed in the thesis:

(i) Are there sex- and species-specific differences in the seasonal variation of the energetic condition (glycogen, triglyceride) in natural *Gammarus* populations? (**Chapter 3**)

(ii) Does the metabolic activity and behaviour differ between different amphipod species? (**Chapter 4**)

(iii) What are the direct effects of ambient ammonia on the physiology and behaviour of *D. villosus*? (**Chapter 5**)

Depending on the specific question the conducted field and laboratory studies apply physiological methods ranging from measurements of whole organism responses (e.g. determination of metabolic rate *in vivo*) down to the determination of macromolecules (e.g. the concentration of specific energy reserves or adenosine-phosphates). **Chapter 6** comprises a discussion of the fundamental conclusions drawn from the conducted studies, as well as their relevance and general implications for the application of physiological indicators in freshwater ecological research.

Chapter 2

Physiological indicators of fitness in benthic invertebrates: a useful measure for ecological health assessment and experimental ecology

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Unpublished supplementary material to this chapter is provided in the appendix of the thesis: Material and method used for the determination of the adenylate energy charge (AEC, shown in Box 2).

Note by the author:

This chapter is based on the following journal publication. Due to copyright issues, the text of the chapter was replaced by the reference information. Thus, the interested reader is kindly asked to read the published paper via the following reference:

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DOI: 10.1007/s10452-011-9375-7

Abstract

Physiological indicators of fitness present a measure of an organism’s response to a changing environment. An analysis of how these organisms allocate and store their energy resources provides an understanding of how they cope with such environmental changes. Each individual has to balance the investment necessary to acquire a certain resource with the energy gained by it. This trade-off can be monitored by measuring several physiological indicators of fitness such as energy storage components, metabolic state or RNA/DNA ratio. Because environmental adaptations and ecological strategies of survival are best examined within the natural environment, our research has to rely on the physiological indicators that are easily accessible in the field. The physiological indicators presented here are significant for an individual’s fitness and in turn lead to reliable values in field-collected samples. Based on our own expertise and on a literature survey, the physiological relevance of the presented indicators is explained. Furthermore, some consideration to the analytical methods used to obtain the physiological indicators is given, and possible errors introduced at the sampling site and during the laboratory procedures are discussed. This work demonstrates that the integration of ecological and physiological expertise facilitates the identification of future ecological problems much earlier than separate approaches of both disciplines alone.

Keywords: Fitness; physiological indicators; aquatic invertebrates; methods; stream ecology

Chapter 3

Mate guarding in relation to seasonal changes in the energy reserves of two freshwater amphipods (*Gammarus fossarum* and *G. pulex*)

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Jochen Becker, Christian Ortmann, Markus A. Wetzel, Carola Winkelmann, Jochen H. E. Koop, *Freshwater Biology* (2013) 58, 372–381.

DOI: 10.1111/fwb.12065

Summary

1. We assessed sex-specific seasonal changes in major energy storage compounds (triglycerides, glycogen) in *Gammarus fossarum* and *Gammarus pulex* collected from the field, with respect to their reproductive activity.

2. The dynamics of stored energy followed a seasonal pattern in both species and sexes. Moreover, over a 4-year period, these changes were independent of the year in which they were investigated. Stored energy reached a peak in late winter, but was depleted in late summer and early autumn, coinciding with the reproductive periods.

3. Triglyceride (annual mean ± SD) accounted for 79.7 ± 11.9% of the total stored energy and was responsible for the seasonal pattern. In contrast, glycogen contributed a lesser percentage (20.3 ± 11.9%). Over the study period, the amount of stored energy ranged between 0.39 and 4.08 kJ g^{-1} dry mass (triglyceride: 0.19–3.69 kJ g^{-1} dry mass; glycogen: 0.14–0.80 kJ g^{-1} dry mass).

4. In both species, the energy reserves of males were drastically depleted shortly before the cessation of precopulatory mate guarding in the field, thus offering a bioenergetic explanation for the reproductive period in these two widespread species.

Keywords: *Gammarus* spp., glycogen, reproductive period, seasonality, triglyceride

Chapter 4

Metabolic activity and behavior of the invasive amphipod *Dikerogammarus villosus* and two common Central European gammarid species (*Gammarus fossarum, Gammarus roeselii*): Low metabolic rates may favor the invader

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DOI: 10.1016/j.cbpa.2015.10.015

Abstract

The Ponto-Caspian amphipod *Dikerogammarus villosus* is one of the most successful invaders in Central European rivers. Contrary to studies on its ecology, ecophysiological studies comparing the species' physiological traits are scarce. In this context, in particular the metabolic activity of the invasive species has rarely been considered and, moreover, the few existing studies on this species report strongly deviating results. The purpose of this study was to assess the metabolic activity and behavior of *D. villosus* and other common European amphipod species (*Gammarus fossarum, Gammarus roeselii*) in relation to temperatures covering the thermal regime of the invaded habitats. Based on direct calorimetric measurements of metabolic heat dissipation at three temperature levels (5 °C, 15 °C and 25 °C), we found the routine metabolic rate of *D. villosus* to be significantly lower than that of the other studied gammarid species at the medium temperature level. The estimated resting metabolic rate indicated a similar trend. At 5 °C and 25 °C, both routine and resting metabolic rate did not differ between species. Compared to *G. fossarum* and *G. roeselii, D. villosus* exhibited lower locomotor activity at the low and medium temperatures (5 °C and 15 °C). In contrast, its locomotor activity increased at the high experimental temperature (25 °C). *G. fossarum* and *G. roeselii* were apparently more active than *D. villosus* at all studied temperatures. We conclude that *D. villosus* has both physiological and behavioral adaptations that lead to a reduction in metabolic energy expenditure, which is assumed to be beneficial and might contribute to its invasive success.

Keywords: *Dikerogammarus villosus*; *Gammarus fossarum*; *Gammarus roeselii*; glycogen; heat production; invasive species; metabolic rate; locomotor activity; triglyceride

Chapter 5

Physiological and behavioral responses of the invasive amphipod, *Dikerogammarus villosus*, to ammonia

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Abstract

We studied the physiological and behavioral responses of the Ponto-Caspian amphipod *Dikerogammarus villosus* during exposure to four total ammonia concentrations (NH3 + $NH4^+$; Tot_{Amm}): 0.003 (control), 0.06, 1.6, and 7.0 mmol L^{-1} (0.042, 0.84, 22.4, and 98.0 mg L^{-1}) for a period of up to 12 h at 21 °C. During the transition period from the control to treatment concentration as well as during the first hour of exposure to 0.06 and 1.6 mmol L^{-1}, gammarids increased their locomotor activity, which was manifested in significantly higher routine metabolic rates compared to control conditions. At control conditions, the resting metabolic rate amounted to 0.98 ± 0.26 mW g^{-1} and significantly increased by 19 and 37% at 0.06 and 1.6 mmol L^{-1}, respectively. The highest examined [Tot_{Amm}] caused a rapid and significant increase in resting metabolic rate by 37% within the first 4 h of exposure before gammarids died. The exposure to elevated [Tot_{Amm}] also resulted in a significant decreased RNA:DNA ratio and significantly higher glycogen concentrations compared to the control. We conclude that even a short exposure to Tot_{Amm} of 0.06 mmol L^{-1}, which may occur in natural habitats, disturbs the physiology and behavior of *D. villosus* and leads to increased metabolic costs of the maintenance and reduced protein synthesis.

Keywords: Ammonia; metabolism; locomotor activity; heat production; RNA:DNA ratio; glycogen; triglyceride; *Dikerogammarus villosus*; invasive amphipod

Abbreviations: Tot_{Amm}, total ammonia

Chapter 6

General Discussion (Synthesis)

Assessing the response of organisms to their biotic and abiotic environment by analysing measures at the sub-individual level of biological organization is a promising approach. It has the potential to increase the effectiveness of freshwater ecological studies, e.g. in the context environmental health assessment, but also to detect sub-lethal effects in experimental ecology. By determining physiological indicators of fitness the focus is directed towards the physiological response of organisms rather than on the ultimate responses which mostly manifest in changes in the benthic community (Chapter 2). Compared to traditional ecological indicators, physiological indicators of fitness are characterized by a shorter response time, ranging from seconds to several weeks depending on the specific measures (see Chapter 2). Hence, physiological indicators bear potential to assess effects in benthic communities faster than it is possible using common approaches such as the biomonitoring of the benthic community or the determination of the reproductive success of organisms. Nevertheless, even though the applicability of physiological indicators in the context of stream ecology has been highlighted by recent studies as mentioned previously they have been rarely applied up to now.

My thesis provides a theoretical and practical contribution for successful implementation of physiological indicators in freshwater ecological studies. In **Chapter 2**, the basic assumptions of assessing the physiological fitness of organisms by means of physiological indicators have been highlighted and indicators suitable to be determined in almost every modern ecological laboratory suggested. Moreover within this chapter, the relevant theoretical and methodological fundamentals for a determination of these indicators using methods well established in animal physiology and biochemistry have been addressed. Further, as a part of several complementing case studies, Chapter 2 includes the results of a brief experimental study in which I applied the determination of the adenylate energy charge (AEC, based on the concentration of adenosine-phosphates, Atkinson, 1968) as an indicator of the cellular energy status of organisms.

The ecophysiological research within my thesis focused on native and non-native freshwater amphipods because of their function as keystone species in aquatic habitats. Even though, *G. fossarum* and *G. pulex* are considered potential reference species for ecotoxicological freshwater monitoring (Chaumot *et al.*, 2015), to date, only few studies investigated the baseline variation of physiological indicators of these in central Europe commonly distributed native species in natural, undisturbed populations or intentionally conducted comparative studies between related species. Therefore, the field study presented in **Chapter 3** complements the limited knowledge on the variation of physiological indicators of these freshwater gammarids. Unlike previous studies, the one presented here considers the analysis of specific energy storage compounds, namely glycogen and triglyceride, instead of more general measures, such as the total lipid content (e.g. Gee, 1988; Gismondi *et al.*, 2012; Sroda and Cossu-Leguille, 2011) or the total caloric value (e.g. Nilsson, 1974). Excluding substrates with other functions, e.g. structural lipids (phospholipids), was assumed to provide a more realistic assessment of the actual amount of energy available for metabolism and for ecological relevant processes like growth, reproduction and movements as well as for compensatory mechanisms in response to changing environmental conditions. The comparatively high temporal resolution of the study (2−4 week intervals) enabled the correlation of energetic condition with the characteristic reproductive cycles of both species. The resulting knowledge on the specific variation of energy storage compounds over the course of the year led to an energetic explanation for the reproductive resting period of *Gammarus* spp. commonly observed in late autumn. As a general result the study clearly revealed a profound seasonal variation as well as sex-specific differences in the amount of stored triglyceride in both *Gammarus* species. Further, the results of the study suggested that inter-annual variation in the concentration of stored triglyceride and glycogen is negligible. The general seasonal succession of stored triglyceride was characterized by a high concentration of this storage compound in late winter and a continuous depletion over the course of the year until the following late summer/early autumn.

While Chapter 3 focused on determining the energetic condition of organisms based on energy storage compounds, the laboratory studies presented in **Chapters 4 and 5** mainly focused on the actual energy consumption of organisms by quantifying the metabolic rate *in vivo*. Therefore direct calorimetry, i.e. the measurement of metabolic heat dissipation, was applied (1) to compare the metabolic and behavioural activity between different amphipod species occurring in central European streams or rivers (Chapter 4) and (2) to determine the direct effects of ambient ammonia as one relevant environmental factor on *D. villosus* (Chapter 5). The study presented in Chapter 4 represents the first ecophysiological study directly comparing the metabolic activity of the invasive amphipod *D. villosus* and the native or naturalized central European gammarid species *G. fossarum* and *G. roeselii*, respectively. It provided evidence for species-specific differences in routine metabolic rate, as was indicated by a lower rate of the invader compared to both other species at a medium experimental temperature (15 °C). The observed difference was limited to this medium temperature level and no differences were found at the other studied temperature levels (5 and 25 °C). Considering the behavioural activity the present study revealed that the invader is less active than *G. fossarum* and *G. roeselii* at a low and medium temperature (5 and 15 °C). At the highest experimental temperature (25 °C) however, no differences between species were observed. Within the second laboratory study (Chapter 5) the direct effects of ammonia on the metabolic activity, RNA:DNA ratio, and locomotor activity of *D. villosus* have been investigated for the first time in this respective species. Based on direct calorimetric measurements we found that increased ammonia concentrations always caused higher routine metabolic rate compared to control conditions. While even short-time exposure to an artificially high ammonia concentration (7.0 mmol L^{-1}) was always lethal for the experimental organism after a period of increased metabolic activity (c. 4 h), a comparatively low ammonia concentration (0.06 mmol L^{-1}), which indeed occurs under natural conditions, already caused changes in the physiology and behaviour of *D. villosus* and resulted in increased metabolic costs for maintenance and reduced protein synthesis.

Implications of the experimental results for the implementation of physiological indicators in ecological studies

While the general aspects concerning the choice of appropriate physiological indicators, the required sampling procedures in ecological studies and the basic analytical methods have been addressed and discussed in Chapter 2 of the thesis, the results of the conduced experiments emphasize further aspects which need to be considered for a successful implementation of physiological indicators of fitness in ecological studies.

Indicators at low levels of biological organization, i.e. the individual level and lower, have been applied in other disciplines, e.g. in ecotoxicology or marine ecology. In ecotoxicology, the determination of sub-individual ‘biomarkers’ is used to indicate the exposure of organisms to environmental pollutants or to analyse their sub-lethal effects.

In this context, ‘biomarkers’ are defined as any biochemical, cellular, physiological or behavioural variation which can be measured in tissue, body fluid samples or at the whole organisms level (Depledge, 1994). Considering this definition, the ‘physiological indicators’ that the present thesis was focussed on comply with it − although they have been named differently. Nevertheless, with respect to their general function as indicators, there appear to be differences between their use in ecology and ecotoxicology. The application of physiological indicators within an ecological context, as outlined in Chapter 2, focuses on the individual health or physiological condition of an organism. They should indicate impairments in the energy metabolism, which may cause changes at the population or community level due to a reduced fitness. By contrast, although exposure to pollutants can certainly cause impairments of the energy metabolism, e.g. through increased metabolic costs for maintenance or reduced feeding (Amiard-Triquet *et al.*, 2012), the focus within the ecotoxicological application of biomarkers is mostly directed towards the determination of environmental pollution.

With respect to an application in ecological studies the analysis of sub-individual indicators is a valuable tool to detect changes in habitat conditions or effects of specific environmental factors. Focusing on the physiological condition of organisms within a community rather than on changes in community characteristics (e.g. species abundance, community structure) can overcome the intrinsic time lag, i.e. the time needed until changes in reproductive success of species translate into observable changes within the population or community (see Chapter 2). This time saving appears to be attractive for several applications in freshwater ecosystems. For example, it could contribute to a more effective management of freshwater ecosystems, because it enables early intervention and establishment of conservation measures in response to changing habitat condition. Moreover, physiological indicators can provide additional endpoints in experimental ecology that respond faster than traditional ones (e.g. individual growth rate). Hence, the analysis of physiological indicators could either complement traditional ecological approaches (e.g. the biomonitoring of benthic communities) or be used separately. In fact, the physiological indicators that have been described (cf. Chapter 2) and applied (cf. Chapters 2–5) within the present thesis are mostly related to the energy metabolism, because the energetic condition plays a central role in organism’s survival and function, as well as in stress adaptation and tolerance (Sokolova *et al.*, 2012). These indicators either focus on directly quantifiable measures, such as the concentration of main energy reserves which provide the energy needed to cover most of the costs for maintenance and ecological relevant processes (e.g. movement, growth and reproduction, cf. Chapter 2), the metabolic rate as a measure of the actual energy consumption of an organism (Willmer *et al.*, 2005), or on derived indices, such as the current cellular energy status (Adenylate energy charge, Atkinson, 1968) and the RNA:DNA ratio as an indicator of growth (Grimm *et al.*, 2015).

Dealing with variation in physiological indicators

Temporal and spatial variability appears to be frequent when studying organisms within their natural habitat in general but also when field-collected individuals are studied in the laboratory environment. This variability could be observed in different situations, ranging from varying species abundances during field sampling to the intra-specific variation in the concentration of physiological indicators. It is well known, that the physiological response of organisms is affected by various biotic and abiotic factors within their natural environment, but also individuals themselves can differ in their response to environmental stress due to differences in e.g. individual nutritional status, developmental stage or reproductive cycle. To account for this variability ‘active biomonitoring’, i.e. the transplantation of caged organisms reared in the laboratory or collected from non-polluted sites, is advocated in ecotoxicology. It allows to use standardized organisms e.g. with respect to sex, age, size, nutritional state (Amiard-Triquet *et al.*, 2012). However, in ecological studies the condition of a natural field population is of particular interest and intra-specific traits variation, e.g. related to phenotypic plasticity, is considered as an important factor affecting the outcome of ecological interactions (Bolnick *et al.*, 2011). Hence, active-biomonitoring appears not to be expedient in any ecological context. Consequently, procedures accounting for the apparent natural variation should be applied rather than eliminating it.

The most obvious variation observed in the present thesis was **seasonal variation**. It was particularly evident in the temporal pattern of stored triglycerides in the studied *Gammarus* species (*G. fossarum, G. pulex*, Chapter 3). Due to its high specific energy content, stored triglycerides caused the total amount of stored energy to vary greatly over the course of the year within both species. Seasonal variation of energy storage compounds has also been reported for *G. roeselii* (Gismondi *et al.*, 2012; Sroda and Cossu-Leguille, 2011b). Further, seasonal variation might not only be common in gammarids, but also in other benthic invertebrates, as was evident e.g. in mayflies nymphs (*Hexagenia* spp. in Cavaletto *et al.*, 2003; *Ephemera danica*, *Rhithrogena semicolorata* in Winkelmann and Koop, 2007). Irrespective of the specific factors contributing to this variation, the general fact should be considered as a natural characteristic (‘baseline’) of the studied species in future studies assessing energy reserves as a physiological indicator and needs to be considered as one important factor confounding the interpretation of measured absolute values across seasons. It is obvious, that the assessment of one distinct measured value would depend on the season due to the changing baseline level of the respective indicator. Consequently, in order to avoid misinterpretation, determined values of physiological indicators cannot be evaluated *per se* on the basis of a general reference value of the respective physiological indicator, but rather need to be related to the existing baseline level that is present in the natural population at the moment of sampling.

There are different practical solutions to account for seasonal variation. Considering a benthic invertebrate sampling campaign assessing the physiological condition of aquatic invertebrates over an extended period, e.g. several years, one solution could be timely coordinated sampling campaigns that are conducted during a standardized time of year. Such defined sampling seasons are commonly applied within ecological assessment approaches, e.g. the monitoring of the benthic community in the context of the European Water Framework Directive (Haase *et al.*, 2004) to account for seasonal changing species abundances in EPT-taxa (Ephemeroptera, Plecoptera, and Trichoptera) which only spend parts of their life in aquatic habitats. Nevertheless, concentrating sampling campaigns within a short temporal interval has a potential drawback, because comparisons are only possible on a yearly basis and therefore drastically reduce the time-savings achievable by the application of fast-responding physiological indicators. Another practical solution, that provides results within a shorter timeframe, is the application of physiological indicators within a comparative context. This approach could involve a simultaneous assessment of reference and treatment conditions within the same sampling campaign, which consequently enables an immediate comparison between both conditions without the effect of seasonal variation. Lastly, it is conceivable to account for seasonal variation during data analysis following the actual sampling campaign. In this respect, the derivation of specific reference values for distinct periods can be a solution. This can be particularly considered appropriate when independent sampling campaigns at quite different dates should acquire a snapshot of the condition of the studied populations. However, in order to derive those specific reference values, a profound knowledge about the seasonal variation is required as a prerequisite for the actual study. Therefore, this is only possible when time and other factors, e.g. financial resources, permit the necessary extensive preliminary studies in this regard. Nevertheless, this should not be a reason for exclusion, but it limits the practical feasibility to some extent. Thus, it is only recommended in cases where extensive knowledge on the variation of the potentially applied physiological indicators in the respective habitat is available in advance of the actual assessment.

In addition to the seasonal variation, the present thesis provides evidence for **sex-specific** and **species-specific differences** in the analysed physiological indicators. Sex-specific differences were observed in the triglyceride concentration of both studied gammarid species in this thesis (cf. Chapter 3) and have also been reported for the lipid and glycogen content of *G. roeselii* (Gismondi *et al.*, 2012). Consequently, the sex of the organisms should be determined during the field sampling whenever possible and taken into account during analysis. Basically it would be advantageous to select those organisms for a study that allow an easy and immediate sex-determination under field conditions, e.g. based on sexual dimorphisms. However, this is not always possible and, thus, stress resulting from the handling time or mechanical manipulation of the respective organisms during sex-determination in the field, has to be expected as a potential error affecting the physiological status of the organisms. While this is particularly true for fast responding molecular indicators, most of the physiological indicators proposed within this thesis respond more slowly (see Fig. 3 in Chapter 2) and are therefore less prone to be affected. Considering ‘starvation’ as severe stress, the work of Hervant *et al.* (1999) support this assumption by reporting the triglyceride and glycogen content of *G. fossarum* to be significantly reduced after 14 and 7 days, respectively.

With respect to the experiments conducted for the present thesis, the sampling of male individuals from precopulatory pairs permitted an immediate sex-determination based on the characteristic female carrying during mate-guarding of male individuals (cf. Sutcliffe, 1992) However, when planning a potential study, differences in reproductive periods even between closely related species need to be considered. This has been observed in *G. fossarum* and *G. pulex* in the Tännichtgrundbach and Gauernitzbach, respectively (cf. Chapter 3) − even though these streams were characterised by similar habitat conditions (Schmidt *et al.*, 2009) and both species generally exhibit a very similar lifestyle (Schmedtje and Colling, 1996). Further, the sampling of individuals that were apparently reproductively active ensured that individuals of at least similar developmental stages were used for the analysis of physiological indicators. This can be considered as another possibility to minimize the natural variability in the determined physiological measures (Winkelmann and Koop, 2007). Of course, some taxa do not permit fast sex-determination, because sex-specific characteristics are absent or cannot be discerned in living animals under field conditions (e.g. holometabolic insects or molluscs). Nevertheless, if these taxa are relevant for the study, they should not be excluded from the actual sampling campaign, but increased variation in the determined values due to sex-specific differences needs to be considered. Moreover, when investigating responses across different species, e.g. native and non-native species, inter-specific differences need to be considered as indicated by the different behavioural and metabolic activity of *G. fossarum*, *G. roeselii* and *D. villous* (cf. Chapter 4).

General implications concerning the application of physiological indicators in freshwater ecological studies and further potential

As previously described, the analysis of physiological indicators of fitness in ecological field studies might be valuable to detect effects in the benthic community faster than it is possible by focusing on traditional ecological measures. However, it needs to be considered that, under field conditions, organisms are affected by various natural and anthropogenic environmental factors simultaneously. Hence, the analysis of physiological indicators in field-collected organisms always integrates the effects of multiple factors over time. By contrast, laboratory experiments within an ecological context provide an opportunity to study the effects of specific environmental factors individually, while keeping other factors constant or at least unaltered (Resetarits and Bernardo, 1998). Even though such experimental settings do not fully reflect natural habitat conditions, they can provide complementary and essential information to understand the mechanisms leading or contributing to changes in the abundance and distribution of species in the field, or to determine general physiological traits of organisms.

In this regard the laboratory environment enables the use of methods which cannot be applied in the field due to practical reasons. One of these laboratory-based methods is ‘calorimetry’. It can be used to determine metabolic activity (metabolic rate) of organisms *in vivo* either directly as metabolic heat dissipation, or indirectly based on oxygen consumption or carbon dioxide production. Within the present thesis direct calorimetry has been applied in two studies (Chapters 4 and 5). While the first study focused on the differences in metabolic and behavioural activity between different amphipod species, the latter investigated the direct effects of ambient ammonia on the physiological and behavioural responses of the invasive amphipod *D. villosus* – a factor which has been assumed to influence the spatial distribution of this species and *G. roeselii* due to the different ammonia tolerance ranges of both species (Gergs *et al.*, 2013).

Unlike the analysis of the other sub-individual physiological indicators, the application of calorimetry provides a non-destructive and non-invasive method to assess the effects of the studied factor in a living organism in almost real-time (Lamprecht and Schmolz, 1999). Direct calorimetry is already a promising method on its own, especially to estimate the actual investments into aerobic and anaerobic energy metabolism under the experimental conditions. Further, the gained knowledge of the actual energy consumption can be used in combination with the determination of stored energy (based on the concentration of energy storage compounds) to estimate the energy budget of a species. This approach allows the identification of any mismatch between energy consumption and energetic condition of an organism. It can be used to calculate a theoretical survival time as was done in Chapter 3 (referring to the discussion section of the chapter). A similar but not directly equivalent approach is the estimation of the cellular energy allocation (CEA, De Coen and Janssen, 1997) which is often used in ecotoxicological studies. In fact, the determination of CEA involves a biochemical determination of the amount of energy stored as carbohydrates, lipids, and proteins and the energy consumption measured as the activity of the mitochondrial electron transport system (ETS). However, by estimating the amount of energy available from the three energy constituents mentioned previously it might be overestimated, because under unstressed conditions glycogen and triglycerides mostly cover the metabolic energy demand, whereas other energy-containing compounds, such as proteins and phospholipids (included in total lipids), are only exploited under adverse conditions (e.g. severe starvation, Hervant *et al.*, 1999). Furthermore, the ETS activity rather provides information on the ‘metabolic potential’ than on the actual energy consumption of an organism. This relates to the fact that the biochemical determination of the ETS activity focuses on the total catalytic activity of all enzymes within the respiratory chain and, as it is determined under optimal experimental conditions, all enzymes function at their maximum (Cammen *et al.*, 1990). Although this approach represents a fundamental difference to direct calorimetric determination of the actual metabolic activity, the ETS activity can provide an additional physiological indicator that can be measured in field-collected individuals (Cammen *et al.*, 1990). Thus, it provides a method to gain at least some insight into the metabolic activity of individuals living in their natural environment which is not possible using calorimetry and, therefore, can contribute to field studies where direct calorimetry cannot be applied due to technical reasons. The determination of the ETS activity has already been applied in *G. fossarum* and other crustaceans (e.g. *Asellus aquaticus, Niphargus sphagnicolus*; Schmidlin *et al.*, 2015; Simčič *et al.*, 2005; Simčič and Anton Brancelj, 2003; Simčič and Brancelj, 2006). However, bearing the comparatively slow response of the ETS activity after the variation of environmental factors (e.g. 3–4 days until equilibrium in the calanoid copepod *Acartia tonsa*, Båmstedt, 1980) in mind, this method is not appropriate to study the direct effects of any environmental factor, e.g. ammonia, as presented in Chapter 5. This question requires methods capable to monitor more immediate responses, e.g. direct calorimetry, even though it is only possible on isolated members of a complex ecosystem without connection to the rest of their environment and, consequently, can only contribute to a basic estimate of the full energetic picture of the system (Lamprecht and Schmolz, 1999). For this reason, the application of direct calorimetry should be regarded as beneficial method to assess the ecophysiological characteristics (traits) of a specific taxon in an ecological laboratory study rather than a method that can be applied routinely in field studies.

Implementing physiological species traits in trait-based approaches in community ecology

Given that the energetic condition of organisms affects their ability to cope with environmental stress, the identification of species-specific differences in physiological indicators of fitness might provide an opportunity to extend the set of biological species traits used in traits-based approaches in community ecology (e.g. Lamouroux *et al.*, 2004; Statzner *et al.*, 2005, 2001). Such trait-based approaches are increasingly applied to explain variation in species assemblages and provide improved mechanistic knowledge of species-environmental relationship compared to taxon-based approaches focusing on structural characteristics of the benthic community. By not focusing on specific taxa, these approaches permit comparisons across regions with different species composition (Verberk *et al.*, 2013). To date these approaches cover many autecological but only few physiological species traits. In this respect the observation that the allocation of energy to different storage compounds apparently reflects the life-style and adaptation of organisms to their environment (e.g. Koop *et al.*, 2008) appears promising for establishing new species traits focusing on the physiological characteristics of organisms. For example, in the aquatic mayfly larvae *Heptagenia flava* energy storage is predominantly covered by triglycerides. The high weight-specific energy value of triglyceride, compared to glycogen, allows these organisms to accumulate a comparatively high amount of energy, which can then be used for emergence as well as flight and reproduction of the non-feeding adult mayflies (Koop *et al.*, 2008). By contrast, my own studies beyond this thesis indicate that energy storage in the freshwater clam *Corbicula fluminea* is dominated by glycogen (Heininger, 2014a, 2014b). Unlike triglycerides, this storage compound can be anabolised even under anaerobic conditions, which are likely to occur during periods of long-lasting valve closure, e.g. in response to environmental stress (Ortmann and Grieshaber, 2003).

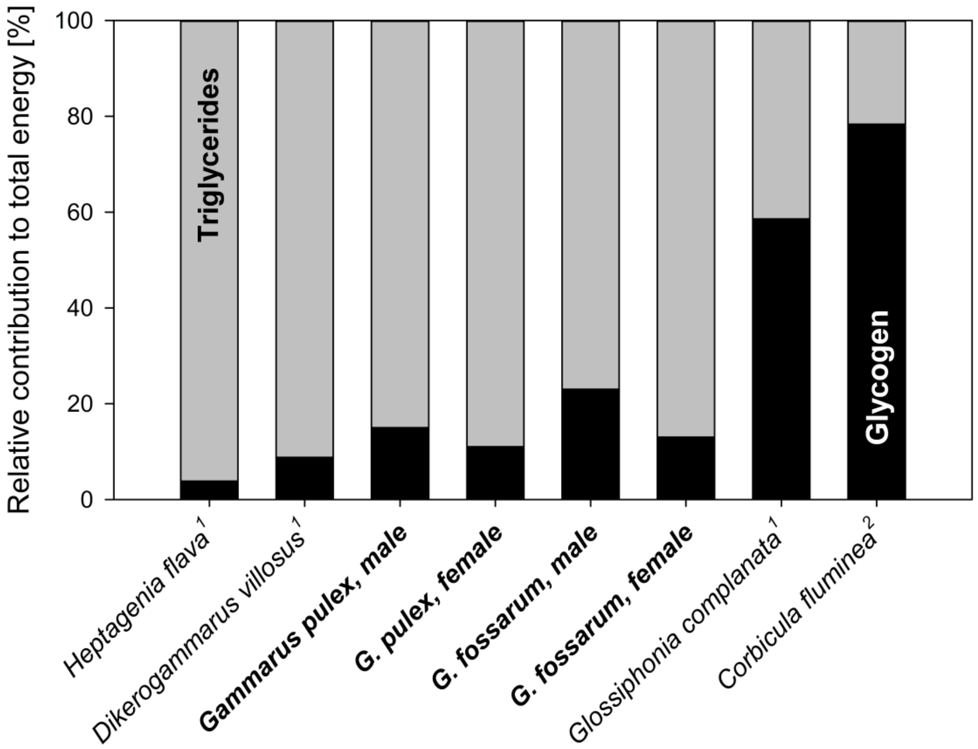

Figure 1 Relative contribution of glycogen and triglycerides (as percentages) to the total energy in field-collected aquatic invertebrates. The underlying data for this comparison have been acquired as a part of the present thesis (*Gammarus sp.*), result from other studies of the author of this thesis (*Corbicula fluminea*) or are cited from literature. Numbers in hyperscript indicate sources as follows: ^{1}Koop *et al.*, 2008; ^{2}Heininger, 2014a, 2014b.

Comparing energy storage pattern between taxa exhibiting a similar life-style, e.g. based on the data which have been acquired for the amphipod species *G. pulex* and *G. fossarum* (cf. Chapter 3) and reported for *D. villosus* by Koop *et al.* (2008), it appears to be consistent as suggested by a rather similar contribution of glycogen and triglyceride to the total stored energy (Figure 1). This may lead to the conclusion, that the dominant energy storage compound in organisms, i.e. glycogen *vs*. triglyceride storage, might represent a potential physiological species trait. Using relative values instead of absolute ones, e.g. by stating the contribution of a specific storage compound to the total stored energy (exemplarily shown in Figure 1), would account for the variation in absolute total energy storage that exists between different taxa. Nevertheless, it needs to be noted that the data exemplarily presented in Figure 1 are based on several independent studies, and consequently differ in respect to sampling period, analytical methods applied as well as data analysis. Hence, they can only provide preliminary results which should be further studied in detail within future studies under more standardised experimental conditions.

Practical considerations concerning the implementation of physiological indicators in freshwater ecological studies

With respect to required analytical equipment and applied methods, the present thesis has shown that several physiological indicators could be relatively easy implemented in ecological studies. Most of the proposed indicators that have been applied in this thesis can be determined photometrically or fluorometrically, i.e. concentration of glycogen and triglycerides, as well as the RNA:DNA ratio. The required devices can be considered as standard within a modern ecological laboratory. Moreover, the enzymatic assays necessary for these biochemical analyses are commercially available, but can often be prepared with little effort and cheaper following published protocols (refer to Bergmeyer, 1984). Even the determination of the adenylate energy charge (AEC), which bases on high performance liquid chromatography (HPLC), seems to be feasible in well-equipped modern ecological laboratories, because there are other methods in freshwater ecology that apply HPLC (e.g. pigment-based chemotaxonomy and quantification of phytoplankton, e.g. Schlüter *et al.*, 2006). Direct calorimetry, however, might be considered a method with limited potential to be routinely determined in ecological laboratories or applied in field ecological studies. This particularly relates to the rather time-consuming measurements, and the fact that suitable instruments are only commercially available at high costs or need to be self-constructed (e.g. described in Normant *et al.*, 2007). Moreover, technical requirements limit direct calorimetry to a use in the laboratory (Normant *et al.*, 2007). However, although not being a direct equivalent to calorimetric approaches, the photometrical determination of the ETS activity might be considered an alternative approach feasible within an ecological context and with common laboratory equipment.

Apart from the technical requirements, practical implementation of physiological indicators is directly possible in any comparative context in field and laboratory experiments. This indeed represents the application type which utilizes the general advantages of physiological indicators, i.e. a fast response time compared to traditional ecological measures, to the largest extent. Nevertheless, the sampling of organisms for the determination of physiological indicators in parallel to traditional biomonitoring campaigns is recommended to gain the most benefit. Even though it only provides information on the actual values of the physiological indicators in the studied organisms, and thus might increase the workload of the actual biomonitoring campaign initially, it offers clear advantages in the long-term. First of all, it provides basic knowledge on the variation of the proposed indicators in natural communities, which is generally scarce in species that are not relevant in the field of ecotoxicology. Secondly, it provides valuable knowledge whenever collected data of the physiological indicators can subsequently be correlated with patterns of abundance, biomass and reproduction of the same taxa obtained from traditional biomonitoring campaigns. This, in the end, would help to define reference values against which other values measured within one-time sampling campaigns could be evaluated, e.g. to acquire a snapshot of the physiological condition (‘physiological fitness’) of a population – which in turn can be considered as one of the ultimate goals of the proposed physiological indicators approach.

Summary

The implementation of physiological indicators reflecting the response of organisms to changes in their environment is assumed to provide potential benefits for ecological studies. By analysing the physiological condition of organisms in freshwater ecological studies rather than their ultimate effects, physiological indicators can contribute to a faster assessment of effects than using traditional ecological indicators, such as the evaluation of the benthic community structure or the determination of the reproductive success of organisms. This can increase the effectiveness of environmental health assessment and experimental ecology. In this respect the thesis focuses on physiological measures characterizing the energetic condition and energy consumption (the concentration of energy storage compounds, the adenylate energy charge, the energy consumption *in vivo*), as well as individual growth (RNA:DNA ratio) of organisms. Although these sub-individual indicators are commonly applied in marine ecology and more recently in ecotoxicology, they have been rarely applied in freshwater ecology to date. With respect to an increased use of physiological indicators in freshwater ecological studies, the objectives of the present thesis are twofold. First, it highlights the potential of assessing the individual fitness by means of physiological indicators in freshwater ecological studies. For that reason, Chapter 2 provides the basic assumptions as well as the theoretical and methodological fundamentals necessary for the application of physiological indicators within freshwater ecology and, furthermore, points out their applicability by several case studies. As second objective, the thesis addresses selected ecophysiological aspects of native and non-native freshwater amphipods, which are considered suitable candidates for the determination of physiological indicators in ecological studies due to their function as keystone species within aquatic habitats. The studies presented in Chapters 3−5 of the thesis provide information on (i) species- and sex-specific seasonal variations within the energetic condition of natural *Gammarus* populations (*G. fossarum*, *G. pulex*), (ii) differences in metabolic activity and behaviour between different amphipod species (*G fossarum*, *G. roeselii* and *D. villosus*), as well as (iii) the direct effects of ambient ammonia on the physiology and behaviour of *D. villosus*. The fundamental conclusions drawn from the conducted field and laboratory studies, as well as their relevance and general implications for the application of physiological indicators in freshwater ecological research are discussed in Chapter 6.

Zusammenfassung

Die Verwendung physiologischer Indikatoren, welche die Reaktion von Organismen auf Veränderungen ihrer Umwelt widerspiegeln, bietet ein großes Potenzial für ökologische Studien. Durch die Analyse des physiologischen Zustandes von Organismen ermöglichen diese Indikatoren eine schnellere Erfassung von Veränderungen in aquatischen Ökosystemen als es durch die Betrachtung ökologischer Indikatoren, wie z. B. der Struktur der Benthosgemeinschaft oder des Reproduktionserfolges einzelner Arten, möglich ist. Dieser Zeitvorteil kann sowohl die Effektivität der Habitatbewertung als auch experimenteller Studien in der aquatischen Ökologie erhöhen. In diesem Zusammenhang konzentriert sich die vorliegende Arbeit auf physiologische Messgrößen, wie die Konzentration von Energiespeicherstoffen, den zellulären Energiestatus (Adenylate Energy Charge) oder die Stoffwechselaktivität *in vivo*, die den energetischen Zustand oder den aktuellen Energieverbrauch von Organismen charakterisieren, sowie auf das RNA:DNA-Verhältnis als Wachstumsindikator. Obwohl diese Indikatoren in der marinen Ökologie, und in jüngerer Zeit auch in der Ökotoxikologie, regelmäßig verwendet werden, haben sie in der limnischen Ökologie bisher wenig Anwendung gefunden. Um die Anwendung physiologischer Indikatoren in diesem Bereich zu verstärken verfolgt die Dissertation zwei Ziele. Zum einen verdeutlicht sie das Potenzial der einzelnen physiologischen Indikatoren, zum anderen stellt sie Grundlagenwissen zu ausgewählten ökophysiologischen Aspekten einheimischer und gebietsfremder Arten der Ordnung Amphipoda bereit, die als Schlüsselarten aquatischer Lebensräume als besonders geeignet für die Bestimmung physiologischer Indikatoren angesehen werden. In diesem Sinne werden in Kapitel 2 sowohl die grundlegenden Annahmen, als auch die theoretischen und methodischen Grundlagen für die Bestimmung physiologischer Indikatoren aufgezeigt und ihre Anwendbarkeit in Fallstudien verdeutlicht. Die durchgeführten Experimentalstudien (Kapitel 3−5) liefern Informationen zu (i) den art- und geschlechtsspezifischen Unterschieden in der saisonalen Variation des energetischen Zustandes natürlicher *Gammarus* Populationen (*G. fossarum*, *G. pulex*), (ii) den Unterschieden in der Stoffwechsel- und Bewegungsaktivität verschiedener Amphipoda-Arten (*G. fossarum*, *G. roeselii* und *D. villosus*), sowie (iii) den direkten Auswirkungen von Ammoniak auf die Physiologie und die Bewegungsaktivität von *D. villosus*. In Kapitel 6 werden abschließend die grundlegenden Schlussfolgerungen aus den durchgeführten Feld- und Laborstudien, sowie ihre Relevanz und Auswirkungen für die Anwendung physiologischer Indikatoren in der limnischen Ökologie diskutiert.

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Appendix

This appendix describes the material and method used for the determination of the adenylate energy charge (AEC) presented in Chapter 2 (Box 2) of the thesis.

Animal collection

Gammarids were collected within a field study in two small detritus-based, second-order streams (Gauernitzbach and Tännichtgrundbach) both being tributaries of the River Elbe, Germany. While *G. fossarum* was sampled between December 2007 and September 2008 in the Tännichtgrundbach, *G. pulex* samples were taken from the Gauernitzbach between February 2008 and September 2008. During this period always 12−20 paired gammarids of each species were sampled in 2−4 week intervals. The precopulatory pairs were immediately separated at the sampling sites and both males and females were placed individually in reaction tubes after removing excess water by blotting with tissue paper. Subsequently, animal samples were flash-frozen and stored in liquid nitrogen (–196 °C) until analyses could be performed in the laboratory. While 3–5 males and 3–5 females of the collected individuals were intended for the determination of triglyceride content (described in Chapter 3) the same count of individuals was used for the analysis of the AEC and glycogen in one extract as described below. General information on the field study as well as animal sampling procedure is provided in Chapter 3 of the thesis.

Sample preparation

The concentration of adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP), as well as glycogen was determined in perchloric acid (PCA) extracts of single deep-frozen individuals using high-performance liquid chromatography (HPLC) in case of adenosine phosphates and enzymatic analysis for glycogen. Subsequent to the preparation of individual PCA extracts for each animal sample following the procedure described in Chapter 3 of the thesis the extract was divided. While 30–100 µL were used for the determination of glycogen concentration (described in Chapter 3), the remainder of the extract was centrifuged for 10 min at 18,200 x g and 4 °C. After neutralization with 5M K_2HPO_4 the supernatant was centrifuged a second time (30 min at 18,200 x g, 4 °C). Finally, 30−40 µL of the supernatant were stored at −20 °C until HPLC analysis was performed in bulks of 24 samples per day. Therefore, the respective samples were defrosted on ice and subsequent to a first centrifugation step (15 min at 18,200 x g, 4 °C) the resulting supernatant was centrifuged a second time (10 min at 18,200 x g, 4 °C) in clean reacting tubes. Afterwards 13−15 µL of the final supernatant were diluted (1:2) with ultrapure water (Astacus Analytical, MembraPure GmbH, Bodenheim, Germany) in HPLC vials on ice and stored in the temperate auto sampler of the HPLC device for analysis overnight.

HPLC conditions and analysis

The analysis of adenosine phosphates was performed on a LaChrome Elite HPLC system (VWR-Hitachi International GmbH, Darmstadt, Germany) using a anion-exchange column (SphereClone, 5 µm SAX 250-4.6 mm, Phenomenex, Torrance, CA, USA) and corresponding pre-column (SAX 4 x 3.0 mm, Phenomenex, Torrance, CA, USA) connected to a low pressure gradient pump system (L-2130). The mobile phases consisted of a potassium dihydrogen phosphate/dipotassium hydrogen phosphate buffer system (eluent A: 40 mM; eluent B: 500 mM) adjusted to pH 5.5 with 5N HCl. All solutions were prepared on a weekly basis, degassed and filtered using vacuum filtration (0.45 µm, cellulose-acetate membrane) and subsequently stored at 4 °C until use. The conditions during the chromatographic separation using a continuous gradient elution were: 0 min 100 % A, 0 % B; 4−30 min continuous change to 0 % A, 100 % B; 30.5−36 min 100 % A, 0 % B to reach initial conditions of the column for the next run. The flow rate of the mobile phases was 1 mL min^{-1} and the injection volume was 10 µL. All samples were kept at 4 °C in a temperate auto sampler (L-2200) and analyses were performed consecutively at 30 °C column temperature (column oven L-2300) and 260 nm using a diode array detector (L-2455).

External standards of AMP, ADP and ATP (3 levels in the range of 5−50 µmol L^{-1} AMP, 10−100 µmol L^{-1} ADP, 20−200 µmol L^{-1} ATP) were analysed on a daily basis for quantification of adenosine phosphates concentrations based on the peak area. Peak identification was based on the retention time of internal standards included in each sequence.

Data analysis and calculations

Data acquisition and analysis was performed with EZChrom Elite software (Version 3.1.6, Scientific Software, Inc., Pleasanton, CA, USA). Based on the specific concentration of adenosine phosphates the adenylate energy charge (AEC) of each individual was calculated according to Atkinson (1977) as:

$$AEC = \frac{[ATP] + 0.5 \cdot [ADP]}{[ATP] + [ADP] + [AMP]}$$

All calculations, figure compilation, as well as statistical analysis were performed using SigmaPlot software (Version 12.3, Systat Software Inc., San Jose, USA). Differences in the mean AEC between male and female individuals (*n* as indicated in Box 2) were assessed using paired t-tests at $\alpha \leq 0.05$ for each species separately.

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