Compound-specific $\delta^{13}\text{C}$ analyses reveal sterol metabolic constraints in an aquatic invertebrate

René Gergs^{1,2*}, Nicole Steinberger¹, Birgit Beck^{3†}, Timo Basen^{3,4}, Elizabeth Yohannes³, Ralf Schulz¹ and Dominik Martin-Creuzburg³

¹Institute for Environmental Sciences, University of Koblenz-Landau, Fortstraße 7, 76829 Landau, Germany

²Federal Environment Agency, Schichauweg 58, 12307 Berlin, Germany

³Limnological Institute, University of Konstanz, Mainaustraße 252, 78464 Konstanz, Germany

⁴Fisheries Research Station BW, Argenweg 50/1, 88085 Langenargen, Germany

RATIONALE: Dietary sterol deficiencies can have severe life history consequences for consumers. Compound-specific stable isotope analysis (CSIA) was applied to the exploration of the sterol metabolic constraints and bioconversion capacities of the amphipod *Gammarus roeselii*. Evaluating structural sterol requirements has great potential to improve our understanding of the ecological relevance of sterols as limiting nutrients.

METHODS: Juvenile *G. roeselii* were reared on food mixtures consisting of different ratios of the two algae *Scenedesmus obliquus* (cultivated with ^{13}C -labeled NaHCO_3) and *Nannochloropsis limnetica* (unlabeled), which have been shown previously to differ in food quality. We measured the sterol content and composition using a gas chromatograph equipped with a flame ionization detector and the $\delta^{13}\text{C}$ values of sterols using compound-specific isotope ratio mass spectrometry to examine potential sterol-mediated nutritional constraints of *G. roeselii*.

RESULTS: In the food mixtures, $\delta^{13}\text{C}$ values of cholesterol, synthesized by *N. limnetica*, were -25‰ and those of the Δ^7 -phytosterols, chondrillasterol and fungisterol, synthesized by *S. obliquus*, were 7 and 18‰ , respectively. Although the cholesterol concentrations in *G. roeselii* decreased with increasing proportion of dietary *S. obliquus*, the $\delta^{13}\text{C}$ values remained constant at -25‰ . Lathosterol, which appeared in *G. roeselii* at high dietary proportions of *S. obliquus*, had a $\delta^{13}\text{C}$ value of 35‰ .

CONCLUSIONS: We provide evidence that the Δ^7 -phytosterols present in *S. obliquus* cannot be metabolized to cholesterol in *G. roeselii*, resulting in the accumulation of lathosterol in the animals and potentially in sterol-limited growth. These findings emphasize the advantage of CSIA in revealing the physiological mechanisms associated with nutritional constraints.

One of the major challenges that animals are frequently confronted with during their life cycle is coping with nutritional constraints. It is well established that dietary deficiencies in essential biochemical nutrients can limit the growth and reproduction of animals.^[1–3] Sterol auxotrophy appears to be widespread among invertebrates and it is generally accepted that all arthropods are incapable of synthesizing sterols from low molecular weight precursors and thus rely on a dietary sterol supply.^[1,2,4,5] Sterols are important for various physiological processes; they are indispensable as structural components of eukaryotic cell membranes, and serve as precursors for steroid hormones, such as the molt-inducing ecdysteroids in arthropods.^[6–8] A deficiency in dietary sterols has serious life history

consequences for invertebrate consumers,^[9–11] which may not only be confronted with low dietary sterol concentrations in general, but also sterols that are unsuitable to cover their physiological demands.^[12,13] In contrast to plants and algae, which contain a great number of phytosterols, most animals predominantly contain cholesterol. Consequently, most herbivorous species take up dietary phytosterols and metabolize them to cholesterol.^[1,2] The capacity to convert dietary phytosterols into cholesterol differs among species. Thus, it is important to explore such sterol-mediated metabolic constraints to better understand the physiological and ecological consequences associated with dietary sterol deficiencies.

Stable carbon isotopic techniques based on naturally occurring differences in bulk ^{13}C signatures are commonly applied to study trophic relationships.^[14,15] A more promising but less frequently applied approach is to study stable isotope signatures in individual compounds, i.e. compound-specific stable isotope analysis (CSIA). In addition to providing information on the dietary sources of individual compounds, CSIA can help to elucidate specific nutrient requirements, bioconversion capacities and associated physiological constraints.^[16–19]

* Correspondence to: R. Gergs, Federal Environment Agency, Schichauweg 58, 12307 Berlin, Germany.
E-mail: rene.gergs@uba.de

† Current address: Environmental Chemistry, Eawag, Ueberlandstrasse 133, 8600 Duebendorf, Switzerland.

Aquatic invertebrates often feed on a variety of food sources but still are susceptible to the predominance of nutritionally inadequate food sources.^[20,21] Amphipods, as key species in benthic food web processes, are often classified as shredders, feeding primarily on deposited leaf litter.^[22,23] However, several studies have demonstrated that amphipods prefer more valuable food sources, such as eukaryotic algae,^[24] and that they also prey on other invertebrates.^[25] Considering the high plasticity in their feeding strategies, gammarids are best characterized as omnivores.^[26,27] Recent findings indicate that *Gammarus roeselii* is able to exploit deposited phytoplankton as a food source and that the growth and survival of *G. roeselii* feeding on deposited phytoplankton are affected by the phytoplankton sterol composition.^[28,29]

The approach that we applied here is based on the finding that juvenile *G. roeselii* feeding on the green alga *Scenedesmus obliquus* exhibit a significantly higher mortality than those feeding on other deposited algae, indicating that this green alga is of poor nutritional quality.^[29] Green algae, such as *S. obliquus*, are characterized by a deficiency in long-chain polyunsaturated fatty acids and often contain only Δ^7 -sterols, which have been shown to constrain growth and/or development of several arthropod species.^[12,30,31] Here, we evaluate sterol metabolic constraints in *G. roeselii* using a compound-specific stable isotope approach. We hypothesized that the poor food quality of *S. obliquus* for *G. roeselii* is due to a deficiency in suitable dietary sterols and we studied the sterol bioconversion capacities of amphipods reared on ¹³C-labeled and unlabeled algae by measuring sterol-specific stable carbon isotope signatures.

EXPERIMENTAL

Test animals: origin and maintenance

Adult gammarids (*G. roeselii*) were obtained from the littoral zone of the oligotrophic prealpine Lake Constance via kick sampling at the shoreline. To hatch juveniles for the experiments, the gammarids were kept in climate chambers with a diurnal dark light cycle of 12 h : 12 h at 15 °C in aerated aquaria containing lake water, gravel of different grain sizes for shelter, and dried alder leaves as a food source.^[28]

Experimental setup

Feeding experiments were conducted with juvenile gammarids (2–3 mm body length) in glass beakers filled with 100 mL of filtered lake water (<1 µm); a small pebble (organic matter removed using a muffle furnace) was provided as shelter in each beaker. Juvenile *G. roeselii* were randomly transferred into the experimental beakers. Gammarids were fed *ad libitum*^[32] and transferred into new beakers three times a week to avoid accumulation of food, fecal pellets and biofilm formation. Food suspensions were added with a pipette near the bottom of the beaker to expedite the sedimentation of algae and to increase the availability of food particles for the amphipods. All experiments were conducted from August 15th to September 5th in 2011. Each replicate (n = 24 per food treatment) consisted of one individual monitored for 3 weeks.

The green alga *Scenedesmus obliquus* (SAG 276-3a) and the eustigmatophyte *Nannochloropsis limnetica* (SAG 18.99) were cultivated semi-continuously at a dilution rate of 0.2 day⁻¹ in aerated 5 L vessels containing Cyano medium (20 °C, illumination at 120 µmol quanta m⁻² s⁻¹).^[33] These species were chosen because they are known to have different sterol compositions and none of the measured sterols are abundant in both species.^[11] Algae were harvested in the late-exponential growth phase. To be able to trace back the dietary origin of the sterols, *S. obliquus* was grown in Cyano medium containing 30% NaH¹³CO₃ (99% ¹³C, Sigma-Aldrich, St. Louis, MO, USA). Five food suspensions, each with a total carbon concentration of 2 mg C L⁻¹, consisting of different proportions of *N. limnetica* and *S. obliquus* (90:10, 80:20, 50:50, 20:80, and 10:90), were prepared by concentrating (centrifugation at 3000 g, 10 min) and resuspending the cells in fresh medium. The carbon concentrations of the food suspensions were estimated by photometric light extinctions (800 nm) and carbon-extinction equations determined prior to the experiment. The carbon light extinction regressions were subsequently confirmed by carbon analysis of the food suspensions using an elemental analyzer (EuroEA3000, HEKAtech GmbH, Wegberg, Germany).

Sterol analyses

Sterols were extracted from pre-combusted GF/F filters (Whatman, 25 mm diameter; GE Healthcare Life Science, Freiburg, Germany) loaded with approximately 1 mg particulate

Table 1. Sterol composition and total sterol content of the different food mixtures (means of n = 3 ± SD; reported as µg/mg C) consisting of different proportions of the two algae *Nannochloropsis limnetica* (Nanno) and *Scenedesmus obliquus* (Scene)

Variable	Origin	Nannochloropsis:Scenedesmus				
		90:10	80:20	50:50	20:80	10:90
Cholesterol	Nanno	5.7 ± 0.5	5.2 ± 0.4	4.3 ± 0.5	1.2 ± 0.3	0.3 ± 0.2
Sito-/clionasterol	Nanno	2.5 ± 0.6	2.0 ± 0.3	1.3 ± 0.4	0.2 ± 0.1	n.d.
Isofucosterol	Nanno	2.1 ± 0.2	1.9 ± 0.5	1.9 ± 0.2	0.5 ± 0.2	n.d.
Fungisterol	Scene	n.d.	0.8 ± 0.2	1.7 ± 0.3	2.4 ± 0.3	2.4 ± 0.4
Chondrillasterol	Scene	0.2 ± 0.1	1.6 ± 0.4	3.6 ± 0.5	4.7 ± 0.4	5.1 ± 0.7
22-Dihydrochondrillasterol	Scene	n.d.	n.d.	1.0 ± 0.1	1.6 ± 0.2	2.0 ± 0.3
Total sterol content		10.5 ± 0.7	11.5 ± 0.9	13.6 ± 0.8	10.5 ± 0.8	9.7 ± 0.6

n.d. = not detectable

organic matter of the food suspensions or of crushed animals ($n = 3$) using a mixture of dichloromethane/methanol (2:1, v/v). For the analysis, the pooled cell-free extracts were dried under a stream of nitrogen and saponified with 0.2 mol L^{-1} methanolic KOH (70°C , 1 h). Subsequently, the sterols were partitioned into iso-hexane/diethyl ether (9:1, v/v), again dried under a stream of nitrogen, and resuspended in a volume of $20 \mu\text{L}$ iso-hexane. The sterols were analyzed by gas chromatography (GC) using a HP 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a HP-5 capillary column (Agilent Technologies). Details of the GC settings are given elsewhere.^[34,35] The sterols were quantified by comparison with the internal standard 5α -cholestane using multipoint calibration curves generated for different sterols. Sterols that were not commercially available were quantified as cholesterol-equivalents using the cholesterol calibration curve. The limit of quantification was 10 ng of sterol. The sterols were identified by their retention times and their mass spectra, which were recorded with a gas chromatograph/quadrupole mass spectrometer (Agilent Technologies, 5975C inert MSD) equipped with a fused-silica capillary column (HP-5MS, Agilent; GC settings as described for FID). The sterols were analyzed in their free form and as their trimethylsilyl derivatives. Mass spectra were recorded between m/z 50 and 600 in the electron ionization (EI) mode.

Compound-specific stable isotope analyses

The stable carbon isotope signatures ($\delta^{13}\text{C}$ values) of the sterols were measured in the algal food mixtures and in surviving gammarids in the stable isotope laboratory of the Limnological Institute, University of Konstanz (Konstanz, Germany). All the isotope values are presented using the δ notation in relation to the international reference standard Vienna PeeDee belemnite. Prior to $\delta^{13}\text{C}$ measurements, the sterols were extracted as described above ($n = 3$, exceptions are given in the appropriate place). Separation of the sterols was carried out on an Agilent 7890A gas chromatograph; the column and all the configurations used were the same as described above for the sterol analyses. The separated sterols were passed into a combustion interface operated in the GC-combustion (GC-C) mode (interface: 350°C ; furnace 850°C) and measured as CO_2 in an isotope ratio mass spectrometer (Micromass Isoprime Ltd, Stockport, UK). Repeated analyses of an internal standard (5α -cholestane) revealed an accuracy of the sterol analyses of 0.3‰ ($\pm 1 \text{ SD}$) for CSIA.

To verify the enrichment of *S. obliquus* with ^{13}C , bulk stable carbon isotope signatures were measured in each food mixture using a vario PYRO cube elemental analyzer (Elementar, Analysensysteme GmbH, Hanau, Germany) connected to the isotope ratio mass spectrometer ($n = 3$; precision of the internal standard casein $\pm 1 \text{ SD}$ of 0.05‰). Measurements of the internal standards were carried out to determine the accuracy of the devices.

Statistical analyses

Statistical analyses were conducted using the statistical software package R.^[36] Differences in the sterol content and stable carbon isotope signature of the sterols from algal mixtures and *G. roesellii* were analyzed using linear regression

Figure 1. Stable carbon signatures (mean $\delta^{13}\text{C} \pm \text{SD}$ representing the biological variances) of the food mixtures consisting of the two algae *Nannochloropsis limnetica* and *Scenedesmus obliquus* (A, bulk signatures), of sterols extracted from these algal mixtures (B), and of sterols extracted from *Gammarus roesellii* (C) reared on these algal mixtures ($n = 3$, exceptions are given in the figure).

models (lm) with the ratio of the two algae (*Nannochloropsis limnetica*:*Scenedesmus obliquus*) as covariate. Homogeneity of variances was tested using Bartlett's test. Data of sterol content were ln transformed to achieve homogeneity of variances.

RESULTS

The sterol composition and the concentrations of particular sterols in the algal food mixtures (*N. limnetica*:*S. obliquus*) and in the gammarids feeding on these food mixtures reflected the proportions in which the two algae were provided (Table 1). With increasing proportions of *S. obliquus* in the food mixtures, the concentrations of cholesterol (F = 45.0; $p < 0.001$) and isofucosterol (F = 60.4; $p < 0.001$) decreased significantly, whereas the concentration of fungisterol increased (F = 100.3; $p < 0.001$). The bulk $\delta^{13}\text{C}$ values in algal mixtures showed that *S. obliquus* was enriched with ^{13}C ; the $\delta^{13}\text{C}$ values increased gradually from -21.2‰ in the 90:10 mixture to $+12.6\text{‰}$ in the 10:90 mixture (Fig. 1(A)). The $\delta^{13}\text{C}$ values of cholesterol, which is abundant in *N. limnetica* but absent in *S. obliquus* (Table 1), did not differ between algal food mixtures ($\sim -25\text{‰}$; F = 0.02; $p = 0.90$). The $\delta^{13}\text{C}$ values of chondrillasterol ($\sim -7\text{‰}$; F = 1.6; $p = 0.24$) and fungisterol ($\sim -18\text{‰}$; F = 0.09; $p = 0.77$), which are abundant in *S. obliquus*, did not change with changing proportions of the two algae (Fig. 1(B)). Isofucosterol from *N. limnetica* and 22-dihydrochondrillasterol from *S. obliquus* were inseparable after combustion, resulting in $\delta^{13}\text{C}$ values that increased from -16.3‰ in the 90:10 mixture to $+17.3\text{‰}$ in the 10:90 mixture (F = 90.3; $p < 0.001$; Fig. 1(B)).

The concentration of cholesterol in the *G. roeselii* reared on the algal mixtures decreased with increasing proportions of *S. obliquus* (F = 6.7; $p = 0.025$; Fig. 2(A)), and hence with the amount of cholesterol in the food. Furthermore, with increasing proportions of *S. obliquus* in the algal mixtures the concentration of lathosterol increased in *G. roeselii* (F = 41.4; $p < 0.001$; Fig. 2(B)). The $\delta^{13}\text{C}$ values of the cholesterol in the *G. roeselii* ($\sim -25\text{‰}$) did not change with the dietary proportions of the two algae and did not differ from the values of cholesterol measured in the algal mixtures ($p = 0.06$, Fig. 1(C)). Lathosterol, which was detectable in *G. roeselii*

when the proportion of *S. obliquus* in the diet exceeded 50% (Fig. 2(B)), had $\delta^{13}\text{C}$ values of $\sim +35\text{‰}$ (the $\delta^{13}\text{C}$ value was below the detection limit in the 50:50 mixture; Fig. 1(C)), indicating a ^{13}C -enriched source.

DISCUSSION

The results of this study indicate that dietary phytosterols differ in their suitability to serve as a cholesterol precursor in the metabolism of *G. roeselii*. The consistently ^{13}C -depleted cholesterol in *G. roeselii* indicates that cholesterol could not be synthesized from the phytosterols present in *S. obliquus* (Fig. 1(C)). This implies that the cholesterol present in *G. roeselii* derived either from *N. limnetica* maternal investments or from dietary sources gathered prior to the growth experiment. These data suggest that the recently reported characterization of *S. obliquus* as a poor quality diet for *G. roeselii*^[29] is due to an inadequate phytosterol composition. *S. obliquus* primarily contains Δ^7 -sterols^[37] and dietary Δ^7 -sterols have been reported previously to be less suitable for a number of arthropods than Δ^5 -sterols,^[12,30,31,38] suggesting that *G. roeselii* requires a dietary source of Δ^5 -sterols for growth and survival. In addition to cholesterol, a second sterol, the Δ^7 -sterol lathosterol, was detected in *G. roeselii* raised on diets containing high proportions of *S. obliquus* ($\geq 80\%$). The highly ^{13}C -enriched carbon signature of lathosterol indicated that this sterol was synthesized by the amphipod from a compound present in *S. obliquus*. Possible precursors in *S. obliquus* are the Δ^7 -sterols chondrillasterol, 22-dihydrochondrillasterol and fungisterol (Fig. 3); a conversion into lathosterol would require a C24 dealkylation and also, in the case of chondrillasterol, a removal of the C22 double bond. C24-dealkylation and the removal of double bonds in the sterol side chain are common processes in the conversion of dietary phytosterols into cholesterol in herbivorous arthropods.^[1,39] As indicated by the stable isotope signatures, lathosterol could not be used by *G. roeselii* to synthesize cholesterol. The highly enriched carbon signature of lathosterol and the increasing lathosterol concentrations in the animals clearly indicate that this sterol was synthesized from dietary sterols present in *S. obliquus*.

Figure 2. Concentrations (mean $\pm$ SD) of cholesterol and lathosterol ($\mu\text{g mg}^{-1}$ dry weight) in *Gammarus roeselii* ($n = 3$) reared on different algal food mixtures consisting of different proportions of the two algae *Nannochloropsis limnetica* and *Scenedesmus obliquus*.

Figure 3. Potential precursors for lathosterol biosynthesis in *Gammarus roeselii*. The highly ^{13}C -enriched carbon signature of lathosterol indicated that this sterol derived from Δ^7 -sterols present in *Scenedesmus obliquus*, i.e. chondrillasterol, 22-dihydrochondrillasterol and/or fungisterol. The conversion of these dietary precursors into lathosterol requires C-24 dealkylation and, in the case of chondrillasterol, removal of the C22 double bond. The stable isotope signatures revealed that lathosterol could not be further processed to cholesterol ($\Delta^7 \rightarrow \Delta^5$) in *G. roeselii*.

Although we cannot exclude that lathosterol was actively synthesized to satisfy specific physiological demands, we propose that the inability to convert lathosterol into cholesterol simply resulted in the accumulation of this unsuitable and potentially detrimental sterol in *G. roeselii*.

CONCLUSIONS

Our results suggest that the gammarid *G. roeselii* is susceptible to an inadequate dietary sterol supply and that dietary Δ^7 -sterols are unsuitable as dietary cholesterol precursors. Although the ecological significance of such sterol-mediated metabolic constraints remains to be established, our data indicate that compound-specific stable carbon isotope signatures can provide valuable information on the physiological mechanisms associated with nutritional constraints. We conclude that the application of CSIA has great potential to advance food quality research both in terrestrial and in aquatic ecosystems.

Acknowledgements

The authors thank P. Merkel for assistance in the laboratory, S. Stehle for comments on a previous version of the manuscript and K.-O. Rothhaupt for scientific support. RG was funded by the German Research Foundation (DFG, GE2219/3-1) and the research initiative "AufLand" at the University of Koblenz-Landau. DMC was supported by the DFG (MA 5005/1-1) and the "Young Scholar Fund" of the University of Konstanz.

REFERENCES

- [1] D. Martin Creuzburg, E. Von Elert, in *Lipids in Aquatic Ecosystems*, (Eds: M. T. Arts, M. Brett, M. Kainz). Springer, New York, 2009, p. 43.
- [2] S. T. Behmer, W. D. Nes. Insect sterol nutrition and physiology: A global overview. *Adv. Insect Physiol.* 2003, 31, 1.
- [3] D. C. Müller Navarra, M. T. Brett, A. M. Liston, C. R. Goldman. A highly unsaturated fatty acid predicts carbon transfer between primary producers and consumers. *Nature* 2000, 403, 74.
- [4] L. J. Goad. Sterol biosynthesis and metabolism in marine invertebrates. *Pure Appl. Chem.* 1981, 51, 837.
- [5] M. Carvalho, D. Schwudke, J. L. Sampaio, W. Palm, I. Riezman, G. Dey, G. D. Gupta, S. Mayor, H. Riezman, A. Shevchenko, T. V. Kurzchalia, S. Eaton. Survival strategies of a sterol auxotroph. *Development* 2010, 137, 3675.
- [6] M. L. Grieneisen. Recent advances in our knowledge of ecdysteroid biosynthesis in insects and crustaceans. *Insect Biochem. Mol. Biol.* 1994, 24, 115.
- [7] H. Ohvo Rekilä, B. Ramstedt, P. Leppimäki, J. P. Slotte. Cholesterol interactions with phospholipids in membranes. *Prog. Lipid Res.* 2002, 41, 66.
- [8] T. H. Haines. Do sterols reduce proton and sodium leaks through lipid bilayers? *Prog. Lipid Res.* 2001, 40, 299.
- [9] D. Martin Creuzburg, E. Von Elert, K. H. Hoffmann. Nutritional constraints at the cyanobacteria *Daphnia magna* interface: The role of sterols. *Limnol. Oceanogr.* 2008, 53, 456.
- [10] D. Martin Creuzburg, A. Wacker, E. von Elert. Life history consequences of sterol availability in the aquatic keystone species. *Daphnia. Oecologia* 2005, 144, 362.
- [11] T. Basen, K. O. Rothhaupt, D. Martin Creuzburg. Absence of sterols constrains food quality of cyanobacteria for an invasive freshwater bivalve. *Oecologia* 2012, 170, 57.

- [12] D. Martin Creuzburg, S. Oede, A. Wacker. Thresholds for sterol limited growth of *Daphnia magna*: a comparative approach using 10 different sterols. *J. Chem. Ecol.* 2014, 40, 1039.
- [13] D. Martin Creuzburg, E. Von Elert. Impact of 10 dietary sterols on growth and reproduction of *Daphnia galeata*. *J. Chem. Ecol.* 2004, 30, 483.
- [14] M. Brauns, D. von Schiller, R. Gergs, in *Handbuch Angewandte Limnologie. 30. Ergänzungslieferung. Methodische Grundlagen III 3.3*, (Eds: M. Hupfer, W. Calmano, H. Klapper, R. D. Wilken). Wiley VCH, Weinheim, 2012, p. 1.
- [15] B. Fry. *Stable Isotope Ecology*, (2nd ed.). Springer Science +Business Media, New York, 2006.
- [16] M. S. Fantle, A. I. Dittel, S. M. Schwalm, C. E. Epifanio, M. L. Fogel. A food web analysis of the juvenile blue crab, *Callinectes sapidus*, using stable isotopes in whole animals and individual amino acids. *Oecologia* 1999, 120, 416.
- [17] D. M. O'Brien, M. L. Fogel, C. L. Boggs. Renewable and nonrenewable resources: amino acid turnover and allocation to reproduction in Lepidoptera. *Proc. Natl. Acad. Sci. USA* 2002, 99, 4413.
- [18] A. Bec, M. E. Perga, A. Koussoroplis, G. Bardoux, C. Desvillettes, G. Bourdier, A. Mariotti. Assessing the reliability of fatty acid specific stable isotope analysis for trophic studies. *Methods Ecol. Evolut.* 2011, 2, 651.
- [19] P. M. Chamberlain, I. D. Bull, H. I. J. Black, P. Ineson, R. P. Evershed. Lipid content and carbon assimilation in Collembola: implications for the use of compound specific carbon isotope analysis in animal dietary studies. *Oecologia* 2004, 139, 325.
- [20] O. Söderström. Effects of temperature and food quality on life history parameters in *Parameletus chelifer* and *P. minor* (Ephemeroptera): a laboratory study. *Freshwater Biol.* 1988, 20, 295.
- [21] L. G. Willoughby, D. W. Sutcliffe. Experiments on feeding and growth of the amphipod *Gammarus pulex* (L.) related to its distribution in the River Duddon. *Freshwater Biol.* 1976, 6, 577.
- [22] N. H. Anderson, J. R. Sedell. Detritus processing by macroinvertebrates in a stream ecosystem. *Annu. Rev. Entomol.* 1979, 24, 351.
- [23] F. Bärlocher, B. Kendrick. Fungi and food preferences of *Gammarus pseudolimnaeus*. *Archiv für Hydrobiologie* 1973, 72, 501.
- [24] N. Friberg, D. Jacobsen. Feeding plasticity of two detritivore shredders. *Freshwater Biol.* 1994, 32, 133.
- [25] D. W. Kelly, J. T. A. Dick, W. I. Montgomery. Predation on mayfly nymph, *Baetis rhodani*, by native and introduced *Gammarus*: direct effects and the facilitation of predation by salmonids. *Freshwater Biol.* 2002, 47, 1257.
- [26] D. W. Kelly, J. T. A. Dick, W. I. Montgomery. The functional role of *Gammarus* (Crustacea, Amphipoda): shredders, predators, or both? *Hydrobiologia* 2002, 485, 199.
- [27] C. MacNeil, J. T. A. Dick, R. W. Elwood. The trophic ecology of freshwater *Gammarus* spp. (Crustacea: Amphipoda): problems and perspectives concerning the functional feeding group concept. *Biol. Rev.* 1997, 72, 349.
- [28] R. Gergs, N. Steinberger, T. Basen, D. Martin Creuzburg. Dietary supply with essential lipids affects growth and survival of the amphipod *Gammarus roeselii*. *Limnologica* 2014, 46, 109.
- [29] T. Basen, R. Gergs, K. O. Rothhaupt, D. Martin Creuzburg. Phytoplankton food quality effects on gammarids: benthic pelagic coupling mediated by an invasive freshwater clam. *Can. J. Fish. Aquat. Sci.* 2013, 70, 198.
- [30] S. T. Behmer, D. O. Elias, R. J. Grebenok. Phytosterol metabolism and absorption in the generalist grasshopper, *Schistocerca americana* (Orthoptera : Acrididae). *Arch. Insect Biochem. Physiol.* 1999, 42, 13.
- [31] W. C. M. Klein Breteler, N. Schogt, M. Baas, S. Schouten, G. W. Kraay. Trophic upgrading of food quality by protozoans enhancing copepod growth: role of essential lipids. *Mar. Biol.* 1999, 135, 191.
- [32] R. Gergs, K. O. Rothhaupt. Feeding rates, assimilation efficiencies and growth of two amphipod species on biodeposited material from zebra mussels. *Freshwater Biol.* 2008, 53, 2494.
- [33] F. Jüttner, J. Leonhardt, S. Möhren. Environmental factors affecting the formation of mesityloxide, dimethylallylic alcohol and other volatile compounds excreted by *Anabaena cylindrica*. *J. Gen. Microbiol.* 1983, 129, 407.
- [34] D. Martin Creuzburg, E. Sperfeld, A. Wacker. Colimitation of a freshwater herbivore by sterols and polyunsaturated fatty acids. *Proc. Roy. Soc. London, Series B* 2009, 276, 1805.
- [35] D. Martin Creuzburg, A. Wacker, T. Basen. Interactions between limiting nutrients: Consequences for somatic and population growth of *Daphnia magna*. *Limnol. Oceanogr.* 2010, 55, 2597.
- [36] R Development Core Team. *R: a Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, 2011. Available: <http://www.R-project.com>.
- [37] D. Martin Creuzburg, A. Wacker, C. Ziese, M. J. Kainz. Dietary lipid quality affects temperature mediated reaction norms of a freshwater key herbivore. *Oecologia* 2012, 168, 901.
- [38] F. G. Prahl, G. Eglinton, E. D. S. Corner, S. C. M. O'Hara, T. E. V. Forsberg. Changes in plant lipids during passage through the gut of *Calanus*. *J. Mar. Biol. Assn. U. K.* 1984, 64, 317.
- [39] N. Ikekawa, M. Morisaki, Y. Fujimoto. Sterolmetabolism in insects: Dealkylation of phytosterol to cholesterol. *Acc. Chem. Res.* 1993, 26, 139.