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Energy reserves in the water louse *Asellus aquaticus* (Isopoda, Crustacea) from surface and cave populations: seasonal and spatial dynamics

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With 4 figures and 1 table

Abstract: The depletion of energy reserves in animals has already been recognized as a useful indicator of environmental pollution with potentially high ecological significance. However, apart from being affected by pollution, energy reserves in organisms also vary depending on natural abiotic and biotic factors. Seasonal and spatial dynamics of energy reserves in *Asellus aquaticus* (Isopoda, Crustacea) are presented here to determine the reference values of energy biomarkers. *A. aquaticus* are abundant and ubiquitous freshwater crustaceans and, as such, very suitable organisms for biomonitoring. Individuals were collected in spring, summer and autumn at two surface localities and one cave locality along the sinking river Pivka (Slovenia), and analysed for lipid, carbohydrate and protein content. The obtained values of energy reserves are discussed together with the physical and chemical parameters of water determined at the time of sampling. The surface-water populations in particular show a seasonal and spatial variability of energy reserves. Carbohydrates follow a distinct seasonal pattern of decreasing values toward autumn in all three populations. The spring protein content in both surface populations is much lower compared with the summer and autumn levels, while no distinct pattern was observed for lipids. The quantity of energy reserves in cave specimens does not differ much from the surface ones, but are less variable. Our data reveal that energy biomarkers differ between both season and locality, which should be taken into consideration in biomonitoring studies. We also suggest the use of only male specimens of comparable size where possible. To enable a valid comparison between different studies, the methods for the analysis of energy reserves should be harmonised.

Keywords: water lice; lipids; carbohydrates; proteins; water quality; biomonitoring

Introduction

The amount of energy reserves in animals has been recognized as a useful indicator of the stress caused by environmental pollution. Schill & Köhler (2004a) found a large reduction in lipids and glycogen in *Oniscus asellus* from a metal-polluted environment. *In situ* bioassay with mussels *Dreissena polymorpha* and *Perna viridis* showed a reduction in glycogen, lipid and protein content in relation to environmental pollution (Smolders et al. 2004a; Smolders et al. 2004b; Yeung et al. 2017). Similarly, a correlation between energy reserves (glycogen and lipids) and the pollution of estuaries was found for *Nereis diversicolor* (Durou et al. 2005). In a polluted environment, organisms presumably spend considerable energy to avoid contaminants, to excrete or detoxify the contaminant, and to repair potential pathological effects instead of investing energy into growth and reproduction (Calow & Sibly 1990; Forbes & Calow 1996). Contaminants

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may also have a strong indirect effect on aquatic organisms by, e.g., reducing the quality of food. Feckler et al. (2016) showed the fungicide contamination of water to lower the microbial colonisation of leaf material, which reduced the growth and lipid content in *Asellus aquaticus*. Some older studies (Holdich & Tolba 1981; Tolba & Holdich 1981) clearly indicated that specimens of *A. aquaticus* from polluted sites are in general smaller compared with specimens from clean sites, while the females produce less eggs, and the rate of development and degree of survival of eggs is lower. These studies also showed that a significantly lower body size, wet weight and reproduction output of *A. aquaticus* from polluted water are undoubtedly related to the increased energy expenditure resulting from pollution, and not from any genetic differences between populations. Therefore, the excessive depletion of energy reserves may also affect the next generations and, thus, can be reflected at a population or even ecosystem level after some time (Depledge & Fossi 1994). The consequences of increased energy expenditure may be most severe in ecosystems where the amount of nutrients is limited, such as in caves (Culver & Pipan 2009).

A. aquaticus is a common and abundant freshwater crustacean inhabiting all types of fresh to slightly brackish waters in most of Europe (Henry & Magniez 1995), and is one of only a few species of the genus also inhabiting subterranean habitats in large populations (Sket 1994). The species is capable of withstanding high fluctuations in temperature and oxygen (Holland 1976; Dehedin et al. 2013), as well as a moderately increased organic load (Liebmann 1962; Pârvulescu 2009), and can be found during all four seasons. As its racial differentiation (Sket 1994; Prevorčnik et al. 2004; Verovnik et al. 2004) and, in the case of surface populations, life history are well known (Økland 1978; Murphy & Learner 1982; Štrus & Blejec 1983; Iversen & Thorup 1988), *A. aquaticus* is a well suited organism for the biomonitoring of surface and cave freshwaters.

However, in the wild, organisms simultaneously respond not only to a variety of contaminants, but also to natural stressors, which cause the temporal and spatial variability of energy biomarkers (Handy et al. 2003). For example, the energy reserves in crustaceans may be influenced by the amount and source of food (Charron et al. 2014), season (Dutra et al. 2007; Sroda & Cossu-Leguille 2011; Gismondi et al. 2012), temperature (Issartel et al. 2005; Sroda & Cossu-Leguille 2011; Gismondi et al. 2012), amount of oxygen (Hervant et al. 1996; Hervant et al. 1999), gender (Sroda & Cossu-Leguille 2011; Gismondi et al. 2012), moulting (Charron et al. 2014), mating (Plaistow et al. 2003; Sparkes et al. 1996; Jormalainen et al. 2001) and source population (Hervant et al. 1999; Donker 1992; Schill & Köhler 2004b). Therefore, to determine the relevant responses of organisms to pollution, more attention should be paid to those confounding factors at a study site with the potential to influence energy reserves.

Therefore, our aim was to determine the range of energy reserves in three closely related populations of *A. aquaticus* (Verovnik et al. 2004) over three seasons in their natural environment, with the collected values of energy reserves presented here together with the physical and chemical parameters of water measured at the time of sampling. These results could serve as reference values of energy biomarkers for further biomonitoring studies. To our knowledge, we provide information on the energy reserves of a subterranean population of *A. aquaticus* for the first time. However, different protocols for the measurement of energy reserves have been described in the literature. While suitable for within-study comparisons, the comparison of data from different studies is difficult and uncertain, since the output between methods can differ, as shown in Berges et al. (1993) and Barnes & Blackstock (1973). Hence, the available protocols have been analysed, with that used here giving the optimal compromise between robustness and efficiency.

Material and methods

Chemicals

We used dibasic and monobasic potassium phosphate, triton X-100, glycerol tripalmitate, glucose, bovine serum albumin, and trichloroacetic acid from Sigma (Germany); chloroform, methanol, sulphuric acid, and glucose from Merck (Germany); BCA Protein Assay Reagents A and B from Pierce (U.S.A.). All chemicals have grades typically 99 % or higher, and, hence, are the highest grades commercially available.

Animal sampling procedure

The water lice were sampled in spring (18 March 2015), summer (17 July 2015) and autumn (12 October 2015) at three sites along the sinking river Pivka (Slovenia, Europe): (i) the cave population in the subterranean section of the Pivka River in the cave Planinska jama (hereafter referred to as locality Planina Cave), (ii) upstream of the Planina Cave just prior to the Pivka River sink in Postojna on Pivka Polje (hereafter referred to as locality Pivka Polje), (iii) downstream of the Planina Cave after the Unica River (i.e. the name of the Pivka River after its confluence with another sinking river) resurgence on Planina Polje near Jakovica (hereafter referred to as locality Planina

Polje). These localities are the same as previously reported (Jemec et al. 2017). Both surface localities are approximately 5 km of straight-line distance from the Planina Cave, which is characterized by total darkness. The river bank is composed of large boulders or bedrock, while the river bed is mainly rocky with scattered sections of shallow sediments. The Pivka polje locality is situated within the park surrounding the entrance to the Postojna Cave. Willows (*Salix* sp.) and maples (*Acer* sp.) grow along the mostly regulated river. The river bed substrate consists of sand and pebbles. At the Planina polje locality, the river is not regulated, and its river bed is densely overgrown with helo- and hydrophytes. Willows and alders (*Alnus* sp.) grow along the river banks. All localities are characterised by strong variability of flow dynamics (i.e. water table fluctuation, differences in flow velocities, flow types, etc.) in response to different hydrologic conditions within a short time period. In the sinking river Pivka, catchment-area land is mainly used for agriculture and forestry. In the wider surroundings of the river, there are several smaller villages and a small town Postojna with less than 10,000 inhabitants.

Both surface populations were sampled with a water-net (mesh size 0.5 mm), while individuals in the Planina Cave were collected using a perforated spoon. All individuals were first rinsed with bottled water with a neutral pH, gently dried with a paper towel, and individually frozen on-site in dry ice, remaining at −30 °C until the completion of the analyses one month after sampling. Individuals of sizes above 0.5 mm and of both genders were collected, with the exception of ovigerous females. This was taken into consideration in the data analysis.

Quantification of energy reserves

Specimens were thawed but kept on ice throughout the procedure, prior to which the fresh weight was measured with a Sartorius MC 210 P analytical scale. The gender of summer and autumn animals was determined by inspecting the shape of the first pleopods (Verovnik et al. 2009). As the specimens were of insufficient size to provide the data of all three energy parameters, lipids and carbohydrates were recorded individually in 15 specimens per sampling locality, while the other 15 were used for the protein measurements (altogether 30 specimens per season and locality).

For proteins: specimens were homogenized in 800 µL of 50 mM potassium–phosphate buffer (pH 7.0) with 0.5 % v/v of triton X-100. Homogenisation was performed using ULTRA-TURRAX IKA T10 basic for 40 s on stage 5. The homogenate was centrifuged at 16,000 g at 4 °C for 15 min. The protein content analysis was conducted with the commercially available BCA Protein Assay method (Pierce). Bovine serum albumin (BSA, Sigma) were used as the standard. Measurements were conducted in three parallels. The protein content was measured spectrophotometrically on microplates at 562 nm (BioTek CYTATION 3, USA).

For lipids and carbohydrates: animals were homogenized in 800 µL of deionized water (dH_2O). Homogenisation was performed using ULTRA-TURRAX IKA T10 basic for 40 s on stage 5. The lipid extraction was conducted according to Bligh & Dyer (1959), and the measurements as described in Marsh & Weinstein (1966), and adapted for microplates as in Ferreira et al. (2010). According to Marsh & Weinstein (1966), the charring method is a nonspecific method for determining the total lipid content in a sample. The lipids were extracted by transferring 300 µL of homogenate, and adding 500 µL of chloroform and methanol, and vortexed for 1.5 min; 250 µL of dH_2O was then added and vortexed for another 1.5 min. The mixture was centrifuged for 10 min at 1000 g. The chloroform phase (100 µL) was transferred to a tube, with 500 µL of sulphuric acid added. The sample was then incubated at 200 °C for 15 min. After cooling down, 500 µL of dH_2O was added after which the mixture was vortexed, with 200 µL of each sample applied on a microplate. The same procedure as for the sample in chloroform phase (processing with sulphuric acid and incubation at 200 °C) was done for the standard glycerol tripalmitate dissolved in chloroform.The lipid content was measured spectrophotometrically at 375 nm (BioTek CYTATION 3, USA).

The carbohydrate content was measured according to the method described in Albalasmeh et al. (2013), which is based on Dubois et al. (1956), where simple and complex carbohydrates are hydrolysed and dehydrated by sulphuric acid into furfural derivatives, which are then measured by comparing the absorbance of a standard solution treated in the same way. Since the reaction is dependent on the quickness of the addition of sulphuric acid, three parallels were used, with glucose used as the standard. Added to the 300 µL of homogenate were 100 µL of 15 % trichloroacetic acid, which was vortexed and incubated at −30 °C for 15 min. The mixture was centrifuged at 1000 g for 10 min, with the resulting supernatant diluted 2-fold, and 150 µL transferred to a glass tube; 450 µL of sulphuric acid was quickly added to the middle of the sample. The mixture was vortexed and cooled before transferring 200 µL of each sample to a microplate. The carbohydrate content was measured spectrophotometrically at 315 nm (BioTek CYTATION 3, USA).

Analysis of water

During each sampling, the *in situ* water temperature, dissolved oxygen, oxygen saturation, conductivity, and pH were measured by the portable multimeter CyberScan 600 (Eutech Instruments). Additionally, 3 L of water was taken from each locality for chemical analyses; water samples were transported to the laboratory on ice in clean plastic bottles and analysed for inorganic nitrogen (sum of ammonium N-NH_4^+, nitrate N-NO_3^- and nitrite N-NO_2^- nitrogen), orthophosphates (P-PO_4^{3-}), chlorides (Cl^-) and total organic carbon (TOC, TOC 5000A, Shimadzu). Only pre-cleaned and acid-washed glassware was used. All the reagents were of analytical grade, and only deionized water was used for the preparation of reagents. Each analysis was performed in duplicate or triplicate, with the standard deviations always within the range given by standard procedures. The quality of analytical procedures was confirmed by incorporating calibration standards and by using blank samples. All measurements were performed according to the protocols described in APHA (2012).

Data analyses

All recorded energy reserve values were normalised by the fresh weight of animals. Data were analysed using R 3.1.2 (R Core Development Team 2015) and R Studio (RStudio Team 2015).

Nonparametric tests were applied because the assumptions of parametric statistical tests were not met. The Kruskal–Wallis test and *post hoc* analyses with Dunn's Multiple Comparison Test using rank sums (Dunn 1964) were performed for compar-

Table 1. Physical and chemical parameters of water at the sampling localities.

Locality	Season	T (°C)	Cond.† (µS cm^{-1})	O_2 (mg L^{-1})	O_2 (%)	pH	Inorg. N‡ (mg L^{-1})	N-NO_3^- (mg L^{-1})	N-NO_2^- (mg L^{-1})	N-NH_4^+ (mg L^{-1})	P-PO_4^{3-} (mg L^{-1})	Cl^- (mg L^{-1})	TOC§ (mg L^{-1})
Planina Cave	Spring	7.1	317.6	12.0	90.0	7.8	2.29	2.07	0.02	0.20	0.04	31.3	41.4
	Summer	9.2	250.7	7.7	68.3	8.0	2.91	2.82	0.01	0.08	0.01	10.3	20.9
	Autumn	10.9	251.7	10.0	91.3	7.7	8.69	8.69	/	/	0.07	25.3	2.1
Pivka Polje	Spring	7.3	361.7	10.2	87.0	7.7	2.53	1.91	0.05	0.57	0.08	42.6	34.1
	Summer	28.0	286.4	10.5	137.0	8.5	1.32	0.77	0.53	0.02	0.10	63.8	5.0
	Autumn	11.5	233.5	8.7	80.5	7.7	2.91	2.88	0.03	/	0.05	33.0	1.5
Planina Polje	Spring	7.8	230.2	6.4	54.5	7.3	1.28	0.90	0.04	0.34	0.09	10.1	75.0
	Summer	28.5	174.3	9.0	115.0	8.0	0.09	/	0.01	0.08	0.02	8.0	3.5
	Autumn	12.2	223.7	7.8	73.0	7.4	0.01	/	0.01	/	0.01	9.3	2.3

† Conductivity; ‡ Inorganic nitrogen; § Total organic carbon; / – below the limit of detection.

ing the groups. Gender differences were tested with the Mann-Whitney test. Tukey box-and-whiskers plots were generated using the ggplot2 package (Wickham 2009), with whiskers representing the lowest and highest data still within 1.5 inter-quartile ranges (IQR) of the 1st and 3rd quartile, respectively; the IQR was also used for comparing variability.

Results

Physical and chemical properties of water

Seasonal variation: A high seasonal variation was recorded in the total organic carbon (TOC), inorganic nitrogen and orthophosphate amounts at all three localities (Table 1). While the TOC concentration is the highest in spring, decreasing closer to autumn, other parameters do not show a specific seasonal trend. The seasonal water temperature is evident only on the surface, remaining relatively constant (7–11 °C) in the cave. A moderate seasonality (summer deviation) was also evident in O_2 concentration, which differs between the cave and surface waters. Hardly any seasonal variation was recorded in the pH, chloride concentration and water conductivity.

Spatial variation: The water temperatures in spring and autumn are similar at all three localities, but a factor of three higher at the surface sites in summer. The spatial variation was also observed in the case of phosphate, chloride and inorganic nitrogen concentrations and, to some extent, also in O_2 concentration and conductivity. Apart from phosphates in spring, all mentioned parameters are lower at the most downstream locality, i.e., Planina Polje.

Wet weight of animals

Seasonal variation: Animals are heaviest in spring at all localities (Fig. 1). The weight of summer and autumn animals is similar in the Planina Cave and in Pivka Polje, while at Planina Polje, animals are significantly heavier in autumn (Dunn test, $p < 0.05$).

Spatial variation: Spring animals are heaviest at Planina Polje (Dunn test, $p < 0.05$), but much heavier in the cave compared with both surface localities in summer and autumn.

Gender variation: Males appear to be slightly heavier than females, but the difference is not statistically significant at any locality.

Lipids

Seasonal variation: While no significant seasonal differences in lipid content were recorded in animals from either the Planina Cave or Pivka Polje (Fig. 2), the lipid content of animals at Planina Polje in the summer

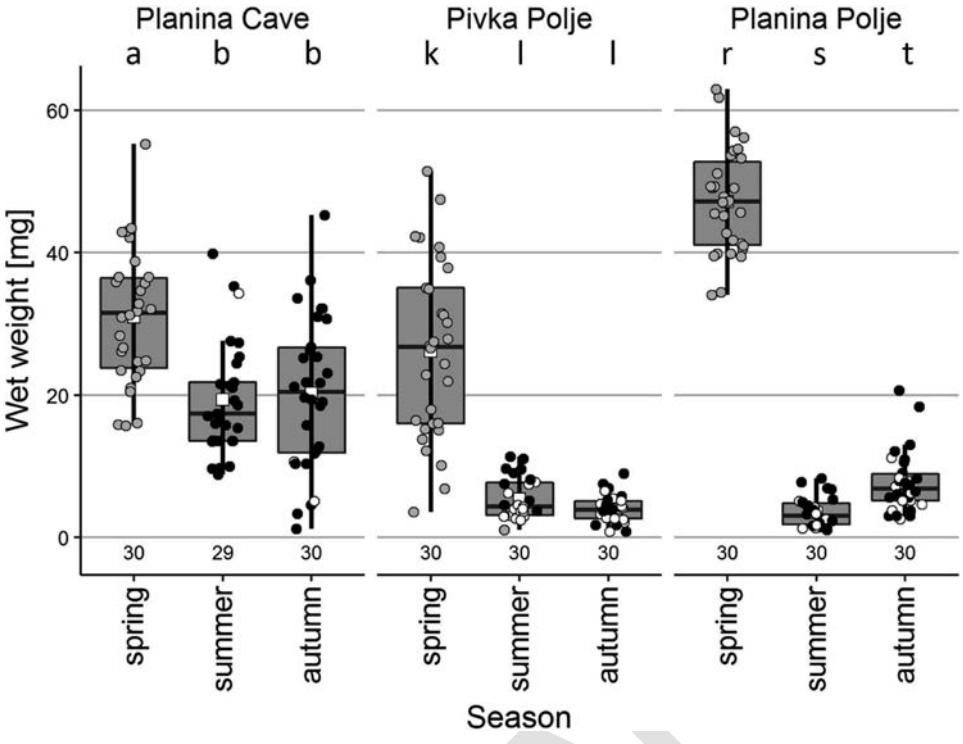

Fig. 1. Wet weight of *Asellus aquaticus* used for the determination of energy reserves. Animals were sampled in three seasons at three localities. Box-plots are shown together with individual values: black circle – male, white circle – female, grey circle – unknown sex, white square – mean value, black horizontal line – median value; numbers below box-plots – number of analysed animals; letters above box-plots indicate significant differences (Dunn test, $p < 0.05$) within each locality.

exceeded the quantities of the other two seasons significantly (Dunn test, $p < 0.05$). The amount of lipids in *A. aquaticus* generally ranged from 4.16–44.39, 2.35–49.18 and 2.59–39.35 µg mg^{-1} wet weight in spring, summer and autumn, respectively. At Pivka Polje, some extreme values were recorded during summer (82.86 µg mg^{-1}, male) and autumn (77.37 µg mg^{-1}, male; 107.6 µg mg^{-1}, female; 137.4 µg mg^{-1}, female), which exceed the median values by factors of 5–8, and are thus excluded from both Figure 2 and further analysis.

Spatial variation: Spring and autumn animals from Pivka Polje contain much more lipids (Dunn test, $p < 0.05$) than animals sampled at the same seasons from the other two localities. In summer, animals from the Planina Polje lipid values exceed (Dunn test, $p < 0.05$) those from the other localities. The lipids of cave animals appear to be much more consistent ($IQR_{Planina\ Cave}$: 3.6–5.8) compared with at the surface localities ($IQR_{Pivka\ Polje}$: 7.1–13.6; $IQR_{Planina\ Polje}$: 1.6–14.1)

Gender variation: Females appear to contain more lipids than males, but the difference is not statistically significant at any locality.

Carbohydrates

Seasonal variation: The amount of carbohydrates was generally the highest in spring, decreasing slightly towards autumn at all three localities (Fig. 3). Nevertheless, only in animals from Planina Cave and Planina Polje are the autumn quantities significantly lower (Dunn test, $p < 0.05$) than the spring and summer values. Carbohydrates in animals in spring, summer and autumn range from (all per wet weight) 4.91–34.34 µg mg^{-1}, 3.89–38.44 µg mg^{-1} and 2.30–25.22 µg mg^{-1}, respectively.

Spatial variation: In spring animals, we observed no differences in the amount of carbohydrates between localities, while summer and autumn animals from Pivka Polje contain significantly more carbohydrates (Dunn test, $p < 0.05$) compared with the cave animals, which also show the lowest variability of data in summer and autumn (IQR < 5) compared with the rest of the samples (IQR = 5–10).

Gender variation: No significant differences between males and females were recorded.

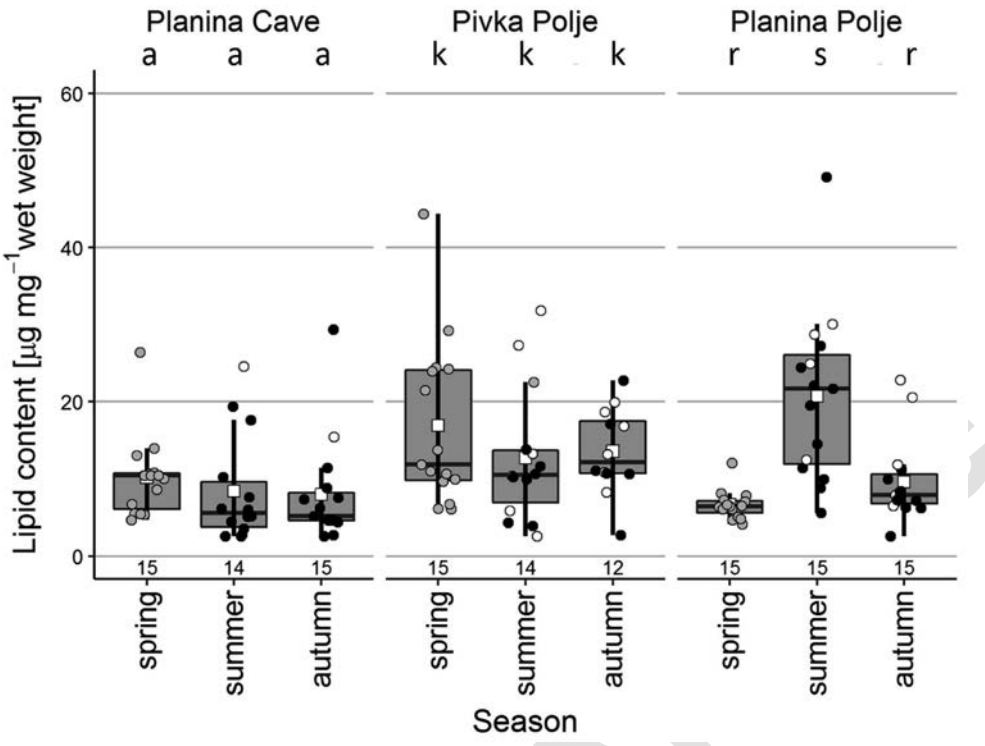

Fig. 2. Lipid content in *Asellus aquaticus* sampled in three seasons at three localities. Box-plots are shown together with individual values: black circle – male, white circle – female, grey circle – unknown sex, white square – mean value, black horizontal line – median value; numbers below box-plots – number of analysed animals; letters above box-plots indicate significant differences (Dunn test, $p < 0.05$) within each locality.

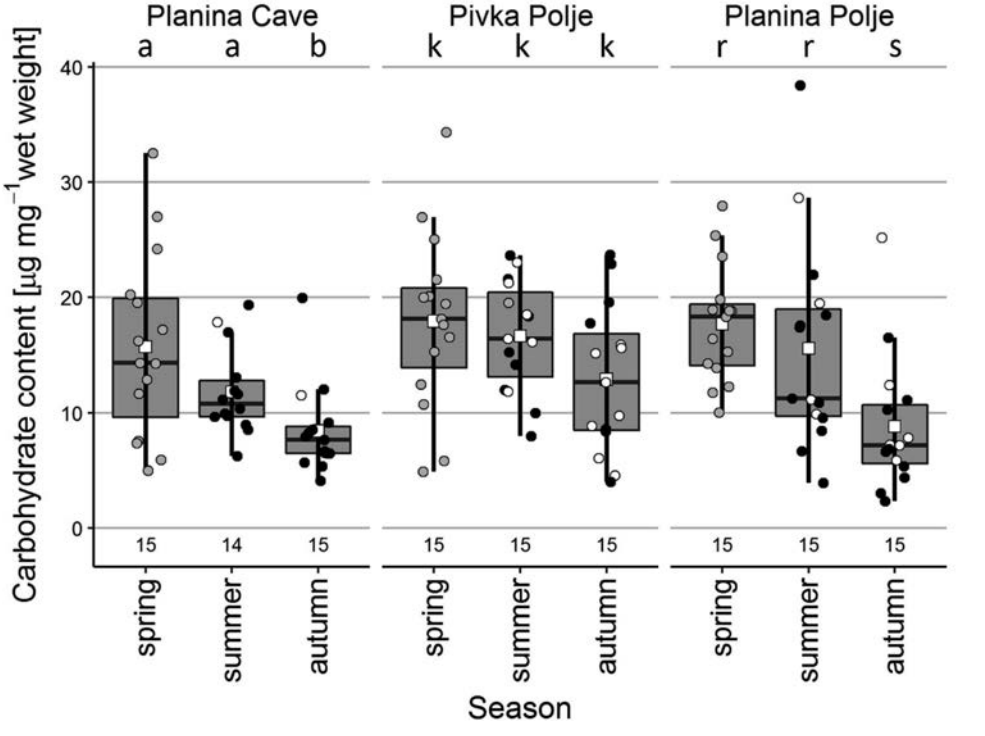

Fig. 3. Carbohydrate content in *Asellus aquaticus* sampled in three seasons at three localities. Box-plots are shown together with individual values: black circle – male, white circle – female, grey circle – unknown sex, white square – mean value, black horizontal line – median value; numbers below box-plots – number of analysed animals; letters above box-plots indicate significant differences (Dunn test, $p < 0.05$) within each locality.

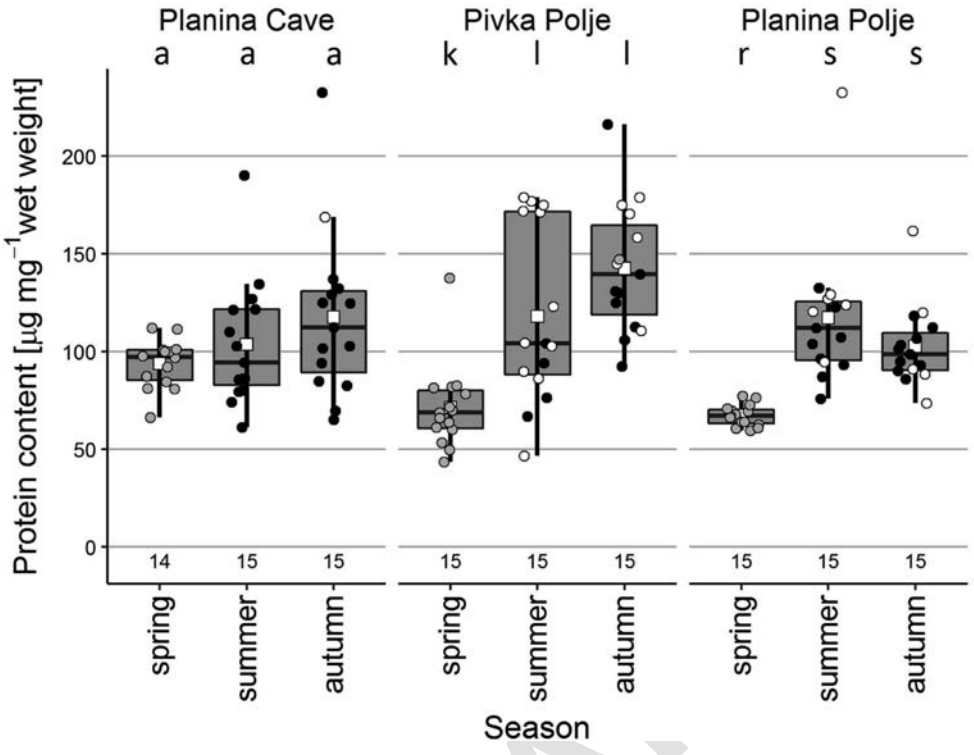

Fig. 4. Protein content in *Asellus aquaticus* sampled in three seasons at three localities. Box-plots are shown together with individual values: black circle – male, white circle – female, grey circle – unknown sex, white square – mean value, black horizontal line – median value; numbers below box-plots – number of analysed animals; letters above box-plots indicate significant differences (Dunn test, $p < 0.05$) within each locality.

Proteins

Seasonal variation: In Planina Cave animals, no significant seasonal differences in protein content were recorded (Fig. 4), although there is a noticeable trend of increasing protein towards autumn. In contrast, the amount of proteins in spring animals from both surface localities is significantly lower (Dunn test, $p < 0.05$) compared with summer and autumn animals. The amount of proteins in animals in spring, summer and autumn range from (all per wet weight) 43.55–137.7 µg mg^{-1}, 46.54–232.5 µg mg^{-1} and 64.97–232.6 µg mg^{-1}, respectively. The spring samples show a lower variability (IQR_{spring}: 7–19.3) compared with samples from the other two seasons (IQR_{summer}: 30–83.5; IQR_{autumn}: 19–45.6).

Spatial variation: Planina Cave spring animals contain more proteins (Dunn test, $p < 0.05$) compared with the surface localities, while the protein content of animals from Pivka Polje exceeds (Dunn test, $p < 0.05$) the values from the other two localities in autumn.

Gender variation: Females appear to contain more proteins than males, but the difference is not statistically significant at any locality.

Discussion

The seasonal and spatial dynamics of lipids, carbohydrates and proteins have been investigated in closely related *A. aquaticus* populations sampled at one cave and two surface localities. The carbohydrate quantity follows a distinct seasonal pattern, decreasing from spring towards autumn in all three populations. In the case of lipids and proteins, no distinct common pattern was observed. In both surface populations, the summer and autumn protein content is significantly higher than the spring level, while the increase in lipid amount is restricted to the summer months in animals from Planina Polje.

When comparing the three energy reserves of *A. aquaticus* from three different localities, some differences are also found. However, the energy reserves of cave animals, especially with regard to lipids, appears to be less variable compared with animals at surface localities.

Energy reserves in relation to abiotic and biotic factors

Energy reserves can be influenced by seasonal changes in factors connected to the environment or the stud-

ied species itself. The temperature, amount of oxygen and food availability are most commonly discussed as abiotic factors determining the energy reserves in freshwater crustaceans (Hervant et al. 1996; Issartel et al. 2005; Dutra et al. 2007; Sroda & Cossu-Leguille 2011; Gismondi et al. 2012). For the amphipod *Gammarus roeseli*, Sroda & Cossu-Leguille (2011) reported that energy reserves negatively correlate with temperature. Generally, the energy reserves in animals are reported to be the highest in autumn and winter, and the lowest in summer (Sroda & Cossu-Leguille 2011; Gismondi et al. 2012). In contrast, we do not find any of the measured energy reserves to be significantly lower in summer in comparison with spring *A. aquaticus*. Furthermore, the lipid content in Planina Polje is actually significantly higher in summer than in the other seasons. Similarly, the protein content of the animals in Pivka Polje and Planina Polje is significantly higher in summer, and does not decrease in autumn. The carbohydrate content trends downwards across the sampling period, regardless of temperature. As *A. aquaticus* is known to be resistant to thermal stress (Korhonen & Lagerspetz 1996; Dehedin et al. 2013), higher temperatures in summer may not influence the energy reserves in the same way as described in amphipods.

According to the measured O_2 concentration, none of the localities can be described as hypoxic or anoxic. Abundant macrophyte beds and algae in well-lit surface river stretches result in high summer oxygen saturation at midday. In such cases, morning hypoxia or even anoxia is not unusual (Allan & Castillo 2007; Sroda & Cossu-Leguille 2011), whereby, in such conditions, *A. aquaticus* spends considerable energy for ventilation, which reduces its energy reserves (Hervant et al. 1997). If periods of hypoxia also occurred at the sampled localities, such periods were probably too short to considerably influence the energy reserves in the *A. aquaticus* sampled in the summer, so that the amounts of lipids, carbohydrates and proteins in summer animals were the same or higher compared with spring animals.

Among the measured water parameters, only the TOC amount shows a seasonal pattern comparable with that of carbohydrates at all three localities. High TOC values in spring are not unusual, since, during frequent rainfall, large quantities of organic particles are washed from land into the streams where they are degraded (Niemirycz et al. 2006). Both dissolved and particulate organic matter and associated microbes are important food sources for surface and cave aquatic primary consumers (Simon et al. 2003).

The seasonal variation in energy reserves could also be to the result of physiological differences among sampled generations. In *A. aquaticus* from the temperate zone, large overwintering females (Murphy & Learner 1982) are known to breed in March to April, release the offspring after about a month (Štrus & Blejec 1983) and die (Økland 1978; Murphy & Learner 1982). Therefore, their offspring must mature fast to produce their own offspring in late July to October, with the latter representing the next overwintering generation (Økland 1978; Štrus & Blejec 1983). We believe that our summer and autumn surface samples represent such fast growing individuals, as they are much lighter than the spring ones. Their significantly higher levels of proteins suggest an increased synthesis due to fast growth and maturation, but further studies are needed to confirm this.

Energy reserves may also depend on the gender, with several authors reporting gammarid females to contain higher amounts of lipids, carbohydrates and proteins compared with males (Plaistow et al. 2003; Sroda & Cossu-Leguille 2011; Gismondi et al. 2012). On the one hand, breeding females have increased lipid content due to yolk formation during vitellogenesis (Meusy 1980; Rosa & Nunes 2002; Sroda & Cossu-Leguille 2011). On the other hand, gammarid and isopod males may lose their energy reserves by searching for receptive mates and by guarding the female prior to copulation (Sparkes et al. 1996; Plaistow et al. 2003; Koop et al. 2008). Thus, energy reserves may differ between males and females, as noted but not statistically proven here. In addition, spatial and temporal differences in breeding intensity, together with the overlapping of generations (Økland 1978), may contribute to the large variability of energy reserves, predominantly with regard to lipids and proteins, as observed here.

When comparing the three energy reserves of *A. aquaticus* from three different localities, no large differences are found in general. We expected differences in particular between the cave and surface populations, since the cave environment is characterised by permanent darkness, a relatively stable climate, and a high dependence on allochthonous surface energy inputs (Culver & Pipan 2009; Culver & Pipan 2014). Cave species are usually adapted to endure starvation over prolonged periods of time (Hervant et al. 1996; Langecker 2000), exhibiting an enhanced capacity of energy storage, lower metabolic rates and lower locomotor activity (Hervant et al. 1996; Hervant et al. 1999; Simčič et al. 2005; Wilhelm et al. 2006; Simčič & Brancelj 2007). However, the spring amount of

TOC in the Planina Cave and its upstream surface locality are similar, with the highest summer amount of TOC recorded in the Planina Cave. Contrary to expectations, food may not be a major limiting factor for the cave water lice in the Planina Cave. Similar trophic conditions in the cave and surface streams may explain the absence of differences in the quantities of energy reserves between both *A. aquaticus* ecomorphs. However, cave water lice have lower growth rates, less frequent moults and mating periods, with most probably not more than one offspring per year and a longer life span (Lattinger-Penko 1979). It is most likely that, in our case, the same generation and predominantly males were sampled in the cave over three seasons. That, together with the stable climate conditions, most probably resulted in the much lower variability of energy reserves compared with surface waters.

On the methodology of energy reserve measurement

For lipids, the most widely used detection method seems to be the sulpho-phospho-vanillin reaction as described by Zöllner & Kirsch (1962) whose variants have been used in several studies (e.g. Sparkes et al. 1996; Ribeiro et al. 2001; Plaistow et al. 2003; Engenheiro et al. 2005; Stanek et al. 2006; Dutra et al. 2007; Sroda & Cossu-Leguille 2011; Gismondi et al. 2012). Although relatively simple, fast and reproducible (Zöllner & Kirsch 1962; Barnes & Blackstock 1973; Van Handel 1985), its accuracy depends on the calibration standard used, and is less suitable for measuring completely saturated lipids (Knight et al. 1972). Thus, a good knowledge of the lipid composition has to be established before choosing the correct calibration standard. Another method for the total lipid quantification is the "simple charring method" (Marsh & Weinstein 1966), which is even simpler that the sulpho-phospho-vanillin method, as it uses only one reagent (sulphuric acid) for visualising the lipids, before measuring in the ultraviolet spectrum. While the latter method is very sensitive and has a high reproducibility, it is nonspecific if different lipids are not separated beforehand (Kritchevsky et al. 1973). Moreover, the method is used in studies concerning lipidomics and energy reserve biomarkers (e.g. Blough & Merlie 1970; Ferreira et al. 2010; Arambourou & Stoks 2015; Helmholz et al. 2016). The measurement of triglycerides alone can be performed by their saponification (Van Handel 1961; Noble & Campbell 1970) or alkoxide transesterification (Soloni 1971) and subsequent quantification of glycerol. Quantification methods of specific lipid groups also employ enzymatic assays (Salè et al. 1984; Carr et al. 1993; Winkelmann & Koop 2007).

The two most widely used methods for the total carbohydrate estimation in the literature are the anthrone (Dreywood 1946; Van Handel 1985) and phenol methods (Dubois et al. 1956), which rely on using concentrated sulphuric acid to turn carbohydrates in an aqueous solution into furfural derivatives, which then react with either anthrone or phenol to develop colour. While the anthrone reaction has been used in several studies (e.g. Trevelyan & Harrison 1956; Sparkes et al. 1996; Plaistow et al. 2003; Gismondi et al. 2012), anthrone is unstable in the presence of sulphuric acid, and the mixed reagent cannot be stored (Giese 1967). The phenol method has also been used in numerous studies (Ribeiro et al. 2001; Stanek et al. 2006; Ferreira et al. 2010; Helmholz et al. 2016), which Masuko et al. (2005) report is easier to use in microplate format that the anthrone method by Laurentin & Edwards (2003). However, we find that, due to the dependence of this method on the rapid addition of sulphuric acid to the sample, it is important to be mindful of the uncertainty with which the human factor introduces to the measurements, since sulphuric acid added to the sample slowly or on the tube wall instead of the centre does not produce the same results. An even simpler method is described by Albalasmeh et al. (2013), which omits phenol or anthrone altogether by directly measuring the ultraviolet absorbance of furfural derivatives produced from simple and complex carbohydrates by the quick addition of concentrated sulphuric acid to an aqueous sample. They report this method to be simpler, faster and more accurate than the phenol method, and without the health hazards involved with using phenol.

The three most common colorimetric methods for estimation of the protein content are those described by Lowry et al. (1951), Bradford (1976) and Smith et al. (1985). The Bradford (1976) method relies on the binding of the dye Coomassie Brilliant Blue G-250 to protein, which shifts the absorption maximum of the dye. While rapid, sensitive and reproducible, detergents can interfere with the method (Chang 2010). The Lowry et al. (1951) and the Smith et al. (1985) methods both take advantage of the "biuret" reaction, where Cu^{2+} ions form coloured complexes with peptide bonds, and then enhance the colour response with adding reagents. While Lowry et al. (1951) describe the use of Folin phenol reagent, Smith et al. (1985) describe a procedure using bichinonic acid (BCA). While the sensitivity of the two is comparable, the BCA method has several advantages, including a more

linear response, greater simplicity, and a more stable reagent. Moreover, the method is not contaminated by detergents, buffer salts and denaturating reagents, but is more susceptible to contamination by reducing sugars (Chang 2010). All three methods are available in commercially sold kits.

Conclusions

To our knowledge, our data is the first regarding the seasonal and spatial variation of energy reserves in cave and surface-dwelling *A. aquaticus*, with surface populations in particular showing a seasonal and spatial variability of energy reserves. Carbohydrates follow the distinct seasonal pattern of decreasing towards autumn in all three sampled populations. The spring protein content in both surface populations is much lower compared with the summer and autumn levels, while no distinct pattern is observed for lipids. While the quantity of energy reserves in cave animals does not significantly differ from surface animals, they are less variable.

Our data reveal energy reserves to differ between both season and locality, which should be taken into consideration in biomonitoring studies. We also suggest the use of only male specimens of comparable size where possible because the breeding cycle of females and the potential overlapping of generations can reflect a higher variability of energy reserves. Although the exclusion of young animals and females (at least ovigerous) would cause a mismatch with the actual situation in the field, in our opinion, this would adequately reduce data variability in routine biomonitoring, along with the number of sampled animals.

To ensure comparison between studies, the methods for the analysis of energy reserves should be harmonised. In our opinion, our protocols deliver the optimal compromise between robustness and efficiency.

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