

RESEARCH ARTICLE

Environmental warming induces behavioral and metabolic changes in a freshwater crustacean – aeglids as a model organism

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Abstract – Ecological processes in small-order streams are dependent on their community. A threat to the appropriate functioning of these processes is the water warming of streams since changes in temperature can modify the behavior, abundance, and distribution of the species. A way to predict the consequences of global warming on ecological processes in these ecosystems is to study their aquatic community. Here we evaluated behavioral and metabolic changes in *Aegla longirostri* at different temperatures (21 °C and 24 °C). Experiments were performed in laboratory conditions. We calculated leaf consumption and quantified glycogen, protein and amino acid composition in the hepatopancreas. We also conducted a behavioral test to investigate the activity level of aeglids. Leaf consumption did not differ between temperatures. However, the amount of protein was higher at 21 °C, and the amino acid and glycogen levels were greater at 24 °C. In the present study we evaluated only the activity of hepatopancreas, so we can assume that the organ may have used glucose through the breakdown of glycogen and also performing some protein break. However, this hypothesis needs to be confirmed by checking for muscle activity. Animals kept at 24 °C showed a lower level of activity. This strategy possibly occurs to save energy, as in elevated temperature crustaceans spend extra energy to maintain their homeostasis. This study indicates that a future increase temperature of streams will impact the populations of aeglids by changing their metabolism and behavior.

Keywords: Aeglidae / energy reserves / glycogen / protein / shredder

1 Introduction

Due to the increase of CO₂ in the atmosphere and its contribution to the greenhouse effect, an increase is estimated in the average global air temperature up to 4.8 °C until 2100 (IPCC, 2014). There is a direct relationship between air temperature and water temperature (Morrill *et al.*, 2005), and an increase of 5 °C in air temperature can lead to an increase of up to 3.3 °C in the temperature of water from rivers and streams (Langan *et al.*, 2001; Koycheva and Karney, 2009). Because of the climate changes that have occurred so far, warming of water has already altered the abundance and distribution of some aquatic species such as fish (Perry *et al.*, 2005) and mussels (Galbraith *et al.*, 2010). For the future, it is believed that these global changes in climate have the potential to cause modifications at the individual, population and community levels (Walther *et al.*, 2002). Ecological processes such as carbon mineralization and primary production (Acuña *et al.*,

2008; Demars *et al.*, 2011) also can be affected. These facts point to the importance of understanding how global warming may affect the aquatic communities and the ecosystem processes that occur in these environments (Cole *et al.*, 2007).

In the face of a scenario of climate change, the aquatic communities in small-order streams may be drastically affected, with direct effects on the ecological processes involved (Martínez *et al.*, 2014; Ferreira *et al.*, 2015). The decomposition of allochthonous plant material from riparian vegetation, which is mainly performed by hyphomycetes (Bärlocher, 1992) and invertebrate shredders (Graça, 2001), would be one of these processes with potential deleterious effect on the aquatic community. Changes in water temperature in streams can influence the metabolic rates of these organisms (Brown *et al.*, 2004), thus inducing modifications in their physiology resulting in alterations in their biochemical composition or even suppression of their activities.

The ability of organisms to maintain the stability of an internal environment (homeostasis) is energetically costly. Crustaceans keep the homeostasis through the control of the levels of carbohydrates, lipids and proteins (Dall and Moriarty,

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1983). The relative importance of these reserves and the order of use vary among species, stage of life and type of stress (Jimenez and Kinsey, 2015). Changes in environmental conditions (temperature, O₂ availability and salinity) and life cycle changes (reproduction and molting) can modify the amount of these compounds in the body of animals. In crustaceans, the main monosaccharide present in the hemolymph is glucose (Chang and O'Connor, 1983), which is used in the synthesis of chitin and glycogen and pyruvate formation (Jimenez and Kinsey, 2015). When at a high concentration, glucose is stored in the form of glycogen, particularly in the hepatopancreas and muscles (Vinagre and Da Silva, 1992; Oliveira *et al.*, 2003; Buckup *et al.*, 2008). For the most part, lipids are stored in the hepatopancreas (Chang and O'Connor, 1983; Kucharski and Da Silva, 1991) and proteins, in the hepatopancreas and muscles (Claybrook, 1983; Buckup *et al.*, 2008).

A way to predict the consequences of a future scenario of global warming on ecological processes in small-order streams is to study their aquatic community. In the headwater streams of southern Brazil, *Aegla longirostri* Bond-Buckup and Buckup, 1994 is an important shredder that participates in the process of leaf litter fragmentation (Cogo and Santos, 2013), and it is an essential species in nutrient cycling. These animals live in small-order streams with average annual temperature of 18 °C (unpublished results). They are often under rocks, and leaf litter and their diet consist mainly of vegetable tissue and insect larvae, being considered omnivores (Santos *et al.*, 2008). Given the above, the objective of this study was to test, under laboratory conditions, if an increase in water temperature will modify leaf consumption, the composition of metabolites in the hepatopancreas and movement of *A. longirostri*. Our hypothesis is that at high temperatures, the metabolism of aeglids will be accelerated, causing (1) an increase in leaf consumption, (2) a decrease in glycogen reserves. Assuming that they will use the breaking of glycogen for energy, (3) there will be no changes in the levels of proteins and amino acids. We also expect that higher temperature will (4) reduce the movement and activity of the animals as a way of saving energy.

2 Methods

2.1 Collection and maintenance of shredders

We collected specimens of *A. longirostri* manually and with traps in May and June 2015 in a small-order stream, located in the southern region of Brazil (Santa Maria; RS; 29°39'49"S; 53°44'34"W). The site features a seasonal semideciduous forest with diverse riparian vegetation and an apparent absence of anthropic impact. The stream has areas of pools and riffles, and the substrate is mainly rocky with the presence of leaf litter and sediment deposits (especially in pools). In the experiments, we used only males at the intermolting stage and collected in the same period. This because there are differences between compounds in the hepatopancreas of males and females, aeglids go through a period of fasting during molting, and the reproductive period can change the energy reserves of the animals (Oliveira *et al.*, 2003; Ferreira *et al.*, 2005). After

collection, the animals were transported to the laboratory and individually measured for cephalothorax length with a digital caliper (precision: 0.01 mm).

Before the experiment, the animals remained (for at least seven days) in individual aquariums (2 L) with the water temperature of 18 °C, constant aeration, controlled photoperiod (12 h light; 12 h dark), rock for shelter and *ad libitum* food (leaf litter collected in the stream). After that, they were randomly separated into two groups and acclimated (three days) in a BOD incubator, in the same conditions (see above) but unfed. The first group was adapted at 21 °C and the second one at 24 °C. We chose these temperatures because they are above the average water temperature in streams of the region (18 °C) and because at higher temperatures (such as 26 °C and 27 °C) the aeglids have high mortality (preliminary test). At that time, we also conditioned (three days) leaves of *Ficus luschnathiana* (Miq.) Miq., a plant species used for checking leaf consumption by aeglids. We selected this species because it is abundant in the region and can be found on the banks of debris along the stream. A recent study showed that aeglids consume this species in the natural environment (Cogo and Santos, 2013). The senescent leaves of *F. luschnathiana* were previously collected, open air dried, wrapped in fine mesh litter bags (500 µ) and incubated in the stream for 14 days to allow microbial conditioning, a step in which the leaves become more palatable to shredders (Graça *et al.*, 2015).

2.2 Experiment 1 – fragmentation and biochemical analyses

In the experiment, the animals were kept alone in aquariums (2 L) with constant aeration, controlled photoperiod (12 h light; 12 h dark), rock for shelter. Also, the animals received conditioned leaves discs (12 mm). Each animal received 370.0 ± 15.0 mg of dry mass leaf (DM) for consumption. Overall, we used 12 aquaria/animals at 21 °C and 11 aquaria/animals at 24 °C. Besides, we have kept a control aquarium, where we placed the leaf discs, without the presence of the shredder, to check leaf mass loss as a result of leaching and the action of microorganisms. After two days, the remaining discs (experimental and control) were dried in an oven (60 °C for two days) and weighed for determination of the remaining DM. Leaf consumption corresponded to the leaf mass loss that occurred in the presence of the shredder, subtracting the value of leaf mass loss obtained in the control aquarium (without shredder).

For the analyses of energy reserve of aeglids, we chose to evaluate glycogen, protein and amino acids in the hepatopancreas, because this is a place of storage of reserves in aeglids (Oliveira *et al.*, 2003; Ferreira *et al.*, 2005). At the end of the experiment, we cryoanesthetized the animals to remove the hepatopancreas, which was stored in a freezer (−20 °C) for subsequent analysis. The level of glycogen in the hepatopancreas was determined by the method described by Bidinotto *et al.* (1997) after addition of KOH and ethanol for glycogen precipitation. The tissue was heated with KOH at 100 °C and centrifuged at 10 000 g for 10 min to determine the level of proteins. The supernatant was used to estimate the amount of

Table 1. Description of the behaviors analyzed and their respective scores.

| Behavior | Description | Score |
|-------------------|--|-------|
| Inactive | Absence of any apparent movement or movement only of cephalic appendages (antennae, antennules and/or maxillipods) | 0 |
| Low activity | Movement of the chelipeds, pereopods, pleopods and/or short movements of the animals | 1 |
| Moderate activity | Active movement of the animal along the aquarium | 2 |
| Intense activity | Tail flipping | 3 |

Adapted from Dalosto and Santos (2011).

Table 2. Mean \pm standard error of the variables analyzed in *Aegla longirostri* at 21 °C and 24 °C. CL: cephalothorax length. Time with food: time spent manipulating the food; Time without food: time without manipulating food (videotaped over a period of 600 s, only at night).

| | 21 °C | 24 °C | The test value | <i>p</i> -Value |
|--|------------------|------------------|----------------|-----------------|
| CL (mm) | 19.2 \pm 2.2 | 19.6 \pm 2.4 | $t = -0.37$ | $P = 0.71$ |
| Leaf consumption (mg) | 177.6 \pm 97.7 | 179.6 \pm 94.3 | $t = -0.04$ | $P = 0.96$ |
| Glycogen ($\mu\text{mol g}^{-1}$) | 11.3 \pm 1.8 | 13.3 \pm 2.8 | $U = 29.5$ | $P = 0.02$ |
| Proteins (mg g ⁻¹) | 43.4 \pm 5.2 | 19.9 \pm 2.5 | $t = 15.19$ | $P < 0.0001$ |
| Amino acids ($\mu\text{mol g}^{-1}$) | 15.1 \pm 1.9 | 37.8 \pm 2.1 | $t = -27.20$ | $P < 0.0001$ |
| Score 10 h | 24.2 \pm 8.8 | 23.2 \pm 8.5 | $U = 136$ | $P = 0.44$ |
| Score 22 h | 18.5 \pm 8.3 | 15.8 \pm 4.2 | $U = 86.50$ | $P = 0.02$ |
| Latency (s) | 28.5 \pm 25.2 | 136.7 \pm 93.5 | $U = 3$ | $P = 0.15$ |
| Time with food (s) | 562.5 \pm 23.5 | 286.5 \pm 1.9 | $U = 1$ | $P = 0.04$ |
| Time without food (s) | 9.0 \pm 9.0 | 176.7 \pm 74.7 | $U = 1$ | $P = 0.04$ |

proteins following the method described by Lowry *et al.* (1951). The neutral supernatant was used for colorimetric determination of amino acids according to Spies (1957).

2.3 Experiment 2 – Behavior

We made behavioral observations at 10 h and 22 h for two days. For 10 min (600 s), the animals ($N = 10$ at 21 °C and $N = 8$ at 24 °C) were videotaped (Sony[®] HDR-CX560 handycam) for later analysis. The activity of aeglids was estimated using specific actions, to which we assigned scores, adapted according to Dalosto and Santos (2011) (Tab. 1). To determine the scores, we divided the 600 s at 10-second intervals (totaling 60), and during each interval, we annotated the score of the predominant activity (at least 5 s, except for the tail flipping action, which we took into consideration even if the animal persisted for less than 5 s). The final score is the sum of the scores given in each interval (they may vary from 0: inactive animal to 180: maximum activity).

After the observation at 22 h of the second day, we performed another behavioral observation (videotaped over a period of 600 s, only at night). On this occasion, we offered food to the animals and checked latency, time spent manipulating the food (time with food) and the time without manipulating food (time without food). For this purpose, we chose animals ($N = 4$) at random in each treatment and offered a mix of four pieces of leaves previously collected in the stream. We opted to observe only at 22 h because aeglids are

mostly nocturnal animals (Sokolowicz *et al.*, 2007). We did these observations at night with the use of incandescent red-light bulbs, because crustaceans have low sensitivity to this wavelength (Turra and Denadai, 2003).

2.4 Statistical analysis

To test if there was a difference in cephalothorax length, leaf consumption, levels of glycogen, protein and amino acids among treatments, either the *t*-test or the Mann-Whitney test was performed, depending on the normality of the data. Differences in behavior between day and night within the same treatment were tested using the Wilcoxon signed-rank test for related samples and differences between treatments were tested with the Mann-Whitney test for independent samples. Mann-Whitney test was used to test differences in latency and time spent manipulating the food. The analyses were performed in the software BioEstat 5.0.

3 Results

3.1 Experiment 1 – fragmentation and biochemical analyses

The cephalothorax length of the animals and leaf consumption did not differ between the temperatures (Tab. 2). However, the level of protein was higher at 21 °C while the levels of the amino acids and glycogen were higher at 24 °C (Tab. 2; Fig. 1).

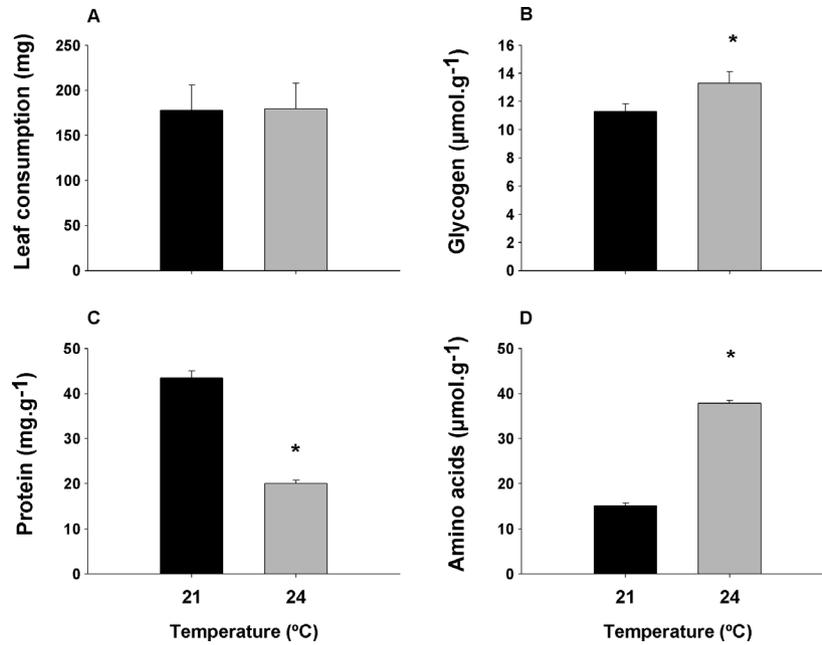


Fig. 1. Consumption and biochemical analyses. Mean \pm standard error of leaf consumption (A), the level of glycogen (B), protein (C) and amino acids (D) in the hepatopancreas of *Aegla longirostri* at 21°C and 24°C. Asterisks indicate significant differences between the groups.

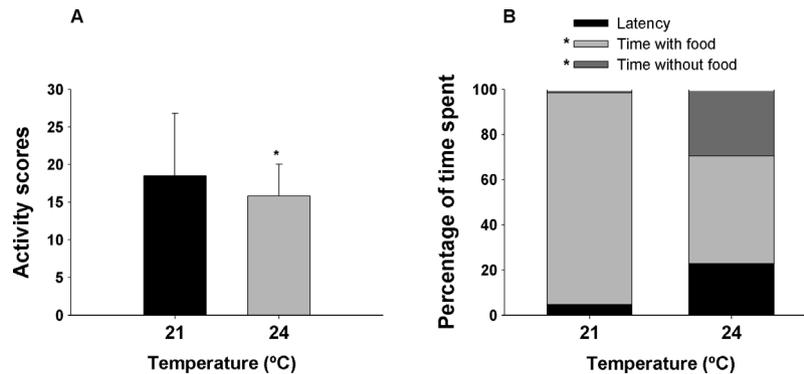


Fig. 2. Behavioral observations. Mean \pm standard error of the average activity scores at 22 h (A) and % of time spent in categories (i) latency, (ii) manipulate the food and (iii) without food in 21°C and 24°C (B). Asterisks indicate significant differences between the groups.

3.2 Experiment 2 – Behavior

There was no difference in the activity of the animals between day and night both at 21°C ($Z=1.18$, $df=19$; $P=0.24$), and in 24°C ($Z=0.38$, $df=15$; $P=0.71$), as well as between the two days of experiments at different temperatures (Tab. 2). However, during the night (22 h), the animals at the lowest temperature showed a higher level of activity (Tab. 2; Fig. 2).

Concerning observation of animals after the offer of food, we found that the latency period did not differ between the different temperatures, but the animals at a temperature of 21°C spent more time with food than animals at 24°C (Tab. 2; Fig. 2B).

4 Discussion

Nutrient cycling in small streams that use allochthonous plant material as the main source of energy depends on their aquatic community. Therefore, changes in these organisms due to heating of the water can cause changes in the decomposition of leaf litter and consequently in nutrient cycling. Experiments with higher temperatures have confirmed this hypothesis (Martínez *et al.*, 2014). Increases in water temperature increase the rate of decomposition of leaf litter due to the increase in (i) leaching of soluble compounds (Chergui and Pattee, 1990), (ii) fragmentation and consumption by invertebrates (González and Graça, 2003; Azevedo-Pereira *et al.*, 2006), and (iii) microbial activity (Buzby and Perry, 2000; Canhoto *et al.*, 2016).

Contrary to these results and our initial prediction, the increase in water temperature did not cause an increase in leaf consumption by *A. longirostri*. At the highest temperature, the aeglids metabolism may not have been speeded up enough to foster an increase in leaf consumption, hence using the energy from food and their energy reserves was sufficient. The absence of differences in leaf consumption at different temperatures may also be related to the chemical quality of the plant species. This characteristic must be considered in a scenario of climate change vs. leaf decomposition because interactions with water temperature can modify the quality of the leaf litter as a function of leaching and mainly of microbial conditioning (Ferreira *et al.*, 2015). The leaves of *F. luschnathiana* were placed in a natural environment, and only after that, they were offered to the aeglids, under experimental conditions. So, the similar results in both experimental conditions tested in this study may be explained by the similar characteristics of the leaves.

Although we have not confirmed our first hypothesis, on leaf consumption, our results show that changes in water temperature produce differences in the biochemical metabolism and the behavior of aeglids. Crustaceans have no particular or specialized organ to regulate their body temperature; hence their metabolic rates vary according to environmental temperature changes (Vernberg, 1982; Lagerspetz and Vainio, 2006) and consequently their energy reserves may be altered. In this study, we observed that the levels of glycogen in the hepatopancreas of aeglids were greater at the highest temperature, and the levels of protein and amino acids differed between the temperatures. Thus, we can say that the aeglids have adopted different strategies for support adverse situation at the analyzed temperatures. At 21 °C, the animals used glucose probably due to glycogen breakdown. On the other hand, at 24 °C, glycogen levels were higher than in aeglids kept at 21 °C, so we suppose that they used some protein hydrolysis. The increase in the level of amino acids at the temperature of 24 °C suggests that some proteolysis occurred at this temperature. However, the hypothesis needs confirmation through muscle activity measurements. As the supply and concentration of glucose can be derived both from glycogenolysis and gluconeogenesis (Sánchez-Paz *et al.*, 2007), we diagnosed that aeglids used different metabolic pathways. At 21 °C, glucose was produced through the breakdown of glycogen (glycogenolysis), while at 24 °C, amino acids were the source of energy for metabolism. The use of the gluconeogenesis pathway (generation of glucose from breakdown of proteins or amino acids) has already been reported for *A. platensis* fed on some diet rich in protein and low in carbohydrates (Ferreira *et al.*, 2005) and for *Neohelice granulata* in different diets (Oliveira and Da Silva, 1997).

In different stress conditions, crustaceans use different energy reserves. In this study, at a high temperature, *A. longirostri* decreases its level of proteins, and the same occurs to *Carcinus aestuarii*, which features lower levels of protein in the hemolymph in this condition (Matozzo *et al.*, 2011). By contrast, *Gammarus pulex* showed a decrease of its level of glycogen at high temperatures (Foucreau *et al.*, 2014). Glycogen and proteins are typically used during periods of intense activity or changes in environmental conditions for crustaceans (Dutra *et al.*, 2008). The glycogen is used during the process of molting, in periods of fasting, during hypoxia

and anoxia, in osmoregulation and growth (Hu, 1958; Chang and O'Connor, 1983; Kucharski and Da Silva, 1991; Oliveira and Da Silva, 2000; Oliveira *et al.*, 2001; Oliveira *et al.*, 2004). In fasting, amphipod crustaceans primarily use the reserves of glycogen and protein and finally the reserves of lipids, while *G. fossarum* uses the reserves of proteins and lipids (Hervant *et al.*, 1999).

Other studies have also found a relation between levels of glycogen and variations in environmental conditions. For example, specimens of *Hyaella curvispina* have lower levels of glycogen under conditions of increased salinity and lower water volume and temperature (*e.g.* Dutra *et al.*, 2008). The estuarine crab (*N. granulata*) also depends on glycogen for their survival during significant variations in environmental parameters such as temperature, salinity and periods of food shortage (Oliveira and Da Silva, 1997). Some studies indicate that seasonal variation in energy reserves of aeglids seems to be species-dependent and related to the reproductive period and food availability (Oliveira *et al.*, 2003, 2007). Oliveira *et al.* (2007) suggested that the physiological characteristics of aeglids are related to their success in exploiting resources available in the environment as well as to the ability to survive in some varied environmental conditions.

The thermal control of crustaceans is based only on behavioral mechanisms, such as stimulus-based changes in locomotion. We know that they avoid extreme temperatures due to the changes in their locomotor activity (*e.g.* Lozán, 2000) and their movements increase because of thermal preference (Lagerspetz and Vainio, 2006). Aeglids showed less activity at higher temperatures, corroborating our prediction. These animals were dependent on environmental conditions in several studies, such as availability of oxygen (Dalosto and Santos, 2011), availability of particulate organic matter (Bücker *et al.*, 2008) and different land uses, such as urbanization and agriculture (Trevisan *et al.*, 2009). We observed that variations in water temperature influenced the behavior of aeglids, indicating that an increase in global temperature can affect the populations in distinct ways, such as changing the metabolism of animals as well as their behavior (locomotor activity). This strategy may have been used to save energy, because at high temperatures the animals spend extra energy to maintain their homeostasis.

Water temperature is one of the main abiotic factors influencing the survival and growth of decapod crustaceans (Hartnoll, 2001). In the short term, the temperature of 24 °C caused changes in the metabolism and activity of aeglids but did not affect the survival of these animals. If aeglids become less active at higher temperatures, they may present greater difficulty in finding food, mainly animal material, since they actively search for food resources. However, additional studies in the long term are required to verify the survival of these animals at high temperatures and the possible consequences for their growth and fitness.

The physiological processes of animals comprise a connection between the characteristics of life history and current environmental conditions. Understanding how these processes respond to climate changes can provide evidence about the implications of global warming predicted in biological communities (Small *et al.*, 2015). The present study showed that, as a consequence of global warming, changes might occur in the metabolism and behavior of

aeglids. Since these crustaceans are strategic consumers of leaf litter and predators in the streams where they live (Cogo *et al.*, 2014; Cerezer *et al.*, 2016), changes in their niche can lead to imbalances in biogeochemical cycles and aquatic trophic chains. However, further long-term studies are necessary for a complete understanding of the consequences of the warming of water resulting from climate change in organisms and ecosystem processes of aquatic environments.

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