J. Limnol., 2018; 77(2): 189-198
DOI: 10.4081/jlimnol.2017.1658

ORIGINAL ARTICLE

Effect of temperature on behavior, glycogen content, and mortality in *Limnoperna fortunei* (Dunker, 1857) (Bivalvia: Mytilidae)

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ABSTRACT

Limnoperna fortunei (Dunker, 1857) is a freshwater mussel with physiological tolerance to different environmental conditions, which may explain its success as an invasive species. The role of abiotic factors in its establishment, abundance and projections of risk of further spread into several areas has been studied. These mussels may respond to multiple environmental stressors, such as temperature, through physiological mechanisms, behavioral responses, mortality or some combination of these. The aim of this study was to investigate the behavioral responses (valve closing), glycogen concentrations and mortality of *L. fortunei* under four different temperatures (5°C, 10°C, 20°C and 30°C) during a chronic test (30 days). Two-way analysis of variance (ANOVA) was used to compare glycogen concentrations across days of the experiment and at the different temperatures. Differences in valve-closing behavior and mortality among temperatures were tested using repeated-measures ANOVA. We observed that most of the mussels maintained at 5°C closed their valves (74.7±15.3%), indicating that they remain inactive at low temperatures. The glycogen levels significantly differed among the temperatures tested. These differences occurred mainly due to the high glycogen values observed in mussels exposed to 10°C. Stability in glycogen concentrations was observed within each particular temperature. The cumulative mortality was higher at extreme temperatures (5°C and 30°C). The ideal temperature for laboratory maintenance and tests is approximately 20°C. Our data also show that *L. fortunei* can survive and maintain their energy reserves (glycogen) for several days at 5°C, an important feature related to its invasion success.

Key words: Bioinvasion; golden mussel; valve closing; physiology.

Received: April 2017. **Accepted:** November 2017.

INTRODUCTION

Limnoperna fortunei (Dunker, 1857), or the golden mussel, is an invasive species native to Southeast Asia that has spread to Taiwan, Korea, Japan and South America. This species has successfully increased its geographic distribution in South America, chiefly through its physiological plasticity, rapid growth and sexual maturation and its ability to colonize several natural and artificial substrates (Darrigran and Damborenea, 2011; Boltovskoy *et al*., 2015; Nakano *et al*., 2015; Barbosa *et al*., 2016; Cordeiro *et al*., 2016). Considering these factors, several studies investigated the roles of abiotic factors (*e.g*., calcium, temperature and salinity) on the establishment, abundance and risk of further spread of the golden mussel to several areas (Darrigran and Damborenea, 2005, 2011; Oliveira *et al*., 2010, 2011; Darrigran *et al*., 2012).

Temperature is one of the most important abiotic factors influencing the ability of species to survive (Bradie and Leung, 2017). Hence, species distributions are related to this variable (Bradie and Leung, 2017). Species distribution models frequently include temperature, and temperature is usually one of the variables that most contributes to the predictions (Bradie and Leung, 2017). Temperature affects bivalve development, growth, and survival rates in captivity and in the wild due to its influence on physiological

processes (Gabbot and Bayne, 1973; Oliveira *et al*., 2011; Carey *et al*., 2013; Boltovskoy *et al*., 2015). Water temperature plays a key role in the reproductive cycle of golden mussels (Boltovskoy *et al*., 2015) and was considered to be an important variable associated with the invasion capability of macrofouling bivalves (Oliveira *et al*., 2010, 2011).

Dreissenid bivalves, such as the zebra mussel, *Dreissena polymorpha* (Pallas, 1771), and the quagga mussel, *Dreissena rostriformis bugensis* (Andrusov, 1897), present ecological characteristics that are very similar to those of the golden mussel and are also some of the most aggressive freshwater invaders (Karatayev *et al*., 2015). However, *L. fortunei* is considered a more aggressive invader than both species of *Dreissena* (Karatayev *et al*., 2015). Previous studies have examined the minimum and maximum temperatures of the water bodies where golden mussels are found, and the upper thermal limit is approximately 35°C, which is slightly higher than that observed for *Dreissena* spp. (32-33°C) (Karatayev *et al*., 2015). The lower temperature limit for the growth of *L. fortunei* in Asia (Korea) is close to 0°C, the same as that observed for the two *Dreissena* species (Karatayev *et al*., 2015). In Japan, golden mussels survive at water temperatures of 5-6°C (Karatayev *et al*., 2015), while the minimum winter temperature of the water bodies colonized by *L. fortunei* in South America is approximately 10°C (Oliveira *et al*., 2011). In fact, field data have revealed that *L. fortunei* has an extremely wide thermal tolerance range, from 0 to 35°C (Oliveira *et al*., 2011; Karatayev *et al*., 2015).

Several laboratory experiments have described the effects of different temperatures on the mortality, behavior, and development of the golden mussel; however, some essential methodological details were not provided (*e.g*., acclimation, feeding, water renovation and monitoring of physical and chemical parameters, such as pH, dissolved oxygen and ammonia). In an experiment conducted for 7 to 10 days, the mortality of juveniles and adults exposed to 5°C was observed to be lower than that of individuals exposed to 35°C (Montalto and Marchese, 2003). A 30-day experiment showed that adult mussels survive up to 15 days at 0-1°C (although they are inactive during this time) and that the mortality at 5-7°C was >80% (Oliveira *et al*., 2010). In addition, when exposed to temperatures above 10°C, the mussels were active, and 80% of them survived for 30 days (Oliveira *et al*., 2010). Thus, despite their high mortality rates, golden mussels can survive at low temperatures. However, the physiological alterations related to this stressor have not yet been investigated.

Experimental research indicates that *L. fortunei* appears to be more resistant to heat than *D. polymorpha* (Perepelizin and Boltovskoy, 2011). The acute upper lethal temperature that resulted in the 100% mortality of *L. fortunei* varied from 43.5±0.0 to 50.3±0.6°C, and the time to achieve total mortality at such temperatures varied from 1.8±0.1 to 15.3±0.1 h (Perepelizin and Boltovskoy, 2011). Rolla and Mota (2010) also observed 100% mortality after 24 h at 37 and 40°C, 72 h at 35°C and 96 h at 32°C. At temperatures above 40°C, *L. fortunei* stopped all feeding activities and closed their valves until death (Perepelizin and Boltovskoy, 2011). Perepelizin and Boltovskoy (2011) demonstrated that the highest filtration activity of golden mussels was detected at 31°C and argue that *L. fortunei* is more resistant to higher temperatures than *Dreissena*. Slower growth rates and larval development were documented by Cataldo (2015) and Nakano *et al*. (2015) at lower temperatures. Information on mortality rates, filtration rates and growth rates reinforces the *L. fortunei* affinity for warmer waters (Perepelizin and Boltovskoy, 2011; Cataldo, 2015; Nakano *et al*., 2015).

In freshwater ecosystems in tropical and subtropical areas, temperatures can vary during the year, exposing mussels to several stressors related to it, such as different dissolved oxygen levels and limited food availability (Boltovskoy, 2015). The metabolic activities of mussels usually increase with higher temperatures, and oxygen and food resources can become scarce with increasing water temperature (Carey *et al*., 2013). Thus, as a consequence of environmental fluctuations and other stressors, marine and freshwater mussels have developed a physiological and behavioral strategy (*i.e.*, valve closing) necessary for survival in several waterbodies (Kittner and Riisgård, 2005; Palais *et al*., 2011; Dowd and Somero, 2013).

Valve closing is an important response related to defense against predators and to physiological disturbance caused by poor water quality. Valve movement is easily measurable and is one of the most important bivalve behaviors; it was monitored in experiments involving chemical (Di Fiori *et al*., 2012; Montresor *et al*., 2013) and nutritional stressors (Riisgård and Larsen, 2015; Cordeiro *et al*., 2016) and temperature changes (Perepelizin and Boltoskoy, 2011). In general, filter-feeding bivalves such as *L. fortunei* reduce their filtration rate by closing their valves when exposed to lower temperatures or abrupt temperature changes (Kittner and Riisgård, 2005; Perepelizin and Boltoskoy, 2011). Thus, the valve opening-closing mechanism is considered an efficient physiologically regulated mechanism that allows the mussel to save energy during a stress event (Tang and Riisgård, 2016). The use of metabolic reserves, or the depletion of glycogen concentrations, has been associated with the nutritional conditions of the mussels and is a good parameter for assessing their physiological conditions (Widdows and Bayne, 1971; Gabbott and Bayne, 1973; Patterson *et al*., 1999; Chen *et al*., 2001;

Nandurkar and Zambare, 2012; Anacleto *et al*., 2013; Cordeiro *et al*., 2016, 2017). In fact, stressors (*e.g*., extreme temperatures, starvation) cause severe changes in their metabolism and contribute to mortality (Patterson *et al*., 1999; Lee *et al*., 2008; Anacleto *et al*., 2013; Cordeiro *et al*., 2016).

To gain a more comprehensive understanding of the effects of temperature and considering that mussels may respond differently to stressors (*i.e*., physiological response, behavioral response, mortality or some combination of these) (Dowd and Somero, 2010; Palais *et al*., 2011; Perepelizin and Boltovskoy, 2011; Cordeiro *et al*., 2016, 2017), we attempted to examine the effects of different temperatures on *L. fortunei* responses. These data may contribute to i) a better understanding of the physiological and behavioral responses of the golden mussel to different temperatures, which may be related to their ability to adapt to temperature changes; ii) predictions about the geographic expansion of *L. fortunei*; iii) the development of more efficient laboratory rearing protocols through the definition of an optimal temperature; and iv) an enhancement of our knowledge about the interactions between physiological and behavioral responses to environmental stress. Considering the broad temperature tolerance range of *L. fortunei*, we hypothesize that this species employs behavioral and physiological mechanisms to maintain its glycogen reserves even at stressful temperatures.

METHODS

Individuals of *L. fortunei* were manually and carefully collected in September 2015 from the photic zone, right below the water surface, where they were incrusted on the substrate. The reservoir of Itaipu's hydroelectric power plant (Paraná River) is located at Foz do Iguaçu city, Paraná state, Brazil (25°26'17.1"S; 54°30'45.7"W). The water temperature was 23°C. The mussels were collected and transported to the laboratory according to Cordeiro *et al*. (2016). Individuals were acclimatized to laboratory conditions for 15 days in 240 L aquaria containing dechlorinated tap water under a 12:12 h light:dark cycle. The physical and chemical parameters in the aquaria were monitored daily using an optical oximeter (ProODO; YSI Inc., Yellow Springs, OH, USA), a pH meter (HI 3221; HI 1131B; Hanna Instruments, Woonsocket, RI, USA) and an ammonia-selective electrode (HI 4101; Hanna Instruments). The aquaria were maintained under the following conditions (mean±SD): 19.6±0.3°C, pH 8.15±0.6, 7.26±0.19 mg L^{-1} O_2 and ≤0.6 mg L^{-1} total ammonia-N (TA-N). During this period, constant aeration was provided, and the animals were fed daily with live algae (*Scenedesmus* sp. and *Ankistrodesmus* sp.) (Cordeiro *et al*., 2016).

Acclimation to the experimental temperatures

Water temperatures were selected based on the temperature ranges of the main South American waterbodies (Boltosvkoy *et al*., 2015; Oliveira *et al*., 2015). A group of 800 mussels was selected using size as the criterion (21-27 mm in shell length). Later, other criteria were used for selection, and, after visual evaluation, individuals that did not attach to the containers and did not present extended siphons were excluded (Montresor *et al*., 2013; Cordeiro *et al*., 2017). Eight days before the experiment, groups of 200 individuals were randomly distributed among 40 L aquaria and were acclimated to four different temperatures (5°C, 10°C, 20°C and 30°C). During these eight days, acclimation to each experimental temperature was performed at a rate of 2°C/day. Thereafter, each group was maintained at the same temperature for 30 days as described below. Recirculating water chillers (RWCs) or recirculating coolers (Gelaqua 1/3 hp and temperature stability 0.1°C) were used to cool the water (one RWC per aquarium) and maintain it at 5°C, 10°C and 20°C. A thermostat was used to maintain the temperature at 30°C.

During the trials, the animals were maintained under a 12:12 h light:dark cycle and constant aeration and were fed daily with live algae (*Scenedesmus*, Meyen, 1829 (Chlorophyceae: Scenedesmaceae) and *Ankistrodesmus*, Corda, 1838 (Chlorophyceae: Selenastraceae) (Cordeiro *et al*., 2016)). The water physical and chemical parameters were monitored daily. Individuals had their soft tissues removed, stored and maintained at -20°C for quantification of the glycogen concentration (see details in the "Glycogen quantification" section). This procedure was performed to evaluate the glycogen concentrations after acclimation and during the experiments on the 15th and 30th day of treatment.

Experiment

One hundred and fifty animals from each experimental temperature (treatment) were randomly distributed among six plastic containers (4 L), each containing 25 mussels and 2 L of dechlorinated water. These containers were placed in incubators (Nova Ética 411/FPD thermal stability±0.1°C). Each incubator was adjusted to maintain a 12:12 h light:dark cycle and one of the four temperatures tested (5°C, 10°C, 20°C and 30°C). The criteria to select the plastic containers were the same as those presented above. Only the containers with all twenty-five individuals attached and exhibiting the siphon were used in the trials. The other containers were not selected for the trials.

The experimental duration was 30 days. The animals were fed three times a week with live algae as specified above three hours before water replacement (100% daily

water replacement). Algae were added to the container until a final concentration of 10^5 cells/mL (in each container) was reached. Water replacement was carried out with dechlorinated tap water. Before replacement, the water was maintained in aerated tanks (40 L) that were kept at each experimental temperature (using a chiller or thermostat as mentioned above). The physical and chemical parameters of the replacement water were also monitored as mentioned above. Each container was cleaned every day before water replacement, and the organic residues and feces were removed using tap water and a thin paintbrush (Cordeiro *et al*., 2017). The physical and chemical parameters were monitored daily using the equipment specified above, and they were maintained at appropriate levels for *L. fortunei* (Montresor *et al*., 2013; Cordeiro *et al*., 2016, 2017) (Tab. 1). Unionized ammonia concentrations were calculated according to the methodology described by Emerson *et al*. (1975), considering the water pH, temperature and TA-N concentration data for the water samples (Tab. 1).

During the experiment, mortality and valve-closing responses were recorded over 24 h intervals, and dead individuals were removed from the containers daily to prevent water quality degradation (Cordeiro *et al*., 2016). Dead mussels present open valves (without extending the siphon), and they keep them open even after applying mechanical stimuli (Cordeiro *et al*., 2016). The valve-closing response was determined by direct observation, and the percentage of individuals with closed valves was calculated. Open valves and extended siphons indicate that the animals are active (breathing and eating).

At the end of the experiment, the mussels that remained closed were individually inspected, and those exhibiting ciliary beats of the gills were considered alive.

Glycogen quantification

On days 0, 15 and 30 of the experiment, 18 mussels from each treatment were killed, and their soft tissues were removed. These intervals were chosen based on the observation that the glycogen concentration showed significant changes after two weeks (Widdows and Bayne, 1971; Joyner-Matos *et al.*, 2009; Cordeiro *et al.*, 2017).

Samples consisting of one gram of pooled tissues each were collected in triplicate. They were stored at -20°C. The glycogen concentration of the tissues was determined using the 3,5-dinitrosalicylic acid technique (Sumner, 1944; Pinheiro and Gomes, 1994), and the concentrations are expressed as milligrams of glucose per gram of tissue (mg glucose × g tissue^{-1}, wet weight).

Statistical analysis

The ANOVA assumptions were tested using Levene's test (homogeneity of variance) and analysis of the residuals of the probability plot (normality). The glycogen concentration data are independent and unpaired with time. A two-way ANOVA was performed to compare the glycogen concentrations among the different temperatures (5°C, 10°C, 20°C and 30°C) and among days over the timespan of the experiment (Zar, 2009). Data on valve closing and mortality are dependent and paired with time. The differences in valve closing and mortality among temperatures were tested using a repeated-measures ANOVA. For mortality, the first two days were excluded from the analysis because the value was zero for all treatments. When the ANOVA results indicated a group difference, Tukey's honestly significant difference *post hoc* test was used to identify between which pairs of samples the differences were significant. All statistical analyses were performed using STATISTICA 13.0 software (Dell Inc., 2015). Data were not transformed.

RESULTS

Valve-closing behavior

The tested temperatures significantly affected the valve-closing response of *L. fortunei* (ANOVA: $F_{3,20}$=128.18, P<0.001). The mussels exposed to 5°C presented closed valves more frequently (74.7±15.3%) than those at higher temperatures (Fig. 1A and Tab. 2). The mussels kept at 10°C presented greater fluctuation in valve closing throughout the experiment (Fig. 1B), and their response was significantly different from that of those exposed to 20°C and 30°C (28.1±16.7% and 23.6±14.9%, respectively). The valve-closing response was not significantly different between individuals

Tab. 1. Water quality parameters for each temperature during the 30 days of the experiment (mean values±SD).

	Temperature (°C)	DO (mg L^{-1})	pH (mg L^{-1})	TA-N (mg L^{-1})	NH3-N (mg L^{-1})
5°C	4.8±0.5	11.9±0.27	7.5±0.14	0.067±0.025	0.000
10°C	9.9±0.8	10.4±0.14	7.6±0.13	0.042±0.026	0.000
20°C	20.4±0.6	8.3±0.12	7.6±0.18	0.047±0.015	0.001±0.001
30°C	30.2±1.2	6.9±0.27	7.7±0.18	0.052±0.018	0.003±0.001

DO, dissolved oxygen; TA-N, total ammonia nitrogen; NH3-N, unionized ammonia.

maintained at 20°C (28.1±16.1%) and those maintained at 30°C (23.6±14.9%) (Fig. 1A).

Glycogen quantification

The glycogen concentrations were significantly different among the temperatures tested (ANOVA: $F_{3,24}$=5.39, P=0.006). The high glycogen concentration observed in mussels at 10°C (1.48±0.75 mg g^{-1}) was significantly different from that observed at 30°C (0.36±0.31 mg g^{-1}) (Fig. 2A). No significant differences in glycogen concentration were observed within each temperature over the experimental periods analyzed, indicating that there was no interaction between these factors (ANOVA: $F_{6,24}$=2.31, P=0.07) (Fig. 2B and Tab. 2).

Mortality

The effect of the interaction between time and temperature on mortality was significant (ANOVA: $F_{81,540}$=14.38, P<0.0001). Mortality was low at intermediate temperatures (10°C and 20°C) during the entire experiment (Tab. 2), but at extreme temperatures, the mortality increased faster after the 15th day for the 5°C treatment and after the 24th day for the 30°C treatment (Fig. 3).

DISCUSSION

Thermal stress in bivalves may cause combinations of behavioral (valve closing/opening) and physiological responses (Anestis *et al*., 2007; Dowd and Somero, 2013). In the present work, the effects of thermal stress were assessed in terms of valve closing, glycogen concentration in the tissues and mortality. Based on our data, the closing of valves did not significantly differ between mussels subjected to 20°C and 30°C and was lower than the closing of valves in mussels subjected to 5°C and 10°C. In fact, we observed that mussels maintained at 5°C frequently kept their valves closed (74.7±15.3%), reinforcing previous observations of inactivity at lower temperatures (Oliveira *et al*. 2010). When a mussel's valves are closed, they can no longer access food particles and oxygenated water, the total metabolic output consequently decreases, and the ability to excrete wastes is hindered. This inactivity (valve closure) may be associated with a reduction in metabolic rates in mussels.

During prolonged stress, bivalves may readjust their metabolic profile and save energy through these

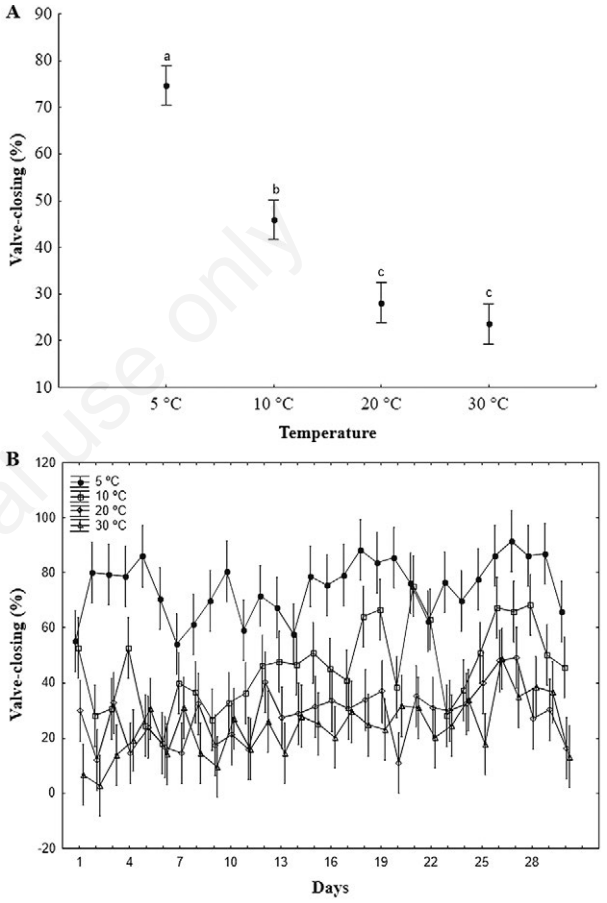

Fig. 1. Valve-closing of *Limnoperna fortunei* (%) (mean±95% confidence intervals). Different temperatures (5°C, 10°C, 20°C and 30°C) (A), and daily values throughout the 30 days for different temperatures (5°C, 10°C, 20°C and 30°C) under laboratory conditions (B). Different letters on the bars indicate significant statistical difference (P<0.05).

Tab. 2. Values of valve-closing behavior (mean±SD) and cumulative relative mortality of *Limnoperna fortunei* maintained at different temperatures and glycogen concentration (mean±SD) after 0, 15 and 30 days of the experiment.

Temperature	Valve-closing (%)	Mortality-final (%)	Glycogen (mg g^{-1})		
			0 day	15th day	30th day
5°C	74.7±15.3	60.2%	1.35±0.71	0.31±0.22	1.76±0.13
10°C	45.8±20.5	1.4%	1.47±0.85	2.26±0.08	0.98±0.50
20°C	28.1±16.7	6.4%	1.33±0.66	0.59±0.16	0.09±0.03
30°C	23.6±14.9	33.1%	0.69±0.25	0.27±0.14	0.13±0.21

mechanisms (Anestis *et al*., 2007; Riisgård and Larsen, 2015). The energy balance in mussels fluctuates with environmental conditions, such as temperature (stressors), which might also adversely affect their mortality (Palais *et al*., 2011). Valve-gaping behavior was related to the differential success of invasive *versus* native species (Nicastro *et al*., 2010). The combined behavioral and physiological repertoires of a particular invasive species could confer a substantial advantage in terms of withstanding stress incurred as a result of abiotic factors, such as elevated temperature (Dowd and Somero, 2010).

Individuals subjected to 10°C, 20°C and 30°C closed their valves less frequently than the mussels exposed to 5°C, indicating that they are capable of maintaining metabolic activities (*e.g*., filtration, excretion and oxygen consumption) at these temperatures. Previous studies have suggested that bivalves remain open in suitable environments (Bayne *et al*., 1976; Riisgård *et al*., 2003; Ortmann and Grieshaber, 2006; Tang and Riisgård, 2016). Higgins (1980) showed that mussels that regularly fed (as shown here) spent more time with open valves and exposed siphons. Our data correspond with the physiological studies of Schurink and Griffiths (1992), which showed that in mytilids, temperatures of 10 to 20°C allow adequate metabolic activity and guarantee their comfort under these conditions, allowing them to maintain their rates of filtration, absorption, and respiration.

Kittner and Riisgård (2005) showed that *Mytilus edulis* Linnaeus, 1758 closed their valves and reduced filtration rates when exposed to 6°C. The freshwater bivalve *Corbicula fluminea* (Müller, 1974) showed a reduction in its metabolic rate of up to 10% when the valves were closed (Ortmann and Grieshaber, 2006). Riisgård and Larsen (2015) reported that during stressful periods, mussels close their valves, reduce their oxygen uptake and save energy. Several studies have revealed that when exposed to lower temperatures, mussels reduce their metabolic activities, such as their reproductive and filtration rates (Choi and Shin, 1985; Kittner and Riisgård, 2005; Boltovskoy *et al*., 2015). This reduction in metabolic rate was associated with a reduction in energy expenditure (saving energy) during stress events (Riisgård *et al*., 2003).

Considering only the glycogen concentration, we observed that in the studied periods (0, 15 and 30 days), variation in concentration occurred among mussels exposed to different temperatures but not within the different temperatures tested. When the experiment began (0 days), the glycogen concentration was similar among the different temperatures tested (Fig. 2B) and was considered to be indicative of adaptation or acclimatization to each temperature by the regulation of metabolism (Widdows and Bayne, 1971). The glycogen concentration in mussels maintained at 10°C was higher than that of those maintained at 30°C. One potential explanation for this increase in glycogen concentration is the fluctuation in valve closing up to the 13th day; from that moment on, the mussels began to keep their valves closed more frequently, which may have favored glycogen accumulation. This phenomenon deserves attention in future research. This change in glycogen concentration and behavior may be related to physiological perturbation during this period elicited by stress (Dowd and Somero, 2013). In this case, the maintenance of glycogen concentrations and the valve closing behavior at the end of the experiment are considered to be indicative of an adaptation to stress conditions (Widdows and Bayne, 1971).

In contrast, the absence of an interaction between the factors of time and temperature indicated stable glycogen concentrations. Stable concentrations occurred when no behavioral alterations were observed (*i.e.*, continued valve

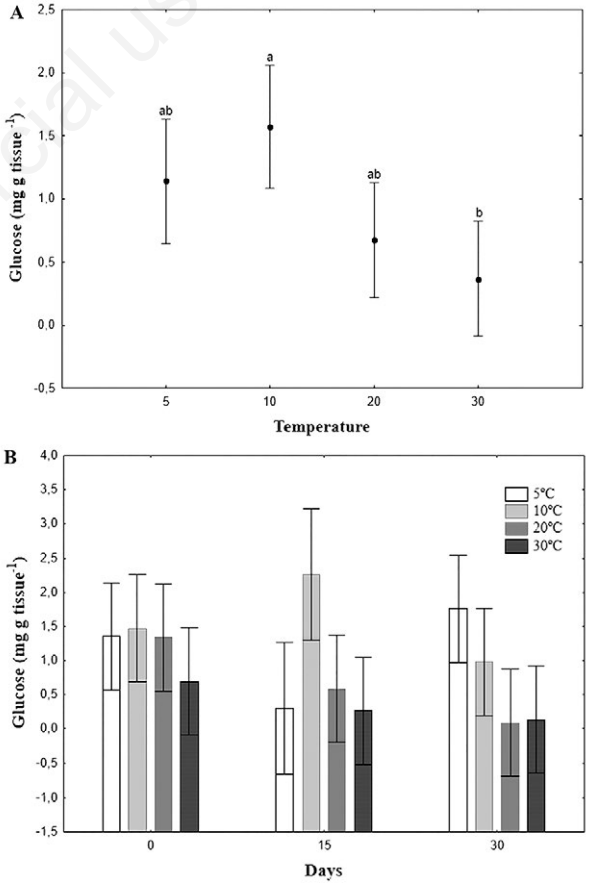

Fig. 2. Average glycogen content (mg glucose g tissue^{-1}, wet weight) and 95% confidence intervals of *Limnoperna fortunei* under different temperatures (5°C, 10°C, 20°C and 30°C) (A); and after 0, 15 and 30 days at different temperatures (5°C, 10°C, 20°C, and 30°C) (B). Different letters on the bars indicate significant statistical difference ($P < 0.05$).

closing behavior at 5°C and continued valve opening behavior at 20 and 30°C). Stable concentrations were also observed by Patterson *et al.* (1999) and Cordeiro *et al*. (2016) when feeding regimes remained the same for the duration of the experimental period. Stable glycogen concentrations were observed during all periods analyzed (0, 15 and 30 days) within each temperature tested (Fig. 2B). These data suggest that *L. fortunei* maintained a stable metabolism throughout the 30 days at the same temperature, indicating that the mussels had already adapted or acclimatized to each temperature. This information is compatible with the observations of Palais *et al*. (2011) regarding the dreissenid *D. polymorpha*, in which the glycogen concentrations were similar when the mussels were maintained over the same seasonal periods or at similar temperatures.

The two extreme temperatures tested here (5°C and 30°C) showed the highest mortality rates (60% and 33%). Previous experimental studies have examined the effects of temperature on the survival rates of the golden mussel (Montalto and Marchese, 2003; Oliveira *et al*., 2010; Rolla and Mota, 2010). However, in these studies, they did not measure water quality parameters, and other factors that could have influenced the results. It is known that some water quality parameters must be kept within a narrow range in order to maintain the survival of freshwater organisms. Thus, the temperature was considered to be the cause of death, when other non-detected factors could be related to these deaths.

Rising temperatures increase the formation of unionized ammonia, which is highly toxic to aquatic animals, such as *L. fortunei*, at very low concentrations

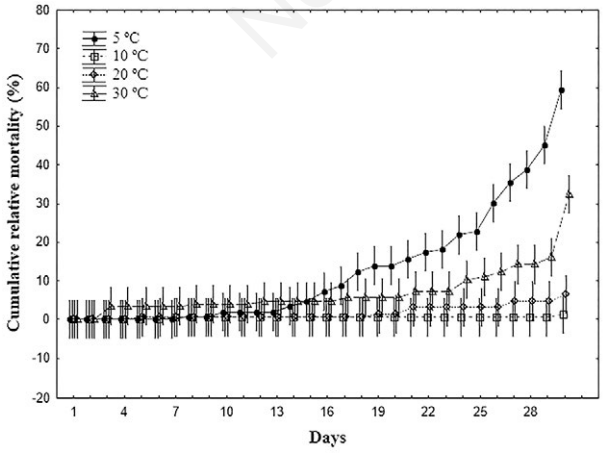

Fig. 3. Cumulative relative mortality (%) of *Limnoperna fortunei* with 95% confidence intervals for different temperatures (5°C, 10°C, 20°C and 30°C) throughout the 30 days under laboratory conditions.

(Montresor *et al.*, 2013). Thus, in the presence of high concentrations of ammonia, temperature increases may lead to death that is not due to the increased temperature itself but due to the increase in toxic ammonia concentrations. In the present work, we monitored water quality parameters and maintained ammonia concentrations at low levels to guarantee that temperature increases would not result in toxic concentrations of unionized ammonia. Thus, the mortality observed here was a direct effect of temperature, and this allowed us to effectively test *L. fortunei* response to temperature. The safe level of unionized ammonia for *L. fortunei* adults is 0.025 mg L^{-1} NH_3-N (Montresor *et al.*, 2013), and concentrations were kept below this range (0.00 to 0.003±0.001 mg L^{-1} NH_3-N) in the present work (Tab. 1). Temperature increases also decrease the concentrations of dissolved gases in the water, and oxygen concentrations thus decrease at high temperatures. The ideal concentration of oxygen is above 5.0 mg L^{-1} (Campos *et al*., 2016), and we kept the concentration above this value during the trials. Water pH is another vital parameter for aquatic animals. The tolerance range of *L. fortunei* lies between pH 6.0 and 9.0 (Darrigran *et al.*, 2011; Campos *et al*., 2016). Moreover, pH increases also induce unionized ammonia formation. During the trials, the pH was kept within the safe level for *L. fortunei*.

Oliveira *et al*. (2010) showed that at lower temperatures (5-7°C), mortality was approximately 80%. This discrepancy in the mortality results may be related to: i) the physiological conditions of the animals before the test; ii) differences in the size range of the mussels tested (21-27 mm used here and 8-13 mm by Oliveira *et al*. 2010); iii) differences among populations; or iv) an elevation in toxic ammonia during the experiment, which was not monitored by Oliveira *et al*. (2010), and inadequate water renovation (the water was replaced every week for the treatments using water from the Paraguay River). All these issues may influence the mortality rates of *L. fortunei* and generate discrepancies in the results among different studies, as reported by Montresor *et al*. (2013).

Our data also showed that the golden mussel tolerates low temperatures (5°C) but presented higher frequencies of closed valves and mortality. When the valves are closed for a prolonged period, the animals are forced to use stored food reserves (*e.g*., glycogen) and anaerobic respiration until energy resources are depleted or metabolic wastes reach a toxic level, causing the mortality of the mussels (Bayne *et al*., 1976). Our results are in accordance with previous data that showed that the lower temperature limits for *L. fortunei* growth are approximately 5 and 10°C (Nakano *et al*., 2011). The mortality rate was also significantly higher at 30°C than at 10°C and 20°C, although the mussels exposed to 30°C

presented lower frequencies of closed valves when compared to those at 10°C and 20°C. Previous studies showed high mortality rates in the bivalves *D. rostriformis bugensis* and *D. polymorpha* at 30°C (Mills *et al*., 1996; White *et al*., 2006), suggesting that high temperatures are harmful to these mussels. Down and Somero (2013) showed that the effect of temperature on the survival rate varies according to mytilid species. *Mytilus trossulus* Gould, 1850 was highly affected by the tested temperature (33°C), presenting a mortality above 80%, while *Mytilus californianus* Conrad, 1837 and *Mytilus galloprovincialis* Lamarck, 1819 showed lower mortality rates (20 and 40%, respectively).

Data in the literature show that high temperatures accelerate bivalve metabolism rates, with an increase in energy expenditure due to increased respiration, filtration and absorption rates (Schurink and Griffiths, 1992; Palais *et al*., 2011; Perepelezin and Boltovskoy, 2011). High temperatures increase the susceptibility of individuals to environmental stressors, which may be natural or anthropic (Palais *et al*., 2011; Montresor *et al*., 2013, White *et al*., 2015), and increase the excessive proliferation of algae and, especially, bacteria, which grow faster in experiments at higher temperatures (Perepelezin and Boltovskoy, 2011). Excretion rates also increase at high temperatures (Schurink and Griffiths, 1992), and the primary product excreted by bivalves is ammonia (Griffiths and Griffiths, 1987). This reinforces the importance of controlling the ammonia concentration in the water, especially when the experiments are conducted at high temperatures.

CONCLUSIONS

Although temperature was considered to be an important factor in the reproduction and establishment of golden mussels in different invaded areas, it was not considered as the single limiting factor for *L. fortunei* establishment in some geographic regions where the temperature ranges from 2°C to 16°C (Karatayev *et al*., 2015). Our physiological data reinforce this view, showing that *L. fortunei* survive under good conditions at 10°C and that, despite the high mortality rate, mussels maintain their energy reserves (glycogen) even when exposed to temperatures as low as 5°C.

The ability to withstand stressful periods during the dispersal process is crucial to invasion success (Mackie and Claudi, 2010). Several stressors can act as a barrier, including temperature (low or high), drought, low oxygen level or other aspects related to poor water quality. Our data show that *L. fortunei* had the ability to endure periods (up to 30 days) of low water temperatures with minimal impact on glycogen concentrations. This feature increases the ability of this species to invade new areas. During the dispersal process, mussels can withstand relatively long periods at low water temperatures and maintain sufficient energy reserves to found new populations. This increases the list of physiological and ecological features that contribute to the dispersal ability of this mussel (Mackie and Claudi, 2010).

Based on our results and the data in the literature, the ideal temperature for rearing and testing mussels in the laboratory is approximately 20°C. In fact, i) most of the individuals remained with their valves open, indicating that vital activities, such as feeding and breathing, were maintained; ii) the glycogen levels remained stable (without physiological stress); and iii) the mortality rate was low. In addition, this temperature also may minimize the disturbance to mussels caused by the excessive proliferation of algae and bacteria, which grow faster at higher temperatures, as observed by Perepelizin and Boltovskoy (2011). We add data to the growing literature on *L. fortunei* demonstrating that combinations of physiology and behavior may have crucial roles in the ecological success of invasive species.

ACKNOWLEDGMENTS

We thank Stênio Augusto de Souza Coelho for technical support and Dr. D. R. Fernandez (Itaipu Binacional), Francisco Ludwig and Dr. S. Ludwig for logistical support during sampling. This work was supported by grants from the foundation for research from the State of Minas Gerais Fapemig and Vale S.A (CRA - RDP-00097-10), CNPq and Capes.

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