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# Effects of L-proline on swimming parameters of *Daphnia magna* subjected to heat stress

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ABSTRACT

L-proline (L-PROL) is an essential amino acid, a constituent of many proteins and the osmoprotective molecule produced and accumulated in higher plants and some freshwater microalgae in response to various environmental stressors. Knowledge on thermoprotective effects of this amino acid on freshwater invertebrates is very scarce. Therefore the aim of our study was to determine the effect of L-PROL at concentrations: 10 mg/L, 20 mg/L and 50 mg/L on swimming behavior (immobilization, swimming track density, swimming speed, turning ability) of *Daphnia magna* subjected to temperatures: 22 °C, 35 °C and 38 °C. We found that L-PROL elevated all the measured swimming parameters at 22 °C when compared to the untreated crustaceans. Furthermore, L-PROL alleviated heat-induced inhibition of these parameters in the experimental animals subjected to 35 °C. The results suggest that L-PROL stimulates swimming performance and alleviates alterations of swimming parameters induced by heat stress in *D. magna*. Moreover, these findings may support the hypothesis that in natural conditions, L-PROL may protect crustaceans against thermal stress.

## 1. Introduction

*Daphnia magna* is a keystone species in freshwater ecosystem with many important roles as filter feeders and as a food source for fish (Filella et al., 2008). Swimming behavior of these crustaceans characterized by several measurable parameters such as swimming speed, track density, vertical migration turning angle ect. has been used as a sensitive indicator of stressful factors (Shimizu et al., 2002; Ren et al., 2017; Bownik et al., 2018).

Physiological and biochemical processes in the crustaceans may be maintained at a certain range of temperature (Mezquita et al., 1999). Excessive cold or heat of the aquatic environment may disrupt the homeostasis of the living organism and may also have ecological impacts such as predator-prey relationships between *Daphnia pulex* and *Chaoborus americanus* (Riesen, 2015; Klumpen et al., 2017). Although *Daphnia* developed genetic and physiological mechanisms to adapt to seasonal and long-term changes of ambient temperature, swimming behavior is one of the fastest endpoints responding to thermal stress (Gerritsen, 1982; De Meester et al., 2011; Ziarek et al., 2011). Escape behavior or vertical migration of the crustaceans from the sunlight-exposed surface to deeper colder parts of the water column are common examples of behavioural responses of crustaceans to the increased temperature of surface water, occurring particularly during hot summer seasons (Pijanowska and Kowalczewski, 1997; Gerritsen, 1982). Daphniid swimming behavior is very complex and comprises many endpoints specifically responding to a given stressor. Therefore, several parameters are typically used in a single study (Bownik et al., 2018). Track density is a novel experimental tool indicating density of trails left by a swimming daphniid in the observation dish (Bownik et al., 2018). This parameter indicates the ability to sense the environment by showing movement trails of a daphniid swimming in various parts of the observation dish. Swimming speed is the next parameter that may be impaired by a variety of factors (Shimizu et al., 2002; Barrozo et al., 2015; Bownik, 2017). This parameter reflects the effectiveness of neuromuscular transmission in the synaptic endplates of the second antennae responsible for swimming movements. Daphniid turning ability is an endpoint indicating spatial orientation by showing changes in the swimming route (Dees et al., 2008; Betini et al., 2016). A decrease or excessive increase of turnings may suggest disturbances in movement coordination.

Little is known about the protective role of natural substances commonly present in the aquatic environment in crustaceans subjected to heat stress. Our previous study showed that the osmoprotective amino acid, ectoine alleviated inhibition of *D. magna* swimming speed subjected to heat stress (Bownik et al., 2014). L-PROL is an essential amino acid being a component of collagen and other proteins (Li and

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Wu, 2018) and also playing an important role in nutrition or wound healing (Wu et al., 2011). This molecule is also an osmoprotectant known to be accumulated in bacteria, algae and yeast in a response to various environmental stressors (Rajendrakumar et al., 1994; Roessler and Müller, 2001; Liang et al., 2014; Mahipant et al., 2017). Heat stress is one of environmental factors stimulating some species of terrestrial plants, freshwater bacteria, yeast and filamentous algae to produce higher levels of L-PROL (Hayat et al., 2012; Cvikrová et al., 2013; Zhang et al., 2015; Vives-Peris et al., 2017; Hildebrandt, 2018). Experimental studies showed thermoprotective effects of this amino acid in fish during bacterial infections or heat stress (Zhao et al., 2015). However, little is known on the influence of L-PROL on swimming activity of zooplankters subjected to elevated temperature. Therefore, our goal was to determine effects of L-PROL on *D. magna* swimming activity (using track density, speed, turning ability as endpoints) subjected to heat stress.

## 2. Material and methods

### 2.1. Animal culture

*D. magna* were cultured in a continuous parthenogenetic reproduction started from a single female hatched from a dormant egg. The culture was maintained for several generations in 6 L tanks with 5 L of aerated medium under light:dark period of 16 h: 8 h with a constant temperature of 22 ± 1 °C. The experimental medium (48 mg of $NaHCO_3$, 30 mg of $CaSO_4{\cdot}2H_2O$, 30 mg of $MgSO_4$ and 2 mg of KCl per liter of deionized water adjusted to a pH of 7.4) was prepared according to ASTM standards (American Society of Testing and Materials, 1986). The number of cultured daphniids was about 30 animals per 1 L. Experimental crustaceans were fed once daily with 5 mL per container of baker's yeast suspension (10 mg/L). Neonates (≤24 h old) were used in the experiment. The animals were not fed 24 h before the study and during the exposure to L-PROL.

### 2.2. Experimental design

L-PROL (> 98% purity) purchased from Sigma-Aldrich (USA) was diluted in *Daphnia* culture medium to the following concentrations: 0 mg/L, 10 mg/L, 20 mg/L and 50 mg/L. Each concentration of L-PROL was added to three (triplicate) observation dishes (35 mm diameter) at a volume of 5 mL. Afterwards, 10 daphniids were transferred to each dish. The dishes were incubated at one of three temperatures: 22 °C, 35 °C or 38 °C in a thermoblock with a built-in calibrated thermometer and automated system maintaining the temperature. Behavioural biomarkers (immobilization, swimming track density, swimming velocity, turning angles) were determined at 2 h, 24 h and 48 h of incubation. All the animals used in the experiment came from the same culture batch. The experiment was done at light: dark conditions of 16 h: 8 h. Culture medium parameters (pH-7.8 ± 0.4, oxygen concentration-9.0 ± 0.9 mg/L, conductivity-386 μS/cm, temperature-22 ± 1 °C) were constant before the experiment. Animals with ceased motility for at least 15 s were treated as immobilized.

### 2.3. Swimming speed

Swimming speed of *D. magna* neonates was determined according to the method previously described by Bownik et al. (2018). Swimming behavior of the animals was video recorded for a minimum of 1 min (with a speed of 30 frames per second) with a digital camera installed on a stand. During video recording the observation dishes containing the experimental animals were kept in the thermoblock to maintain the temperature. Because of small depth of the solution vertical swimming of crustaceans was negligible. Swimming tracks of *D. magna* were visualized and analyzed by a frame-by-frame method with the use of Tracker® 4.11.0 software. In brief, the swimming track left by a single individual (interpreted as a mass point) and mean velocity (v) expressed in millimeters per second was measured by clicking with the cursor on *D. magna* image in each separate frame of the clip. Since the experimental animals in the dish were moving virtually only in two dimensions, analysis of swimming behavior was based on the trajectory represented by x and y coordinates. Average speed for 10 individual daphniids in each experimental group was calculated by the software and plotted as superimposed amplitudograms in separate graphs. The mean speed of 10 individual daphniids from each experimental group was treated as a result.

### 2.4. Imaging of swimming track density

Swimming track density was measured by calculation of the percentage of swimming tracks that covered the area of the observation dish in each experimental group according to the method previously described by Bownik et al. (2018). Briefly, processing of a 1-min video recording with Tracker® 4.11.0 software generated an image with trails left by 10 swimming daphniids on a background with x and y coordinates. The image was then transformed to 1 colour depth with Toupview 3.7 software producing a black and white image with all the tracks treated as black pixels. The result of the analysis was the percentage of black pixels (in relation to white background of the observation dish) from each experimental group was calculated by the histogram analysis.

### 2.5. Turning ability

Turning activity of the swimming daphniids was determined with Tracker® 4.11.0 software. In brief, after visualisation the swimming tracks in the software, a 2-dimensional coordinate system for the observation dish was established to determine the turning angles. The result of the analysis was a graph showing change of angle (θ) visible in the y axis of the graph against time (t) on x axis. The parameter was calculated by the software for 10 individual daphniids in each experimental group and plotted on the graph during 1 min recording. Turning ability was calculated by subtraction of the minimal θ from maximal θ value for an individual daphniid and the data from 10 individuals from each experimental group were averaged.

### 2.6. Statistical analysis

Statistical analyses were done with Statistica® 13.1 software. Data normality and homogeneity of variances were determined by the Shapiro-Wilk and Levene's tests, respectively. The comparisons of means among the groups were done by ANOVA followed by the post-hoc Tukey's. The significance level was set to be at least $p < 0.05$. The results are presented as means ± standard deviation (SD).

## 3. Results

### 3.1. Immobilization

Daphniids treated with the experimental concentrations of L-PROL at 22 °C were not immobilized after 2 h, 24 h and 48 h, however, the heat-stressed animals showed inhibited motility (Fig. 1). Although rapid immobilization was noted in daphniids subjected to 38 °C (100% of immobilized *D. magna* at 50 mg/L of L-PROL) no differences after 2 h and 24 h was observed between the control animals and those treated with 10 mg/L or 20 mg/L of L-PROL. The most pronounced alleviation of immobilization was manifested by daphniids subjected to 35 °C (Fig. 1a). The immobilization rate in both non-treated and the exposed groups to 50 mg/L of L-PROL was 80 ± 5%, however lower rates were at 10 mg/L and 20 mg/L (70 ± 6% and 40 ± 6%, respectively) after 48 h.

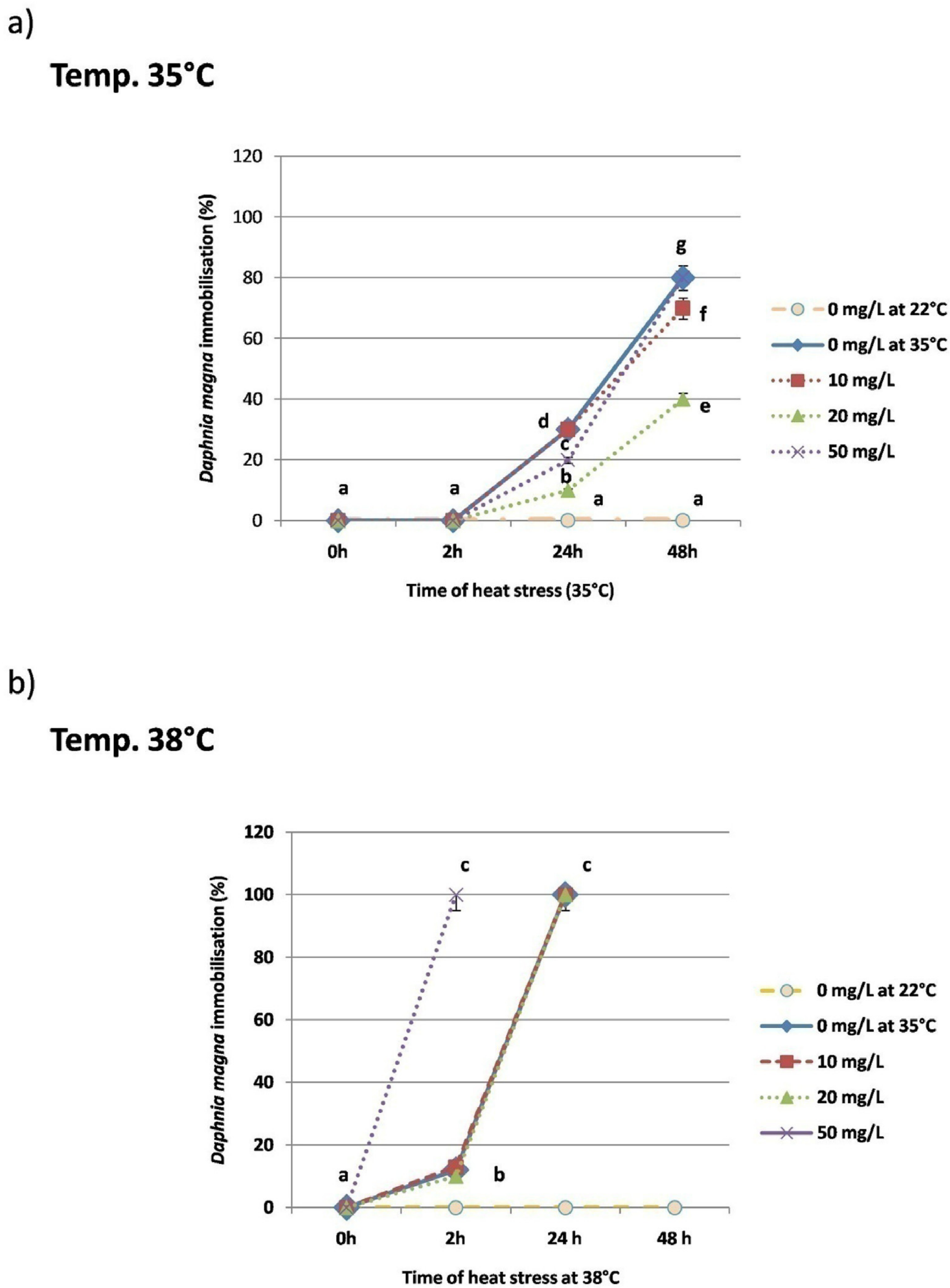


**Fig. 1.** Immobilization of *Daphnia magna* subjected to heat stress at 35 °C (a) and 38 °C (b) and treated with different concentrations of L-proline. Results are presented as means ± SD; n = 30. Values not sharing a common superscript letter (a–g) differ significantly.

### 3.2. Swimming track density

*D. magna* treated with L-PROL at a temperature of 22 °C manifested a concentration-dependent increase of swimming track density (Fig. 2). Augmentation of the parameter was observed after 2 h of the exposure to both 10 mg/L and 20 mg/L (33 ± 5% of BP (Black Pixels) and 35.7 ± 2% of BP, respectively) when compared to the untreated animals (28.56 ± 2% of BP). Swimming track density was also elevated at 50 mg/L increasing rapidly from 29 ± 2 of BP after 2 h to 38.11 ± 2% of BP after 24 h. The untreated daphniids exposed to 35 °C showed the most distinct reduction of swimming track density (from 28.56 ± 2% at 0 h to 12.38 ± 2% of BP) at 48 h. The most significant attenuation of the decrease occurred at 20 mg/L of the amino acid (26.13 ± 2% of BP) at 48 h. Daphniids subjected to 38 °C showed a rapid decrease of swimming track density at 2 h of the exposure. No significant differences were found both between the experimental groups and between the control and the experimental groups. Surprisingly, 50 mg/L of L-PROL induced 100% immobilization of the test animals.

### 3.3. Swimming speed

L-PROL increased swimming speed of daphniids exposed to 22 °C (Fig. 3). The highest stimulation of the parameter was noted in the animals treated with 20 mg/L after 24 h (3.35 ± 0.03 mm/s) when compared to the untreated control (2.56 ± 0.3 mm/s). Lower increase of this parameter at that time of exposure was found in the group exposed to 10 mg/L of the amino acid (2.84 ± 0.4 mm/s). The untreated *D. magna* subjected to a temperature of 35 °C showed a reduction of

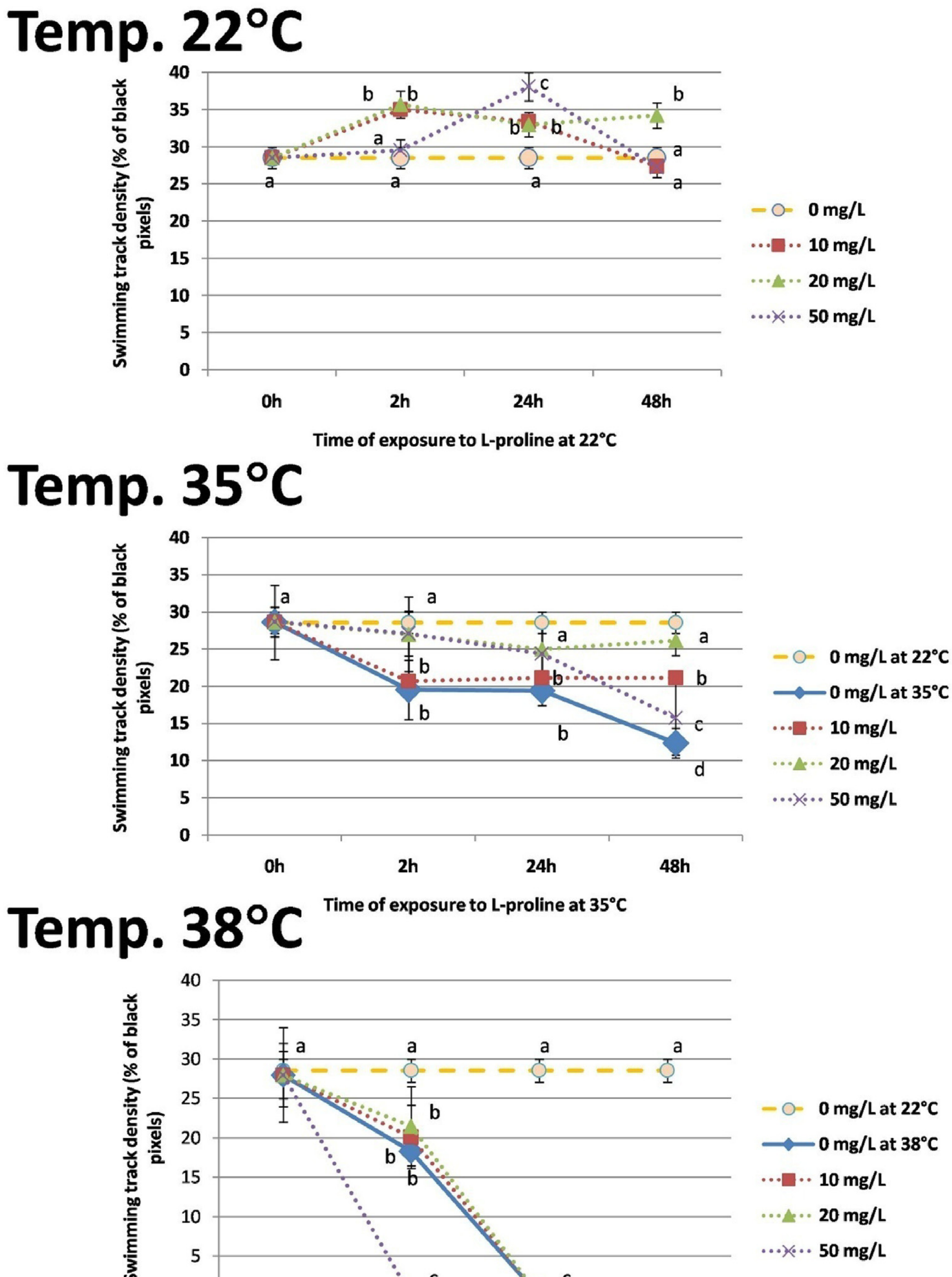


**Fig. 2.** Imaging of swimming track density of *Daphnia magna* exposed to various concentrations of L-proline at 22 °C, 35 °C and 38 °C. The results are presented as means ± SD; n = 30. Values not sharing a common superscript letter (a–d) differ significantly.

swimming speed with a complete cessation of movement after 48 h. On the other hand, L-PROL at 20 mg/L attenuated the inhibition after 2 h and 24 h (1.41 ± 0.1 mm/s and 1.45 ± 0.4 mm/s, respectively when compared to the untreated group (0.93 ± 0.2 mm/s and 1.06 ± 0.1 mm/s at 2 h and 24 h, respectively). However, the parameter was completely depressed after 48 h of the exposure. The untreated crustaceans exposed to 38 °C showed a rapid decrease of swimming speed after 2 h (0.45 ± 0.5 mm/s) and no motility was observed after 24 h. The L-PROL-treated daphniids did not show statistical differences as compared to the untreated group.

### 3.4. Turning ability

*D. magna* exposed to L-PROL at 22 °C showed alteration of turning ability (θ max-θ min) when compared to the untreated animals (Fig. 4a and b). Graphs from Tracker® (Fig. 4 a) showing angle changes (θ) (shown as trail curves) of individual daphnids indicate that the highest turning ability (highly curved trails) was noted after 24 h at 20 mg/L of L-PROL (θ maxθ-min = 59.5 ± 12) as compared to the untreated daphniids (θ max-θ min = 35 ± 15). Although turning ability was decreased in the untreated group after 48 h (θ max-θ min = 27 ± 13) it was augmented at 20 mg/L and 50 mg/L (θ max-θ min = 50.6 ± 12

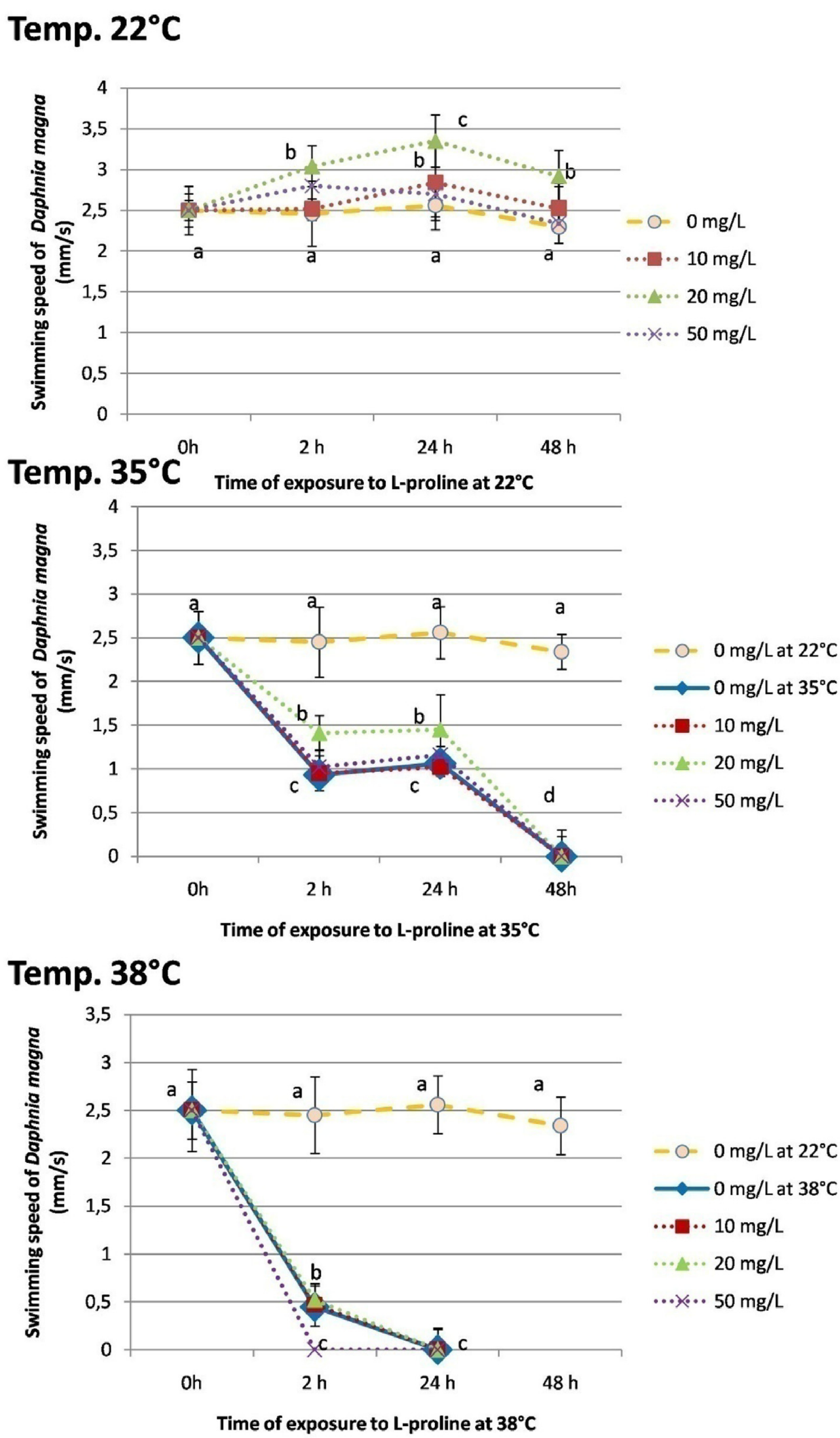


**Fig. 3.** Effects of L-proline on swimming speed of *Daphnia magna* subjected to various temperatures: 22 °C, 35 °C and 38 °C. Results are presented as means ± SD; n = 30, values not sharing a common superscript letter (a–d) differ significantly.

and 47.1 ± 16, respectively). Exposure to a temperature of 35 °C resulted in the inhibition of turning ability of untreated *D. magna* reaching the lowest value after 48 h of the exposure (θ max-θ min = 14 ± 14) (Fig. 5a and b). On the other hand, animals treated with L-PROL at 10 mg/L, 20 mg/L and 50 mg/L showed increased values of this parameter (θ max-θ min = 34 ± 16, 37 ± 33, 36.5 ± 23, respectively). The animals stressed with a temperature of 38 °C manifested a rapid inhibition of turning ability after 2 h (Fig. 6a and b). The complete reduction of the parameter was noted in the group exposed to 50 mg/L of L-PROL. No statistical differences were noted between experimental groups treated with the amino acid and the control at 38 °C.

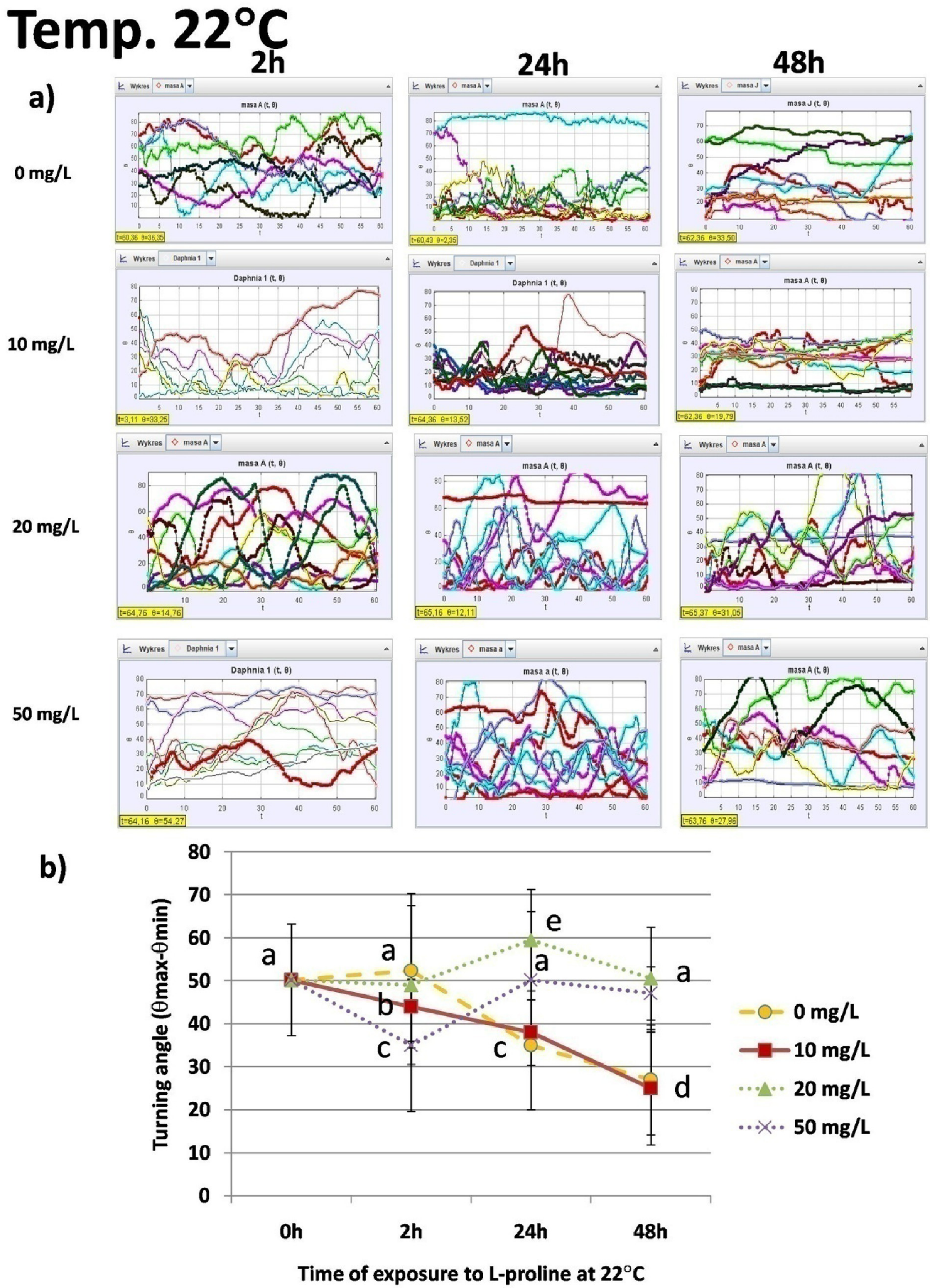


**Fig. 4.** Effects of L-proline on turning ability of *Daphnia magna* at a temp. of 22 °C. Panel a) shows graphs of angle changes for individual daphnids obtained with Tracker® at different times of exposure and concentrations of the amino acid. Panel b) shows the average turning ability. Results are presented as means ± SD, n = 30. Values not sharing a common superscript letter (a–e) differ significantly.

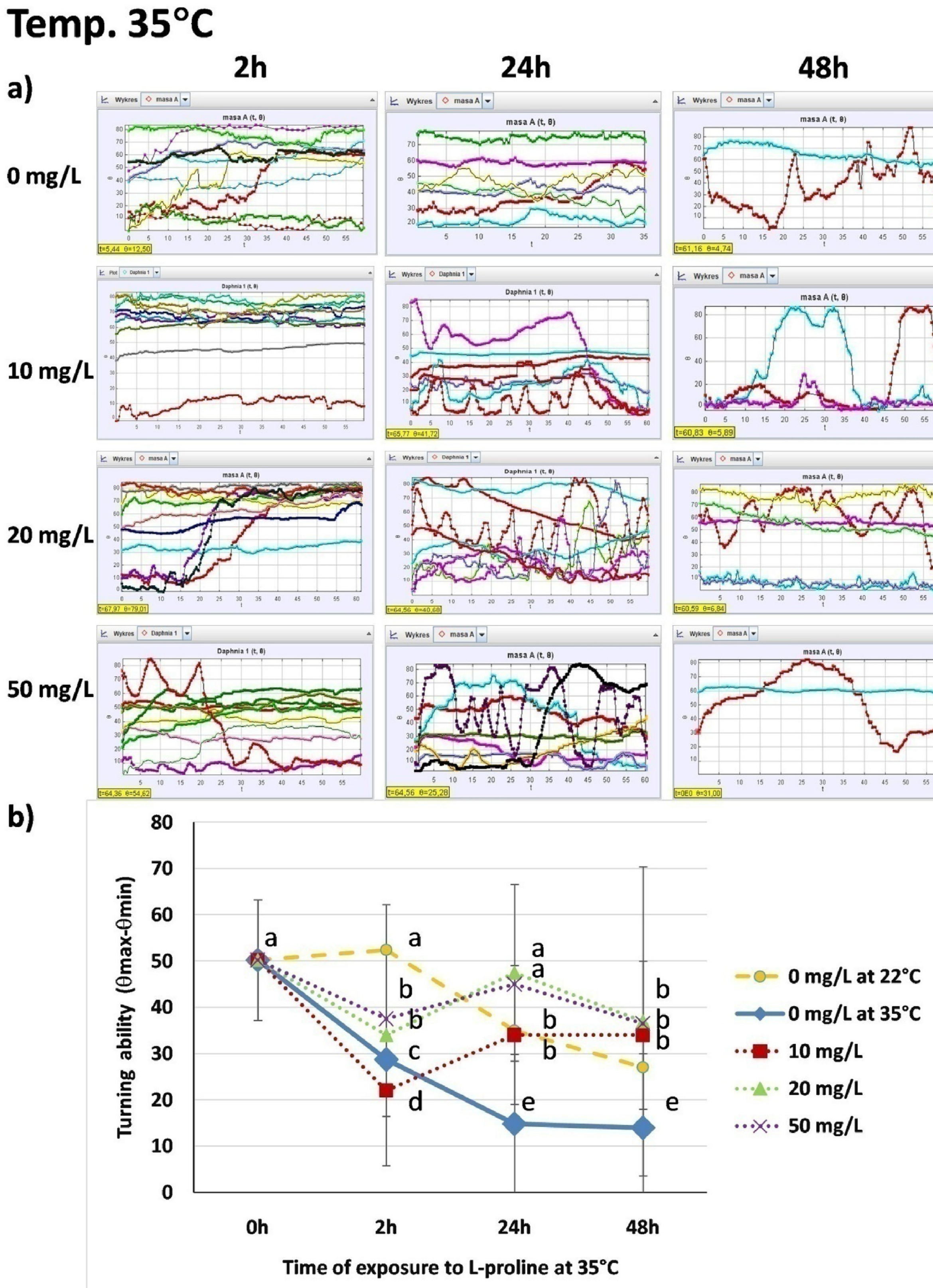


**Fig. 5.** Effects of L-proline on turning ability of *Daphnia magna* subjected to a temp. of 35 °C. Panel a) shows graphs of angle changes for individual daphnids obtained with Tracker® at different times of exposure and concentrations of the amino acid. Panel b) shows the average turning ability. Results are presented as means ± SD, n = 30. Values not sharing a common superscript letter (a–d) differ significantly.

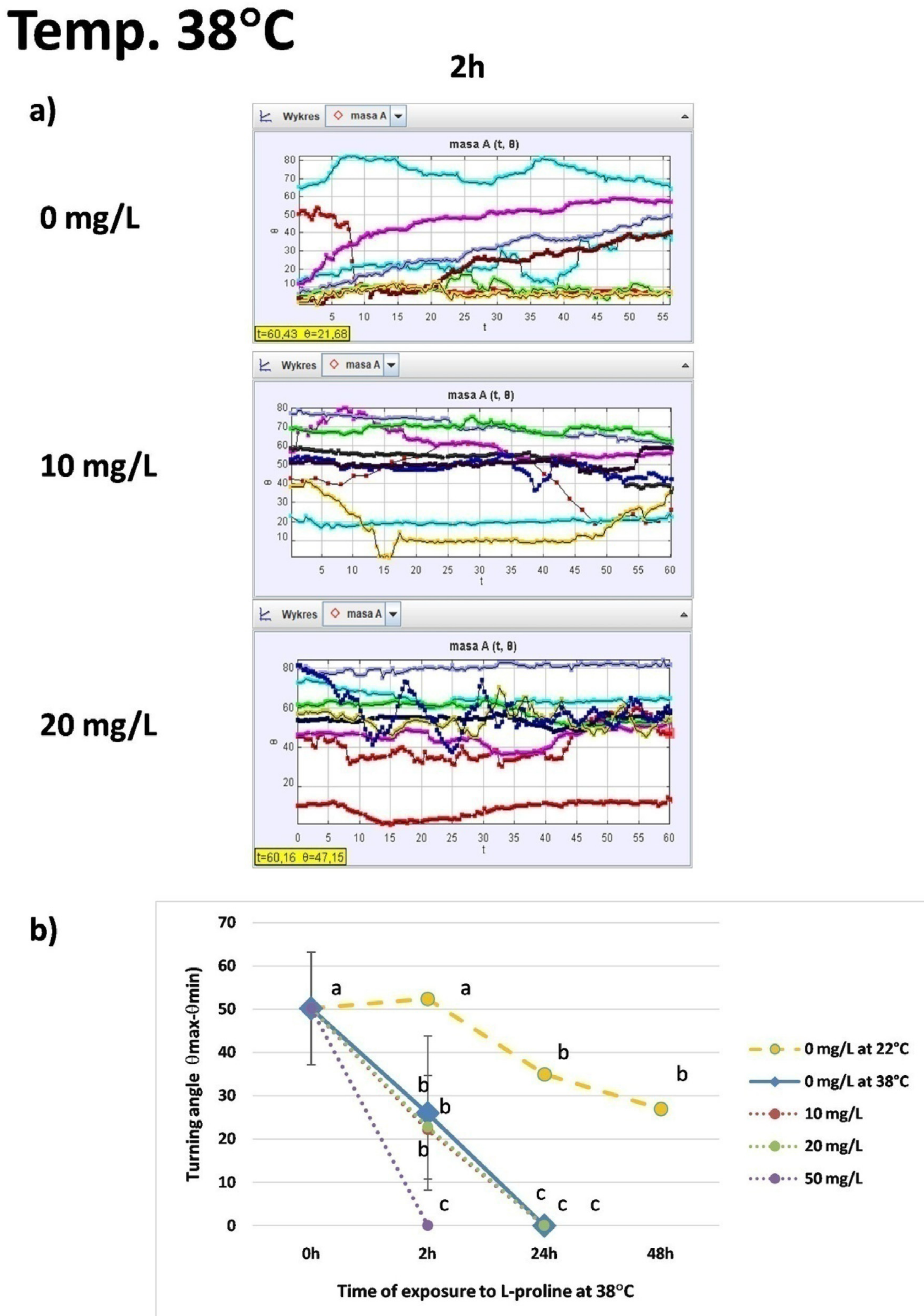


**Fig. 6.** Effects of L-proline on turning ability of *Daphnia magna* subjected to a temp. of 38 °C. Panel a) shows graphs of angle changes for individual daphnids obtained with Tracker® at different times of exposure and concentrations of the amino acid. Panel b) shows the average turning ability. Results are presented as means ± SD; n = 30. Values not sharing a common superscript letter (a–d) differ significantly.

## 4. Discussion

The present study showed that L-PROL increased swimming performance of *D. magna* at a temperature of 22 °C. Swimming track density was previously determined in daphniids exposed to a dopamine agonist, apomorphine (Bownik et al., 2018). Here we show, that this endpoint was elevated in L-PROL-treated experimental groups, which suggests an enhancement of the sensory and locomotory activities and thus improved the daphniid ability to explore the surrounding environment. Daphnid swimming speed is a common biomarker widely used in determination of swimming behavior (Shimizu et al., 2002; Barrozo et al., 2015; Bownik, 2017) Our study revealed that L-PROL increased this endpoint in daphniids which suggests that the amino acid may increase the effectiveness of signal transmission in neuromuscular synapses of the daphniid second antennae. We also found that L-PROL-induced stimulation of turning ability of *D. magna* indicating that the daphnid ability to coordinate their movements was improved. It is to note that the middle level of L-PROL induced a considerable increase of each measured endpoint of the swimming behavior. Although stimulation of swimming performance was found at the highest amount of the amino acid, the indices were lower than those observed in the animals exposed to the middle level. This suggests that an effective augmentation of daphniid swimming activity occurs at a narrow range of concentrations of PROL.

Although amino acids are known to be constituents of structural proteins for animals (Katayama et al., 2016; Li and Wu, 2018) some reports indicate that may also be used as nutrients, neurotransmitters and modulators of neuromuscular transmission in insects, fish and mammals (Brosnan and Brosnan, 2013; Zinellu et al., 2013; Moinard et al., 2017). Therefore, two hypotheses may be proposed as the explanation of stimulatory effects of PROL on *D. magna* swimming performance: i) modulatory activity of L-PROL on the neurotransmission system, ii) exploitation L-PROL by the zooplankters as a nutrient source (Wood, 1988).

The first hypothesis may be supported by studies in mammals (Henzi et al., 1992). For example, a study in mice lacking L-PROL transporter showed a decreased locomotor activity (Shulz, 2011). The second hypothesis is supported by the finding that some bee and wasp species possess a unique feature to oxidize L-PROL used as metabolic fuel for flight (Teulier et al., 2016). On the other hand, L-PROL as an agonist of the glycine receptor and NMDA and non-NMDA-glutamate ionotropic receptors was found to inhibit transmission by rat neurons (Henzi et al., 1992). Inhibitory effects of a bacterial amino acid ectoine on *D. magna* swimming speed was noted by Bownik et al. (2015). Different responses of the crustacean swimming speed to L-PROL and ectoine may result from the fact that ectoine may not be used as a carbon source.

A number of studies showing that exposure of *D. magna* to various stressful factors may lead to complete cessation of movement (Zhang et al., 2014; Heger et al., 2018; Toumi et al., 2018). Our study showed that *D. magna* subjected to heat stress both at 35 °C and 38 °C were immobilized as a consequence of denaturation of cell membrane proteins and enzymes, however considerable alleviation of the immobilization of the L-PROL-treated animals may be associated with the increased stability of structural proteins and enzymes. These findings are similar to those obtained by Bownik et al. (2014) who found a reduction of the number of the immobilized heat-stressed *D. magna* treated with the osmoprotective amino acid, ectoine (Bownik et al., 2014). On the other hand, we did not notice the differences after 24 h-exposure between the L-PROL treated and untreated animals subjected to 38 °C probably because detrimental changes progressed too rapidly. Although the highest concentration of PROL alleviated the immobilization of daphniids exposed to 35 °C after 24 h, the amino acid was not effective after 48 h and also it did not alleviate immobilization at 38 °C. This effect may be explained by the fact that PROL may induce a slighter thermoprotective effect at high levels.

The present study also showed that heat stress at 35 °C decreased swimming track density, speed and turning ability in the non-treated animals, whereas alleviation of the reduction was found in L-PROL-treated animals. Since exposure of *D. magna* to 38 °C induced a very rapid decrease of the parameters in both treated and untreated daphniids, no differences between these groups were noted. Alleviation of the heat stress-induced alterations of behavioural endpoints may be a consequence of the enhanced stabilization of macromolecules and cell membranes in the heat-stressed crustaceans treated with L-PROL (Huang et al., 2015). Additionally, reduction of oxidative damage during heat stress by of the amino acid at the medium concentration may also be a possible reason of the alleviatory effects (Cvikrová et al., 2013). Interestingly, although the reduction of all the measured swimming endpoints was alleviated most effectively at 20 mg/L of PROL, the highest concentration of the amino acid potentiated the inhibition of this parameter in heat-stressed daphniids. This effect may be associated with the fact that PROL at higher levels may induce some detrimental effects.

In summary, the present findings indicated stimulatory effects of L-PROL on swimming performance of *D. magna* in addition to its alleviatory effects during heat stress. L-PROL acts probably via different mechanisms in different thermal conditions. The augmented swimming performance noted at normal temperature may result from the stimulatory potential of the amino acid on the neuromuscular system or from its nutritional enhancement. Furthermore, the alleviation of the inhibition rate and swimming activity should be associated with thermoprotective properties of L-PROL characteristic to most osmolytes. Since L-PROL is synthesized and accumulated in phytoplankton subjected to heat stress (Kumar et al., 2016; Calatrava et al., 2018), it may be hypothesized that in natural conditions exposure to L-PROL or consumption of food rich in L-PROL protein may naturally enhance resistance of *D. magna* to higher temperatures in the aquatic environment, particularly during hot summer periods, however a detailed study is needed to confirm this hypothesis.

## Conflicts of interest

The authors declare no conflict of interest.

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