

Resistance to low oxygen in the Ponto–Caspian amphipod *Pontogammarus robustoides* varies among lentic habitats of its northern invaded range



Eglė Šidagytė^{a,*}, Kęstutis Arbačiauskas^{a,b}

^a Laboratory of Evolutionary Ecology of Hydrobionts, Nature Research Centre, Vilnius, Lithuania

^b Department of Zoology, Faculty of Natural Sciences, Vilnius University, Vilnius, Lithuania

ARTICLE INFO

Article history:

Received 8 April 2016

Received in revised form 29 June 2016

Accepted 6 September 2016

Available online 9 September 2016

Keywords:

Anaerobic metabolism

Hypoxia

Invasive species

Lactate dehydrogenase

Lakes

ABSTRACT

The Ponto–Caspian amphipod *Pontogammarus robustoides* was introduced into Lithuanian inland waters more than 50 years ago and is now among the most successful local crustacean invaders. Existing as an oxyphilic species in its native range, in Lithuania it managed to establish in some lentic eutrophic waters facing long-term hypoxic conditions under winter ice cover, or shorter periods of hypoxia during the warm season. Recently, it has been observed to be further expanding in such waters. The aim of this study was to explore the possible divergence in anaerobic metabolism among introduced populations facing different selective pressures. A closed-bottle experiment was conducted using individuals from three Lithuanian lentic water bodies of different trophic status: mesotrophic, eutrophic and hypertrophic. Severe hypoxia was gradually reached, after which lactate dehydrogenase activity was significantly higher in amphipods from eutrophic and hypertrophic, than from mesotrophic environments, and was well correlated with trophic status of inhabited environments as assessed by chlorophyll *a* concentration. These findings suggest a physiological acclimation response to oxygen deficiency faced by the species in some environments in the northern invaded range, which may have a genetic background. Such resistance to oxygen deficiency may expand the environmental niche and promote species' spread into previously unsuitable habitats, which may pose a threat to native species residing in refuges that are currently free of the invader.

© 2016 Elsevier GmbH. All rights reserved.

1. Introduction

Ponto–Caspian crustaceans are among the most successful invaders in Europe and the North American Great Lakes owing to their wide environmental tolerances and high phenotypic variability arising from the complex geological past of their native region (Reid and Orlova, 2002). In the early 1960s, the amphipod *Pontogammarus robustoides* (Sars, 1894) and several other Ponto–Caspian peracaridans from Ukrainian waters were deliberately introduced into the Kaunas Water Reservoir (WR) located on the Nemunas River in Lithuania to increase fish production. After a successful establishment they naturally spread downstream to the Curonian Lagoon (Gasiūnas, 1972). In more recent years, these introduced peracaridans have been officially transferred to

numerous lakes and reservoirs in Lithuania (and beyond the borders of the country) but have failed to establish in all of them (Arbačiauskas et al., 2010). Most introduced peracaridan species, including *P. robustoides*, also show an ability to spread within and outside Lithuania naturally or at least without the intentional aid of man (Gasiūnas, 1972; Arbačiauskas et al., 2010, 2011b). The initial purpose of the introduction, that is, the improvement of fish production, seems not to have been achieved (Arbačiauskas et al., 2010). Instead, *P. robustoides* proved to have a negative effect on native macroinvertebrate communities (Arbačiauskas, 2005; Gumuliauskaitė and Arbačiauskas, 2008).

The native habitat of *P. robustoides* ranges from the lower reaches of freshwater rivers to large transitional waters (Morduhaj-Boltovskoj et al., 1972); therefore, it is considered an oxyphilic species. However, it has been observed that in native and new ranges, *P. robustoides* is more likely to survive in lentic waters and is less sensitive to adverse oxygen conditions than are other Ponto–Caspian amphipods (Morduhaj-Boltovskoj et al., 1972; Dedyu, 1980; Żytkowicz et al., 2008). Among alien amphipods in

* Corresponding author at: Nature Research Centre, Akademijos Str. 2, LT-08412 Vilnius, Lithuania.

E-mail address: e.sidagyte@gmail.com (E. Šidagytė).

Lithuania, this species is the most widely distributed within lentic habitats and is thus considered the best adapted to such environments (Arbačiauskas, 2005; Arbačiauskas et al., 2011b). Until now its distribution in lentic Lithuanian waters was mostly limited to large open mesotrophic lakes (Arbačiauskas, 2005; Arbačiauskas et al., 2011b), in accordance with its oxyphilic preference.

In contrast, in several eutrophic reservoirs or lakes with high water turnover in which *P. robustoides* nevertheless successfully established, populations have been facing substantial selective pressure with respect to oxygen conditions, for more than 50 years. In highly eutrophic lentic waters, these conditions may seasonally worsen during summer algal blooms and even more so under winter ice cover, which persists for much longer periods than in the southern native range of the species (Arbačiauskas and Gumuliauskaitė, 2007). Such water bodies may have provided conditions for this invasive species to evolve resistance to low oxygen levels. This, and the fact that in Lithuania *P. robustoides* has been recently observed to have naturally spread through eutrophic and even hypertrophic waters (Arbačiauskas, unpublished data), may provide evidence for such a microevolutionary change.

Physiological responses to hypoxia have been broadly studied in invertebrates (Herreid II, 1980; Ellington, 1983; Livingstone, 1983, 1991; Grieshaber et al., 1994). The main pathway of anaerobic metabolism in crustaceans is the lactate pathway (Livingstone, 1983, 1991; Grieshaber et al., 1994); therefore, research on physiological response to hypoxia usually relies on lactate build-up and recovery patterns (Pritchard and Eddy, 1979; Bridges and Brand, 1980; Agnew and Taylor, 1985; Agnew and Jones, 1986; Hill et al., 1991; Anderson et al., 1994; Spicer et al., 2002; Silva-Castiglioni et al., 2010, 2011) or quantification of the activity of the glycolytic enzyme lactate dehydrogenase (LDH) (Pritchard and Eddy, 1979; Gonzalez and Quiñones, 2002; Yaikin et al., 2002; Bishop et al., 2004) catalysing the interconversion of pyruvate and lactate using nicotinamide adenine dinucleotide (NAD) as a coenzyme. Animal response to temporal hypoxia events in the lactate pathway corresponds to a physiological acclimation, a type of phenotypic plasticity with a possibly underlying genetic basis. Such highly divergent inter- (Pritchard and Eddy, 1979; Gonzalez and Quiñones, 2002; Silva-Castiglioni et al., 2010, 2011) and intraspecific (Agnew and Jones, 1986) responses in the lactate pathway have been suggested to be outcomes of evolutionary change, i.e. genetic adaptations to selective pressures.

In this study, we set out to test whether, having been introduced into environments with varying probabilities of emerging adverse oxygen conditions, *P. robustoides* populations differ in LDH activity levels under hypoxic stress, implying different physiological acclimation response and possible microevolutionary change in the northern invaded range. To this end, a closed-bottle experiment was conducted involving *P. robustoides* populations from three Lithuanian lentic water bodies of different trophic status, ranging from mesotrophic to hypertrophic.

2. Materials and methods

2.1. Water bodies and experimental animals

Experimental animals were collected in August 2012, in three Lithuanian water bodies: mesotrophic Lake Dusia, eutrophic Kaunas WR, and hypereutrophic Lake Simnas (see Table 1 for water body characteristics). The species was deliberately introduced in the early 1960s into all of these water bodies (Gasiūnas, 1972; Arbačiauskas et al., 2011b), where it has now been established for at least 50 years. In each water body, sampling was performed in wadeable depths of a 50-m stretch of open stony littoral areas in habitats preferred by the species. Random adult amphipods were

Table 1

Hydrochemical indicators of trophic status and morphological characteristics of water bodies sustaining populations of the amphipod *Pontogammarus robustoides* used in the current study. BOD₇ is the biochemical oxygen demand for 7 days. Multiannual means of hydrochemical data were compiled from available data for the period 2001–14 from the Lithuanian Environmental Protection Agency (<http://vanduo.gamta.lt/cms/index?rubricid=8ea41f73-9742-4d71-aa10-0a5988713fe5>, accessed on 3 March 2016).

Water body	Lake Dusia	Kaunas WR	Lake Simnas
Total P, µg L ⁻¹	34.0	114.0	79.3
Total N, µg L ⁻¹	763.4	1549.1	1591.7
Chlorophyll <i>a</i> , µg L ⁻¹	5.7	24.7	31.6
BOD ₇ , mg L ⁻¹	1.9	3.3	4.2
Transparency, m	5.3	2.1	1.4
Trophic status	Mesotrophic	Eutrophic	Hypereutrophic
Mean depth, m	15.4	10.5	2.3
Area, km ²	23.3	63.5	2.4
Retention, years	16.7	0.2	0.6
Comp. catchment	4.7	~110.2	74.6

caught using a dip net and transported to the laboratory in a cooler. Amphipods from each population were placed in a 5-L volume of bacteria-filtered and constantly aerated (90–100% oxygen saturation) water and kept for 48 h to acclimate to the experimental temperature. Animals were fasted all this time in order to reduce the thermic effect of food on their metabolism and thus on the results of the experiment. Acclimation and the experiment were performed at a constant temperature of 18 °C, which is typical of Lithuanian lakes during summer. A water mixture from the three water bodies was used for animal pre-treatment and experiments.

2.2. Gradually induced hypoxia experiment

To simultaneously simulate poor oxygen conditions and estimate detrimental oxygen levels, the experiment was performed in closed bottles allowing animals to gradually self-induce hypoxia. Avoiding gravid females, six amphipods of visually similar body size from the same population were enclosed in a 250-mL glass vessel of 7-cm bottom diameter filled with bacteria-filtered and oxygenized (100% saturation) water. For each population, three such vessel replicates were created and sealed. An experiment in each vessel was terminated when three amphipods therein, that is, half of the experimental animals, were inactivated (showed no movement of any limb even after gently shaking the vessel). Observations for individual inactivation were made every 30 min. A terminated vessel was then opened and the inactivating oxygen concentration was measured using an optic fibre oximeter (PreSens Precision Oxy-4 mini, Germany) with a dipping probe, over a magnetic stirrer. All the experimental specimens (active and inactive) were identified to be sure of the species, then were filter dried, weighed, frozen in liquid nitrogen and then stored at –70 °C until LDH activity analysis.

One control vessel for each of the three populations filled with 750 mL of constantly aerated bacteria-filtered water (90–100% oxygen saturation) and containing 18 amphipods was kept adjacent to the experimental bottles. At the end of the experiment, to match the number of independent observations (i.e., the number of experimental vessels per population), three random specimens were drawn from each of the control vessels and treated the same way as the experimental animals to determine baseline levels of LDH activity. Possible bacterial consumption of oxygen was measured in three additional sealed vessels filled with filtered water without amphipods. In these vessels, oxygen content dropped from 100% to approximately 97% throughout the experiment; therefore, bacteria were considered to have a negligible effect on the experimental results.

2.3. LDH activity assay

LDH activity was assayed for each specimen individually. Each specimen was ground using a pestle, with the addition of 0.5 mL of 80 mM Na₂HPO₄ buffer (pH 7.4). The mass was then centrifuged (1.5 min, 13,600 rpm, room temperature) and sonicated for 1 min (pulse on 10 s, off 15 s, amplitude 40%) in an Eppendorf tube immersed in an ice bath. The homogenate obtained was centrifuged, and the supernatant was used for the assay. Spectrophotometric measurement of LDH activity in assay mixtures was conducted at 340 nm and 30 °C. The assay mixture was obtained by adding 950 µL of 80 mM Na₂HPO₄ buffer (pH 7.4), 20 µL of 5 mM NADH, and 30 µL of 0.1 mM pyruvate to obtain final concentrations similar to those used by Gonzalez and Quiñones (2002). (Different assay temperatures and mixture concentrations were also tested but had little effect on the results.) The reaction was started by adding 10 µL of the supernatant. LDH activity of each specimen was determined from analytical triplicates. Measurements were corrected for nonspecific NADH oxidation measured individually in the absence of pyruvate. LDH activities were expressed as µmol of NADH oxidized per 1 min in 1 g of amphipod mass (µmol min⁻¹ g⁻¹ wet weight).

2.4. Statistical analyses

Amphipod performance was evaluated as individual time to inactivation and as oxygen concentration in a vessel when half of the individuals were found inactivated. To compare individual time to inactivation of experimental amphipods among populations (Lake Dusia, Kaunas WR, Lake Simnas), a Cox proportional hazards (CPH) model with Efron likelihood adjustment for ties (Efron, 1974) was fitted (N = 3 populations × 3 vessels × 6 individuals = 54 observations). Vessels were used as subjects for data aggregation and thus the robust method for estimation of variance was selected. In order to test the population effect on oxygen concentration causing inactivation of half of the individuals in a vessel, an analysis of covariance (ANCOVA) model was used (3 pop. × 3 ves. = 9 obs.). The total weight of amphipods in a vessel must have had an effect on shared oxygen consumption and therefore the time until a critical level was reached. On the other hand, wet weight may also determine the critical oxygen level. Thus, mean amphipod weight per vessel was included in both of these models (CPH and ANCOVA).

We applied two separate models to compare weight-specific LDH activity in experimental and baseline amphipods. Due to the possible dependency of LDH activity on body size, individual wet weight was used in both of these models. A linear mixed-effects model (LME; 3 pop. × 3 ves. × 6 ind. = 54 obs.) including a random effect of vessel nested within a population was applied for this analysis of experimental activities, and an ANCOVA model (3 pop. × 3 ind. = 9 obs.) was used to compare baseline activities. Finally, an ANCOVA model including independent observations of the baseline treatment (individual values) and the experiment (vessel means) was fitted (2 treatments × 3 pop. × 3 ind./ves. = 18 obs.). Within this model, we tested for effects of treatment (experimental v. baseline), habitat trophic status (assessed as chlorophyll *a* concentration), and body weight on measured LDH activity.

For the ANCOVAs and the LME, homoscedasticity assumptions were tested using Brown's–Forsythe's tests (met in all cases with $P > 0.05$). Pairwise comparisons between populations were performed using Tukey's HSD tests. The assumptions of proportionality of hazards in the CPH model were also tested (met with $P > 0.4$). The population of Lake Dusia was used as a reference because this lake is expected to provide the most favourable conditions for oxyphilic crustaceans (see Table 1). Consequently, model coefficients for the other two populations could be expected to be of the same sign: negative for inactivation hazard (longer resistance to hypoxia) and

Table 2

Results of the Cox proportional hazards model for *Pontogammarus robustoides* inactivation in the experiment. WW_{ves} is the mean wet weight of amphipods per vessel. Population effects for Lake Simnas and Kaunas WR were tested against the reference level of Lake Dusia. Significant probabilities ($P < 0.05$) are indicated in bold italics.

Effect	Hazard ratio (95% CI)	$b \pm SE$	χ^2	P
WW_{ves}	1.29 (1.14–1.46)	0.26 ± 0.06	16.78	<0.001
Lake Simnas	0.63 (0.14–2.83)	−0.46 ± 0.77	0.36	0.54
Kaunas WR	0.90 (0.24–3.47)	−0.10 ± 0.69	0.02	0.88

positive for LDH activity (resistance through higher LDH activity). Interactions among the included predictors were considered in all models but were removed if they were not significant. Statistical analyses were performed using STATISTICA v.12 software (Dell Inc., 2015).

3. Results

3.1. Amphipod performance

Wet weight of experimental animals (mean ± SD) from Lake Dusia, Kaunas WR and Lake Simnas was 41.0 ± 8.9, 52.6 ± 16.1 and 48.2 ± 10.2 mg. On average, an experiment in a vessel lasted for 28.8 ± 2.2 h. During the first half of the experiment the experimental and control amphipods were observed lying individually on the bottom or swimming up and around the vessel. Toward the end of the experiment the experimental animals were more prone to lying calmly on the bottom while the control animals did not show such a change in behaviour. All control amphipods remained active throughout the experiment. No aggressive encounters between individuals were noticed on observation occasions.

The CPH model for amphipod inactivation time in the experiment (Table 2) described 70% of the data. Mean weight of animals in a vessel was a highly significant term increasing the average hazard by 29% with each additional milligram of weight (Fig. 1A). The coefficients for populations were not statistically significant (Fig. 1B). According to ANCOVA model, inactivating oxygen concentration did not differ with population ($F_{2,5} = 2.2$, $P = 0.2$) and neither was it related to mean amphipod weight ($F_{1,5} = 0.4$, $P = 0.6$); overall it was 0.49 ± 0.16 mg L⁻¹.

3.2. LDH activity comparison

The LME model of LDH activity of experimental amphipods (Table 3) described 57% of variation and was overall significant ($F_{9,42} = 6.2$, $P < 0.001$). Only the effect of population was statistically significant. Tukey's HSD tests indicated that LDH levels of amphipods from Lake Dusia were different from the levels reached by the other populations ($P < 0.001$). According to model coefficients, amphipods from Kaunas WR and Lake Simnas were predicted to have, respectively, 22.7 and 23.6 µmol min⁻¹ g⁻¹ higher LDH activity than amphipods from Lake Dusia (Fig. 2).

Baseline amphipods from Lake Dusia, Kaunas WR and Lake Simnas weighed 38.7 ± 6.6, 35.7 ± 10.6 and 52.7 ± 6.0 mg (wet weight), correspondingly. The ANCOVA model of baseline LDH activity (Table 3) described 63% of the variation but barely indicated possibility of any tendencies ($F_{3,5} = 2.8$, $P = 0.15$). Model coefficients (Table 3) indicated similar tendencies as in the model for experimental LDH activities: amphipods from Lake Simnas and Kaunas WR may have higher LDH activity than amphipods from Lake Dusia, by 24.6 and 11.3 µmol min⁻¹ g⁻¹, respectively (Fig. 2). The model also suggested that with each additional milligram of weight, baseline LDH activity on average may decline by 0.9 µmol min⁻¹ g⁻¹.

The ANCOVA model including LDH activity of baseline and experimental amphipods (Table 4) described 74% of the variation and was highly significant ($F_{3,14} = 13.4$, $P < 0.001$). In comparison

Table 3
Results of linear models for LDH activity of experimental (LME) and baseline (ANCOVA) amphipods *Pontogammarus robustoides* from the closed-bottle experiment, testing for population and wet weight (WW_{ind}) effects. In the LME model, a random factor of vessel was nested within a population factor. Significant probabilities ($P < 0.05$) are indicated in bold italics. See Fig. 2 for model predictions for a 47-mg wet-weight amphipod.

Response	Effect	df	$b \pm SE$	MS	F	P
Experimental LDH	WW_{ind}	1	-0.0 ± 0.1	5.0	0.1	0.81
	Population	2		1822.9	23.8	<0.001
	Kaunas WR		22.7 ± 5.4			
	Lake Simnas		23.6 ± 5.4			
	Vessel (Population)	6		76.1	0.9	0.51
	Error	42		85.2		
Baseline LDH	WW_{ind}	1	-0.9 ± 0.5	316.0	4.0	0.10
	Population	2		291.8	3.7	0.10
	Kaunas WR		11.3 ± 7.4			
	Lake Simnas		24.6 ± 9.6			
	Error	5		79.0		

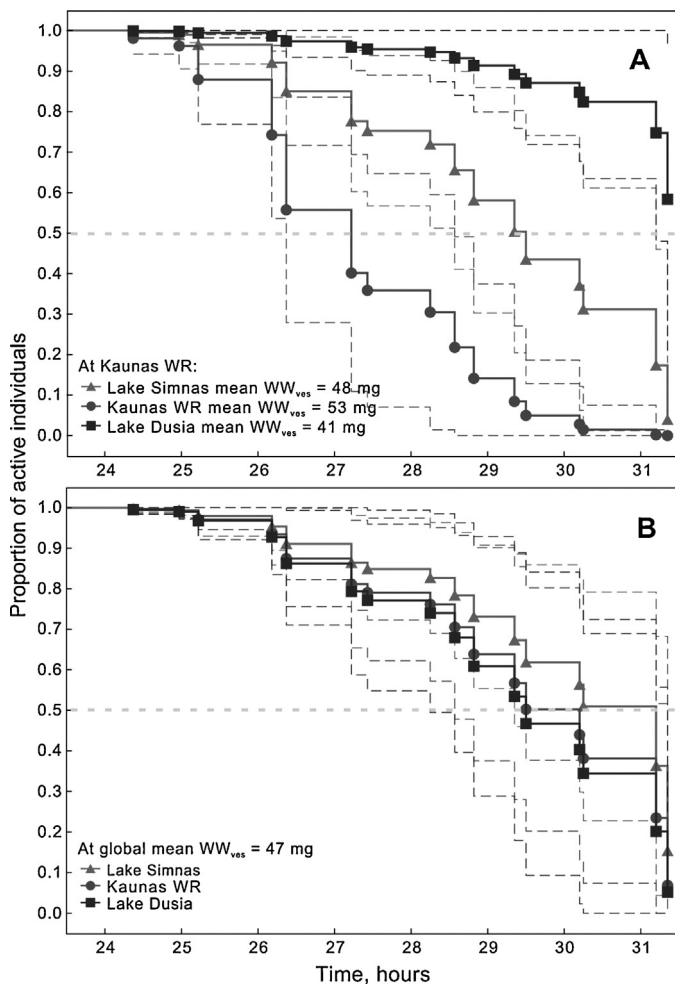


Fig. 1. Survival function plots (Breslow estimation with 95% CI) from the Cox proportional hazards model for the amphipod *Pontogammarus robustoides* in a closed-bottle experiment (see Table 2 for model parameters) showing (A) a significant effect of mean wet weight of amphipods in a vessel ($P < 0.001$; curves produced with the population parameter fixed as Kaunas WR and using amphipod weights characteristic of each population) and (B) an insignificant effect of population ($P > 0.5$; given a fixed mean weight for all experimental amphipods and using different population parameters).

to previous LDH activity models, this analysis was more powerful as twice more independent observations were used (18 v. 9), averaging of the experimental values may have provided more robustness for the data, and fewer degrees of freedom were consumed by the predictor describing population/habitat (1 for trophic status v. 2 for population factor). All three statistically significant

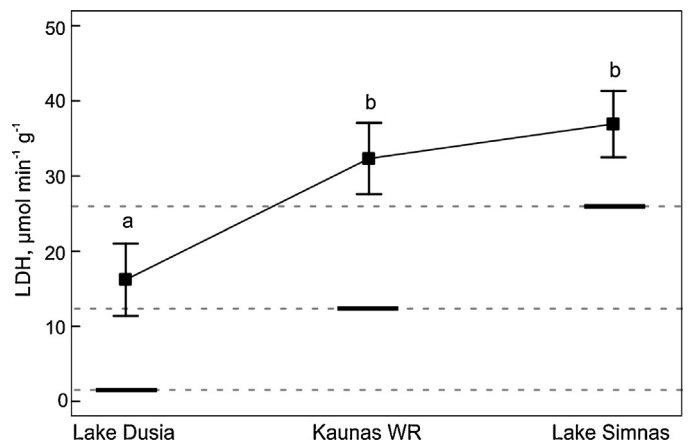


Fig. 2. Experimental LDH activity (mean with 95% CI) predicted for a 47-mg wet-weight amphipod *Pontogammarus robustoides* from three populations inhabiting water bodies of different trophic status: mesotrophic Lake Dusia, eutrophic Kaunas WR and hypertrophic Lake Simnas. Dark horizontal bands denote baseline LDH activity predicted for 47-mg wet-weight individuals. Predictions were made using the models presented in Table 3. Different letters (a, b) denote significant differences (Tukey HSD test, $P < 0.001$) in experimental LDH activity among water bodies.

Table 4
Results of ANCOVA model for amphipod *Pontogammarus robustoides* LDH activity, testing for the effects of wet weight (WW: vessel mean for experimental and individual values for baseline individuals), treatment within the study (experiment v. baseline), and mean multiannual chlorophyll *a* concentration as a proxy for habitat trophic status. Significant probabilities ($P < 0.05$) are indicated in bold italics. See Fig. 3 for partial residual plots.

Effect	df	$b \pm SE$	MS	F	P
WW	1	-0.5 ± 0.2	277.0	6.3	0.03
Treatment	1	13.6 ± 3.3	750.1	17.0	0.001
Chlorophyll <i>a</i>	1	0.9 ± 0.2	1230.2	27.8	<0.001
Error	14		44.2		

coefficients indicated that with each additional milligram of weight, LDH activity on average declined by $0.5 \mu\text{mol min}^{-1} \text{g}^{-1}$ (Fig. 3A), experimental amphipods exhibited $13.6 \mu\text{mol min}^{-1} \text{g}^{-1}$ higher LDH activity than baseline amphipods (Fig. 3B), and the strongest positive effect was that of habitat trophic status (Fig. 3C).

4. Discussion

The results of this study suggest the existence of diverging LDH activity levels under hypoxic conditions in populations of *P. robustoides* inhabiting lentic water bodies of different trophic status in the northern invaded range. Amphipods from highly eutrophic waters exhibited higher LDH activity than amphipods from a mesotrophic lake; thus a higher resistance to hypoxic

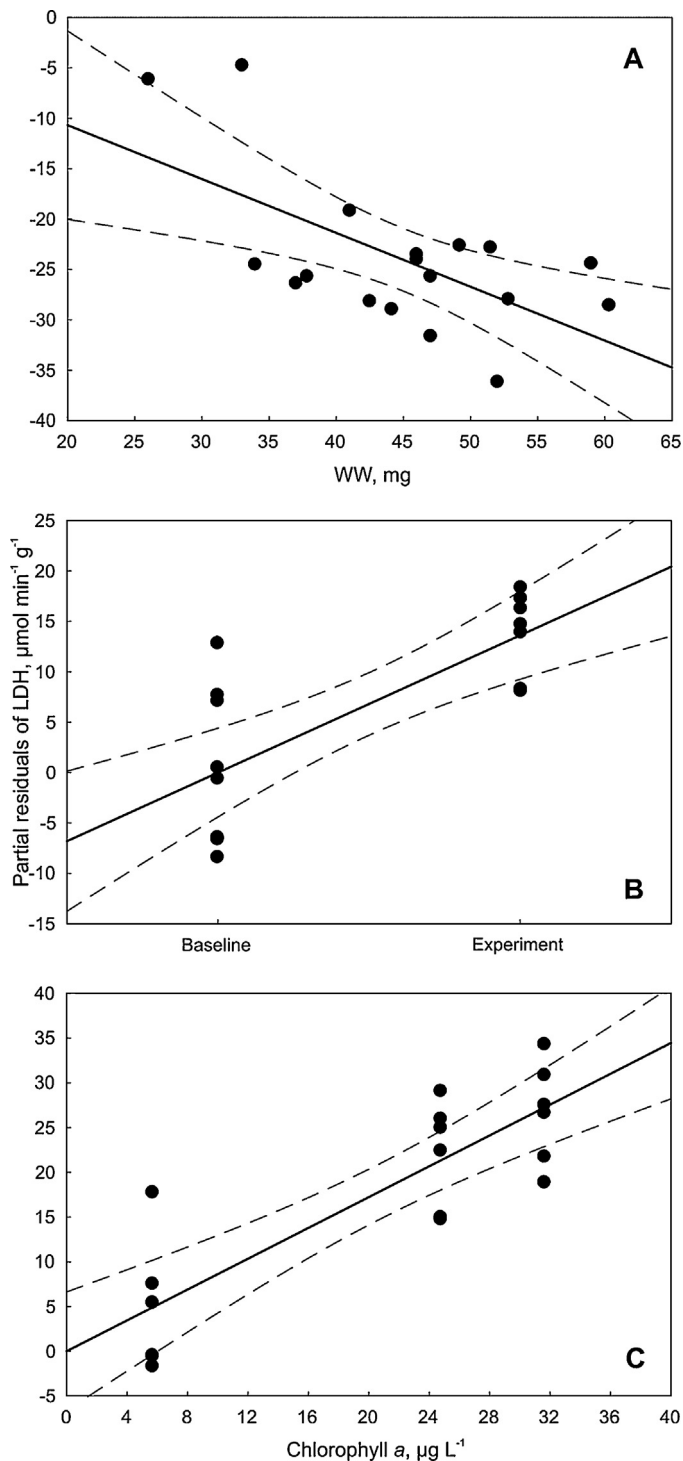


Fig. 3. Partial residual plots (with 95% CI) from the ANCOVA model (see Table 4) illustrating the effects of (A) wet weight (WW), (B) treatment (experiment v. baseline), and (C) mean multiannual chlorophyll *a* concentration of habitat on LDH activity of the amphipod *Pontogammarus robustoides*. In C, the three increasing chlorophyll *a* concentrations indicate Lake Dusia, Kaunas WR and Lake Simnas, respectively.

conditions of individuals inhabiting eutrophic environments, in which they indeed should face periods of detrimental oxygen concentrations, can be anticipated. The strong correlation between LDH activity levels and trophic status of waters inhabited by *P. robustoides* also supports the enhanced resistance to hypoxia in certain introduced populations.

Observed variation in LDH activity levels among populations indicated different physiological acclimation response. It is unlikely that this response may have resulted from different developmental acclimatisation (Angilletta, 2009) as used experimental animals represented the offspring of the spring mating season of the same year (2012), the offspring which were unlikely to encounter detrimental oxygen concentrations during their ontogeny. According to monitoring data from 2006 to 2014 (Lithuanian Environmental Protection Agency; <http://vanduo.gamta.lt/cms/index?rubricId=8ea41f73-9742-4d71-aa10-0a5988713fe5>, accessed on 3 March 2016), during the growing season oxygen concentration in the surface layers (which is also characteristic to the littoral zone) of all studied water bodies did not drop below 8 mg L^{-1} . Such a concentration is obviously above the limiting one for amphipods known to be approximately 2 mg L^{-1} (Dedyu, 1980). Consequently, an ability of a population to respond to decreasing oxygen levels differently may have a genetic basis which has been acquired quite rapidly (in ca. 50 years). Since in our experiment we used adult individuals caught in the field and did not perform a common garden experiment required to rule out maternal effects (Mousseau and Fox, 1998), genetic variation in resistance to hypoxia (sensu Merila and Hendry, 2013) still cannot be proved. However, recent findings in evolutionary ecology suggest that the divergence in LDH activity levels, i.e. an ability to differently respond to decreasing oxygen, can be related to underlying genetic adaptation. The amphipod *P. robustoides* in Lithuanian lakes usually has three generations per year (Arbačiauskas and Gumuliauskaitė, 2007); thus it has already produced around 150 generations under the selective pressure of contrasting oxygen conditions. At least in plants, adaptation to selective pressures in a new environment can occur within 20 generations or even less (Prentis et al., 2008). In vertebrates, measurable evolutionary change can occur less than 50 generations after invasion of a novel habitat (see Lescak et al., 2015 for a fish example). Finally, using common garden experiments contemporary adaptations to new environments have been documented in some invasive crustaceans (Lee et al., 2003, 2012; Sargent and Lodge, 2014).

In normoxic conditions of our experiment, raw (not weight-corrected) LDH activity of *P. robustoides* from lentic waters of different trophic status was measured within the range of $0.9\text{--}37.5 \mu\text{mol min}^{-1} \text{g}^{-1}$. Due to different experimental designs (constant or decreasing oxygen content, termination criteria) and LDH activity assay protocols (whole body or specific tissue, different assay conditions and mixtures, etc.), comparisons of the LDH activity values measured here with those in the literature are complicated. Considering differences in assay pH and temperature, the order of normoxic LDH activity of *P. robustoides* falls within the range of field LDH activity reported for the marine copepod *Calanus chilensis* Brodsky, 1959 ($0.8\text{--}0.9 \mu\text{mol min}^{-1} \text{g}^{-1}$), which is non-resistant to hypoxia, and the euphausiid *Euphausia mucronata* G. O. Sars, 1883 ($131.6\text{--}152.9 \mu\text{mol min}^{-1} \text{g}^{-1}$), which is well adapted to travel through the subsurface oxygen minimum layer (Gonzalez and Quiñones, 2002), closer to the LDH activity range of the former species. This position within the reported activity range is in accordance with the predominant characterization of *P. robustoides* as an oxyphilic species; however, it does not classify it as strictly oxyphilic like *C. chilensis*.

Under hypoxia, a significant divergence in LDH activity levels of *P. robustoides* was found. In individuals from a mesotrophic lake raw LDH activity values varied within a range of $0.8\text{--}28.3 \mu\text{mol min}^{-1} \text{g}^{-1}$, which fell within the overall normoxic LDH activity range recorded in this study. On the other hand, in amphipods from eutrophic and hypertrophic waters the raw LDH activity varied within the range of $20.3\text{--}56.1 \mu\text{mol min}^{-1} \text{g}^{-1}$ and approached the activity measured in the muscles of mud-dwelling thalassinid shrimps incubated in

anoxia ($40.3\text{--}51.8 \mu\text{mol min}^{-1} \text{g}^{-1}$; Pritchard and Eddy, 1979). This suggests that although *P. robustoides* introduced into habitats with good oxygen conditions have remained predominantly oxyphilic, populations residing in new habitats susceptible to hypoxia may have developed a more pronounced resistance to oxygen deprivation.

In this experiment, the mean oxygen concentration resulting in inactivation of half the adult *P. robustoides* was 0.49 mg L^{-1} , which, as expected, was higher than the previously reported lethal oxygen concentration of 0.21 mg L^{-1} obtained for adult individuals from the native range at 20°C (Dedyu, 1980). To avoid traumatizing the animals before the experiment we only weighed them after it, and failed to fully standardize their weight. The observed strong influence of body weight on the time for which animals remained active may be explained by the higher oxygen consumption rate of larger individuals, and thus shorter time to reach inactivating concentrations. It has been shown that critical oxygen limits may also depend on amphipod age and sex even within the same population (Hoback and Barnhart, 1996). We conclude that variation in amphipod performance related to divergent hypoxia resistance among populations may be not that large and would require a more sensitive experimental design to be detected.

In this study, a closed-bottle experiment was chosen as a technically simple method that can simultaneously allow gradual transition to hypoxic–anoxic conditions and provide approximate measures of oxygen consumption rate and its critical concentrations, survival and metabolic response. Such a design provided sufficient data to detect intraspecific differences in LDH activity; however, it did not enable detection of differences in critical oxygen concentrations and time to inactivation. It should be also noted that extrapolation of the results to ambient winter temperatures (4°C) should be done with caution due to possible temperature influences on kinetics of the lactate pathway (e.g. Trausch, 1976; Thebault and Le Gal, 1978). Thus, the hypothesis of long-term winter hypoxia limiting the distribution of *P. robustoides* within temperate lakes (Arbačiauskas and Gumuliauskaitė, 2007) may warrant additional testing.

Resistance to low oxygen in the northern invaded range where hypoxic conditions often develop would definitely be beneficial for *P. robustoides*. Such resistance may allow the species to expand its environmental niche and invade previously unsuitable habitats such as eutrophic lentic waters. An example of such an expansion might be the recent invasion of *P. robustoides* from Lake Simnas into Lake Žuvintas, located only 5.3 km downstream. The species was introduced into Lake Simnas in 1963 but was not recorded in Lake Žuvintas until 50 years later in 2014, despite regular investigations of the lake over the last intervening decades (Arbačiauskas et al., 2010; Arbačiauskas, unpublished data). Lake Žuvintas is a shallow and highly eutrophic overgrowing lake of continuously decreasing ecological status (Linkevičienė et al., 2007), and it is an even less suitable habitat for *P. robustoides* than Lake Simnas. Recently, Lake Simnas has also become highly eutrophicated but its high water turnover rate may allow it to support Ponto–Caspian amphipods in the long term (Arbačiauskas, 2005).

An expansion in the environmental niche of *P. robustoides* may have another ecological implication. Due to the specific intolerances of invasive peracaridan species, proximate waters of lower salinity (Grabowski et al., 2009), higher ammonia (Gergs et al., 2013) or lower oxygen levels (Arbačiauskas, 2005) have been considered as refuges for native macroinvertebrate species. In Lithuania, *P. robustoides* is already known for eradicating native amphipods (*Gammarus* spp.; Arbačiauskas, 2005, 2008) and detrimentally affecting various macroinvertebrates (Gumuliauskaitė and Arbačiauskas, 2008) including organisms sensitive to pollution, thus affecting conventional ecological monitoring of rivers

(Arbačiauskas et al., 2011a) and lakes (Šidagytė et al., 2013). It has been recently revealed that the predatory behaviour of *P. robustoides* can be more pronounced in waters of higher trophic (Arbačiauskas et al., 2013). Further, it has been shown that the species may occupy a trophic position similar to that of other invasive amphipods including the killer shrimp *Dikerogammarus villosus* (Sowinsky, 1894) and the daemon shrimp *D. haemobaphes* (Eichwald, 1841), and higher than that of other alien and native amphipods (Bacela-Spychalska and Van Der Velde, 2013). *Pontogammarus robustoides* was even able to outcompete the preceding invader *D. haemobaphes* (Jażdżewska and Jażdżewski, 2008). Thus, severe impacts of *P. robustoides* on macroinvertebrate communities of lentic waters—primarily lakes of higher trophic status—may be expected if the suggested adaptive response to hypoxia could facilitate a massive invasion of the species.

5. Conclusions

We conclude that populations of *P. robustoides* show a divergent physiological response within its lactate pathway that could contribute to their resistance to long-term hypoxia under winter ice cover or summer hypoxic episodes in eutrophic lakes and other lentic waters of the northern invaded range. The time introduced populations have existed under novel selective pressure is sufficient for microevolutionary change underlying such divergent resistance to decreased oxygen levels. However, common garden experiments or other means of search for a genetic basis of this divergence in LDH levels is warranted to confirm the observed phenomenon as a genetic adaptation. Enhanced resistance to hypoxic environments should promote species dispersal across inland waters and may pose an even greater threat to native species, leaving them no safe refuge.

Acknowledgements

This study was supported by the Research Council of Lithuania, Project No. LEK-10/2012. We are grateful to Rolandas Meškys and Rasa Rutkienė for allowing us to use their laboratory facilities and advising on LDH activity assessment, and Asta Audzijonytė for comments on the manuscript. We also appreciate the work of two anonymous reviewers who provided critical comments that considerably improved the manuscript.

References

- Agnew, D.J., Jones, M.B., 1986. Metabolic adaptations of *Gammarus duebeni* Liljeborg (Crustacea Amphipoda) to hypoxia in a sewage treatment plant. *Comp. Biochem. Physiol. A: Physiol.* 84, 475–478. [http://dx.doi.org/10.1016/0300-9629\(86\)90351-8](http://dx.doi.org/10.1016/0300-9629(86)90351-8).
- Agnew, D.J., Taylor, A.C., 1985. The effect of oxygen tension on the physiology and distribution of *Echinogammarus pirloti* (Sexton & Spooner) and *E. obtusatus* (Dahl) (Crustacea: Amphipoda). *J. Exp. Mar. Biol. Ecol.* 87, 169–190. [http://dx.doi.org/10.1016/0022-0981\(85\)90089-9](http://dx.doi.org/10.1016/0022-0981(85)90089-9).
- Anderson, S.J., Taylor, A.C., Atkinson, R.J.A., 1994. Anaerobic metabolism during anoxia in the burrowing shrimp *Calocaris macandreae* Bell (Crustacea: Thalassinidea). *Comp. Biochem. Physiol. A: Physiol.* 108, 515–522. [http://dx.doi.org/10.1016/0300-9629\(94\)90335-2](http://dx.doi.org/10.1016/0300-9629(94)90335-2).
- Angilletta, M.J., 2009. *Thermal Adaptation: A Theoretical and Empirical Synthesis*. Oxford University Press Inc, New York. <http://dx.doi.org/10.1093/acprof:oso/9780198570875.001.1>.
- Arbačiauskas, K., 2005. The distribution and local dispersal of Ponto–Caspian Peracarida in Lithuanian fresh waters with notes on *Pontogammarus robustoides* population establishment, abundance and impact. *Oceanol. Hydrobiol. Stud.* 34, 93–111.
- Arbačiauskas, K., Gumuliauskaitė, S., 2007. Invasion of the Baltic Sea basin by the Ponto–Caspian amphipod *Pontogammarus robustoides* and its ecological impact. In: Gherardi, F. (Ed.), *Biological Invaders in Inland Waters: Profiles, Distribution, and Threats*. Springer, Dordrecht, pp. 463–477. http://dx.doi.org/10.1007/978-1-4020-6029-8_25.
- Arbačiauskas, K., Rakauskas, V., Virbickas, T., 2010. Initial and long-term consequences of attempts to improve fish-food resources in Lithuanian waters

- by introducing alien peracaridan species: a retrospective overview. *J. Appl. Ichthyol.* 26, 28–37, <http://dx.doi.org/10.1111/j.1439-0426.2010.01492.x>.
- Arbačiauskas, K., Višinskienė, G., Smilgevičienė, S., 2011a. Non-indigenous macroinvertebrate species in Lithuanian fresh waters, Part 2: macroinvertebrate assemblage deviation from naturalness in lotic systems and the consequent potential impacts on ecological quality assessment. *Knowl. Manag. Aquat. Ecosyst.* 402, 13, <http://dx.doi.org/10.1051/kmae/2011076>.
- Arbačiauskas, K., Višinskienė, G., Smilgevičienė, S., Rakauskas, V., 2011b. Non-indigenous macroinvertebrate species in Lithuanian fresh waters Part 1: Distributions, dispersal and future. *Knowl. Manag. Aquat. Ecosyst.* 402, 12, <http://dx.doi.org/10.1051/kmae/2011075>.
- Arbačiauskas, K., Lesutienė, J., Gasiūnaitė, Z.R., 2013. Feeding strategies and elemental composition in Ponto-Caspian peracaridans from contrasting environments: can stoichiometric plasticity promote invasion success? *Freshw. Biol.* 58, 1052–1068, <http://dx.doi.org/10.1111/fwb.12108>.
- Arbačiauskas, K., 2008. Amphipods of the Nemunas River and the Curonian Lagoon, the Baltic Sea basin: where and which native freshwater amphipods persist? *Acta Zool. Lit.* 18, 10–16, <http://dx.doi.org/10.2478/v10043-008-0002-3>.
- Bacela-Spychalska, K., Van Der Velde, G., 2013. There is more than one killer shrimp: trophic positions and predatory abilities of invasive amphipods of Ponto-Caspian origin. *Freshw. Biol.* 58, 730–741, <http://dx.doi.org/10.1111/fwb.12078>.
- Bishop, R.E., Kakuk, B., Torres, J.J., 2004. Life in the hypoxic and anoxic zones: metabolism and proximate composition of Caribbean troglitic crustaceans with observations on the water chemistry of two anchialine caves. *J. Crust. Biol.* 24, 379–392, <http://dx.doi.org/10.1651/C-2459>.
- Bridges, C.R., Brand, A.R., 1980. The effect of hypoxia on oxygen consumption and blood lactate levels of some marine Crustacea. *Comp. Biochem. Physiol. A: Physiol.* 65, 399–409, [http://dx.doi.org/10.1016/0300-9629\(80\)90051-1](http://dx.doi.org/10.1016/0300-9629(80)90051-1).
- Dedyu, I.I., 1980. Amphipods of Fresh and Salt Waters of the South-West Part of the USSR. Shtiintsa Publishers, Kishinev (In Russian).
- Dell Inc, 2015. STATISTICA (data Analysis Software System), Version 12. www.statsoft.com.
- Efron, B., 1974. The efficiency of cox's likelihood function for censored data. *J. Am. Stat. Assoc.* 72, 557–565, <http://dx.doi.org/10.1080/01621459.1977.10480613>.
- Ellington, W.R., 1983. The recovery from anaerobic metabolism in invertebrates. *J. Exp. Zool.* 228, 431–444, <http://dx.doi.org/10.1002/jez.1402280305>.
- Gasiūnas, I., 1972. Enrichment of fodder basis of water bodies of Lithuania by acclimatized crustaceans-like organisms from the Caspian Sea complex. In: Maniukas, J., Virbickas, J. (Eds.), *On the Breeding of Fish and Crustacean-Like Organisms in the Water Bodies of Lithuania.*, pp. 57–68, Mintis Vilnius (In Russian).
- Gergs, R., Schlag, L., Rothhaupt, K.O., 2013. Different ammonia tolerances may facilitate spatial coexistence of *Gammarus roeselii* and the strong invader *Dikerogammarus villosus*. *Biol. Invasions* 15, 1783–1793, <http://dx.doi.org/10.1007/s10530-013-0408-0>.
- Gonzalez, R.R., Quiñones, R.A., 2002. Ldh activity in *Euphausia mucronata* and *Calanus chilensis*: implications for vertical migration behaviour. *J. Plankton Res.* 24, 1349–1356, <http://dx.doi.org/10.1093/plankt/24.12.1349>.
- Grabowski, M., Bacela, K., Konopacka, A., Jazdzewski, K., 2009. Salinity-related distribution of alien amphipods in rivers provides refugia for native species. *Biol. Invasions* 11, 2107–2117, <http://dx.doi.org/10.1007/s10530-009-9502-8>.
- Grieshaber, M.K., Hardewig, I., Kreutzer, U., Pörtner, H.-O., 1994. Physiological and metabolic responses to hypoxia in invertebrates. *Rev. Physiol. Biochem. Pharmacol.* 125, 43–147, <http://dx.doi.org/10.1007/BFb0030909>.
- Gumuliauskaitė, S., Arbačiauskas, K., 2008. The impact of the invasive Ponto-Caspian amphipod *Pontogammarus robustoides* on littoral communities in Lithuanian lakes. *Hydrobiologia* 599, 127–134, <http://dx.doi.org/10.1007/s10750-007-9209-8>.
- Herreid II, C.F., 1980. Hypoxia in invertebrates. *Comp. Biochem. Physiol. A: Physiol.* 67, 311–320, [http://dx.doi.org/10.1016/S0300-9629\(80\)80002-8](http://dx.doi.org/10.1016/S0300-9629(80)80002-8).
- Hill, A.D., Taylor, A.C., Strang, R.H.C., 1991. Physiological and metabolic responses of the shore crab *Carcinus maenas* (L.) during environmental anoxia and subsequent recovery. *J. Exp. Mar. Biol. Ecol.* 150, 31–50, [http://dx.doi.org/10.1016/0022-0981\(91\)90104-5](http://dx.doi.org/10.1016/0022-0981(91)90104-5).
- Hoback, W.W., Barnhart, M.C., 1996. Lethal limits and sublethal effects of hypoxia on the amphipod *Gammarus pseudolimnaeus*. *J. North Am. Benthol. Soc.* 15, 117–126, <http://dx.doi.org/10.2307/1467437>.
- Jazdzewska, A., Jazdzewski, K., 2008. *Pontogammarus robustoides* (G. O. Sars 1894) (Crustacea, Amphipoda), a new Ponto-Caspian invader in Great Masurian Lakes (NE Poland). *Fragm. Faun.* 51, 1–7, <http://dx.doi.org/10.3161/00159301FF2008.51.1.001>.
- Lee, C.E., Remfert, J.L., Gelembiuk, G.W., 2003. Evolution of physiological tolerance and performance during freshwater invasions. *Integr. Comp. Biol.* 43, 439–449, <http://dx.doi.org/10.1093/icb/43.3.439>.
- Lee, C.E., Posavi, M., Charmantier, G., 2012. Rapid evolution of body fluid regulation following independent invasions into freshwater habitats. *J. Evol. Biol.* 25, 625–633, <http://dx.doi.org/10.1111/j.1420-9101.2012.02459.x>.
- Lescak, E.A., Bassham, S.L., Catchen, J., Gelmond, O., Sherbick, M.L., von Hippel, F.A., Cresko, W.A., 2015. Evolution of stickleback in 50 years on earthquake-uplifted islands. *Proc. Natl. Acad. Sci. U. S. A.* 112, E7204–E7212, <http://dx.doi.org/10.1073/pnas.1512020112>.
- Linkevičienė, R., Taminskas, J., Šimanasienė, R., 2007. Protected areas in regions of intensive economical activity: conflict of nature protection and nature use (case study of Dovinė river catchment). *Acta Geogr. Debr. Landsc. Environ.* 1, 43–56.
- Livingstone, D.R., 1983. Invertebrate and vertebrate pathways of anaerobic metabolism: evolutionary considerations. *J. Geol. Soc.* 140, 27–37, <http://dx.doi.org/10.1144/gsjgs.140.1.0027>.
- Livingstone, D.R., 1991. Origins and evolution of pathways of anaerobic metabolism in the animal kingdom. *Am. Zool.* 31, 522–534, <http://dx.doi.org/10.1093/icb/31.3.522>.
- Merila, J., Hendry, A.P., 2013. Climate change, adaptation, and phenotypic plasticity: the problem and the evidence. *Evol. Appl.* 7, 1–14, <http://dx.doi.org/10.1111/eva.12137>.
- Morduhaj-Boltovskoj, F.D., Greze, I.I., Vasilenko, S.V., 1972. Otrjad amfipody, ili raznogie—amfipoda. In: Vodjanitskij, V.A. (Ed.), *Opredelitel Fauny Chernogo I Azovskogo Morej: Svobodnozhivushhie Bespozvochnnye Rakoobraznye. Naukova dumka, Kiev*, pp. 440–524 (In Russian).
- Mousseau, T.A., Fox, C.W., 1998. The adaptive significance of maternal effects. *Trends Ecol. Evol.* 13, 403–407, [http://dx.doi.org/10.1016/S0169-5347\(98\)01472-4](http://dx.doi.org/10.1016/S0169-5347(98)01472-4).
- Prentis, P.J., Wilson, J.R.U., Dormontt, E.E., Richardson, D.M., Lowe, A.J., 2008. Adaptive evolution in invasive species. *Trends Plant Sci.* 13, 288–294, <http://dx.doi.org/10.1016/j.tplants.2008.03.004>.
- Pritchard, A.W., Eddy, S., 1979. Lactate formation in *Callinassa californiensis* and *Upogebia pugetensis* (Crustacea: Thalassinidea). *Mar. Biol.* 50, 249–253, <http://dx.doi.org/10.1007/BF00394206>.
- Reid, D.F., Orlova, M.I., 2002. Geological and evolutionary underpinnings for the success of Ponto-Caspian species invasions in the Baltic Sea and North American Great Lakes. *Can. J. Fish. Aquat. Sci.* 59, 1144–1158, <http://dx.doi.org/10.1139/f02-099>.
- Sargent, L.W., Lodge, D.M., 2014. Evolution of invasive traits in nonindigenous species: increased survival and faster growth in invasive populations of rusty crayfish (*Orconectes rusticus*). *Evol. Appl.* 7, 949–961, <http://dx.doi.org/10.1111/eva.12198>.
- Šidagytė, E., Višinskienė, G., Arbačiauskas, K., 2013. Macroinvertebrate metrics and their integration for assessing the ecological status and biocontamination of Lithuanian lakes. *Limnologica* 43, 308–318, <http://dx.doi.org/10.1016/j.limno.2013.01.003>.
- Silva-Castiglioni, D., Oliveira, G.T., Buckup, L., 2010. Metabolic responses of *Parastacus defossus* and *Parastacus brasiliensis* (Crustacea, Decapoda Parastacidae) to hypoxia. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* 156, 436–444, <http://dx.doi.org/10.1016/j.cbpa.2010.03.025>.
- Silva-Castiglioni, D., Oliveira, G.T., Buckup, L., 2011. Metabolic responses in two species of crayfish (*Parastacus defossus* and *Parastacus brasiliensis*) to post-hypoxia recovery. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* 159, 332–338, <http://dx.doi.org/10.1016/j.cbpa.2011.03.030>.
- Spicer, J.L., Dando, C.L., Maltby, L., 2002. Anaerobic capacity of a crustacean sensitive to low environmental oxygen tensions, the freshwater amphipod *Gammarus pulex* (L.). *Hydrobiologia* 477, 189–194, <http://dx.doi.org/10.1023/A:1021063225668>.
- Thebault, M.-T., Le Gal, Y., 1978. Studies on lactate dehydrogenase activity in *Palaeomon serratus*. Effect of the incubation temperature on the kinetic properties of the enzyme system from the caudal muscle. *Biochem. Syst. Ecol.* 6, 141–144, [http://dx.doi.org/10.1016/0305-1978\(78\)90041-8](http://dx.doi.org/10.1016/0305-1978(78)90041-8).
- Trausch, G., 1976. Effect of temperature upon catalytic properties of lactate dehydrogenase in the lobster. *Biochem. Syst. Ecol.* 4, 65–68, [http://dx.doi.org/10.1016/0305-1978\(76\)90013-2](http://dx.doi.org/10.1016/0305-1978(76)90013-2).
- Yaikin, J., Quiñones, R.A., González, R.R., 2002. Aerobic respiration rate and anaerobic enzymatic activity of *Petrolisthes laevigatus* (Anomura Porcellanidae) under laboratory conditions. *J. Crust. Biol.* 22, 345–352, [http://dx.doi.org/10.1651/0278-0372\(2002\)022](http://dx.doi.org/10.1651/0278-0372(2002)022).
- Żytkowicz, J., Kobak, J., Kakareko, T., Kentzer, A., 2008. Species composition and distribution of invasive Ponto-Caspian amphipods in the off-channel microhabitats of a temperate, lowland dam reservoir. *Int. Rev. Hydrobiol.* 93, 62–72, <http://dx.doi.org/10.1002/iroh.200710966>.