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Salinity tolerance of marbled crayfish *Procambarus fallax* f. *virginalis*

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Abstract – Eastern Europe comprises a significant part of the native ranges for indigenous crayfish species (ICS) belonging to the genus *Astacus*. This region has been largely overlooked by astacologists and considered relatively immune to the impacts of non-indigenous crayfish species (NICS). The recent discovery of two marbled crayfish *Procambarus fallax* f. *virginalis* populations in Ukraine has changed this view. Increased propagule pressure (mainly due to pet trade) has raised concerns of NICS which may negatively impair the ecosystems of Azov, Black and Caspian Seas and their tributaries inhabited by ICS. In this study, we provide the first insight into salinity tolerance of marbled crayfish. We performed a 155-day experiment using 5 different salinities (6, 9, 12, 15, and 18 ppt) and a freshwater control. Evaluation of survival, growth and reproduction suggests that marbled crayfish have a lower salinity tolerance than other crayfish species, which may limit their invasive potential in brackish environments. However, its ability to survive for more than 80 days at 18 ppt opens up the possibility of gradual dispersion and adaptation to brackish conditions. Our study highlights the need for further studies elucidating the potential for marbled crayfish to negatively impair these ecosystems.

Keywords: brackish environment / growth / reproduction / moult / survival

Résumé – **Tolérance à la salinité de l'écrevisse marbrée *Procambarus fallax* f. *virginalis*.** L'Europe de l'Est comprend une partie importante des régions abritant des espèces indigènes d'écrevisses (ICS) appartenant au genre *Astacus*. Cette région a été largement ignorée par les astacologues et considérée comme relativement protégée des impacts des espèces d'écrevisses non indigènes (NICS). La découverte récente de deux populations d'écrevisses marbrées *Procambarus fallax* f. *virginalis* en Ukraine ont changé cette vision. Le potentiel d'augmentation de la pression des propagules (principalement en raison du commerce des animaux de compagnie) a soulevé des préoccupations concernant les NICS qui pourraient nuire aux écosystèmes des mers d'Azov, Noire et Caspienne et à leurs affluents habités par des ICS. Dans cette étude, nous fournissons le premier aperçu de la tolérance à la salinité des écrevisses marbrées. Nous avons effectué une expérience de 155 jours en utilisant 5 salinités différentes (6, 9, 12, 15, 18 ppt) et un contrôle d'eau douce. L'évaluation de la survie, de la croissance et de la reproduction suggère que les écrevisses marbrées ont une tolérance à la salinité plus faible que les autres espèces d'écrevisses, ce qui peut limiter leur potentiel invasif dans les milieux saumâtres. Cependant, sa capacité à survivre pendant plus de 80 jours à 18 ppt ouvre la possibilité d'une dispersion progressive et d'une adaptation aux conditions saumâtres. Notre étude souligne la nécessité de poursuivre les études permettant d'éclaircir le potentiel de l'écrevisse marbrée à nuire négativement à ces écosystèmes.

Mots-clés : environnement saumâtre / croissance / reproduction / mue / survie

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1 Introduction

Salinity is an important abiotic factor influencing crucial processes of animals such as feeding, growth, and reproduction, which determines their long-term survival, distribution and success in ecosystems (Snell, 1986; Ball, 1998; Sousa *et al.*, 2006, 2007, 2008; Costa-Dias *et al.*, 2010). Species are generally divided into euryhaline and stenohaline organisms, which specify their ability to adapt to a wide or narrow range of salinities, respectively (Croghan, 1976). Basically, freshwater species do not invade marine environments. However, species with marine ancestry may tolerate a wide range of salinities, while this pattern strongly varies across and within taxa (Hart *et al.*, 1991). Despite physiological changes and potential detrimental effects of elevated salinities to freshwater organisms (Heugens *et al.*, 2001; Nielsen *et al.*, 2003), they can migrate through saline ecosystems (van Ginneken and Maes, 2005; Jaszczołt and Szaniawska, 2011; Kornis *et al.*, 2012). However, this window of opportunity is often limited, and related to a specific part of their life cycle (juvenile stages and reproduction are usually vulnerable to higher salinity) due to physiological constrains (Holdich *et al.*, 1997; Anger, 2003). Thus salinity is of paramount importance for the spread of freshwater animals into new areas (Croghan, 1976; Leppäkoski and Olenin, 2000) due to, for example, possible dispersion to new watersheds through different estuaries. Also, the ionic composition of water, *i.e.*, the ratio of cations to anions together with the pH, strongly affects the magnitude of saline toxicity to freshwater organisms (Frey, 1993; Bailey and James, 2000). Therefore, life history features together with general ecological information (*e.g.*, distribution, abundance and population structure) should be taken into account when evaluating the effects of salinity.

Biological invasions are a significant threat to native biodiversity worldwide, with important ecological and economic impacts (Simberloff *et al.*, 2013; Seebens *et al.*, 2017). These impacts are particularly important in freshwater ecosystems (Strayer, 2010; Sousa *et al.*, 2014; Moorhouse and Macdonald, 2015). Conversely, estuaries and coastal areas have been overlooked in this regard within the last decades (Cohen and Carlton, 1998; Grosholz, 2002). Nowadays, these heavily invaded ecosystems are used as biological corridors for species that are able to withstand saline conditions (Grosholz, 2002). To our knowledge there are only a few studies dealing with invasion of freshwater species into saline environments (Leppäkoski and Olenin, 2000; Gonçalves *et al.*, 2007). Interestingly, some of these few studies assess the colonization of brackish waters by red swamp crayfish *Procambarus clarkii* in Europe, *e.g.*, Sousa *et al.* (2013) and Meineri *et al.* (2014). Considering biological invasions, the EU parliament and Council have listed some non-indigenous invasive species considered to be of high concern to European biodiversity (EU Regulation No. 1143/2014; Commission Implementing Regulation No. 2016/1141). It lists 23 animals of which freshwater crayfish form a remarkable group of five representatives. This clearly highlights the invasive potential, ecological and economic importance of at least some members in this animal group, as documented mainly in Europe and various parts of North America (Lodge *et al.*, 2000; Holdich *et al.*, 2009). Marbled crayfish *Procambarus fallax* f. *virginalis* is one of these listed animals. Its all-female stocks exclusively reproduce via apomictic parthenogenesis, thus producing clones that exhibit fast growth, early maturation, and high fecundity (Seitz *et al.*, 2005; Martin *et al.*, 2010). As presumed from its North American origin, it has also been proven to be a chronic carrier of crayfish plague *Aphanomyces astaci* pathogen (Keller *et al.*, 2014; Mrugała *et al.*, 2014). Marbled crayfish is a capable burrower (Kouba *et al.*, 2016) with the ability to overwinter in the temperate zone (Veselý *et al.*, 2015; Lipták *et al.*, 2016). Marbled crayfish were first discovered in the German aquarist trade in the mid-1990s, from where it dispersed (Scholtz *et al.*, 2003). The pet trade is an important pathway for the spread of non-indigenous taxa, and marbled crayfish are one of the most frequent and environmentally risky crayfish traded (Patoka *et al.*, 2014). Due to irresponsible or uninformed hobbyists, it may intentionally or accidentally be introduced into the wild (Chucholl, 2013; Patoka *et al.*, 2014). Indeed, reports on the presence of single specimens in the wild occurred at the beginning of the new millennium, followed by confirmed established populations in Germany and Slovakia in 2010. Since then, the number of invaded European countries has substantially increased (Patoka *et al.*, 2016, and references cited therein).

Eastern Europe possesses the entire native ranges, or at least their significant parts, for indigenous crayfish species (ICS) belonging to the genus *Astacus*, especially thick-clawed crayfish *Astacus pachypus* (Kouba *et al.*, 2014). This region has been largely overlooked by astacologists and considered relatively safe from the adverse impacts of gradually expanding non-indigenous crayfish species (NICS) (Perdikaris *et al.*, 2012). Discovery of two distant marbled crayfish *Procambarus fallax* f. *virginalis* populations in Dnipropetrovsk and Odessa, Ukraine in 2015 drastically changed this view (Novitsky and Son, 2016). Pet trade surveys provide extended lists of NICS, often of North American origin, both in the Ukraine (Kotovska *et al.*, 2016) and Lower Volga region of the Russian Federation (Vodovsky *et al.*, 2017). This has raised concerns of NICS potential to negatively impact the unique ecosystems of Azov, Black and Caspian Seas as well as their tributaries that are inhabited by ICS. Therefore, our goal was to investigate survival, growth, and reproduction of marbled crayfish in a range of salinities. This information will be important for implementation of possible management measures regarding the spread of this species in Eastern Europe, but also in estuaries elsewhere.

2 Material and methods

2.1 Experimental design and data acquisition

We conducted a 155-day experiment, lasting from the second half of May to the second half of October 2016, on salinity tolerance in marbled crayfish *Procambarus fallax* f. *virginalis*. This time of year normally includes a seasonal peak in reproduction (Vogt, 2015). The experiment was conducted at the Research Institute of Fish Culture and Hydrobiology in Vodňany, Czech Republic and we used animals from our own laboratory culture. Ten specimens (five in two replicates) for each of 6 experimental treatments, 60 animals in total, were used. The experiment was divided into two parts. Animals were first acclimated in a step-wise manner for 5 days to the target levels of salinity (final salinities of 6, 9, 12, 15, and 18 ppt, respectively). On the first day of acclimation crayfish were moved from fresh water to a saline environment of 6 ppt.

Subsequently, salinities were gradually elevated by 3 ppt per day until the target levels were reached. During the acclimation period all crayfish were divided into 12 static aquaria (described below) with 5 specimens per aquarium (10 aquaria with saline conditions and two aquaria with fresh water serving as a control). The aquaria (36 × 29.5 × 54 cm) were always filled with 16 L of aged tap water with or without salt added depending on the treatment. For ion composition of source water, see Table 1. Aquaria were covered by a plastic lid to limit water evaporation and aerated. To minimize aggression, the shelters were provided. For this, two blocks of joined polypropylene tubes, each containing five tubes (length 10 cm, inner diameter 35 mm), were added to each aquarium. The base of each block was represented by three longitudinally joined tubes with a further two tubes positioned pyramidal in the second layer.

The second part of the experiment started immediately after acclimation. Crayfish were held in the same aquaria as in the acclimation period and individually marked with nail polish on specific places on the carapace (Buřič *et al.*, 2008). Every day observations noting the number of individuals alive in each lot, the number of moulting and the presence of eggs were performed. After visual checking, impurities (*e.g.*, faeces and unconsumed food) were gently siphoned. Offspring was counted at the second developmental stage. When crayfish moulted, the marking was renewed when the animal re-calcified their exoskeleton. To maintain water quality, all baths were changed twice per week (Tue and Fri). The light regime was 12L:12D. Water temperature was recorded hourly by means of a temperature sensor MINIKIN (Environmental measuring systems, Brno, Czech Republic) and kept at 20.6 ± 0.7 °C. Crayfish were fed daily in excess with commercial dry feed for aquarium fish enriched with algae (Sera Granugreen, Sera GmbH, Germany) and frozen chironomid *Chironomus* sp. larvae.

We decided to generalize our experimental design by adding common salt (NaCl p.a., Penta s.r.o., Czech Republic), considering that marine water has specific profiles of salts, and these ions represent the bulk of the compositions. The range of tested salinities was chosen based on specific conditions in the target region and by the continental context. Average salinities of 10–12, 12–13 and 17–18 ppt correspond to the Azov, Caspian and Black Seas, respectively (Berdnikov *et al.*, 1999; Jazdzewski and Konopacka, 2002; Pourkazemi, 2006). Thus, salinity values tested in this experiment are relevant both for the mentioned Seas and estuaries elsewhere.

Prior to the experiment, crayfish were measured (digital calliper; Proma CZ Ltd., Mělčany, Czech Republic) and weighed (analytical balance; Kern & Sohn GmbH, Balingen, Germany) to the nearest 0.1 mm and 0.1 g, respectively. Mean ± SD carapace length (29.9 ± 2.3 mm) and weight (5.3 ± 1.5 g) of marbled crayfish did not differ among all experimental groups, *i.e.*, saline and control groups ($F_{5,54} = 0.39$, $p = 0.95$ and $F_{5,54} = 0.53$, $p = 0.87$, respectively). Following re-calcification (usually 2–3 days after moulting), crayfish biometry was re-measured. For assessment of growth, the following indices were calculated:

$$\mathrm{SGR} = \frac{(\ln(W_t) - \ln(W_i) \times 100)}{T}\ (\%\,\mathrm{day}^{-1}). \qquad (1)$$

Firstly, we counted specific growth rate (SGR; Eq. (1)), where W_t is weight at time t, W_i is initial weight and T is time in days.

Table 1. Ion composition of aged tap water used in experiment. Water analyses conducted in the accredited laboratory of the AGRO-LA, spol. s r.o., Jindřichuv Hradec, Czech Republic.

Ion	Concentration (mg L^{-1})
Bicarbonates	67.1
Sulphates	45.5
Calcium	31.6
Chlorides	9.44
Nitrates	8.65
Sodium	8.6
Magnesium	2.92
Potassium	2.42
Iron	0.113
Manganese	0.05
Nitrites	0.01
Total	**176.4**

$$L_m = \frac{(L_a - L_b) \times 100}{L_b}\ (\mathrm{mm}). \qquad (2)$$

Secondly, we counted absolute carapace length increment at moult (L_m; Eq. (2)), specifically for each moult separately. L_a is carapace length after moult and L_b is length before moult.

2.2 Statistical analysis

Non-parametric survival analysis (Kaplan–Meier method) was performed for all groups, using survival package (Therneau and Grambsch, 2000). To confirm normality in data (Kolmogorov–Smirnov test) one-way ANOVA followed by Tukey's HSD test was performed to compare initial biometry, and absolute length increments and SGR values among groups if applicable. Relationships between initiated and successful moult as well as ovulation were evaluated by means of a Spearman-rank correlation. All analyses were conducted in R version 3.2.5 (R Core Team, 2016). The null hypothesis was rejected at $\alpha < 0.05$ in all tests in this study.

3 Results

Marbled crayfish survival rate differed among tested conditions ($\chi^2 = 31.3$, df = 5, $p \leq 0.001$; Fig. 1). At the end of the experiment, survivors were found only in three experimental groups – control ($n = 8$), 9 and 12 ppt, each having a single specimen after 155 days. In other salinity conditions (6, 15, and 18 ppt) no crayfish survived but the last specimens died at different times (146, 87, and 91 days, respectively). When control was removed from survival analysis, we found no differences among salinity conditions ($\chi^2 = 7$, df = 4, $p = 0.13$). Additionally, neither carapace length ($F_{5,54} = 1.03$, $p = 0.31$) nor weight ($F_{5,54} = 0.66$, $p = 0.41$) significantly influenced marbled crayfish survival among salinity conditions.

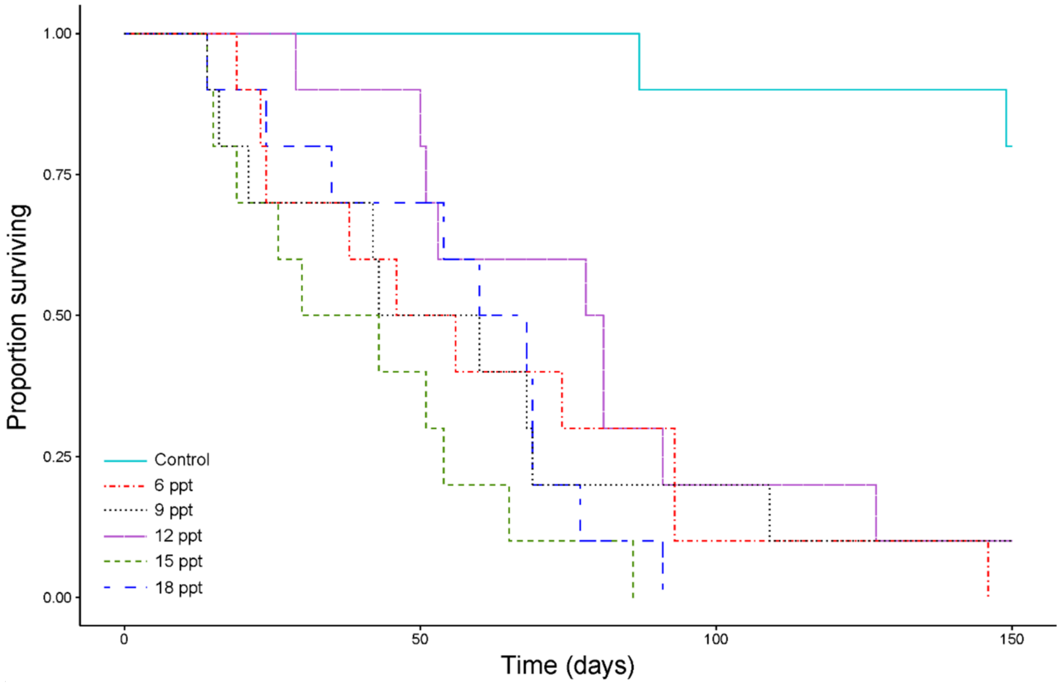

Fig. 1. Survival analysis plot of marbled crayfish *Procambarus fallax* f. *virginalis* kept under different salinities.

Table 2. Growth and reproduction indices of marbled crayfish *Procambarus fallax* f. *virginalis* kept under different salinities. Growth indices presented as mean ± SD. Reproduction indices refer to number of specimens. Values with differing superscripts in given column are significantly different (one-way ANOVA, Tukey's HSD test, $p < 0.05$).

Group	SGR (% day^{-1})	Absolute length increment at moult (mm)		Moult		Ovulation		Juveniles
		1st	2nd	1st initiated/ success	2nd initiated/ success	1st initiated/ success	2nd initiated/ success	
Control	0.22 ± 0.12^{b}	7.28 ± 3.97^{b}	7.33 ± 4.43	9/8	5/5	9/8	1/1	44–141
6 ppt	0.04 ± 0.09^{a}	2.17 ± 3.23^{a}	0	10/3	0/0	3/0	0	0
9 ppt	0	0	0	2/0	0/0	3/0	0	0
12 ppt	0.02 ± 0.08^{a}	0.85 ± 2.56^{a}	0	2/1	0/0	4/0	0	0
15 ppt	0	0	0	2/0	0/0	1/0	0	0
18 ppt	0	0	0	1/0	0/0	1/0	0	0

Salinity negatively influenced physiological processes such as growth, moulting and reproduction of marbled crayfish (Tab. 2). Salinity significantly decreased all measures of moulting (number of initiated, number of successful; Spearman-rank correlation, $p < 0.05$), SGR ($F_{1,30} = 10.74$, $p < 0.001$), and L_m ($F_{1,30} = 9.37$, $p < 0.001$). Only in the control five specimens successfully moulted twice. Salinity negatively influenced ovulation rate and reproduction success of marbled crayfish (Spearman-rank correlation, $p < 0.05$). Females ovulated in all experimental groups, while successful reproduction (reaching 2nd developmental stage) was confirmed only in the control. One female reproduced twice. No apparent cannibalism was observed during the experiment.

4 Discussion

Growth and reproduction are the most important processes expressing fitness and adaptation of species (Guan and Wiles, 1999). Most crayfish are able to survive in saline environments from a few days to a few months, while the effects of salinity on physiological processes differ among crayfish species and families (Jones, 1989; Holdich *et al.*, 1997; Alcorlo *et al.*, 2008). According to Jaszczołt and Szaniawska (2011) spiny-cheek crayfish *Orconectes limosus* are able to successfully reproduce and grow at salinity up to 7 ppt, but growth could be limited in more saline conditions. These results are in line with Holdich *et al.* (1997) who assessed growth in signal crayfish

Pacifastacus leniusculus and narrow-clawed crayfish *Astacus leptodactylus* sensu lato at salinity of 7 ppt. Nevertheless, salinity levels higher than 14 ppt were lethal for eggs in both species. According to Casellato and Masiero (2011), red swamp crayfish reproduce at salinities up to 25 ppt, but there is a negative correlation between salinity and the number of eggs (Alcorlo *et al.*, 2008). Newsom and Davis (1994) suggest elevated salinity as a factor causing higher growth in red swamp crayfish, due to lower energy spent on osmoregulation, which concurs with Sharfstein and Chafin (1979) suggesting salinity of 3–9 ppt as possible for culture of this species. Similarly, Australian species belonging to Parastacidae show analogous patterns where they are generally capable of growth and reproduction under saline conditions (Jones, 1989). For example, Anson and Rouse (1994) found the hatching ability of redclaw *Cherax quadricarinatus* to be from 1 to 20 ppt but hatching rate reduces as salinity increases. Additionally, high salinity (up to 18 ppt) reduced growth and caused lethargy of tested redclaw specimens (Jones, 1989). In comparison to the above-mentioned studies, marbled crayfish exhibited lower survival, growth and no reproduction even in the lowest salinity (6 ppt). Furthermore, increasing salinity contributed to high direct mortality during moulting. It is likely that osmotic stress negatively influenced moulting for which Na^+ and Cl^- are particularly important (Wheatly and Gannon, 1995; Bissattini *et al.*, 2015). The imbalance in ions composition, in particular, might have altered osmoregulation, resulting in a high mortality rate during moulting in our experiment. Nevertheless, it should be taken into account that all mentioned studies had different acclimation periods at each salinity level or used different methods of salt addition (gradual application *vs.* salt shock) and maintenance making possible comparisons among studies very difficult and highly context dependent.

Currently, marbled crayfish is considered a possible result of either hybridization between slough crayfish *Procambarus fallax* and other species of the genus *Procambarus*, or rather of autopolyploid origin (Martin *et al.*, 2016). It is usually regarded as *P. fallax* (Hagen, 1870) f. *virginalis* (Martin *et al.*, 2010). However, Vogt *et al.* (2015) suggest elevation of marbled crayfish to the species level. Slough crayfish and Everglades crayfish *Procambarus alleni* are North American species with a distribution centre in Florida, USA (Taylor *et al.*, 2007). They are probably the closest relatives to marbled crayfish (Martin *et al.*, 2016). Sometimes they live in sympatry in fresh waters (Hendrix and Loftus, 2000; Martin *et al.*, 2010), but it seems that salinity is an important factor in the separation of these two species in brackish conditions (Hendrix and Loftus, 2000). Everglades crayfish can inhabit saline environments in a range of 0–18 ppt (Hendrix and Loftus, 2000), but we are not aware of any study evaluating salinity tolerance in slough crayfish. Considering the relationship of marbled crayfish with the later may partly explains its low salinity tolerance. We found no differences in survival among salinities. It seems that long-term establishment in saline environments such as estuaries or coastal areas are not possible for marbled crayfish. Nevertheless, the ability to withstand saline environments at least for more than 80 days suggests that the species might inhabit watersheds in the vicinity and gradually adapt to more saline conditions using brackish waters as a biological corridor. This might promote its spread to coastal areas and estuaries and then colonisation of different river basins. Also, different water compositions could either reduce or enhance physiological or survival conditions of marbled crayfish, depending on pH, and cations and anions composition (Frey, 1993; Bailey and James, 2000). Furthermore, the short generation time of marbled crayfish might promote its quick adaptation to local conditions. Nevertheless, taking our experimental design as a whole (duration of adaptation and own experiment, numbers and size class of crayfish and salinity values used, etc.), further research is needed since salinity stress and salinity fluctuations may be amplified by other environmental conditions such as temperature, oxygen, and pH (Gilles and Pequeux, 1983).

5 Conclusion

Marbled crayfish is a successful invader with high ecological plasticity, capable of colonizing new habitats (Martin, 2015). We provide first insights into salinity tolerance of marbled crayfish. To sum up, marbled crayfish are probably unable to invade saline ecosystems due to their low survival, reduced growth and prevented reproduction. However, acclimation to natural conditions might lead to higher salinity tolerance due to the broader range of ions which are regulated by different pathways. Even so, its long-term survival in saline conditions has been proved. This might enable its spread in saline ecosystems, which in addition to their short generation time, could potentially lead to its local adaptation in the future.

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