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a ochrany vod
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Burrowing behaviour in crayfish

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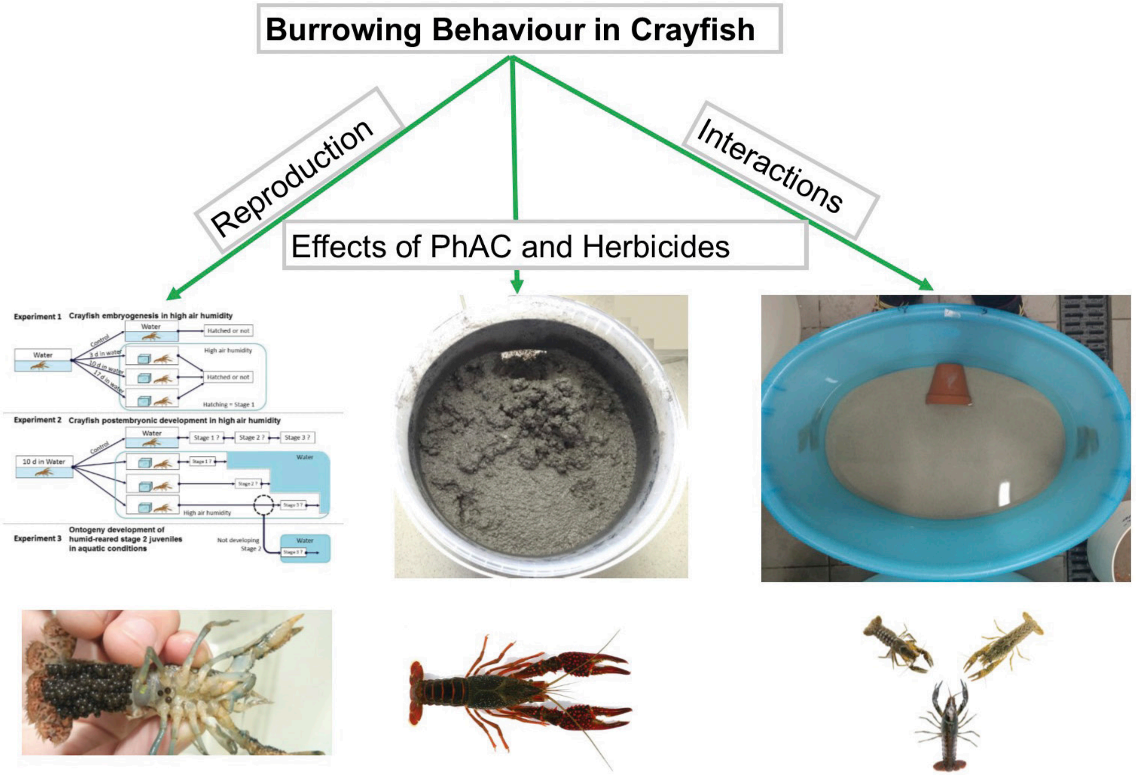
Norovací chování u raků



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Czech Republic, Vodňany, 2020

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

Burrowing behaviour of freshwater crayfish

The crayfish, a member of Decapoda with worldwide distribution (Kawai and Crandall, 2016), is the largest mobile invertebrate in freshwater ecosystems, and often considered to be a keystone species. Crayfish inhabit a broad array of environments ranging from rivers, streams, and lakes to temporary ponds and areas that are seasonally flooded, such as rice-fields. More than 669 species, in four families, have been identified and play important ecological roles as predators and processors of vegetation and carrion as well as being prey for species higher in the food-web (Dorn and Wojdak, 2004; MacNeil et al., 1997). The flow of energy in aquatic ecosystems is often strongly influenced by their presence (Momot, 1995). Crayfish are commercially important (Crandall and De Grave, 2017), in the aquarium trade (Faulkes, 2015), as live bait for fishing (DiStefano et al., 2009), and for human consumption (Ackefors, 2000). Crayfish are also important as model animals for science and research (Oficialdegui, 2000).

The ability to excavate a burrow or to find a suitable shelter is crucial to crayfish survival, allowing them to avoid predation and cannibalism by intraspecifics and to survive winter or periods of drought. Furthermore, burrows are important also for females carrying eggs. A truly non-burrowing crayfish does not exist (Hobbs, 1942; Riek, 1972). Following complex criteria for categorization, crayfish have been be classified as primary, secondary, and tertiary burrowers (Hobbs, 1942; Riek, 1972).

Primary burrowers are those that spend most of their life underground, occasionally moving above the surface for mating, foraging, or migration to new habitats. Most primary burrowers construct complex burrow systems with multiple tunnels, chambers, and entrances (Grow, 1981; Stoeckel et al., 2011; Suter and Richardson, 1977; Welch et al., 2008). The habitats of primary burrowing crayfish range from previously forested residential or industrial areas to agricultural flood plains like rice fields (Loughman et al., 2013). They are typically present in eastern North America, Australia, and South America (Jones et al., 2007; Noro and Buckup, 2010). Several primary burrowing crayfish species are considered threatened (Bryant and Jackson, 1999). It is estimated that 30% of critically imperilled crayfish in North America are primary burrowers, despite representing just 15% of the region's crayfish species (Hopper and Huryn, 2012). Secondary burrowers inhabit open water in areas that are seasonally inundated, but remain in burrows when water levels recede. Compared with the primary burrowers, they construct fewer tunnels and smaller chambers (Correia and Ferreira, 1995; Noro and Buckup, 2010). Tertiary burrowers excavate burrows only in the winter or during drought conditions and, in rare cases, during the breeding season. The occupation of burrows in tertiary burrowers is low compared with that of primary and secondary burrows (Hobbs, 1942; Riek, 1972).

The red swamp crayfish *Procambarus clarkii* (Girard, 1852) has been categorized as both a secondary and a tertiary burrower (Correia and Ferreira, 1995; Huner et al., 1984). It usually constructs a simple burrow having a single tunnel and entrance, but more complex systems have been observed (Barbaresi et al., 2004; Correia and Ferreira, 1995; Huner et al., 1984). Burrows are used seasonally, mostly for reproduction and for shelter during extreme temperatures and drought conditions (Hobbs, 1981; Stoeckel et al., 2011). In Australia, crayfish of the genus *Engaeus* excavate burrows or chambers on slopes without connection to the water table (Horwitz et al., 1985a; Suter and Richardson, 1977) and cannot be assigned to any category proposed by Hobbs (1942). Based on the relationship of burrows with the water-table, Horwitz and Richardson (1986) proposed an alternative classification of burrows constructed by crayfish in Australia: Type 1, burrows associated with lotic or lentic surface waterbodies; Type 2, burrows not contacting surface waters but penetrating the water table; and Type 3, burrows that obtain water from surface run-off or percolation and store that water in chambers above the water table (Horwitz and Richardson, 1986). As the research reported in this thesis did not include Australian species, I have followed the classification of Hobbs (1942) and Riek (1972).

Unresolved ecological gaps in freshwater crayfish burrowing behaviour

Generally, crayfish construct burrows or seek shelter as protection against conspecifics, predators, and adverse environmental conditions (Horwitz and Richardson, 1986; Riek, 1969). In addition to shelter, crayfish burrows may be a source of water; refuge during moulting, oviposition, and egg incubation; and protection of offspring (Atkinson and Taylor, 1988; Barbaresi et al., 2004; Bergman and Moore, 2003; Growns and Richardson, 1988; Huner et al., 1984; Richardson and Swain, 1980). Burrows or shelters are critical to crayfish survival, and their availability may represent a principle bottleneck in crayfish populations (Hobbs, 1981). Although some crayfish species are abundant and widespread globally, many are seriously threatened. Crayfish are being threatened by overharvesting, habitat destruction, biological invasion, and water pollution (Kawai and Crandall, 2016) and face decline or extinction of populations (Richman et al., 2015).

The potential effects of drought on crayfish reproduction

Seasonal fluctuations in water conditions can have a close relationship with faunal life cycles, with critical species life history events synchronized to favourable periods (Gasith and Resh, 1999). The ability to avoid desiccation by excavating vertical burrows into the hyporheic zone might play a significant role in the success of a crayfish species during drought (Kouba et al., 2016). In this situation, juveniles of burrow-dwelling crayfish may not survive outside parent burrows (Anne and Rasa, 1995; Richardson, 2007). Hence, juveniles of some species tend to stay with parents until conditions improve or until reaching sufficient size to excavate their own burrows (Hamr and Richardson, 1994; Horwitz et al., 1985b; Linsenmair, 2007).

Long-term drought is challenging for many freshwater animals, and numerous crayfish species evolved to reproduce in burrows (Crandall and De Grave, 2017; Hobbs, 1942; Riek, 1972). It is often difficult to obtain accurate information of life history traits and behaviours of these species (Richardson, 2007). Water quality and levels in crayfish burrows can fluctuate widely, and how these conditions impact crayfish survival and reproductive success is little known. It is suggested that some crayfish in burrows spend up to 70% of their time in air, due to poor water quality (McMahon and Stuart, 1995), and that water in natural burrows serves mainly to supply humidity (Huner, 1994). McMahon and Stuart (1995) present evidence that crayfish exposed to long periods out of water to escape severely depleted oxygen and enriched carbon dioxide levels in the small pools of water typical of burrows eventually so affected that recovery may be unlikely. It has recently been documented that *P. clarkii* do not spawn in the absence of free-standing water, and oviposition was delayed until replenishment of water in a simulated burrow study (McClain, 2013). Reproductive failure may explain the decline of some crayfish species under prolonged drought (Lodge et al., 2000; Taylor et al., 2007). To survive drought, crayfish tend to inhabit shelters or burrows where they can remain in contact with water or high humidity environments. However, it is not known whether embryogenesis or post-embryonic development can occur without free-standing water.

The objective of the research reported in Chapter 2 was to assess the effects of long-term drought conditions on crayfish embryogenesis and postembryonic development in burrows, when free-standing water was not always available or of suitable quality. Using the marbled crayfish *Procambarus virginalis* as a model species, experiments were conducted to 1) determine whether embryogenesis in crayfish progresses to the hatching stage in a high humidity environment in absence of standing water, 2) assess postembryonic development in a high humidity environment, and 3) determine success of late Stage 2 juvenile metamorphosis when animals are re-immersed.

Effects of aquatic contamination on the freshwater crayfish burrowing behaviour

In addition to extreme weather conditions, the quality of surface waters plays an essential role in maintaining ecosystem biodiversity and function (Bakker, 2012). Water pollution is considered an important driver of global biodiversity decline (Bayen, 2012; Dudgeon et al., 2006; Manosa et al., 2001), with contamination and deterioration of water quality negatively impacting aquatic ecosystems (Affum et al., 2018; Aliko et al., 2018; Sehonova et al., 2018; Yao et al., 2015). Pesticides, pharmaceuticals, chemical fertilizers, detergents, petrochemicals, and plastics are the most common synthetic pollutants detected and studied (Amoatey and Baawain, 2019; Guzzetti et al., 2018; Yang et al., 2017). The negative effects of chemical contamination on non-target organisms in the natural environment is the focus of increasing attention (Arsand et al., 2018; Costa et al., 2018; Moe et al., 2019).

The contamination of aquatic environments by pharmaceutically active compounds (PhAC) (Ebele et al., 2017) is an increasing concern worldwide (Burkina et al., 2015; Shaaban, 2017). Pharmaceuticals reported to pose a serious threat to non-target aquatic organisms (Biel-Maeso et al., 2018; Boxall et al., 2012; Corcoran et al., 2010) include psychoactive compounds, as well as antihistamines and cardiovascular drugs (Berset et al., 2010; Ebele et al., 2017; Irvine et al., 2011; Lindberg et al., 2014; Schafhauser et al., 2018). Their primary source in the environment is outflow from wastewater treatment plants (Cunha et al., 2017; Li et al., 2011) following their incomplete removal (Buchberger, 2007; Golovko et al., 2014; Petrović et al., 2003). Reported aquatic concentrations of maternal compounds and metabolites of PhACs range from ng to μg/L (Aymerich et al., 2016; Grabicova et al., 2015). In excess of 600 pharmaceutical substances have been detected worldwide (Küster and Adler, 2014) in wastewater, surface water, groundwater, and drinking water (Balakrishna et al., 2017; Li, 2014; Simazaki et al., 2015; Sui et al., 2015; Zhang et al., 2018). Although it is widely considered that the risk of these compounds to aquatic biota is minimal because of their sub-lethal concentrations and rapid degradation in the environment (Richmond et al., 2017), some PhACs are reported to show detrimental effects on aquatic organisms, including modification of behaviour and disruption of reproduction and development (Furlong et al., 2011; Imeh-Nathaniel et al., 2017; Mishra et al., 2017). Impacts are documented on algae, zooplankton, mussels, shrimp, crayfish, and fish (Brodin et al., 2013; Buřič et al., 2018; Crane et al., 2006; Diniz et al., 2015; Douda et al., 2019; González-Ortegón et al., 2016).

Herbicides have long been heavily used in agriculture and horticulture (El-Nahhal, 2003; Goncalves and Alpendurada, 2005; Silva et al., 2019) and are detected in natural waters worldwide (Peng et al., 2018; Prosser et al., 2016; Székács et al., 2015; Thurman et al., 2000), typically at concentrations well below levels lethal to aquatic organisms (Amoatey and Baawain, 2019). Nevertheless, sublethal concentrations of herbicides and their metabolites may have detrimental effects on non-target organisms (Cook and Moore, 2008; Velisek et al., 2016; Velisek et al., 2017), impairing physiology and behaviour (Cook and Moore, 2008; Scott and Sloman, 2004). Impacts include alterations to the nervous system (Benli et al., 2007; Sarikaya and Yılmaz, 2003), biochemical and haematological changes, and impaired reproduction and development (Velisek et al., 2014).

Recently, the crayfish has been suggested to be a valuable model for studies of drug addiction (Imeh-Nathaniel et al., 2017; Kubec et al., 2019). Crayfish may become conditioned to prefer environments in which they obtain drugs (Imeh-Nathaniel et al., 2016; Nathaniel et al., 2012a; Panksepp and Huber, 2004), and the innate reward system of crayfish is sensitive to therapeutic drugs used in humans (Nathaniel et al., 2012b). When injected with a low dose of methamphetamine, the rusty crayfish *Faxonius rusticus* (Girard 1852) showed mobility increase to a greater extent than with higher doses, while the higher dose was associated with a decrease in mobility (Imeh-Nathaniel et al., 2017).

The red swamp crayfish *Procambarus clarkii* is a primary model species that has been used in ecotoxicology studies for decades (Fong and Ford, 2014; Stara et al., 2014; Yu et al., 2017). In studies reported in Chapters 3 and 4, we tested the hypothesis that burrowing behaviour of the red swamp crayfish would be altered with exposure to PhACs and herbicides at concentrations frequently detected in aquatic environments. As representative PhACs, we chose methamphetamine (METH) and tramadol (TRA) (1 μg/L), and, as herbicides, terbuthylazine (TERB) and metazachlor (META) (2 μg/L). The reported concentration of methamphetamine in discharge water in the Czech Republic and Slovakia ranges from 13 to 1805 ng/L (Mackul'ak et al., 2016) and tramadol from 3.7 to 5300 ng/L (Grabicova et al., 2017). The concentration of terbuthylazine in European surface and groundwaters has been detected at a range from 0.01 to 13.0 μg/L (Bossi et al., 2002; Chary et al., 2012; Fait et al., 2010; Jurado et al., 2012; Lacorte et al., 1998; Rodriguez-Mozaz et al., 2004; Vega et al., 2005), and the concentration of metazachlor recorded in natural waterbodies can reach 100 μg/L (Mohr et al., 2007) with long degradation time, having a half-life in soil of 2.8–114 days, and in water of 20.6–216 days (Hertfordshire, 2019).

Crayfish competition for shelter as a primary resource

Finding shelter is critical to the life history of tertiary burrowers that excavate burrows only in winter or during drought conditions and, rarely, during the breeding season. It is usually a shallow depression under a rock, stone, or log or an abandoned burrow of another species.

Biological invasions represent a threat to both native crayfish and entire communities, which are closely related to their introduction event. In Europe, prior to 1990, these were largely driven by the presumed value of non-native species for aquaculture. The involved species are often referred to as old non-indigenous crayfish species––Old NICS (Holdich et al., 2009). Later, development of the aquarium pet trade resulted introductions into the wild, intentionally and accidentally (Chucholl, 2013; Patoka et al., 2014), of species classified as New NICS (Furse and Coughran, 2011; Kawai and Crandall, 2016; Richman et al., 2015).

Invasions are increasing in both number of species and their abundance around the world (Early et al., 2016), and aquatic ecosystems are often invaded by multiple species that interact with one another as well as with native biota (Hudina et al., 2011), a process known as over-invasion (Russell et al., 2014). Life history, niche preference, and adaptation strategies of invasive species may overlap, but interactions among them remain largely unexplored. In a novel environment, multiple invaders may alter the ecosystem through additive or multiplicative effects (Jackson, 2015; Roy et al., 2014), compounding the impact of interactions of individual invasive species with incumbents (Preisser and Elkinton, 2008). However, the consequences of multiple invasions is little studied (Russell et al., 2014). Successful invaders can be identified by comparative analysis of invading species (Van Kleunen et al., 2010). Mechanisms may become clear when animals share identical ecological niches and compete for common resources such as food and shelter (Huntingford, 2013). Its current distribution provides growing evidence that the emerging marbled crayfish *Procambarus virginalis* Lyko, 2017 can compete successfully with established spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817) (Chucholl, 2015; Hossain et al., 2018; Lipták et al., 2017; Patoka et al., 2016). The calico crayfish *Faxonius immunis* (Hagen, 1870) is also reported to dominate during direct competition for shelter with the spiny-cheek, previously established in the Rhine River (Chucholl et al., 2008). Marbled and calico crayfish may currently co-exist in an ecological niche, as several reports have confirmed marbled crayfish occurrence in the Rhine River system (Chucholl et al., 2012). Linzmaier et al. (2018) found that marbled crayfish were generally more aggressive than carapace length–matched spiny-cheek crayfish in agonistic encounters, even against larger opponents. Outcome of competition of weight-matched marbled crayfish with calico crayfish and spiny-cheek crayfish and direct competition with these species for shelter has not been assessed.

In this study (Chapter 5 and 6) aggression of marbled crayfish during interspecific interactions under laboratory conditions was quantified by characterizing their direct agonistic interactions and competition for shelter with calico and spiny-cheek crayfish. Differences in aggression may predict interactions and outcomes under natural conditions.

Crayfish burrowing behaviour

Evaluation of methods of casting crayfish burrows

To investigate the basic structure, volume, surface area, and morphology of crayfish burrow systems in natural environments, casts of the burrow can be made and removed from the surrounding soil. The material used for casts of different species is shown in Table 1.

Plaster and concrete are inexpensive and readily available, and plaster has been used to cast crab burrows (Dembowski, 1926; Hayasaka, 1935), but these materials can only provide a cast of the lower portion of burrows and chambers (Lawrence et al., 2001). Gypsum produces good results, but it is a challenge to remove complete casts from the soil because of its fragility (Noro and Buckup, 2010). Polyurethane foam burrow casts are considerably lighter than the concrete and plaster, making them easier to handle and transport (Lawrence et al., 2001). However, its rapid reaction with the chemical reagent can produce hardened foam before the primary liquid can completely fill the burrow system, and it does not set up well when water is present, leading to an incomplete cast (Lawrence et al., 2001; Noro and Buckup, 2010; Welch et al., 2008). Polyester resin is useful for studying the burrows of benthic animals (Atkinson and Chapman, 1984; Hamano, 1990). In particular, non-polyester resin with the appropriate catalyst (Methyl-Ethyl-Acetone Peroxide; Brasnox DM50) can produce a superior cast that is robust and easily removed in one piece (Noro and Buckup, 2010). Polyester resin seems to be the optimal choice for making burrow casts. Removal of the burrow cast from the soil is most commonly accomplished by digging with a small tool (Growns and Richardson, 1988; Noro and Buckup, 2010; Stieglitz et al., 2000; Welch et al., 2008). Water pressure is effective and can preserve the shape of casts, but some digging is required to remove the burrow cast (Lawrence et al., 2001). An industrial high-pressure spray unit can also be used, but has the disadvantage of requiring a source of filtered water that is not always available in the field (Lawrence et al., 2001).

The volume of a burrow cast can be measured by water displacement (Growns and Richardson, 1988; Katz, 1980; Lim, 2006), mass-density analysis (Atkinson et al., 1987; James et al., 1990; Shimoda and Tamaki, 2004), or three-dimensional (3D) laser scanning (Platt et al., 2010). Water displacement is usually the simplest method: the cast is fully immersed in water, and the volume of the displaced water is that of the burrow cast. However, it is not suitable for a plaster cast that will be damaged by water (Tschinkel, 2010). The mass-density method requires a casting medium that is stable in density. The weight of the burrow cast relative to the density of the material are used to obtain the volume of the cast. The 3D laser scanning method has been integrated into software (Bednarz and McIlroy, 2011; DeSilva, 2010; Rossi et al., 2008; Strait et al., 2007). An instrument capable of recording and visually displaying 3D data is well suited for studies of three-dimensional trace fossils, especially burrows. The volume of the burrow cast can be calculated by software from data collected by a 3D scanner (Platt et al., 2010). Surface area is an important parameter of the cast, since it provides the extent of water-sediment interface (Kinoshita, 2002) and can represent redox potential and microbial biomass on walls of subaqueous burrows (Dworschak, 2001). Surface area can be calculated by wrapping the cast in a single layer of foil of known weight per unit area and weighing the foil (Atkinson and Nash, 1990) or by using software to analyse the data collected by the 3D scanner (Platt et al., 2010).

Laboratory protocol for study of crayfish burrowing behaviour

Studying burrowing crayfish in a subterranean habitat can present challenges in locating burrows, determining whether a burrow is active, identifying species inhabiting the burrow, directly observing burrow structure and crayfish behaviour, and manipulating conditions in and around burrows. Simulated setups and experimental systems to study burrowing behaviour of crayfish in laboratories need to be easy to build, inexpensive, replicable, and amenable to manipulation of soil type and water flow, level, and quality. Crayfish within a well-designed and executed system should burrow readily, create easily observed burrows and chimney structures similar to those found in the wild, and exhibit high survival and growth under favourable conditions (Stoeckel et al., 2011). Crayfish survival and behaviour in experimental systems should ideally be similar to that in the wild. As an example, to study the burrowing behaviour of *P. clarkii*, McClain (2010) placed a single mature crayfish in a polyethylene container filled with soil and water from its previous habitat with an artificial burrow mimicking that in the natural world. A more complex artificial setup was devised to study how the burrowing activity of *P. clarkii* altered seepage progression in a river levee, using the species preferred soil for burrowing (Haubrock et al., 2019).

Methods employed in the reported research

Methodological approaches used in the research reported herein were derived from systems established at the Laboratory of Freshwater Ecosystems (formerly Laboratory of Ethology of Fish and Crayfish), combining evaluation of burrows using gypsum casts and 3D scanning with direct observation of species interactions aided by Ethovision software. Studies involving environmental contamination included participation of faculty colleagues skilled in ecotoxicology and chemistry.

Aims of the research

Despite the more than one-hundred years of studying burrowing behaviour of individual crayfish species, there is much to be discovered about the behaviour ecology of burrowing crayfish compared to open water species (Helms et al., 2013). For many species, the basic parameters of life history including life span, reproduction frequency and seasonality, brood size, and survival rates remain unknown, and extrapolation of information obtained from a few well-studied species to such a phylogenetically and geographically diverse group should be done with caution. The number of newly described crayfish is increasing yearly. Thus, knowledge gaps exist that require deeper investigation.

Goals and expected results of the research were to:

- review recent literature with respect to burrowing in crayfish to compile a comprehensive database of current knowledge.

- evaluate crayfish reproductive success under drought conditions. The array of factors threatening biodiversity and aquatic ecosystems is broad and complex, including climate change and severe events such as floods, fires, storms, and long-term drought, with far-reaching consequences. Drought is of key importance for hyporheic dwellers like crayfish.

- assess the burrowing behaviour of crayfish exposed to a range of contaminants found in natural waters. Crayfish are recognized bio-indicators of environmental conditions, and are increasingly utilized for assessment of its status. The effects of pollution under natural conditions on crayfish has been scarcely studied.

- analyse interspecific interactions under laboratory conditions of the emerging invader marbled crayfish *P. virginalis* and widely established invasive spiny-cheek *F. limosus* and calico crayfish *F. immunis* by characterizing their direct agonistic interactions and competition for shelter. In crayfish, aggressive behaviour during interspecific interactions is a major trait associated with their invasion potential that can be used to estimate the ecological consequences of interaction of species sharing a similar niche. Differences in aggression may potentially impact their interactions under natural conditions.

Table 1. *Materials for preparing burrow casts with respect to taxonomic groups.*

Parameter	Aluminium	Paraffin wax	Concrete	Gypsum	Polyester resin	Polyurethane foam	Plaster	Zinc
Taxonomic groups	Ant	Ant, Crab	Crayfish	Crayfish	Crayfish, Shrimp, Crab	Crayfish, Shrimp	Crab, Crayfish	Ant
Advantages	light; strong; not broken; excellent for study and display	easy availability; few requirements	inexpensive; readily available	produces good cast	produces superior casts; light	light; easy to extricate from soil	inexpensive; readily available	suitable for nest with narrow tunnels and fin structure, 1mm or less
Disadvantages	Costly; requires special apparatus	weak; easily bent and distorted; penetrates into the soil	heavy; removal difficult; poor detail	fragile	needs to be mixed with hardener	rapid set up; water in burrow interferes	useful only in lower segments of burrows and chambers	brittle; difficult to obtain; expensive
References	(Tschinkel, 2010)	(Lim and Diong, 2003; Tschinkel, 2010)	(Lawrence et al., 2001)	(Lawrence et al., 2001)	(Lawrence et al., 2001; Stieglitz et al., 2000; Wang et al., 2014)	(Lawrence et al., 2001; Sepahvand et al., 2014; Welch et al., 2008)	(Growns and Richardson, 1988; Katz, 1980; Lawrence et al., 2001)	(Tschinkel, 2010)

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CHAPTER 2

HIGH AIR HUMIDITY IS SUFFICIENT FOR SUCCESSFUL EGG INCUBATION AND EARLY POST-EMBRYONIC DEVELOPMENT IN THE MARBLED CRAYFISH (*PROCAMBARUS VIRGINALIS*)

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High air humidity is sufficient for successful egg incubation and early post-embryonic development in the marbled crayfish (*Procambarus virginalis*)

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Abstract

1. Severe weather events, such as long-term droughts, are challenging for many freshwater species. To survive drought, freshwater crayfish tend to inhabit shelters or burrows where they can remain in contact with water or high humidity environments. However, it is not known whether embryogenesis or post-embryonic development can occur without free standing water.
2. To address this question, three experiments were conducted using artificial burrows with high air humidity and using marbled crayfish (*Procambarus virginalis* Lyko, 2017) as a model species. Marbled crayfish are capable of parthenogenetic reproduction, burrow extensively and are able to travel long distances over land. In the first experiment, ovigerous females were transferred to simulated burrows without free water, but with high air humidity. A control group of females were kept in burrows with free water. Successful hatching was achieved in both groups.
3. In the second experiment, ovigerous females were transferred to simulated burrows with no free water but high air humidity and post-embryonic development were observed. Following successful hatching, offspring moulted to the second developmental stage (stage 2 juveniles). Stage 2 juveniles remained viable without free water for 20 days, but further development was not observed. However, when some of these stage 2 juveniles were placed back into fully aquatic conditions (experiment 3), they moulted to stage 3 within 4 to 8 days.
4. These results demonstrated the ability of marbled crayfish to undergo terminal phases of embryogenesis, including hatching, as well as early post-embryonic development under high air humidity conditions only. Post-embryonic development was suspended in the absence of free water, and successfully resumed when re-immersed.

5. This similar ability to tolerate drought-like conditions during post-embryonic development may also occur in other crayfish species, especially primary burrowers. This unprecedented life history trait may be crucial for inhabiting ecosystems with rapidly changing water regimes. In drying climates, it may confer advantages on some crayfish species (including some invasive species) over others.

KEYWORDS
burrow, drought, hyporheic dweller, macroinvertebrate, ontogeny

1 | INTRODUCTION

Freshwater crayfish (Crustacea, Decapoda, Astacidea) are often considered keystone species in aquatic ecosystems, and several genera are effective ecosystem engineers, with the ability to modify the environment (Creed, 1994; Geiger, Alcorlo, Baltanas, & Montes, 2005; Sanford, 1999). They frequently represent particularly successful invasive species, such as the red swamp crayfish *Procambarus clarkii* (Girard, 1852) and marbled crayfish *Procambarus virginalis* Lyko, 2017 (Momot, 1995; Reynolds, Souty-Grosset, & Richardson, 2013; Kouba, Petrusek, & Kozák 2014). Generally, crayfish have evolved an ability to seek shelter or construct burrows as protection against conspecifics, predators, and adverse environmental conditions (e.g. droughts and temperature extremes) (Horwitz & Richardson, 1986; Riek, 1969). They also use these refuges as a source of water or cover during moulting, oviposition, and incubation of eggs, as well as protection of the offspring (Atkinson & Taylor, 1988; Barbaresi, Tricarico, & Gherardi, 2004; Bergman & Moore, 2003; Growns & Richardson, 1988; Huner, Barr, & Coleman, 1991; Richardson & Swain, 1980). Shelters or burrows are therefore often critical for crayfish survival, and their availability is the *principle bottleneck* in crayfish populations (Hobbs, 1991). Extreme seasonal fluctuations in water regime can have a close relationship with the faunal life cycles, whose important life history events may correlate with favourable periods (Gasith & Resh, 1999). Severe weather events such as long-term drought may be detrimental to aquatic biota (Dudgeon et al., 2006). During severe drought, juveniles of burrow-dwelling crayfish may die outside parental burrows (Anne & Rasa, 1995; Richardson, 2007). As a result, juveniles of some species tend to stay with parents until external conditions improve or until their body reaches sufficient size to enable their own burrowing activity (Hamr & Richardson, 1994; Horwitz, Richardson, & Cramp, 1985; Linsenmair, 2007).

Presence of water, or at least high air humidity in burrows, may be important during dry periods. As an example of successful adaptation, crayfish of the genus *Engaeus* in south-eastern Australia and Tasmania, dig burrows in habitats ranging from open water of permanent water bodies to terrestrial habitats with no free surface water. They must maintain at least high air humidity conditions in their deep burrows to survive in the latter case, only leaving the burrow to search for mates and food, usually at night (Horwitz et al., 1985).

With many species of crayfish having evolved to reproduce within burrows (Crandall & De Grave, 2017; Hobbs, 1942; Riek, 1972), it is often difficult to obtain accurate information on life history traits and behaviours of these species (Richardson, 2007). Water quality and levels in crayfish burrows can fluctuate widely, and crayfish survival and reproductive success under these conditions are poorly known. It is suggested that crayfish in burrows spend up to 70% of their time in air due to the poor water quality (McMahon & Stuart, 1995) and that water in natural burrows serves mainly to supply 100% humidity (Huner, 1994). McMahon and Stuart (1995) present evidence that crayfish exposed to long periods out of water as a result of severely depleted oxygen levels and enriched carbon dioxide levels in small pools of water (typical of water in burrows) eventually become taxed to the point that recovery is difficult. It has recently been documented that spawning in *P. clarkii* cannot occur in the absence of free water and oviposition was delayed for a period of time until free water was returned in a simulated burrow study (McClain, 2013). As a result, reproductive failure may explain the decline of some crayfish species (Lodge, Taylor, Holdich, & Skurdal, 2000; Taylor et al., 2007) under prolonged drought.

Given the physical changes (e.g. ultraviolet light, high temperature, large fluctuations in salinity, pH, and oxygen) that occur during drying (Alekseev, De Stasio, & Gilbert, 2007), invertebrates living in intermittent aquatic environments often show adaptive traits (Lytle & Poff, 2004; Robson, Chester, & Austin, 2011). Freshwater crayfish are capable of aerial respiration, thanks to the structure of gills that do not collapse in air, and thus, provide a bimodal gas exchange surface (McMahon, 2002). Although the number of studies remains limited, McMahon and Hankinson (1993) and McMahon and Stuart (1995) concluded that the burrowing crayfish spent the majority of time utilising aerial respiration. There is also some evidence for possible aerial respiration of crayfish eggs during incubation. *Procambarus clarkii*, among others (Crandall & De Grave, 2017; Hobbs, 1942; Riek, 1972), frequently use burrows for oviposition and egg incubation (Eversole & McClain, 2000; Huner, 1994), but it seems that their eggs are not always submerged in the groundwater within burrows due to low available levels of dissolved oxygen and overall water quality. The oxygen diffuses directly from the burrow atmosphere while eggs are fanned by swimmeret movements (Huner et al., 1991). In the case of astaciculture of signal crayfish, *Pacifastacus leniusculus* (Dana, 1852), and white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858), egg storage is a technique whereby fertilised eggs can be removed from the female's pleopods and artificially stored in a high humid environment for extended periods of time. Clearly, aerial respiration is occurring under these circumstances. However, when the 12th stage of embryonic development (embryo with pulsating heart, Zehnder, 1934) is reached, the eggs need to be immersed for further progress in embryogenesis (Celada et al., 2000; Pérez et al., 2003). This critical phase is presumably determined by the elevated metabolic needs, such as metabolic waste removal as well as maintaining ionic and acid–base balance (McMahon, 2002), in terminal phases of embryogenesis. The ability to respire in air under high humidity conditions in crayfish eggs and early juveniles may be species-specific, which calls for deeper understanding of mechanisms enabling this to occur.

While it is becoming more obvious that oviposition in crayfish is hampered or delayed in burrows lacking free standing water, the effects of high humidity on embryogenesis is largely unknown (McClain, 2013; Niksirat, Kouba, & Kozák, 2014, 2015). However, survival and reproduction of crayfish individuals in burrows over extended periods of time without free water have been demonstrated (Jordan, Babbitt, McIvor, & Miller, 2000; Kouba et al., 2016; McClain, 2013; McClain & Romaire, 2004). Due to its exclusively parthenogenetic mode of reproduction, burrowing behaviour and potential invasive abilities (Gutekunst et al., 2018; Scholtz et al., 2003), *P. virginalis* is a suitable model species to study the effect of seasonal water level fluctuations on the early life-history traits of crayfish living in intermittent aquatic environments (Hossain, Patoka, Kouba, & Buřič, 2018).

Our objective was to assess the effects of long-term drought conditions on crayfish embryogenesis and post-embryonic development in burrows when free water was not always available or of suitable quality. Using *P. virginalis* as a model species, three experiments were conducted: (1) assess the ability of embryogenesis in crayfish to proceed to the hatching stage in highly humid environments in absence of free standing water; (2) assess post-embryonic development in highly humid environments; and (3) determine effectiveness of late stage 2 juvenile metamorphosis when animals are re-immersed.

2 | MATERIALS AND METHODS

2.1 | Study species and experimental design

Mature females with well-developed glair glands were held communally under laboratory conditions at the Research Institute of Fish Culture and Hydrobiology in Vodňany, FFPW USB, Czech Republic. Three experiments (crayfish embryogenesis in high air humidity—Experiment 1, crayfish post-embryonic development in high air humidity—Experiment 2, and ontogeny development of humid-reared stage 2 juveniles in aquatic conditions—Experiment 3) were carried out in laboratory conditions by keeping spawning females inside artificial burrows mimicking burrow habitats (Figure 1).

The animals were maintained in aquaria within a recirculation system at 21–22°C, in a 14:10 light: dark regime, and fed frozen chironomid larvae and sliced carrot in excess. Crayfish were checked for oviposition on a daily basis from August to October 2016 (Experiment 1) and from February to June 2017 (Experiments 2 and 3). These periods include seasonal peaks in reproduction of the species (Vogt, 2015). Females with well-developed broods only (i.e. numerous healthy-appearing eggs) were collected for the experiments. One day after observation of oviposition, ovigerous females were gently taken from the culture system and held individually in polypropylene boxes (18 × 13 × 7.5 cm) covered with plastic lids, representing artificial burrows mimicking burrow habitats or experimental units in the subsequent experiments.

Artificial burrows contained 0.5 L of aged tap water that was exchanged twice a week. Some experimental groups were thereafter transferred from an aquatic environment to experimental burrows with high (>99%) air humidity (measured by a portable weather station WSA-502, EMOS). High air humidity was maintained in the enclosed burrows by the presence of a plastic sponge (dimensions ca. 50 × 35 × 45 mm) containing 50 ml of water and positioned in the centre of the burrow. No free water was accessible in these burrows. Burrows were kept in darkness at ambient laboratory temperatures (21–22°C). Females with their brood were assigned randomly to the various experimental treatments described below. To minimise disturbances, and thus stress, during embryogenesis, the burrows were checked on a daily basis starting 3 days before presumed hatching and continued daily until viable eggs were no longer present. Hatching was noted (when present) and hatchlings quantified under the various treatments.

2.2 | Experimental procedures

2.2.1 | Crayfish embryogenesis in high air humidity—Experiment 1

The first experiment tested the ability of crayfish to successfully complete embryogenesis under high air humidity. Ovigerous females (*n* = 7) maintained under aquatic conditions throughout embryo development (until hatching) represented a control group. Three further groups (*n* = 7 in all cases) simulated situations of free water scarcity with females transferred to artificial burrows with high air humidity at different periods of embryonic development. Females were transferred from a fully aquatic environment to the humidity-only burrows at 3, 10, and 17 days following oviposition. These treatment groups are identified as W3d, W10d, and W17d, respectively. If present, hatchlings (stage 1) were gently removed from pleopods and quantified. The experiment was terminated once viable eggs were no longer present. Female biometry range was 15.6–27.9 mm in carapace length and 0.9–5.7 g in weight. The temperature was 21.3 ± 0.2°C.

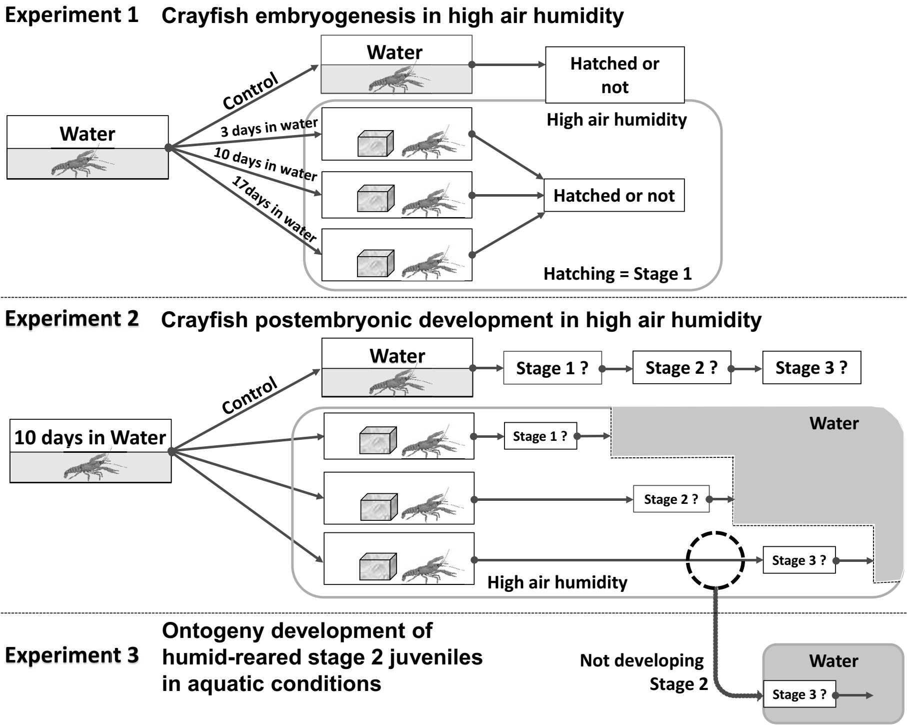

FIGURE 1 Schematic figure of experimental setup [Colour figure can be viewed at wileyonlinelibrary.com]

2.2.2 | Crayfish post-embryonic development in high air humidity—Experiment 2

The aim of Experiment 2 was to assess the ability of juveniles to advance through the post-embryonic developmental stages in high air humidity. Handling of experimental animals was similar to that in the previous experiment (Experiment 1). The control group in this experiment consisted of ovigerous females (n = 17) that were maintained in fully aquatic conditions within the artificial burrows until independent juveniles in stage 3 (Vogt & Tolley, 2004) appeared. Experimental groups simulating situations of water scarcity were all represented with ovigerous females that were initially kept in fully aquatic conditions of artificial burrows for 10 days, followed by repositioning to high air humidity burrows after 10 days as in the group W10d of Experiment 1. Since the ability of offspring to reach stage 1 (hatching), stage 2 (first moult), and stage 3 (independent juveniles) under high humidity conditions was tested, the experimental groups are indicated as W10d-stage 1, W10d-stage 2, and W10d-stage 3, respectively. These groups were represented by 14, 14, and 13 females, respectively.

Eggs and developing juveniles of the control group were not quantified since their enumeration on maternal pleopods would be disturbing for both the female and the offspring. This is unfortunately also true for stage 3 juveniles that we failed to quantify, although their offspring were generally numerous. Our long-term experience with the species suggests that comparable culturing conditions have been sufficient for successful reproduction under laboratory conditions. Commonly observed fecundity of similar-sized females often exceeds 100 eggs, with survival rates of hatchlings reaching stage 2 juveniles in a range of 75–95% in aquatic conditions. Meanwhile, attaining stage 3 and 4 is most often represented by a similar value of a broad range between 35 and 75%. Juveniles of the other experimental groups reaching the respective target endpoint in high air humidity were gently separated (using entomological forceps) from the female pleopods and placed back in artificial burrows (without the female or plastic sponge) containing 0.5 L of aged tap water, exchanged every other day. Transferred juveniles remained under the original experimental treatment designation. In each of these groups, survival rate and developmental stage of the juveniles were checked daily once the juveniles were returned to aquatic

TABLE 1 Proportion (and percentage; %) of stocked female crayfish that successfully hatched eggs, and mean number (± *SD*) and range of hatchlings in successfully reproducing females in Experiment 1. Within a column, values with differing letters differ statistically ($p < 0.05$)

Group	Proportion (and percentage) of stocked female crayfish with successfully hatching eggs	Mean number (± *SD*) of hatchlings in all females	Mean number (± *SD*) and range of hatchlings in successfully reproducing females
Control	7/7 (100%)^{b}	65.4 ± 44.6^{c}	65.4 ± 44.6^{a} (18–128)
W3d	2/7 (28.6%)^{a}	19.3 ± 37.1^{a}	64.5 ± 37.5^{a} (38–97)
W10d	4/7 (57.1%)^{ab}	40.6 ± 45.4^{b}	71.0 ± 35.2^{a} (22–98)
W17d	7/7 (100%)^{b}	68.9 ± 39.2^{c}	68.9 ± 39.2^{a} (24–123)

conditions. Stage 3 juveniles were offered feed (defrosted chironomid larvae) on a daily basis and were monitored until reaching stage 4, which is signified by successful onset of exogenous feeding (Vogt & Tolley, 2004). Achievement of stage 4 in this experiment was considered completion of juvenile ontogeny of this species (Vogt, Tolley, & Scholtz, 2004), which implies no detrimental effects from the experimental conditions. Females with juveniles failing to advance to the next stage in post-embryogenic development after 20 days were terminated. Time from oviposition to earliest observation of each stage of ontogeny was recorded. Female biometry for this experiment ranged 15.3–31.3 mm in carapace length and 2.1–5.2 g in weight. The temperature was 21.7 ± 0.3°C.

2.2.3 | Ontogeny of humid-reared stage 2 juveniles in aquatic conditions—Experiment 3

The last experiment was a follow up to Experiment 2. The inability of stage 2 juveniles to progress to stage 3 under high humidity conditions (group W10d-stage 3 in Experiment 2; see Results below) led us to investigate the ability of stalled stage 2 juveniles to advance to stage 3 when re-immersed (Figure 1). Therefore, 42 stage 2 juveniles (10, 15, and 17 specimens from three females, respectively) were collected from the stalled development group after 20 days in Experiment 2 and returned to artificial burrows with 0.5 L of aged tap water (changed every other day). These were observed as described for further post-embryonic development in an aquatic environment in Experiment 2.

2.3 | Data analysis

Due to the binary nature of the data (0, 1 values, meaning unsuccessful versus successful hatching), we implemented Firth's bias-reduced penalised-likelihood logistic regression to test differences between proportion of successful hatching among the groups (Control, W3d, W10, and W17 in Experiment 1; and Control, W10d-stage 1, W10d-stage 2, and W10d-stage 3 in Experiment 2) with weight of females as a covariate. In the same manner, general linear model with Poisson distribution was used to analyse number of hatchlings in all reproducing females, which was followed by Tukey post hoc test to determine statistical significance between groups. Also, only successfully reproducing females were assessed in this way in Experiment 1. In Experiment 2, survival rate of juveniles successfully reaching secondary target stages 3 and 4 was analysed similarly, using quasi-binomial distribution that accounts for data overdispersion. All analyses were implemented in R version 3.2.5 (R Core Team, 2016).

3 | RESULTS

3.1 | Crayfish embryogenesis in high air humidity—Experiment 1

Successful hatching was observed in all treatments of Experiment 1 (Table 1). Eggs of all females in the control and W17d groups hatched successfully, producing viable juveniles. The proportion of females with hatched eggs declined significantly ((Likelihood Ratio Test), LRT = 10.59; *df* = 4; $p = 0.03$) with reduced duration of aquatic incubation period, being 28.6 and 57.1% in groups W3d and W10d, respectively. Weight of females did not influence proportion of successful hatching ($p = 0.74$). Considering all females involved in the test, the number of hatchlings (stage 1 juveniles) per female significantly declined with the duration of emersion (LRT = 313.25; *df* = 3; $p < 0.001$), while fecundity in successfully hatching females did not

TABLE 2 Proportion (and percentage; %) of stocked females that successfully hatched eggs, and proportion (and percentage; %) of successfully reproducing females with offspring that reached the target post-embryonic developmental stage (stage 1, stage 2, and stage 3) in Experiment 2. Within a column, values with differing letters differ statistically ($p < 0.05$)

Group	Proportion (and %) of females successfully hatching eggs	Proportion (and %) of females with offspring reaching target developmental stage
Control	13/17 (76.5%)^{a}	13/13 (100%)^{b}
W10d-stage 1	9/14 (64.3%)^{a}	9/9 (100%)^{b}
W10d-stage 2	8/14 (57.1%)^{a}	8/8 (100%)^{b}
W10d-stage 3	7/13 (53.8%)^{a}	0/7 (0%)^{a}

differ significantly among groups (LRT = 1.29; *df* = 3; $p = 0.73$), ranging from 64.5 to 71.0. Weight of females positively influenced number of hatchlings ($p < 0.001$).

3.2 | Crayfish post-embryonic development in high air humidity—Experiment 2

Three quarters (76.5%) of females in the control group successfully hatched their eggs, thus attaining stage 1 juveniles in Experiment 2 (Table 2). Hatching was also successful in the other experimental groups that were exposed to humid air following the first 10 days of oviposition in water. Embryogenesis was completed with the hatching of eggs in 53.8 to 64.3% of those females. The proportion of females with hatched eggs did not differ among groups (LRT = 2.22; *df* = 4; $p = 0.69$). Weight of females did not influence proportion of successful hatching ($p = 0.58$). In the control (full aquatic) and W10d-stage 1 (aquatic conditions after stage 1) treatment groups, all hatched offspring advanced through post-embryonic development under aquatic conditions, reaching stage 4. All stocks of successfully hatching juveniles in the W10d-stage 2 treatment group were capable of moulting to stage 2 juveniles under high air humidity conditions, followed by normal post-embryonic development in aquatic conditions. Similarly, hatchlings of the W10d-stage 3 group were capable of moulting to stage 2 juveniles under high humidity but were unable to develop further under continued high humidity (Table 2). Therefore, the proportion of females reaching target developmental stage differed between W10d-stage 3 and other groups (LRT = 26.09; *df* = 4; $p < 0.001$). As for all females in the experiment, weight of females reaching target developmental stage did not influence proportion of successful hatching ($p = 0.98$).

Mean survival rates (% ± *SD*) of marbled crayfish hatchlings that successfully advanced beyond the air humidity target stage when transferred to an aquatic environment are presented in Table 3. In the control group it took, on average, 21.4 and 26.3 days total following oviposition for the earliest observation of stage 1 and stage 2 juveniles to appear, respectively. Stage 3 juveniles were first observed after 31.2 days on average (Table 4). In the W10d-stage 1 group, the mean survival of this treatment group declined from 44.9% of juveniles advancing past stage 1 after transfer to aquatic conditions to 29.0% for those completing the last post-embryonic development stage. The time to first observance of successful development achievement was 21.5, 27.1, and 33.3 days following oviposition for stage 1, 2, and 3, respectively. For the W10d-stage 2 treatment group, mean survival rates of juvenile transferees (to aquatic environment) were 66.2 and 62.1% for those advancing beyond stage 2 and 3, respectively (Table 3). Time to earliest observation of secondary target stage was non-significant among groups (LRT = 0.10–1.55; $p = 0.46–0.99$; Table 4). Juveniles that moulted to stage 2 under high air humidity in the group W10d-stage 3 were unable to further progress in ontogeny under humidity conditions (Table 3). Those offspring eventually died after remaining viable for 35.3 days on average. Significant differences existed between survival rates of groups exposed to highly humid conditions through stage 2 ($F_{2, 21} = 42.65$, $p < 0.001$) and stage 3 development ($F_{1, 15} = 14.26$, $p = 0.002$; Table 3). Weight of females did not influence survival rate and time to the earliest observation of particular stages.

TABLE 3 Survival rates (mean ± *SE*; %) of marbled crayfish hatchings successfully reaching secondary target (Sec.) stage 2, stage 3, and stage 4 in further developmental after transfer to an aquatic environment following the primary treatment period in Experiment 2. Within a column, values with differing letters differ statistically ($p < 0.05$)

Group	Sec. Stage 2	Sec. Stage 3	Sec. Stage 4
Control	n.d.	n.d.	n.d.
W10d-stage 1	44.9 ± 20.8	33.8 ± 21.2^{b}	29.0 ± 19.8^{a}
W10d-stage 2	n.d.	66.2 ± 13.4^{c}	62.1 ± 11.3^{b}
W10d-stage 3	n.d.	0^{a}	NA

Abbreviations: n.d., not determined; NA, not applicable.
Grey highlight denotes periods of aquatic conditions.

3.3 | Ontogeny of humid-reared stage 2 juveniles in aquatic conditions—Experiment 3

In this experiment, where a total of 42 stage 2 juveniles unable to proceed to the next moult were transferred to fully aquatic conditions after 20 days, and most were able to resume development. Eighty-eight percent of the transferred animals moulted within 4–8 days, and further progressed normally to stage 4 with feed.

4 | DISCUSSION

Crayfish have evolved to construct or use burrows not only to survive under fluctuating aquatic habitats, but perhaps also to reproduce

TABLE 4 Time (mean ± *SE*; days) from oviposition to the earliest observation of stage 1 (hatching), stage 2, and stage 3 juveniles whether in high humidity or aquatic conditions in Experiment 2. Within a column, values with differing letters differ statistically ($p < 0.05$)

Group	Stage 1 (Hatching)		Stage 2	Stage 3
Control	10	11.4 ± 0.6^{a}	26.3 (21.4 + 4.9) ± 1.5^{a}	31.2 (26.3 + 4.9) ± 1.4^{a}
W10d-stage 1	10	11.5 ± 0.5^{a}	27.1 (21.5 + 5.6) ± 1.1^{a}	33.3 (27.1 + 6.2) ± 1.0^{a}
W10d-stage 2	10	11.5 ± 0.5^{a}	27.4 (21.5 + 5.9) ± 1.2^{a}	35.3 (27.4 + 7.9) ± 3.7^{a}
W10d-stage 3	10	11.5 ± 0.5^{a}	28.0 (21.5 + 6.5) ± 2.9^{a}	NA

Abbreviation: NA, not applicable.
Grey highlight denotes periods of aquatic conditions.

when water is scarce. However, given the nature of burrowing crayfish and the difficulty of working with naturally constructed and sealed burrows, little is known about how early life history of crayfish in burrows is influenced by environmental factors. Our results clearly demonstrate that high air humidity alone is sufficient during terminal phases of embryogenesis, including the hatching period, in marbled crayfish. It is expected that such adaptation may be a characteristic of other crayfish species that have evolved within seasonal aquatic ecosystems.

We proved that hatchlings are capable of moulting to the second developmental stage under high air humidity conditions. The stage 2 juveniles remained viable for extended periods of time but did not develop further without immersion. When transferred to aquatic conditions, they moulted to the third developmental stage in the course of several days and further progressed normally. This survival trait enables marbled crayfish to spread into a wide range of habitats that experience situations of water scarcity that might otherwise be unsuitable for other, less adapted crayfish species as well as other aquatic taxa (Feria & Faulkes, 2011; Kouba et al., 2016). However, we expect that other crayfish species such as the primary burrowers and perhaps some secondary burrowers, especially the highly invasive *P. clarkii*, are also capable of maintaining ontogeny to some degree without free standing water under humid conditions in a burrow. Such adaptations are particularly valuable in ecosystems with highly variable water availability (for example intermittent freshwater ecosystems). Parts of Australia and North America (e.g. the south-east of the continent) have experienced an increase in the frequency and duration of dry periods (Hughes, 2003) and are also crayfish biodiversity hotspots. Thus, the capacity of adaptations such as that described here will probably be of key importance as climatic drying progresses, possibly eliminating species that lack adaptations that maintain reproduction during prolonged drying (Johnston & Robson, 2009; Robson et al., 2011). Such drying events may also occur in previously unexpected places (Kouba et al., 2016), which requires further knowledge of the effects of drying conditions on macroinvertebrate communities (Robson et al., 2011).

The variety of adaptations used by crustaceans for surviving drying are diverse. In freshwater crayfish, females frequently use burrows for egg incubation, especially in the case of primary and secondary burrowers (Hobbs, 1942; Riek, 1972). Females tend to construct deeper burrows compared to males and frequently occupy the deepest parts of the burrow during egg incubation (Hazlett, Rittschof, & Rubenstein, 1974; Payne, 1972). Seasonal fluctuations of groundwater can influence the depth of burrows (Helms et al., 2013; Stoeckel, Cash, & Helms, 2011). Also, it seems that the water table is often below the bottom of burrows at drying localities, e.g. in the case of *P. clarkii*, which frequently reproduces under these conditions (McClain & Romaire, 2004; Souty-Grosset et al., 2014). Crayfish abilities to reach the water table under laboratory conditions are species-dependent (Bovbjerg, 1970; Dyer, Worthington, & Brewer, 2015). In crayfish of the genus *Engaeus*, which excavate burrows or chambers on hill slopes, they often do not reach the water table (Horwitz & Richardson, 1986; Suter & Richardson, 1977). In cases of burrows above the water table, water present in the burrows is most likely to be perched from the time of their construction and settlement, or perhaps periodically refreshed from seepage or percolation from rainfall. Chimney-like structures built by many species from the genera *Cambarus*, *Engaeus*, and *Geocharax* (Grow & Merchant, 1980; Horwitz et al., 1985; March & Robson, 2006; Noro, Fonseca, Buckup, & Bond-Buckup, 2007; Suter & Richardson, 1977) or closing of the burrow entrance by a mud plug, particularly in females (Barbaresi & Gherardi, 2000; Correia & Ferreira, 1995; Kouba et al., 2016), help in maintaining suitable living condition inside. Aestivation chambers to maintain humidity during desiccation in streambeds are built by adult *Geocharax* sp. (Chester & Robson, 2011; Chester et al., 2014). Furthermore, the non-burrowing crayfish *Gramastacus insolitus* Riek, 1972 are commensal upon larger crayfish species' burrows to survive the seasonal drying of their habitat (Johnston & Robson, 2009). Such behavioural adaptations highlight the importance of maintaining humidity within burrows.

It is reasonable to expect that immersion of eggs is essential, at least at the very early stages of embryonic development (Niksirat et al., 2014, 2015), and it has been documented that oviposition cannot occur in *P. clarkii* without free water (McClain, 2013). Artificially stored eggs of the signal crayfish and white-clawed crayfish need to be transferred to aquatic conditions once the 12th stage of embryonic development is attained (Celada et al., 2000; Pérez et al., 2003). However, these astacids are cold-water species, carry larger eggs and the embryogenesis period is much longer than in marbled crayfish and other warm-water species possessing a faster lifecycle (Kouba, Buřič, & Petrusek, 2015). Moreover, those astacids do not exhibit the ability to burrow extensively vertically as an effective adaptive tool in situations of water scarcity, as can be seen in numerous cambarids and parastacids (Kouba et al., 2016).

Findings here of late embryonic and early post-embryonic crayfish development in the absence of free water for marbled crayfish in particular are noteworthy, as it is a unique species with a high invasion potential due to its parthenogenetic mode of reproduction (Gutekunst et al., 2018; Scholtz et al., 2003). Crayfish males and females are sometimes found sharing a single burrow in sexually reproducing species (Horwitz et al., 1985; Johnston & Robson, 2009). However, presence of a male in the burrow is not a necessary prerequisite for continuity of reproduction in burrows during droughts, given that spermatophores are attached on the ventral side of females (in Astacidae and Parastacidae) or deposited inside the *annulus ventralis* (in Cambaridae and Cambaroididae), often a long time before ovulation (Albaugh, 1973; Buřič, Kouba, & Kozák, 2013; Butler & Stein, 1985). Considering that our trials utilised marbled crayfish females with attached and developing eggs, we believe that our findings are valid also for sexually reproducing species, thus covering both modes of reproduction. Our findings suggests that successful ontogeny under drought-like conditions for this species is primarily related to the tolerance of crayfish eggs to periods without free water, which is well in line with adaptations of other drought-tolerant species, such as construction of special aestivation burrows (Chester & Robson, 2011; Chester et al., 2014), burrow commensalism with other crayfish (Johnston & Robson, 2009), and digging deeper burrows to the water-level during dry seasons (Horwitz et al., 1985; Riek, 1969). The tolerance to drought using various traits is probably crucial for inhabiting ecosystems with high water level fluctuations in freshwater crayfish.

5 | CONCLUSION

Our study revealed an ability of marbled crayfish to undergo terminal phases of embryogenesis, including hatching, as well as early post-embryonic development in high air humidity alone. Importantly, our finding that stage 2 juveniles were able to persist for a prolonged duration at this stage under humid conditions, and that they were able to resume normal ontogeny once re-immersed, greatly increases our understanding of this species' resilience in habitats with variable hydrology. A similar ability to tolerate and reproduce during drought is also expected to occur in other crayfish species such as the primary burrowers or some secondary burrowers, especially the highly invasive *P. clarkii*. This unprecedented life history trait in freshwater crayfish is probably crucial for inhabiting ecosystems with high water level fluctuations and severe drought. In the context of biological invasions, the magnitude of these adaptations to drought will probably also contribute to species displacements, favouring species that are better adapted to such environmental conditions.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: A.K., R.M., and W.G. Performed the experiments: W.G., M.S.H., A.K., M.B., and J.K. Analysed the data: W.G., L.V., M.B., and A.K. Wrote the first draft: W.G. All authors contributed to and edited the manuscript.

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CHAPTER 3

PSYCHOACTIVE COMPOUNDS AT ENVIRONMENTAL CONCENTRATION ALTER BURROWING BEHAVIOR IN THE FRESHWATER CRAYFISH

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Psychoactive compounds at environmental concentration alter burrowing behavior in the freshwater crayfish

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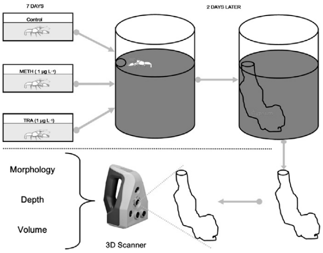
HIGHLIGHTS

- First study evaluating burrowing activity of crayfish exposed to two PhACs.
- Effects on it were detected at an environmentally relevant concentration of 1 µg/L.
- METH-exposed female built smaller burrow than control by relative depth and volume.

GRAPHICAL ABSTRACT



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ABSTRACT

Pharmaceutically active compounds (PhAC) have been increasingly detected in freshwater and marine waterbodies worldwide and are recognized as major emerging micropollutant threat to the aquatic environment. Despite their low concentrations in the environment, there is evidence of effects on non-target aquatic organisms in natural habitats. To assess the potential effects of PhACs on its burrowing behavior, we exposed the red swamp crayfish *Procambarus clarkii* to methamphetamine or tramadol at the environmentally relevant concentration of 1 µg/L. Methamphetamine-exposed females constructed burrows of lower depth and volume relative to individual weight than did controls. Tramadol-exposed females consistently exhibited a tendency for smaller burrows, but this difference was not significant. Exposed males showed a non-significant tendency to excavate larger burrows compared with the control. Control and tramadol-treated females maintained the natural tendency of constructing relatively deeper and/or larger-volume burrows compared with males. This sex-related pattern was not detected in the methamphetamine group. The rate of human therapeutic PhAC usage is relatively stable year-round, and impacts on crayfish burrowing can be particularly damaging during periods of drought, when the dilution of waste waters is reduced, and burrowing becomes a critical survival strategy. Our results suggest that an increasingly broad range of environmental impacts of PhACs on non-target organisms can be expected in natural ecosystems.

1. Introduction

The contamination of aquatic environments by pharmaceutically active compounds (PhAC) (Ebele et al., 2017) is an increasing concern worldwide (Burkina et al., 2015; Shaaban, 2017).

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Pharmaceutically active compounds that pose a serious threat to non-target aquatic organisms (Biel-Maeso et al., 2018; Boxall et al., 2012; Corcoran et al., 2010) include psychoactive compounds such as illicit drugs as well as antihistamines, and cardiovascular drugs (Berset et al., 2010; Ebele et al., 2017; Irvine et al., 2011; Lindberg et al., 2014; Schafhauser et al., 2018). Their primary source in the environment is outflow from wastewater treatment plants (Cunha et al., 2017; Li et al., 2011) following their incomplete removal (Buchberger, 2007; Golovko et al., 2014; Petrović et al., 2003). Reported aquatic concentrations of maternal compounds and metabolites range from ng to µg/L (Aymerich et al., 2016; Grabicova et al., 2015). In excess of 600 pharmaceutical substances have been detected worldwide (Küster and Adler, 2014) in wastewater, surface water, groundwater, and drinking water (Balakrishna et al., 2017; Li, 2014; Simazaki et al., 2015; Sui et al., 2015; Zhang et al., 2018). Although it has been widely considered that the risk of these compounds to aquatic biota is minimal because of their sub-lethal concentrations and rapid degradation in the environment (Richmond et al., 2017), some PhACs are reported to show detrimental effects on aquatic organisms including modification of behavior and disruption of reproduction and ontogeny (Furlong et al., 2011; Imeh-Nathaniel et al., 2017; Mishra et al., 2017). Impacts are documented in algae, zooplankton, mussels, shrimp, crayfish, and fish (Brodin et al., 2013; Buřič et al., 2018; Crane et al., 2006; Diniz et al., 2015; Douda et al., 2019; González-Ortegón et al., 2016) Figure 1.

Crayfish (Crustacea, Decapoda) are distributed throughout the world, including in Europe, and are considered keystone species in the environment (Crandall and De Grave, 2017; Hossain et al., 2018; Kouba et al., 2014; Perdikaris et al., 2017). They have evolved the ability to seek shelter and to construct burrows (Horwitz and Richardson, 1986; Riek, 1969) that provide protection against conspecifics and predators during vulnerable life stages such as molting and reproduction (Atkinson and Taylor, 1988; Bergman and Moore, 2003; Growns and Richardson, 1988; Guo et al., 2019) and serve as a valuable resource in adverse environmental conditions such as drought and temperature extremes (Haubrock et al., 2019; Kouba et al., 2016; Richardson and Swain, 1980). The ability to withstand desiccation by excavating vertical burrows in the hyporheic zone might play a significant role in the success of particular crayfish species (Kouba et al., 2016). Crayfish have been reported to be impacted by PhACs at environmentally relevant concentrations (Buřič et al., 2018; Hossain et al., 2019;Ložek et al., 2019), but PhAC effects on burrowing behavior remain unknown.

The red swamp crayfish *Procambarus clarkii* (Girard, 1852) is a primary model species, and is used in research fields including ecotoxicology (Fong and Ford, 2014; Stara et al., 2014; Yu et al., 2017). Recently, the value of crayfish as a model for studies of drug addiction has been recognized (Imeh-Nathaniel et al., 2017; Kubec et al., 2019). Crayfish may become conditioned to prefer environments in which they obtain drugs (Imeh-Nathaniel et al., 2016; Nathaniel et al., 2012a; Panksepp and Huber, 2004) and the natural reward system of crayfish is sensitive to therapeutic drugs used in humans (Nathaniel et al., 2012b). Rusty crayfish *Faxonius rusticus* (Girard, 1852) administered a low dose of methamphetamine by injection showed mobility increased to a greater extent than with higher doses, while the higher dose exerted a more prominent effect in increasing immobility (Imeh-Nathaniel et al., 2017).

In this study, we assessed the burrowing behavior of red swamp crayfish exposed to environmentally relevant concentrations of the psychoactive compounds methamphetamine and tramadol, both of which have been frequently detected in aquatic environments (Baker and Kasprzyk-Hordern, 2013; Grabicova et al., 2017; Koba et al., 2018). The reported concentration of methamphetamine in discharge water in the Czech Republic and Slovakia ranged from 13 to 1805 ng/L (Mackul'ak et al., 2016) and tramadol from 3.7 to 5300 ng/L (Grabicova et al., 2017). The wastewater treatment plants located on small and middle-sized watercourses typically contribute by 10–25% to the flow rate in the recipients in the Czech Republic. However, in summer and events of severe droughts, the flow rates in recipients are dominated by effluents (R. Grabic, pers. comm.). Thus, we assessed the hypothesis that the burrowing behavior of the red swamp crayfish is altered after their exposure to PhACs, using methamphetamine and tramadol at environmentally relevant concentrations as model compounds.

2. Materials and methods

2.1. Test pharmaceuticals

Methamphetamine (METH) and tramadol hydrochloride (TRA) were obtained from Sigma-Aldrich (USA). Individual stock solutions were prepared in ultra-pure water (aqua-MAX-Ultra system, Younglin, Kyounggi-do, South Korea) at 10 mg/L and stored at 4 °C. The exposure solutions of 1 µg/L were prepared by dilution of the stock solution with aged tap-water. Ultra-pure water and LC/MS grade acetonitrile (Merck, Germany), both acidified with formic acid (Sigma-Aldrich), were used as mobile phases for liquid chromatography. For calculation of concentration of the studied compounds in water, isotopically labelled methamphetamine-D_5 (Lipomed, USA) and tramadol-D_3 (Toronto Research Chemicals, Canada) were used as internal standards. The solubility of methamphetamine and tramadol in water is 928 mg/L and 750 mg/L, respectively (DrugBank, 2019).

2.2. Experimental animals

Red swamp crayfish were laboratory-cultured at the Research Institute of Fish Culture and Hydrobiology in Vodňany, FFPW USB, Czech Republic, where the experimental work was carried out. Individuals with fully developed chelipeds and no missing pereiopods were randomly selected from the culture tanks. Carapace length and post-orbital carapace length were measured to the nearest 0.1 mm using digital Vernier calipers and weighed to the nearest 0.1 g with an electronic balance (Kern & Sohn GmbH, Balingen, Germany). No significant differences were found in the biometry among groups regardless of sex (Table 1).

2.3. Experimental design

2.3.1. Exposure to drugs

The crayfish were exposed for seven days to either METH or TRA at the concentration of ~1 µg/L of pure compound (Table 2), a level considered environmentally relevant (Baker et al., 2012; Fedorova et al., 2014; Grabic et al., 2012; Grabicova et al., 2015; Mackul'ak et al., 2016; Rúa-Gómez and Püttmann, 2012). Individuals maintained in pharmaceutical-free aged tap-water served as controls and were handled in the same way as exposed animals. Crayfish were stocked individually into clear plastic 2.5 L (250 × 190 × 70 mm) boxes with lids in ~ 1.0 L of exposure solution or aged tap-water, temperature 18.3 ± 0.5 °C.

Crayfish were fed commercial feed (Sera Granugreen, Sera, Heinsberg, Germany) at the rate of 5–7 pellets/animal/day. Boxes were cleaned daily during aged tap-water or solution change. The control group boxes were cleaned first to avoid contamination with tested compounds. Animals that molted or spawned during the exposure period were removed from the experiment, leaving 22 females and 17 males for the burrowing trial.

The concentration of METH and TRA in exposure solutions and aged tap-water when freshly prepared and after 24 h in the plastic

Table 1
Sex, carapace length (CL), post-orbital carapace length (POCL), and weight (W) of red swamp crayfish *P. clarkii* specimens used in the study. METH (methamphetamine), TRA (tramadol). Data are presented as mean ± standard deviation; $p < 0.05$. *F*, One-Way ANOVA; *t*, Student's *t*-test.

Experimental setup	Sex	n	CL (mm)		p	POCL (mm)		p	W (g)		p
Control	M	6	34.6 ± 3.1	$F_{group} = 0.078$	0.925	26.2 ± 2.4	$F_{group} = 0.209$	0.813	11.2 ± 3.6	$F_{group} = 0.588$	0.561
				$F_{male} = 0.393$	0.682		$F_{male} = 0.483$	0.627		$F_{male} = 1.017$	0.387
	F	7	35.4 ± 3.2	$F_{female} = 0.242$	0.788	27.5 ± 2.7	$F_{female} = 0.197$	0.823	13.3 ± 3.9	$F_{female} = 0.498$	0.616
METH	M	6	34.5 ± 7.1	$t_{7,6,Control} = 0.431$	0.675	26.7 ± 5.1	$t_{7,6,\ Control} = 0.874$	0.401	14.3 ± 10.0	$t_{7,6,\ Control} = 0.995$	0.341
	F	7	36.2 ± 5.6	$t_{7,6,\ METH} = 0.474$	0.646	28.2 ± 4.6	$t_{7,6,\ METH} = 0.494$	0.633	15.0 ± 7.9	$t_{7,6,\ METH} = 0.137$	0.894
TRA	M	5	37.1 ± 5.2	$t_{8,5,\ TRA} = -0.932$	0.397	28.8 ± 4.6	$t_{8,5,\ TRA} = -0.795$	0.488	19.7 ± 7.6	$t_{8,5,\ TRA} = -1.507$	0.198

Table 2
Mean concentration of methamphetamine and tramadol in red swamp crayfish *P. clarkii* exposure solutions and control water at 0 and 24 h of Control, METH (methamphetamine), TRA (tramadol). Data are presented as mean ± standard deviation; $p < 0.05$. *t*, Paired *t*-test.

Group	n	Compound tested	Time 0 h (μg/L)	Time 24 h (μg/L)	*t*	*p*
Control	3	methamphetamine	<LOQ	<LOQ	–	–
		tramadol	<LOQ	<LOQ	–	–
METH	3	methamphetamine	1.0 ± 0.1	1.0 ± 0.1	0.730	0.248
TRA	3	tramadol	1.0 ± 0.1	1.0 ± 0.1	0.423	0.311

LOQ, limit of quantification. Values for METH ranged from 0.023 to 0.035 μg/L and for TRA 0.025–0.047 μg/L.

boxes was determined by liquid chromatography with tandem mass spectrometry (LC–MS/MS) three times over the exposure period. Water samples (5 mL) were filtered through regenerated cellulose, 0.20 μm pore size (Labicom, Czech Republic), and stored at −20 °C until analysis.

For analysis, water samples were thawed at room temperature, the internal standard was added, and samples were analyzed using Hypersil Gold a Q column (50 × 2.1 mm; 5 mm particles) coupled with an Accela 1250 pump and a triple stage quadrupole MS/MS TSQ Quantiva Mass Spectrometer (Thermo Fisher Scientific, USA) using selected reaction monitoring in positive mode as described by Douda et al. (2019). The LC-MS/MS parameters are described in supplementary material (Table S1). Trace Finder 3.3 (Thermo Fisher Scientific) was used for LC-MS/MS data processing.

The concentration of METH, its metabolite amphetamine, and TRA in the hepatopancreas of red swamp crayfish was determined in fresh tissue immediately after removal of crayfish from burrows. The sample was prepared according to (Grabicova et al., 2018). Briefly, a 0.5 g sample of tissue was placed 2.0 mL Eppendorf tubes, and internal standard and extraction solvent were added. After homogenization (1800 oscillations for 10 min; homogenizer TissueLyser II, Quiagen, Germany) and centrifugation at 4472 × g for 5 min (Minispin, Eppendorf, Germany), the supernatant was filtered through 0.45 μm regenerated cellulose (Labicom, CR) and analyzed by LC-high resolution mass spectrometry using a Q-Exactive mass spectrometer (Thermo Fisher Scientific, USA) in parallel reaction monitoring positive mode. The analytical method is summarized in supplementary material (Table S2).

2.3.2. Substrate and container preparation for assessment of burrowing behavior

To create a suitable test substrate, 18.2 kg of sand with moisture content of 2.1% and 8.4 kg of clay, moisture content 8.6%, were thoroughly mixed by hand (30% clay wet weight). Aged tap-water (3.4 L) was added to reach a final moisture content of 15%. In total, about 270 kg substrate were prepared for the whole experiment in this way. Size distribution of sand and clay particles is presented in Table S3. Seven kilograms of the resultant mixture was placed in each of 39 plastic containers (inner diameter = 19.2 cm, height = 19.7 cm) to a depth of ~ 15.0 cm. To better simulate natural conditions in which areas of residual water persist in dried localities (Souty-Grosset et al., 2014), a shallow initial burrow (diameter 2.6 cm, depth 1.5 cm; volume 8.0 cm^3) was created at the edge of the container and 5 mL aged tap-water was added to stimulate burrowing in the suggested position, following Kouba et al. (2016). One crayfish was placed in each container, and the container was covered by a plastic lid to prevent acute desiccation. Five 1.0 cm holes, one in the center and four equidistant from one another at 6.5 cm from the center, allowed ventilation. The air and sand-clay mixture temperatures were 18.6 ± 0.6 °C and 18.5 ± 0.6 °C, respectively, recorded hourly using Minikin loggers (Environmental Measuring Systems, Brno, Czech Republic).

2.3.3. Creation and measurement of burrow casts

If removal of crayfish from burrows by hand was not feasible, a small quantity of carbonated water was added to the burrow to evict animals. Excess water was removed from the burrow bottom by blotting with absorbent tissue paper, and gypsum casts were created of all excavated burrows. Depth of casts was measured to the nearest 0.1 mm using digital Vernier calipers. Casts were further scanned by an Artec Spider™ hand-held 3D laser scanner (Artec Group, Luxembourg) with a stated resolution of 0.1 mm and accuracy of 0.03 mm. The scanner is based on the structured light principle and provides a 3D mesh of the object as an output, generated in real world coordinates (mm). The resulting stereolithography mesh was imported to Artec Studio v. 10 (Artec Group, Luxembourg) to calculate the volume of the 3D mesh.

2.4. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics v. 22.0. As size varies among individual crayfish, volume and depth of the burrow was assessed relative to specimen weight, following Kouba et al. (2016). Normality and homoscedasticity of the biometry of the sampled animals by group and sex and the relative depth and volume of burrows by group and sex were tested using Kolmogorov-Smirnov (Ghasemi and Zahediasl, 2012) and Breusch-Pagan tests (Long and Ervin, 2000), respectively. The differences in biometry of the samples (by group; by sex) and the difference of mean relative depth and volume of burrows (by group; by sex) were analyzed by one-way ANOVA, and statistical difference in means were determined using multiple comparisons as a post hoc test. Sex differences in animal biometry and relative depth and volume of burrows were compared through Student's *t*-test in each group. A paired *t*-test was used to compare concentrations of tested compounds in the water. In assessing sex differences,

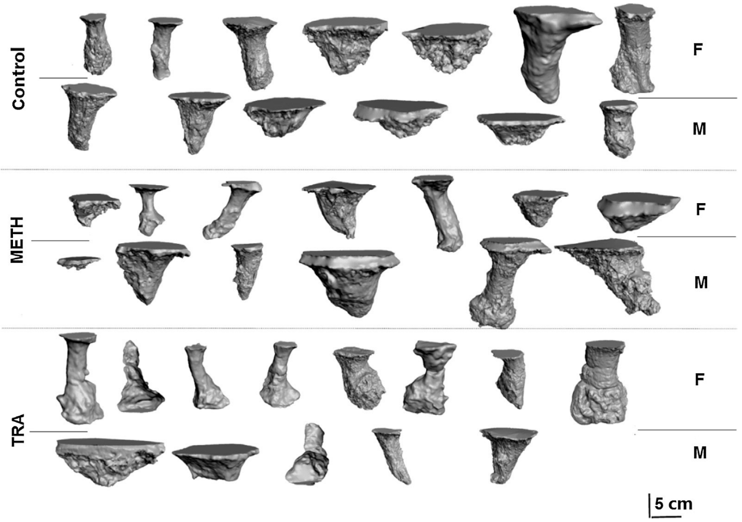

Fig. 1. Three dimensional scans of red swamp crayfish *P. clarkii* burrows. METH = methamphetamine, TRA = tramadol. F = female; M = male.

closing of the burrow entrance with a mud plug was assessed as 1 or 0, and we implemented Firth's bias-reduced penalized-likelihood logistic regression to test differences among the proportion of females with mud plug in the groups. The null hypothesis was rejected at $p < 0.05$ in all cases.

3. Results

All red swamp crayfish in the experiment exhibited burrowing activity (Fig. 1 and S1). They usually constructed a single vertical burrow at the site of the provided burrow. Two control females and one METH male created two burrows, with the deeper one in the suggested position. No significant difference was found among groups in closing the burrow entrance with mud plug (likelihood ratio = 3.125, df = 2, $p = 0.210$). The number of females creating a mud plug to close the burrow entrance was one, one, and four in Control, METH, and TRA, respectively. No male crayfish constructed a mud plug.

There was no significant difference among Control, METH, and TRA in relative volume and depth of burrow ($F_{13,13,13,\ \text{relative}\ volume} = 1.140$, df = 2, $p = 0.331$; $F_{13,13,13,\ \text{relative}\ depth} = 0.165$, df = 2, $p = 0.849$). Significant differences among experimental groups were detected in the relative volume of burrows created by females ($F_{7,7,8} = 3.811$, $p = 0.041$), but relative depth remained similar ($F_{7,7,8} = 2.828$, $p = 0.084$). Females in Control and TRA groups usually excavated deeper burrows than in METH ($p = 0.035$; $p = 0.021$) and larger ($p = 0.033$) and comparable ($p = 0.519$) burrows compared with that in METH (Fig. 2), respectively. Relative depth and volume of male burrows was similar among groups ($F_{6,6,5,\ \text{relative}\ depth} = 0.200$, $p = 0.821$; $F_{6,6,5,\ \text{relative}\ volume} = 0.541$, $p = 0.594$) (Fig. 2).

In controls, significant differences in relative volume of burrows was detected between sexes ($t_{7,6} = -2.569$, $p = 0.037$) (Fig. 2), with the females usually constructing slightly deeper burrows compared with males, without significant difference ($t_{7,6} = -1.325$, $p = 0.212$). A similar pattern was seen in the TRA group both for relative depth ($t_{8,5} = -1.319$, $p = 0.256$) and volume ($t_{8,5} = -2.973$, $p = 0.026$), while sex-related differences were not seen with the METH treatment ($t_{7,6} = 0.615$, $p = 0.555$; $t_{7,6} = -0.197$, $p = 0.848$, respectively) (Fig. 2).

The concentrations of methamphetamine, its metabolite amphetamine, and tramadol in the hepatopancreas of red swamp crayfish of control and exposed groups at the end of burrowing were all below limits of quantification with values of METH ranging from 0.18 to 2.8 ng/g, of amphetamine 0.17–0.89 ng/g, and of TRA 0.2–3.4 ng/g).

4. Discussion

The quality of surface waters plays an essential role in maintaining ecosystem biodiversity and function (Bakker, 2012). The effect of PhACs on freshwater crayfish may be devastating, because the need to find shelter is an essential life history trait, especially when faced with situations such as long-term drought (Kouba et al., 2016). Exposure to PhACs is elevated in these situations, since reduced dilution with lower flow rates are experienced by the animals (Ma et al., 2017). This is a critical issue for population dynamics of species. Females of many primary and secondary burrowers (Hobbs, 1942; Riek, 1972) use burrows for oviposition and/or incubation. Thus, likely to maintain contact with ground water or remain in an environment sufficiently humid for successful reproduction (Guo et al., 2019), females tend to construct deeper burrows than do males and frequently occupy the deepest parts of the burrow during egg incubation (Hazlett et al., 1974; Kouba et al., 2016; Payne, 1972). This was observed in the control group in this study. The relative volume and depth of female red swamp crayfish burrows was significantly lower in the METH group compared with controls, but this was not observed in the TRA-exposed group. Considering that the red swamp crayfish is more resistant to desiccation and possesses burrowing abilities superior to the native European crayfish species noble crayfish *Astacus astacus* (L., 1758), narrow-clawed crayfish *Pontastacus leptodactylus* s.l.

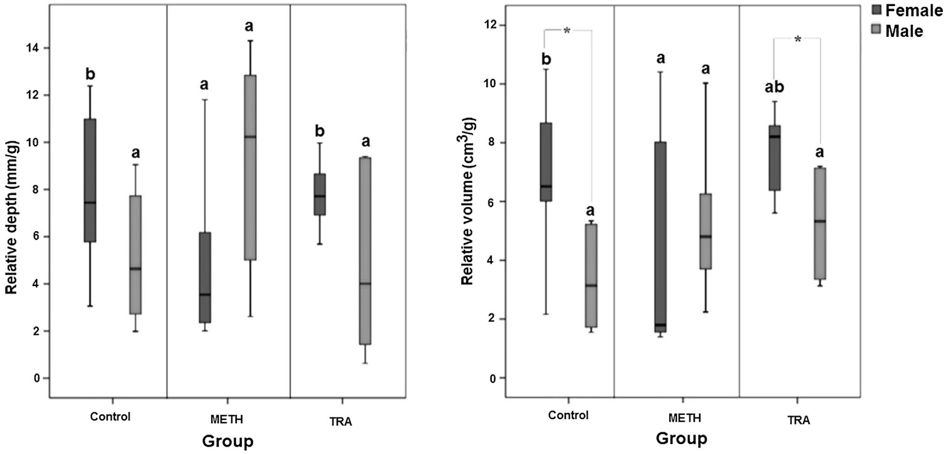

Fig. 2. Box-whisker plot of the depth and volume of red swamp crayfish *P. clarkii* burrows of Control, methamphetamine (METH), and tramadol (TRA) groups relative to individual crayfish weight. Box limits correspond to upper and lower quartiles, horizontal bar to the median. The * indicates significant differences between sexes in a given group. Values with differing letters are significantly different within sex among three groups ($p < 0.05$).

(Eschscholtz, 1823), stone crayfish *Austropotamobius torrentium* (von Paula Schrank, 1803), and invasive species yabby *Cherax destructor* Clark, 1936, spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817), and marbled crayfish *Procambarus virginalis* Lyko, 2017 (Kouba et al., 2016), they might be more resistant to the effects of PhACs in drought conditions.

Marbled crayfish were found to exhibit significantly lower velocity and shorter distance moved than controls after exposure to tramadol at environmentally relevant concentrations (Buřič et al., 2018). Lower velocity and reduced availability of shelter or burrows often leads to higher predation risk in the wild (Holdich et al., 1999; Pecor and Hazlett, 2003; Underwood, 2015), and predicted unstable weather conditions such as severe drought may increase these situations in the future (Cruz-McDonnell and Wolf, 2016; Kouba et al., 2016). The imbalance in sex ratio resulting from reduced availability of shelter or burrows to female crayfish in the wild may lead to a precipitous decline in crayfish populations (Richman et al., 2015; Sanchez-Bayo and Wyckhuys, 2019) compounded by the numerous PhACs in water bodies.

Over 60 PhACs have been reported in tissues of aquatic invertebrates as well as some riparian spiders in Australia (Richmond et al., 2018). Eleven selected PhACs, including tramadol, were detected in liver and kidney of fish inhabiting a contaminated stream in the Czech Republic (Grabicova et al., 2017). Behavioral changes including differences in velocity and distance moved of shrimp, crayfish, killifish, and European perch exposed to PhACs have recently been detected at low and environmental concentrations (Barry, 2013; Brodin et al., 2013; Buřič et al., 2018; González-Ortegón et al., 2016; Hossain et al., 2019). A significant increase in heart rate was observed in signal crayfish *Pacifastacus leniusculus* (Dana, 1852) after a three-week exposure to an environmentally relevant (1 μg/L) concentration of tramadol (Ložek et al., 2019). The predatory fish *Perca fluviatilis* (L., 1758) became more active after preying upon the invertebrate *Coenagrion hastulatum* (Charpentier, 1825) contaminated with a benzodiazepine (Brodin et al., 2014).

Effects of methamphetamine and tramadol at environmentally relevant concentrations were evident. Given the short exposure period (7 days) and low concentration (~1 μg/L) of tested compounds, we suggest that the observed effects are of serious concern due to the long-term exposure, fluctuating and potentially higher concentrations, and multiple sources of PhACs incurred in the natural environment. The concentration of pharmaceuticals such as antidepressants consumed by some representative aquatic top-predators through bioaccumulation may be comparable to as much as half the recommended human therapeutic dose (Richmond et al., 2018). The concentration of PhACs in natural ecosystems is relatively stable year-round, sometimes declining or eliminated in the short-term due to fluctuation in flowrates (Ma et al., 2017; Radjenovic et al., 2009). Concentrations may increase during severe drought because of reduced dilution (K'Oreje et al., 2018) or sorption into sediment (Aljeboree and Alshirifi, 2018), resulting in severe impact on non-target aquatic organisms.

5. Conclusions

We documented for the first time a difference in burrowing behavior of freshwater crayfish exposed to PhACs at an environmentally relevant concentration, which is applicable to other aquatic organisms. Notable, the relative depth and volume of burrows constructed by methamphetamine-exposed females was significantly lower when compared to controls. Tramadol-exposed females consistently exhibited a tendency to construct smaller burrows although this was not significant. Owing to the critical role of surface waters for human use in agriculture, aquaculture, and domestic water (Bakker, 2012), the potential impact of PhACs on freshwater biodiversity should not be ignored. Based on crayfish as a model group and a keystone species of freshwater organisms, a similar mode of action like burrowing can be expected in other hyporheic-dwelling aquatic biota such as unionid mussels and clams and a wide range of aquatic insects. The effects of environmentally relevant concentrations of selected PhACs are evident when tested as single compounds. As aquatic organisms in contaminated environments are usually exposed to multiple chemicals (Backhaus, 2014; Schoenfuss et al., 2016), more research is needed to explore the effect of PhAC cocktails together with abiotic factors such as temperature and pH on aquatic animals.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

Conceived and designed the experiments: A.K., W.G., and M.B.; Performed the experiments: W.G., A.K., M.S.H., M.B. and J.K.; Analyzed the data: W.G. and K.G.; Stock solutions prepared: K.G., T. R.; Wrote the first draft: W.G.; All authors contributed to and edited the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135138.

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CHAPTER 4

EFFECT OF THE HERBICIDES TERBUTHYLAZINE AND METAZACHLOR AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS ON BURROWING BEHAVIOUR OF RED SWAMP CRAYFISH *PROCAMBARUS CLARKII*

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My share on this work was about 35%.

Effect of the herbicides terbuthylazine and metazachlor at environmentally relevant concentrations on burrowing behaviour of red swamp crayfish *Procambarus clarkii*

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Abstract

In recent decades, chemical herbicide compounds have become increasingly detected in fresh and marine waters worldwide and are recognized as a major micropollutant threat to aquatic ecosystems. Despite their low concentrations in the environment, there is evidence of effects on non-target organisms in natural habitats. To assess their potential effects on crayfish burrowing behaviour, we exposed the red swamp crayfish *Procambarus clarkii* to terbuthylazine and metazachlor (representative of triazines and chloroacetanilides, respectively) at the environmentally relevant concentration of 2.0 μg/L, with a 28 days exposure followed by a 2 days period of burrowing observation. The metazachlor-exposed males excavated a higher number of burrows than other tested groups, with comparable depth and volume relative to individual specimen weight. Terbuthylazine-exposed males exhibited a tendency to excavate larger burrows compared with the control, but this difference was not significant. Terbuthylazine-exposed females showed a non-significant tendency to excavate larger burrows compared to those of control. The reported characteristic of female crayfish in the wild to construct deeper and/or larger-volume burrows compared to males was not demonstrated by any group in this experiment, suggesting that our results may have been affected by the low number of observations. These results, along with reported evidence of negative effects of herbicides at environmental concentrations, indicate a need for further research.

Keywords: *pollution; ecological impact; Procambarus clarkii; macroinvertebrate; terbuthylazine; metazachlor*

Introduction

In addition to overexploitation of natural resources and habitat loss/degradation (Arthington et al., 2016; Kominoski et al., 2018), water pollution is considered among the most important drivers of global biodiversity decline (Bayen, 2012; Dudgeon et al., 2006; Manosa et al., 2001), with frequent incidents of pollution and deterioration of water quality impacting aquatic ecosystems (Affum et al., 2018; Aliko et al., 2018; Sehonova et al., 2018; Yao et al., 2015). Pesticides, pharmaceuticals, chemical fertilizers, detergents, petrochemicals, and plastics are common synthetic pollutants (Amoatey and Baawain, 2019; Guzzetti et al., 2018; Yang et al., 2017).

Herbicides have been, and continue to be, heavily used in agriculture and horticulture (El-Nahhal, 2003; Goncalves and Alpendurada, 2005; Silva et al., 2019) and are detected in natural waterbodies worldwide (Peng et al., 2018; Prosser et al., 2016; Székács et al., 2015; Thurman et al., 2000). The triazine family of herbicides is widely used in Europe, but some, including atrazine, simazine, and propazine, were banned in 2003 due to environmental concerns (European Commission, 2003). Nevertheless, these herbicides or their metabolites remain detectable in the environment along with other triazines in current use, including terbuthylazine, due to their long retention time, with a half-life in biologically active soil of 6.5–149 days and in natural waters of 44–196 days (PubChem, 2019). This leads to perdurable leaching and detectable levels years after their use is discontinued (Fuhrmann et al., 2014; Tasca et al., 2018). Triazine herbicides have been largely replaced by supposedly safer chloroacetanilides herbicides such as metazachlor, a class commonly used to control weeds in field crops (El-Nahhal, 2003). Their high water solubility and persistence makes them potentially mobile in soil and water phases; hence chloroacetanilide, similar to triazine, contaminates the aquatic environment through agricultural run-off and leaching (Lopez-Pineiro et al., 2011; Milan et al., 2015; Otto et al., 2007; Tasca et al., 2018). Both are detectable in surface and groundwater for decades (Hvezdova et al., 2018; Kalkhoff et al., 1998).

The concentration of terbuthylazine in European surface and groundwaters is reported to range from 0.01 to 13.0 μg/L (Bossi et al., 2002; Chary et al., 2012; Fait et al., 2010; Jurado et al., 2012; Lacorte et al., 1998; Rodriguez-Mozaz et al., 2004; Vega et al., 2005). The concentration of metazachlor in natural waters can reach 100 μg/L (Mohr et al., 2007) with a long degradation time, having a half-life in soil of 2.8–114 days, and in water of 20.6–216 days (Hertfordshire, 2019). Contamination of aquatic ecosystems by herbicides has become a pressing environmental problem worldwide (Liu et al., 2000; Silva et al., 2019; von der Ohe et al., 2011; Zheng, 2001). Concentrations of the mentioned herbicides in aquatic ecosystems are typically well below levels reported lethal to aquatic organisms (Amoatey and Baawain, 2019), but sublethal concentrations and their metabolites may have shown detrimental effects on non-target organisms (Cook and Moore, 2008; Velisek et al., 2016; Velisek et al., 2017). They may impair the physiology and the behaviour of aquatic animals (Cook and Moore, 2008; Scott and Sloman, 2004), inducing alterations to the nervous system (Benli et al., 2007; Sarikaya and Yılmaz, 2003) and biochemical and hematological changes, as well as impacting reproduction and development (Velisek et al., 2014).

Crayfish are cosmopolitan and play a prominent role in ecosystems (Crandall and De Grave, 2017; Hossain et al., 2018; Perdikaris et al., 2017). They have evolved the ability to seek shelter and to construct burrows (Horwitz and Richardson, 1986; Riek, 1969) that provide protection against conspecifics and predators during vulnerable life stages such as moulting and reproduction (Atkinson and Taylor, 1988; Bergman and Moore, 2003; Growns and Richardson, 1988; Guo et al., 2019b) and serve as shelter in adverse environmental conditions (Guo et al., 2019b; Richardson and Swain, 1980). The ability to withstand desiccation by excavating vertical burrows into the hyporheic zone is crucial to the survival of some crayfish species during severe drought (Kouba et al., 2016). Mobility and burrowing behaviour of crayfish are known to be influenced by psychoactive compounds (Buřič et al., 2018; Guo et al., 2019a; Hossain et al., 2019; Ložek et al., 2019), but the potential effects of herbicides at environmentally relevant levels on burrowing behaviour have not been studied.

We hypothesized that the burrowing behaviour of the red swamp crayfish *Procambarus clarkii* (Girard, 1852) might be impaired by exposure to two classes of widely used herbicides and tested this by analysing effects of terbuthylazine and metazachlor at environmentally relevant concentrations under simulated drought conditions.

Material and Methods

Herbicide preparation

Terbuthylazine (TERB, purity 99.4%) and metazachlor (META, purity 99.7%) were obtained from Sigma-Aldrich (USA). Individual stock solutions stored at 4 °C were prepared in ultra-pure water (aqua-MAX-ULTRA system, Younglin, Kyounggi-do, South Korea) at concentrations of 6.7 mg/L (TERB) and 7.2 mg/L (META). The exposure concentrations of these herbicides of 2 µg/L were prepared by dilution of the stock solution with aged tap water. Ultra-pure water and LC/MS grade acetonitrile (Merck, Germany), both acidified with formic acid (Sigma-Aldrich), were used as mobile phases for liquid chromatography. The concentration of the compounds in water was calculated with isotopically labelled terbuthylazine-D5 (Toronto Research Chemicals, Canada), carbendazim-D3 (Chiron, Norway), metolachlor-13C6 (Cambridge Isotope Laboratories, USA), and metazachlor-D6 ESA (HPC Standards, Germany) used as internal standards (Table 1).

Crayfish

Red swamp crayfish *Procambarus clarkii* were collected from the Sulák brook (47.3855° N, 18.9435° E) in Érd, Hungary, on 18 July 2019, and transported to the Faculty of Fisheries and Protection of Waters, Vodňany, Czech Republic. Crayfish with fully developed chelipeds and no missing pereiopods were selected. Carapace length and post-orbital carapace length were measured to the nearest 0.1 mm using digital Vernier calipers, and crayfish were weighed to the nearest 0.1 g on an electronic balance (Kern and Sohn GmbH, Balingen, Germany).

4.2.3 Experimental protocol

Crayfish handling and exposure to herbicides

The crayfish following a one-day acclimation were then exposed to either TERB or META for 28 days at a level considered environmentally relevant (Bossi et al., 2002; Chary et al., 2012; Fait et al., 2010; Jurado et al., 2012; Lacorte et al., 1998; Mohr et al., 2007; Rodriguez-Mozaz et al., 2004; Vega et al., 2005). Specimens maintained in herbicide-free aged tap water served as controls and were handled in the same way as exposed animals. Crayfish were stocked individually into clear covered plastic 2.5 L boxes (250×190×70 mm) with two ~1 cm openings in one 190 mm wall allowing ventilation. Each box was filled with 1 L exposure solution or aged tap water. Water temperature was recorded regularly and was 19.7 ± 1.3 °C.

Crayfish were fed commercial feed (Sera Granugreen; Sera, Heinsberg, Germany) at the rate of 5–7 pellets/animal/day and carrot chips at the rate of 0.6 g/animal/day. Boxes were cleaned every other day during the tap water/solution change. The control group boxes were cleaned first to avoid contamination with tested compounds. Crayfish that moulted or spawned during the exposure period were removed from the experiment, leaving 26 females and 32 males for the burrowing trial.

The concentration of META and TERB in exposure solutions and aged tap water was determined by inline SPE-liquid chromatography with tandem mass spectrometry (SPE/LC–MS/MS) four times during the exposure period. Five mL samples of water were taken from boxes at time 0 (before water or solution exchange) and at 48 h (after water or solution exchange), filtered through 0.20 µm pore-size regenerated cellulose (Labicom, Czech Republic), and stored at -20 °C until analysis. For analysis, water samples were thawed and brought to room temperature, the internal standard was added, and samples were analysed using SPE/LC-MS/MS with triple stage quadrupole TSQ Quantiva Mass Spectrometer (Thermo Fisher Scientific, USA) using selected reaction monitoring in positive and negative modes. The SPE/LC-MS/MS parameters are described in supplementary material (Table S1). Trace Finder 3.3 software (Thermo Fisher Scientific) was used for data processing.

At the conclusion of the two-day burrowing observation, crayfish were removed from burrows, euthanized in an ice bath, and dissected. Tissue was stored at -20 °C, and the concentrations of TERB and META in the hepatopancreas and abdominal muscle were determined. Sample preparation and analysis followed methods described by Grabicova et al. (2018). Briefly, 0.25 g of tissue with addition of internal standard was extracted by a homogenizer (TissueLyser II, Quiagen, Germany) at 1800 oscillations/min for 10 min, centrifuged (Minispin, Eppendorf, Germany) at 4472g for 5 min, and the supernatant was filtered through 0.45 µm regenerated cellulose (Labicom, Czech Republic). Tissue extracts were analysed by LC-high resolution mass spectrometry (LC-HRMS) using Q-Exactive mass spectrometer (Thermo Fisher Scientific, USA) in parallel monitoring in positive and negative modes. Analytical methods are summarized in supplementary material (Table S2).

Substrate and container preparation for assessment of burrowing behaviour

To create a suitable test substrate, 13.8 kg of sand with a moisture content of 0.3 % and 11.5 kg of clay, moisture content 1.6%, were thoroughly mixed by hand (45 % clay wet weight), and aged tap water (4.7 L) was added to the mixture (25.3 kg) to reach final moisture content of 16.5 %. For size distribution of sand and clay particles see Table S3. The resultant mixture (~ 15.0 kg) was placed in each of 58 plastic buckets (inner diameter = 21.5 cm, height = 27.5 cm) to a depth of ~ 22.0 cm. To better simulate natural conditions in which areas of residual water persist in arid sites (Souty-Grosset et al., 2014), a shallow initial burrow (diameter 2.5 cm, depth 1.5 cm; volume 7.3 cm^3) was created at the edge of the container, and 5 mL aged tap water was added to stimulate burrowing in this suggested position, following Kouba et al. (2016).

A single crayfish was placed in each container, and the container was covered with a plastic lid with a 2.8 cm opening to prevent acute desiccation of the crayfish and allow ventilation. The burrowing observation of the crayfish was two days and then they were removed from the burrows for tissues sampling. The air and substrate temperatures were 20.0 ± 1.4 °C and 20.2 ± 0.2 °C, respectively, recorded hourly using Minikin loggers (Environmental Measuring Systems, Brno, Czech Republic). No significant differences were found in the biometry of the crayfish used in the burrowing experiment among groups, regardless of sex (Table 2).

Creation and measurement of burrowing casts

If manual removal of crayfish from burrows was not possible, a small quantity of carbonated water was added to the burrow to evict the animal. Excess water was removed from the burrow bottom by blotting with absorbent tissue, and gypsum casts were created. Depth of the casts was measured using digital Vernier calipers to the nearest 0.1 mm. Casts were further scanned by an Artec Spider™ hand-held 3D laser scanner (Artec Group, Luxembourg) with a stated resolution of 0.1 mm and accuracy of 0.03 mm. The scanner is based on the structured light principle and provides a 3D mesh image of the object as an output, generated in real world coordinates. The resulting stereolithography mesh was imported to Artec Studio v. 10 (Artec Group, Luxembourg) to calculate the volume of the 3D mesh.

Statistical analysis

Statistical analyses were performed using Statistica Version 13.0. The volume was the sum of all burrows (if present) made by individual crayfish, but only the deepest burrow was included in the analysis of depth. As size varies among individual crayfish, volume and depth of the burrow were assessed relative to specimen weight, following Kouba et al. (2016). Normality and homoscedasticity of the crayfish biometry group and sex, together with the relative depth and volume of burrows by group and sex were tested using Kolmogorov-Smirnov (Ghasemi and Zahediasl, 2012) and Breusch-Pagan tests (Long and Ervin, 2000), respectively. The differences were analysed by one-way ANOVA, followed by multiple comparison of mean ranks for all groups as a post hoc test. Sex differences in animal biometry and relative depth and volume of burrows were compared in each group using Students t-test. A paired t-test was used to compare the concentrations of tested compounds in the water. A general linear model (nested) was used to detect differences in the number of burrows made by individual crayfish in a given group. The null hypothesis was rejected at $p < 0.05$ in all cases.

Results

All red swamp crayfish in the experiment exhibited burrowing activity (Fig. S1). They usually constructed a single vertical burrow at the site of the suggested initial shallow burrow. One control female, one TERB female, one TERB male, one META female, and six META males each created two burrows. A single META male constructed three burrows, with the deepest in the suggested position. Mean number of burrows excavated by males in the META group was higher than in other groups ($F_{3,52} = 3.495$, $p = 0.022$) (Fig. 1). The number of females creating a mud plug to close the burrow entrance was two, four, and one in control, TERB, and META, respectively. One control and one META male were also observed to construct a mud plug. A chimney-like structure located at the entrance of the burrow was made by five TERB females and two males.

There were no significant differences among groups regarding the relative depth and volume of burrow ($F_{18,19,21}$, relative depth = 1.184, df = 2, p = 0.314; $F_{18,19,21}$, relative volume = 0.662, df = 2, p = 0.520). This result was consistent in both males ($F_{10,10,9}$, relative depth = 0.318, p = 0.730; $F_{10,10,9}$ relative volume = 1.293, p = 0.290) (Fig. 2) and females ($F_{8,9,9}$, relative depth = 1.742, p = 0.198; $F_{8,9,9}$, relative volume = 0.249, p = 0.782) (Fig. 2).

In controls, the significant differences in burrow relative depth ($t_{8,10} = 4.685$, p = 0.051) and relative volume ($t_{8,10} = 0.511$, p = 0.485) between sexes observed in the wild was not detected (Fig. 2). This was also the case for TERB ($t_{9,10}$ relative depth = 4.368, p = 0.063; $t_{9,10}$ relative volume = 0.270, p = 0.610) and META groups ($t_{9,12}$relative depth = 0.025, p = 0.876; $t_{9,12}$ relative volume = 1.617, p = 0.219) (Fig. 2).

Concentrations of terbuthylazine and its metabolites (terbuthylazine_desethyl, terbuthylazine_desethyl-2-hydroxy, and terbuthylazine_hydroxy) as well as concentrations of metazachlor and its metabolites (metazachlor ESA and metazachlor OA) were all below limits of quantification in crayfish hepatopancreas and abdominal muscle at the end of the observation period. The limits of quantification of all chemicals are provided in Table S4.

Discussion

The quality of surface waters plays an essential role in maintaining ecosystem biodiversity and function (Bakker, 2012). The negative effects of pesticides on non-target organisms in the natural environment have drawn attention (Amoatey and Baawain, 2019; Mohr et al., 2007; Stadlinger et al., 2018). Effects of herbicides on the burrowing behaviour of freshwater animals is lacking. In the present study, we found that the relative depth and volume of burrows created by red swamp crayfish exposed to metazachlor and terbuthylazine were comparable to that of control.

Metazachlor-exposed males excavated a higher number of burrows than other groups, possibly a stress response (Ložek et al., 2019; Matozzo et al., 2004) manifested as anxiety behaviour (Buřič et al., 2013). In the wild, the availability of shelter and resources such as suitably moist conditions is critical to crayfish (Guo et al., 2019b; Kouba et al., 2016), as survival depends not only on health status but also on indirect consequences to behaviour patterns influencing reproduction, social interaction, and decision making (Kubec et al., 2019). Any effects of herbicides under drought conditions may have disproportionally high impact on the red swamp crayfish, if it results in impairment of its greater resistance to desiccation and superior burrowing abilities compared to native European crayfish species, as well as to some invasive crayfish (Kouba et al., 2016).

Herbicides considered safe by regulatory agencies, like the glyphosate-based, might exert cytotoxic effects on the hepatopancreas of non-target crustaceans (de Melo et al., 2019). Gill and hepatopancreas pathology, alterations in biochemical parameters of haemolymph, oxidative damage to hepatopancreas, and changes in antioxidant biomarkers in muscle and hepatopancreas of red swamp crayfish were recorded after 14 days exposure to terbuthylazine-desethyl, a terbuthylazine degradation product, at the environmental concentration of 2.9 µg/L (Stara et al., 2016). Differences from controls in behaviour, including significantly lower values of speed and velocity of movement, as well as damage to gill and hepatopancreas, of marbled crayfish *Procambarus virginalis* were found after a 28-day exposure to the environmentally relevant concentration of 4.2 µg/L S-metolachlor and its degradation product metolachlor OA (Stara et al., 2019). Significantly lower growth and delayed ontogenetic development of marbled crayfish were also detected at environmentally relevant concentrations of metazachlor and metazachlor OA (Velisek et al., 2020).

The hepatopancreas is the main organ of detoxification of xenobiotics in crustaceans (Vogt, 2002), and the crayfish abdomen is the key organ of forward swimming and other activities (Mulloney and Hall, 2000). We found concentrations of selected herbicides in the hepatopancreas and abdomen of both control and exposure groups were well below limits of quantification. Literature regarding detection of herbicides in hepatopancreas and abdomen of crayfish is scarce. Our observation suggests the concentration of these compounds in target tissues was below limits of quantification.

Females of many crayfish species (Hobbs, 1942; Riek, 1972) use burrows for oviposition and/or egg incubation, and contact with groundwater or sufficient humidity is crucial to their successful reproduction (Guo et al., 2019b). Females of these species tend to construct larger and/or deeper burrows than do males and frequently occupy the deepest parts of the burrow during egg incubation (Guo et al., 2019a; Hazlett et al., 1974; Payne, 1972). This pattern was not observed in any tested group, including the controls, suggesting that some effects of herbicide exposure might remain hidden due to the relatively low number of observations.

The characteristics and morphology of the burrows excavated by crayfish are closely associated with their life history traits (Hartzell, 2019; Palaoro et al., 2016; Williams et al., 1974). The chimney-like structure is important in enhancing air circulation in the burrow through action on wind currents (Hobbs Jr, 1981; Thoma and Armitage, 2008). This type of burrowing was observed in five female and two male TREB-exposed crayfish. Mud plug closing of the burrow entrance to afford protection from desiccation or predation is also a key characteristic (Ilhéu et al., 2003; Souty-Grosset et al., 2014). In a previous study, the mud plug construction was found only in burrows of female crayfish (Guo et al., 2019a), but was seen in both sexes in the present study, as well as reported by Kouba et al. (2016).

The only apparent manifestation of terbuthylazine and metazachlor on the red swamp crayfish behaviour at environmentally relevant concentrations was the significantly higher mean number of the burrows excavated by META-exposed males. In burrowing studies, a sufficient number of replicates is critical, considering the substantial variation in individual specimen responses. Based on crayfish as a model species, a similar stress response might be expected in other hyporheic-dwelling aquatic biota, such as unionid mussels and clams and a wide range of aquatic insects. These results and those of Guo et al. (2019a) suggest that sublethal effects of herbicides and other aquatic contaminants on non-target organisms should be further examined. The observed trends in burrowing and the observed effects of herbicides at environmental concentrations call for further research, and studies of effects on organism behaviour should be an obligatory component of the herbicide registration process.

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Conflicts of interest

The authors declare no conflicts of interest relative to any of the internal or external funding sources.

Author contributions

Conceived and designed the experiments: A.K., and W.G.; Collected animals and performed the experiment: W.G., A.K., A.W, M.S.H., M.Bu., F.L., L.V., M.Bl., and J.K.; Analysed the data: W.G., M.S.H., and K.G.; Stock solutions prepared: K.G., J.V.; Wrote the first draft: W.G.; All authors contributed to and edited the manuscript.

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Table 1. *Concentrations of terbuthylazine and metazachlor in red swamp crayfish exposure solutions and control water at 0 and 48 h. TERB, terbuthylazine and META, metazachlor. Data are presented as mean ± standard deviation; p < 0.05. t, paired t-test.*

Group	n	Compound tested	Time 0 h (μg/L)	Time 48 h (μg/L)	*t*	p
Control	4	terbuthylazine	< LOQ	< LOQ	----	----
		metazachlor	< LOQ	< LOQ	----	----
TERB	4	terbuthylazine	1.2 ± 0.2	1.4 ± 0.3	-1.732	0.182
META	4	metazachlor	1.4 ± 0.1	1.4 ± 0.1	0.225	0.836

LOQ, limit of quantification, values for META ranged from 0.003 to 0.008 μg/L and for TERB 0.002 to 0.009 μg/L.

Table 2. *Sex, carapace length (CL), and weight (W) of red swamp crayfish Procambarus clarkii used in the study. Data are presented as mean ± standard deviation; p < 0.05. F One-Way ANOVA; t, Student's t-test. TERB, terbuthylazine; META, metazachlor.*

Experimental setup	Sex	n	CL (mm)		p	W (g)		p
Control	M	10	44.1 ± 6.3	$F_{group} = 0.911$	0.408	22.8 ± 10.0	$F_{group} = 0.571$	0.569
	F	8	45.5 ± 6.3	$F_{male} = 0.218$	0.806	22.3 ± 8.8	$F_{male} = 0.175$	0.840
TERB	M	10	43.3 ± 6.3	$F_{female} = 1.136$	0.339	22.6 ± 11.0	$F_{female} = 1.636$	0.217
	F	9	41.5 ± 5.6	$t_{10,8, Control} = 0.685$	0.736	16.0 ± 4.4	$t_{10,8, Control} = 0.422$	0.994
META	M	12	42.3 ± 6.1	$t_{10,9, TERB} = 0.822$	0.508	20.5 ± 8.9	$t_{10,9, TERB} = 0.871$	0.435
	F	9	42.7 ± 4.6	$t_{12,9, META} = 0.504$	0.961	20.6 ± 8.7	$t_{12,9, META} = 0.504$	0.961

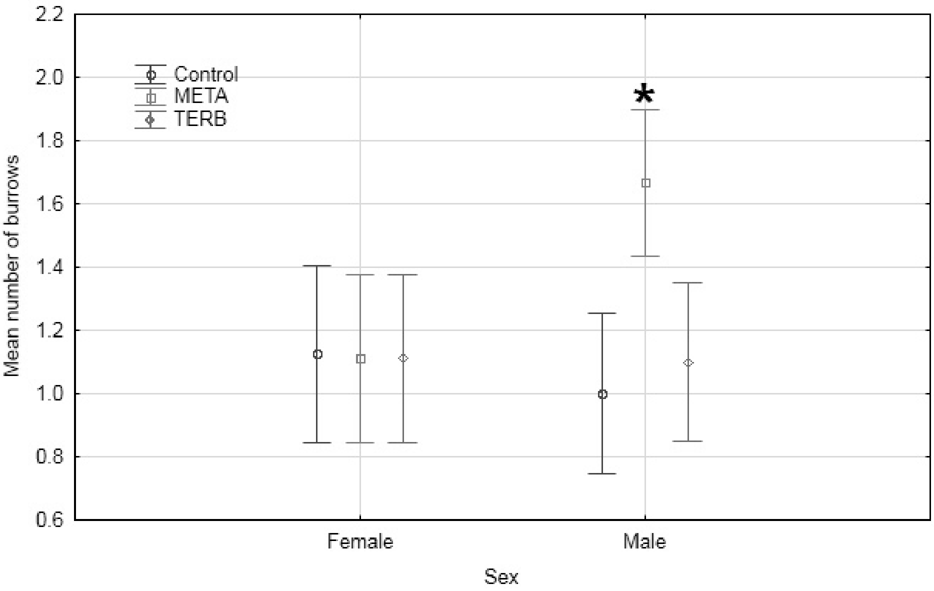

Figure 1. *Number of red swamp crayfish P. clarkii burrows in each treatment by sex. Control (8 females, 10 males), TERB (terbuthylazine; 9 females, 10 males), and META (metazachlor; 9 females, 12 males). Data are presented as mean ± standard deviation. The asterisk indicates significant differences within the given group (p < 0.05).*

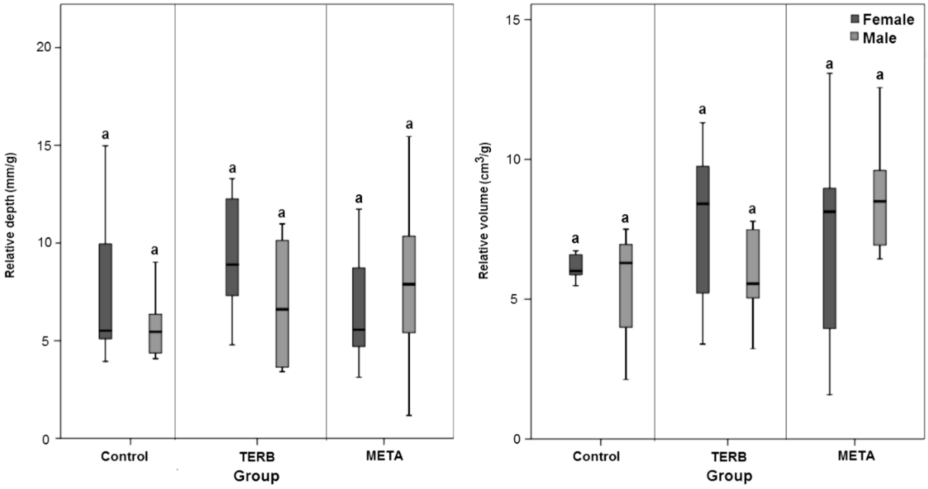

Figure 2. *Box-whisker plot of the depth and volume of red swamp crayfish P. clarkii burrows of control, terbuthylazine (TERB), and metazachlor (META) groups relative to individual crayfish weight. Box limits correspond to upper and lower quartiles, horizontal bar to the median. Values with Differing letters indicate significant among-group differences within sex (p < 0.05).*

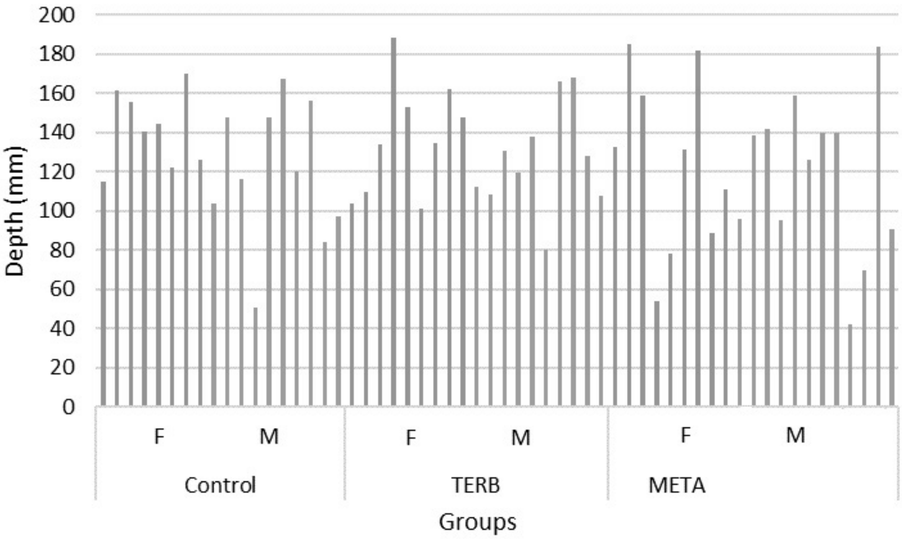

Figure S1. *Depth of red swamp crayfish P. clarkii burrows. TERB, terbuthylazine; META, metazachlor; F, female; M, male. Each column represents a single specimen.*

Table S1. *SPE/LC-MS/MS parameters of water samples analysis.*

- SPE/LC-MS/MS parameters of water samples.
- Columns: SPE extraction column, YMC C8 (20 x 2.1 mm, 10 µm particles, YMC, Chromservis, ČR); analytical column, Hypersil Gold aQ (50 x 2.1 mm, 5 µm particles, Thermo Fisher Scientific, USA); LC gradient: Mobile phase A, acidified ultra-pure water; mobile phase B, acidified acetonitrile (both 0.1% formic acid); Accela 1250 and 600 pumps

Accela 1250 pump				**Accela 600 pump**			
Time [min]	**A** [%]	**B** [%]	**Flow** [µL/min]	**Time** [min]	**A** [%]	**B** [%]	**Flow** [µL/min]
0	95	5	350	0	100	0	1100
1	95	5	350	1.05	100	0	1100
3	75	25	400	1.07	0	100	100
8	0	100	400	9	0	100	100
10	0	100	400	10	0	100	1500
10.01	95	5	400	10.1	100	0	1000
13	95	5	350	13	100	0	1000

- MS transitions:

Compound	**Mode**	**Precursor m/z**	**Quan m/z**	**Qual m/z**	**RT [min]**	**IS used**
Metazachlor	+	278.1	134.11	210.07	6.0	Metolachlor_$^{13}C_6$
Metazachlor ESA	-	322.1	121.14	148.18	4.4	Metazachlor ESA_D_6
Metazachlor OA	+	274.1	134.11	162.11	4.2	Carbendazim_D_3
Terbuthylazine	+	230.1	104.00	174.07	6.2	Terbuthylazine_D_5
Terbuthylazine_ desethyl	+	202.1	146.07	104.00	5.3	Terbuthylazine_D_5
Terbuthylazine_ desethyl-2-hydroxy	+	184.1	128.11	86.11	2.9	Carbendazim_D_3
Terbuthylazine_ hydroxy	+	212.1	156.11	114.11	4.2	Terbuthylazine_D_5

RT, retention time; IS, internal standard

Duration of method: 13 minutes

Table S2. *LC-HRMS parameters of crayfish hepatopancreas, abdominal muscle, and food (pellets and carrot) samples.*

- Analytical column: Hypersil Gold aQ (50 x 2.1 mm, 5 µm particles, Thermo Fisher Scientific, USA)
- LC gradient: Mobile phase A, acidified ultra-pure water; mobile phase B, acidified acetonitrile (both 0.1% formic acid); Accela 1250 pump

Accela 1250 pump			
Time	**A**	**B**	**Flow**
[min]	[%]	[%]	[µL/min]
0	100	0	350
3	70	30	350
5	40	60	350
7	0	100	400
8	0	100	400
8.05	100	0	350
10	100	0	350

- MS transitions:

Compound	**Mode**	**Precursor m/z**	**Quan m/z**	**Qual m/z**	**RT [min]**	**IS used**
Metazachlor	+	278.106	210.0680	134.0965	5.7	Metolachlor_$^{13}C_6$
Metazachlor ESA	-	322.100	120.9603	148.0768	3.8	Metazachlor ESA_D_6
Metazachlor OA	+	274.130	134.0963	206.0805	3.6	Carbendazim_D_3
Terbuthylazine	+	230.100	174.0540	230.1164	6.0	Terbuthylazine_D_5
Terbuthylazine_desethyl	+	202.100	146.0225	202.0850	5.0	Terbuthylazine_D_5
Terbuthylazine_desethyl-2-hydroxy	+	184.100	128.0567	184.1191	2.9	Carbendazim_D_3
Terbuthylazine_hydroxy	+	212.130	156.0878	212.1501	3.9	Terbuthylazine_D_5

RT, retention time; IS, internal standard

Duration of method: 10 minutes

Table S3. *Size distribution of sand and clay particles (analytical laboratory of AGRO-LA Inc. Jindřichův Hradec, Czech Republic).*

Sand **Size class (mm)**	**Proportion (%)**	**Clay** **Size class (mm)**	**Proportion (%)**
0.63–0.8	16.6	< 0.4	52.6
0.8–1.0	75.6	0.4–0.5	9.2
1.0–1.25	3.2	0.5–0.63	7.4
1.4–2.0	0.4	0.63–0.8	14.8
>2	0.2	0.8–1.0	16.0

Table S4. *Limits of quantification (min–max, ng/L) of the target chemicals (META, metazachlor and TERB, terbuthylazine) and their relevant metabolites in food and tissue samples of red swamp crayfish P. clarkii.*

	META			**TERB**			
	Metazachlor	**Metazachlor_ESA**	**Metazachlor_OA**	**Terbutylazine**	**Terbutylazine_ desethyl**	**Terbutylazine_ desethyl-2-hydroxy**	**Terbutylazine_ hydroxy**
carrot	0.13–0.15	0.58–0.73	0.74–0.85	0.28–0.34	0.11–0.13	0.08–0.39	0.09–0.11
pellets	0.12–0.15	0.96–1.40	0.53–0.69	0.26–0.36	0.10–0.14	0.06–0.10	0.08–0.11
muscle	0.12–0.18	0.58–1.10	0.59–0.90	0.20–0.32	0.10–0.15	0.08–0.13	0.10–0.15
hepatopancreas	0.10–0.32	1.40–9.8	1.10–3.6	0.36–0.65	0.22–0.41	0.08–0.23	0.13–0.24

CHAPTER 5

POTENTIAL OF MARBLED CRAYFISH *PROCAMBARUS VIRGINALIS* TO SUPPLANT INVASIVE *FAXONIUS IMMUNIS*

Hossain, M.S., Guo, W., Martens, A., Adamek, Z., Kouba, A., Buric, M., 2020. Potential of marbled crayfish *Procambarus virginalis* to supplant invasive *Faxonius immunis*. Aquatic Ecology 54, 45–56.

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Potential of marbled crayfish *Procambarus virginalis* to supplant invasive *Faxonius immunis*

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Abstract Biological invasions are a growing threat to global biodiversity due to negative impacts on native biota and ecosystem functioning. Research has expanded from investigating native and alien species interactions to examining relationships among alien species. Invasive crayfish may have similar life histories, niche preferences, and adaptation strategies, but their mutual interactions are little understood. This study aimed to quantify interaction patterns of size-matched calico crayfish *Faxonius immunis*, established in the Rhine River catchment, and the parthenogenetic marbled crayfish *Procambarus virginalis*, currently spreading throughout Europe. During agonistic interactions in the absence of shelter, marbled crayfish won a significant majority of fights against calico crayfish, but in the presence of shelter there was no significant difference. When sex of calico crayfish was considered in the analysis without shelter, marbled crayfish won a significantly higher number of fights with female calico crayfish. In the absence of shelter, marbled crayfish dominated calico crayfish females in 83.3% and males in 60% of pairs. With available shelter, the dominance of marbled crayfish was 100% and 54.5% over female and male calico crayfish, respectively. The results suggested that sex and resource availability influence agonistic behaviour in the studied crayfish. Marbled crayfish are confirmed to be competitive against the calico crayfish, which has been shown to be dominant over another serious invader in the Rhine River catchment, the spiny-cheek crayfish *Faxonius limosus*. In natural sympatric populations, the situation may be affected by factors such as size, reproductive variables, water temperature, and predation pressure.

Keywords Biological invasion · Calico crayfish · Competition · Dominance · Interaction · Marbled crayfish

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Introduction

Global biodiversity is threatened by various factors, with biological invasions of critical importance (Lodge 1993; Chapin et al. 2000; Catford et al. 2012). Species introductions outside their native ranges are fundamentally driven by human activity, often related to agriculture, aquaculture, forestry, and biological control programs (Pimentel et al. 1992; Savini et al. 2010) as well as to increasing mobility, tourism, and transportation (Drake and Lodge 2004; Hall 2010). The number of translocated species is increasing globally with no signs of abatement (Early et al. 2016; Seebens et al. 2017). Aquatic ecosystems are particularly vulnerable, often being invaded by multiple alien species that interact with native biota as well as with one another (Ricciardi and Rasmussen 1999; Hudina et al. 2011), a process referred to as over-invasion (Russell et al. 2014). In a novel environment, over-invasion can alter an ecosystem through additive or multiplying effects (Roy et al. 2014; Jackson 2015) and may modify the interaction of invaders with native species (Preisser and Elkinton 2008). The consequences of multiple invasions are understudied (Russell et al. 2014). Successful invasions can be elucidated by comparative analysis of invading species (Van Kleunen et al. 2010), particularly important when organisms share identical ecological niches and resources such as food and shelter (Wilson 2000; Huntingford 2013).

The invasion mechanisms and potential impact of invading species may be best assessed by determining behaviour flexibility and ability to adapt to a new environment (Wright et al. 2010) as well as the survival, growth rate, and reproduction processes of interacting species (Sih et al. 2012; Penk et al. 2017; Hossain et al. 2019b). Antagonism and aggression are important features of animal behaviour (Parker 1974; Manning and Dawkins 2012) that have direct effects on invader abundance, distribution, and dispersion rate in a new environment (Hudina et al. 2014). Agonistic behaviour is principally represented by aggressiveness in combat (Gherardi 2002) and affects success in coping with new predators and competitors (Duckworth 2008; Hudina and Hock 2012).

Crayfish are considered ecosystem engineers and ecologically important benthic, polytrophic, omnivorous species in aquatic environments (Momot 1995; Nyström 2002; Lipták et al. 2019) due to their relatively large body size and overall biomass, long lifespan, and grazing and burrowing activity (Usio et al. 2001; Matsuzaki et al. 2009). Crayfish exhibit aggressive behaviour, which is closely linked to invasion success, throughout their lifespan (Sih et al. 2012). Alien crayfish are among the most destructive of invasive taxa with documented negative impacts at multiple levels of ecological organization (Twardochleb et al. 2013) and represent a major hazard to freshwater biodiversity globally (Lodge et al. 2012; Twardochleb et al. 2013).

In Europe, the Rhine River catchment was historically inhabited by the native noble crayfish *Astacus astacus* (L., 1758), as well as the white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) and, particularly in its upper sections, the stone crayfish *Austropotamobius torrentium* (von Paula Schrank, 1803). These species have largely disappeared as a result of crayfish plague outbreaks caused by an oomycete *Aphanomyces astaci* Schikora. The spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817) was introduced into present-day western Poland as an alternative to endangered native stocks in 1890 with secondary translocations to Germany and France to compensate for the loss of the commercially valuable noble crayfish (Holdich et al. 2006). Thanks to human-assisted translocation and its own dispersal, spiny-cheek crayfish has reached the Rhine River system and become dominant in the area. It is not well documented to what extent the spiny-cheek crayfish was directly or indirectly responsible for the disappearance of native stock, chiefly noble crayfish (Tittizer et al. 2000; Chucholl and Dehus 2011), but it is assumed that its introduction exacerbated the pathogenic crayfish plague (Holdich et al. 2006). The spiny-cheek crayfish dominance in the Rhine River has changed in recent years with the introduction of the calico crayfish *Faxonius immunis* (Hagen, 1870), first recorded in 1993 (Gelmar et al. 2006). The calico is currently the dominant alien crayfish in the Upper Rhine system (Herrmann et al. 2018) and has successfully invaded lentic and lotic habitats including gravel pit lakes and small canals and brooks. The initial introduction was assumed to be as fishing bait used by Canadian soldiers (Gelmar et al. 2006). It is reported that the calico crayfish dominates spiny-cheek crayfish during direct competition for shelter and has displaced it from the areas in which the species overlapped (Chucholl et al. 2008).

In the meantime, the parthenogenic apomictic marbled crayfish *Procambarus virginalis* Lyko, 2017, following its first appearance in aquarium shop in Germany in the mid-1990 s (Lukhaup 2001), became popular in the pet trade. It has subsequently become established in the wild, particularly in Europe, through intentional or unintentional releases (Hossain et al. 2018). Marbled crayfish is listed among the most invasive species in the European Union (EU Regulation No. 1143/2014, Commission Implementing Regulation No. 2016/1141). In recent studies, marbled crayfish showed competitiveness in combat with the highly aggressive red swamp crayfish *Procambarus clarkii* (Girard, 1852) (Jimenez and Faulkes 2011; Hossain et al. 2019b), itself a prominent invader worldwide (Souty-Grosset et al. 2016), and with spiny-cheek crayfish (Linzmaier et al. 2018), although it exhibits lower aggressiveness towards conspecifics (Vogt et al. 2004).

Reports confirming marbled crayfish occurrence in the Rhine River systems and other parts of Germany (Chucholl et al. 2012; Herrmann et al. 2018) suggest the possibility of its coexistence with the calico crayfish or the potential for displacement of one species by the other from their ecological system/niche. No information regarding interactions of marbled and calico crayfish in nature or in laboratory conditions is available. In the present study, we characterized agonistic behaviour of calico versus marbled crayfish to identify differences in aggression that may affect their interactions in field conditions potentially causing displacement and over-invasion.

Materials and methods

Experimental animals

Marbled crayfish originated from our laboratory culture and experienced a similar social structure in their communal culture tanks. Calico crayfish were captured during a hydrobiological survey on 11 July 2017 from a pond in proximity to the Rhine River (Rheinstetten, Germany; 48°58′09.6″N, 8°17′44.0″E) (Ondračková et al. 2018), transferred to a facility of South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, University of South Bohemia in České Budějovice and acclimated to laboratory conditions for 30 days before selection for experiments.

Acclimation and experimental set-up

To eliminate hierarchical status established in the stock tanks, all specimens were held individually in one 250-mL plastic box (163 × 118 × 62 mm), screened from other crayfish, for 7 days prior to initiation of the trials. Water temperature was maintained at $\sim$ 20 °C, and crayfish were fed daily ad libitum on commercial dry aquarium fish feed enriched with algae (Sera Granugreen, Sera GmbH, Germany).

Marbled/calico crayfish pairs were randomly divided into groups with (17 marbled, 11 male, six female calico) and without (18 marbled, ten male, eight female calico) shelter. Pairs were size-matched to within $< 5\%$ wet weight (Aquiloni and Gherardi 2008; Momohara et al. 2013) (Table 1). Sex of calico crayfish was identified, since it has shown significant effect on dominance status in adults (Bovbjerg 1956; Pavey and Fielder 1996).

Crayfish pairs were stocked into oval experimental arenas (600 L × 400 W × 250 H mm) filled with 12 L aged tap water ($\sim$ 20 °C) and 750 g (dry weight) of fine aquarium sand. The shelter group was provided with a halved ceramic plant pot, 94 mm depth with a 60-mm-diameter entrance, placed in the centre of one side of the arena. Crayfish were placed under transparent perforated plastic cups on opposite sides of the arena. After 5 min, the cups were removed to enable crayfish to move and freely interact for a 15 min period that was recorded using digital video camera (Sony HDR-CX240, Sony, Japan) mounted on the lid of the arena. Obtained data were used to quantify the number of contacts, threats, fights, fight duration, and avoidance behaviours as well as to identify the fight initiator and winner (Table S1). Visual evaluation was carried out according to the methods proposed by Hossain et al. (2019b) and the ethogram modified from Hossain et al. (2019b) and Fořt et al. (2019). Dominance was established as the time at which an agonistic event led to one crayfish's persistent avoidance of fighting, contact, or the proximity of (one body length) the other.

Table 1 Carapace length (CL) and weight (*W*) of marbled crayfish and calico crayfish used in interaction pairs in groups with and without shelter

Experimental group	Crayfish (n)	CL (mm)	*W* (g)	*D* (%)
Without shelter	MC (10)	27.9 ± 4.6	6.4 ± 2.6	2.5 ± 1.7
	CC (10, male)	28.1 ± 3.8	6.3 ± 2.6	
	MC (8)	25.4 ± 2.4	4.9 ± 1.2	3.3 ± 1.5
	CC (8, female)	26.0 ± 2.0	4.9 ± 1.3	
	MC (combined)	26.9 ± 3.8	5.8 ± 2.1	2.8 ± 1.6
	CC (combined)	27.2 ± 3.2	5.7 ± 2.2	
With shelter	MC (11)	29.0 ± 5.7	7.0 ± 3.1	2.0 ± 1.8
	CC (11, male)	29.3 ± 5.1	7.1 ± 3.3	
	MC (6)	23.6 ± 2.7	4.0 ± 1.5	2.6 ± 2.1
	CC (6, female)	24.4 ± 2.3	3.9 ± 1.5	
	MC (combined)	27.1 ± 5.6	5.9 ± 3.1	2.2 ± 1.6
	CC (combined)	27.6 ± 5.0	6.0 ± 3.3	

Last column shows the average per cent weight difference (*D*) of paired specimens. Data are presented as mean ± SD

MC marbled crayfish, *CC* calico crayfish

Statistical analysis

The interaction outcomes of pairs with and without shelter were compared using Wilcoxon matched pairs test. Chi-squared (χ^2) tests were used to assess the dominance status of pairs. Fight frequency and duration and latency to fight initiation of pairs with and without shelter were compared with Kruskal–Wallis test. The total number of short and long fights was analysed with Mann–Whitney *U* test. A multiple comparison test was used to determine significant differences among groups. The null hypothesis was rejected at $\alpha < 0.05$ in all tests. Statistical analyses were performed using Statistica 13.2 software (StatSoft Inc., Tulsa, USA).

Results

In the absence of shelter when sex of the calico crayfish was not included in the analysis, there were no significant differences in contact initiation, fight initiation, and avoidance behaviours of marbled crayfish and calico crayfish ($Z = 0.67$, $P = 0.499$; $Z = 1.33$, $P = 0.184$; and $Z = 1.53$, $P = 0.124$, respectively). Marbled crayfish won a significant ($Z = 1.96$, $P = 0.049$) majority of fights against calico crayfish (Table 2) and also won a significantly higher number of fights against calico crayfish females ($Z = 2.52$, $P = 0.012$) (Fig. 1).

In the presence of shelter and without considering the sex of the calico crayfish, the species did not significantly vary in avoidance behaviours ($Z = 0.73$, $P = 0.463$), fights won ($Z = 0.98$, $P = 0.328$), or contact initiation ($Z = 1.82$, $P = 0.068$). Fight initiation was marginally significant ($Z = 1.95$, $P = 0.051$) with marbled crayfish initiating a higher number of contacts and fights than calico crayfish (Table 2). There were no significant differences between marbled crayfish and calico crayfish males in initiating contact ($Z = 1.01$, $P = 0.310$), avoidance ($Z = 0.0$, $P = 1.000$), fight initiation ($Z = 1.213$, $P = 0.225$), and fights won ($Z = 0.34$, $P = 0.735$) (Fig. 2). Marbled crayfish showed a tendency to initiate more frequently the contact ($Z = 1.83$, $P = 0.067$) and fights ($Z = 1.83$, $P = 0.067$); however, these remained statistically insignificant. There were no significant differences in avoidance behaviours ($Z = 1.46$, $P = 0.144$) and fights won ($Z = 1.46$, $P = 0.144$) (Fig. 2).

When calico crayfish sex was excluded from the analysis, 16 of 18 pairs with shelter and 14 of 17 pairs without shelter established dominance, with marbled crayfish dominant significantly more often (χ^2 test$_{\text{without shelter}} = 14.1$; $P < 10^{-3}$; χ^2 test$_{\text{with shelter}} = 8.2$; $P = 0.004$). Pairs with no defined dominance were observed only in interactions of marbled crayfish with female calico crayfish. Marbled crayfish were significantly more frequently dominant over female calico crayfish both with shelter (χ^2 test = 100.0; $P < 10^{-5}$) and without shelter (χ^2 test = 44.4; $P < 10^{-4}$). In the presence of shelter, no significant differences were observed between dominance of marbled crayfish and calico crayfish males (χ^2 test = 0.81; $P = 0.400$). In the absence of shelter, the dominance of marbled crayfish was significant (χ^2 test = 4.0; $P = 0.045$) (Table 3).

Table 2 Agonistic interactions (%) of MC, marbled crayfish and CC, calico crayfish during interaction in the presence and absence of shelter and without considering the sex of calico crayfish

Group	Crayfish	Contact initiator (%)	Avoidance (%)	Fight initiator (%)	Winner (%)
Without shelter	MC	54.9 ± 22.3	38.7 ± 31.6	58.0 ± 18.9	71.0 ± 34.2^{a}
	CC	45.1 ± 22.3	61.3 ± 31.6	42.0 ± 18.9	29.0 ± 34.2^{b}
With shelter	MC	66.9 ± 29.4	37.5 ± 48.8	71.2 ± 30.9	61.3 ± 40.8
	CC	33.1 ± 29.4	62.5 ± 48.8	28.8 ± 30.9	38.7 ± 40.8

Different superscripts indicate significant differences at $P < 0.05$. Values are presented as mean ± SD

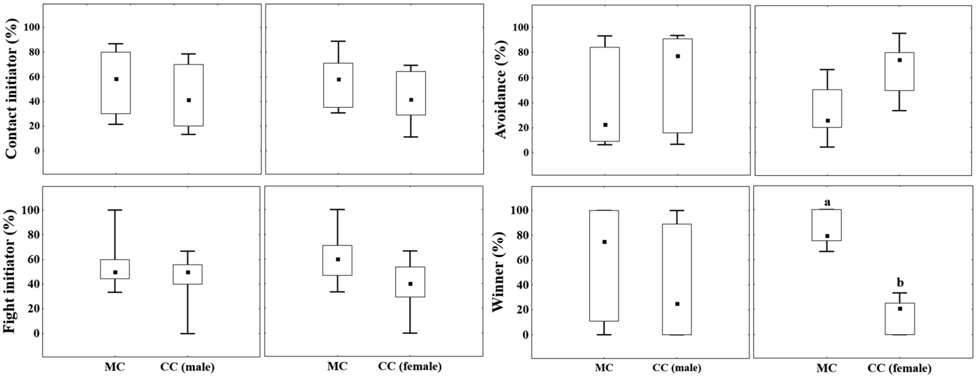

Fig. 1 Comparison of agnostic interactions (%) of MC, marbled crayfish and CC, calico crayfish males and females in the absence of shelter. Values are presented as median (small square), percentiles (large box, 25–75%) and min–max (error bar). Different superscripts indicate significant differences at $P < 0.05$

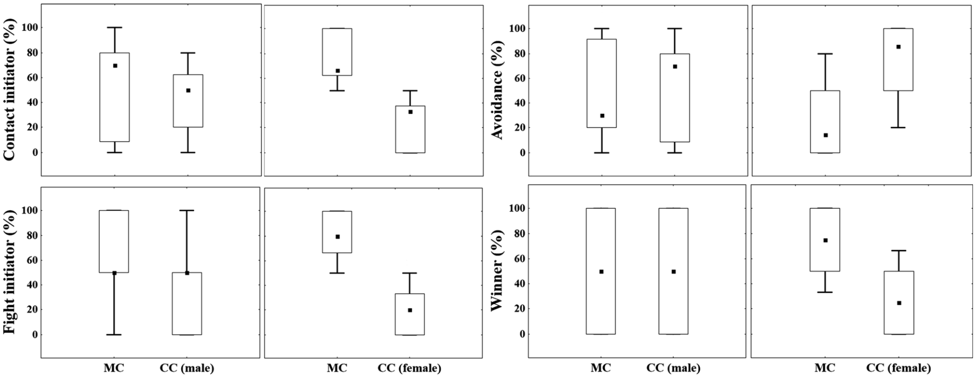

Fig. 2 Comparison of agnostic interactions (%) of MC, marbled crayfish and CC, calico crayfish. Male and female in the presence of shelter. Values are presented as median (small square), percentiles (large box, 25–75%), and min–max (error bar). Different superscripts indicate significant differences at $P < 0.05$

Table 3 Dominance percentage of marbled crayfish and calico crayfish in interactions with the presence and absence of shelter

Group	Crayfish	Established dominance *n*, (%)	Not established *n*, (%)	Per cent established
Without shelter	MC	6 (60.0)	0 (0.0)	60.0^{a}
	CC (male)	4 (40.0)		40.0^{b}
	MC	5 (62.5)	2 (25.0)	83.3^{a}
	CC (female)	1 (12.5)		16.7^{b}
	MC	11 (61.1)	2 (11.1)	68.8^{a}
	CC (combined)	5 (27.8)		31.2^{b}
With shelter	MC	6 (54.5)	0 (0.0)	54.5
	CC (male)	5 (45.5)		45.5
	MC	3 (50.0)	3 (50.0)	100^{a}
	CC (female)	0 (0.0)		0.0^{b}
	MC	9 (52.9)	3 (17.6)	64.3^{a}
	CC (combined)	5 (29.5)		35.7^{b}

Different superscripts indicate significant differences at $P < 0.05$

MC marbled crayfish, *CC* calico crayfish, *n* number of pairs

In addition to total fights per pair, latency to initial fight, total fight duration, and mean duration of fights did not vary significantly within or between groups regardless of sex of calico crayfish and presence or absence of shelter (Table 4). The number of fights categorized as short (< 60 s) and long (> 60 s) did not significantly vary among groups (Fig. 3).

Discussion

In addition to increasing knowledge of ecological strategies and adaptability (Lindqvist and Huner 1999; Holdich et al. 2009), observations of agonistic behaviour during interspecific interactions can estimate the potential success of an invader (Gherardi 2002; Hudina et al. 2016). Aggressive behaviour is relevant to ecological consequences, even when two phylogenetically unrelated species interact in a functionally similar niche (Church et al. 2017). Current dispersion of marbled crayfish in natural waters amplifies the importance of assessing its competitiveness with previously established invaders (Kawai et al. 2016; Hossain et al. 2018). In spite of its placid disposition during interactions with conspecifics (Vogt 2008; Kawai et al. 2016), marbled crayfish

Table 4 Number of fights per pair, time to fight initiation, total fight duration per pair, and mean duration per fight in marbled crayfish versus calico crayfish with the presence and absence of shelter

Group*	Crayfish*	Total fights (n)	Time to fight (s) initiation	Total fight duration (s)	Mean fight duration (s)
Without shelter	MC × CC (male)	4.3 ± 3.1	157.3 ± 164	395.9 ± 241.1	133.4 ± 137.6
	MC × CC (female)	3.6 ± 1.6	140.7 ± 65.8	439.6 ± 267.4	137.4 ± 127.3
	MC × CC (combined)	3.9 ± 2.5	150.5 ± 129.7	425.2 ± 242.4	138.9 ± 126.6
With shelter	MC × CC (male)	2.5 ± 1.1	159.6 ± 174.2	327.5 ± 322.3	188.7 ± 153.2
	MC × CC (female)	3.0 ± 1.2	176.6 ± 108.1	395.3 ± 299.6	219.2 ± 211.7
	MC × CC (combined)	2.7 ± 1.1	166.2 ± 147.2	351.5 ± 306.7	200.4 ± 169.9

MC marbled crayfish, *CC* calico crayfish

*No significant difference observed between groups at $P < 0.05$. Values are presented as mean ± SD

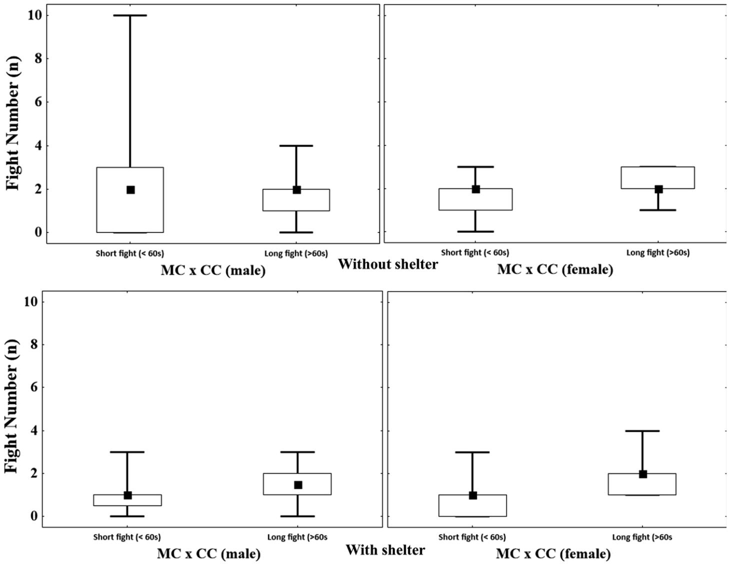

Fig. 3 Comparison of fight types (%) of MC, marbled crayfish and CC, calico crayfish in the presence and absence of shelter with considering the sex of calico crayfish. Values are presented as median (small black square), percentiles (large box, 25–75%), and min–max (error bar)

have shown competitiveness against wild spiny-cheek crayfish (Linzmaier et al. 2018) and the red swamp crayfish (Jimenez and Faulkes 2011; Hossain et al. 2019b), the most successful introduced crayfish species worldwide (Souty-Grosset et al. 2016; Hossain et al. 2018).

We found the marbled crayfish to be dominant in interactions with calico crayfish, which have an established population of the Rhine River system. Marbled crayfish won significantly more fights with calico crayfish females in the absence of shelter. With available shelter, marbled crayfish were significantly more active in initiating contact and combat than were calico crayfish females. This might be due to different strategies of male and female crayfish during agonistic interactions (Wofford 2013). However, both with and without shelter, marbled crayfish were equally active in interactions with calico crayfish males; hence, they could overcome lack of sexual dimorphism and successfully combat either sex of calico crayfish. This combat success has also been observed in previous studies in which marbled crayfish showed significantly higher aggression when compared to both sexes of red swamp crayfish (Hossain et al. 2019b) as well as size-matched and larger spiny-cheek crayfish (Linzmaier et al. 2018).

With and without shelter, the dominance pattern was more pronounced in pairings with calico crayfish males than with females. Compared to males (0%), undefined hierarchy status was higher with calico crayfish females in the absence (25%) and presence of shelter (50%). These differences may have been due to the more aggressive nature (Gherardi 2002) and higher resource-holding potential of males compared to females (Dissanayake et al. 2009). The establishment of hierarchy was also more rapid in pairings with calico crayfish males than females (Table 3). Females may show a propensity to conserve energy for reproduction and care of offspring (Trivers 1972). It is evident from our findings of dominance establishment that shelter occupancy of marbled crayfish was equivalent to that of calico crayfish males and significantly higher than calico crayfish females (Table 3). Aggressive encounters in laboratory conditions have been shown similar to combat dynamics of crayfish in nature (Bergman et al. 2003); hence, we can expect key resources such as shelter to be frequently seized by marbled crayfish in an area in which both species occur. This hypothesis may also be applicable to red swamp and spiny-cheek crayfish, against which marbled crayfish have shown significant competition success in laboratory trials (Jimenez and

Faulkes 2011; Linzmaier et al. 2018; Hossain et al. 2019b).

Our observations and results of Linzmaier et al. (2018) and Hossain et al. (2019b) were in contrast to Fořt et al. (2019) who reported marbled crayfish to be less successful in interspecific interactions with signal crayfish *Pacifastacus leniusculus* (Dana, 1852) and yabby *Cherax destructor* Clark, 1936. The disparities in aggressiveness might be due to phylogenetic differences or to variation in chemical communication systems (Kubec et al. 2018; Fořt et al. 2019). Linzmaier et al. (2018) found that time spent in shelter by marbled crayfish compared to spiny-cheek crayfish varied more with origin of crayfish (cultured/wild) than with sex. Our study using aquarium-reared marbled crayfish showed opponent sex to have an effect on contact initiation, fight initiation, fights won, and dominance establishment. Total number of fights, time to initiation of combat, and mean fight duration did not significantly differ among the crayfish pairs (Table 4). These outcomes could differ in species of homogenous origin.

In competition of spiny-cheek crayfish versus calico crayfish for shelter, calico crayfish were more successful (Chucholl et al. 2008), whereas, in the present study, marbled crayfish outcompeted calico crayfish as they have the highly invasive red swamp crayfish (Hossain et al. 2019b) and the larger spiny-cheek crayfish (Linzmaier et al. 2018). The higher activity and aggressiveness of marbled crayfish shown against calico, red swamp, and spiny-cheek crayfish could accelerate their invasiveness by both direct interactions and competition for resources. Despite the findings of Bergman et al. (2003), outcomes may differ in the natural environment, as natural conditions are more complex in all parameters affecting crayfish. Linzmaier et al. (2018) found that wild marbled crayfish were less active than aquarium-reared, and Chucholl and Pfeiffer (2010) suggested likelihood of syntopic occurrence of marbled and spiny-cheek crayfish without displacement. This situation could soon be apparent, as sympatries are presumed more likely in the future, with some currently existing (Lipták et al. 2017; Szendőfi et al. 2018). So far poorly understood aspects such as different utilization of microhabitats or food resources might contribute to the outcomes of these co-occurrences.

The studied crayfish species exhibit high invasive potential due to their *r*-selected life history traits, particularly when compared with European native counterparts (Kouba et al. 2014; Hossain et al. 2018). Both show substantial burrowing ability (Chucholl 2012; Kouba et al. 2016), overland dispersal, omnivorous feeding habits (Lipták et al. 2017; Herrmann et al. 2018) and are carriers of crayfish plague (Filipova et al. 2013; Keller et al. 2014; Andriantsoa et al. 2019). Marbled crayfish may have higher invasion potential based on low demands for living conditions, short life cycle (Jimenez and Faulkes 2010), parthenogenetic reproduction, prolific breeding habits, behaviour plasticity (Linzmaier et al. 2018), and genetic uniformity (Vodovsky et al. 2017; Hossain et al. 2018, 2019a). Genetic uniformity usually translates to lower survival in nature due to interactions of lethal alleles, but in marbled crayfish this is overcome by heterozygous triploidy (Vogt 2015). A single individual is theoretically sufficient to establish a new population (Martin et al. 2010). The higher number of progeny resulting from early maturation can enhance overspreading (Hossain et al. 2019a). In Madagascar, marbled crayfish have multiplied in the invasion area 100-fold in the past decade. Dispersal was largely driven by human-mediated introductions and now produces economic value as a food resource for the local human population, promoting further translocations. These stocks are carrying the causative of crayfish plague (Andriantsoa et al. 2019) to which endemic *Astacoides* species are presumably sensitive (Svoboda et al. 2017). Similarly to the red swamp crayfish, marbled crayfish can become a serious threat to the aquatic ecosystems worldwide (Putra et al. 2018; Lipták et al. 2019). Besides Europe, the species appeared in Japan (Kawai and Takahata 2010) and is thriving in Israel (http://marmorkrebs.blogspot.com/2019/05/marmorkrebs-in-middle-east.html). Further records are expected due its persisting popularity in the pet trade (Faulkes 2015) including Asian countries (Uderbayev et al. 2017; Guo et al. 2019). Therefore, strategies limiting further introductions and eradicating established populations whenever possible are urgently needed (Patoka et al. 2018; Lidova et al. 2019; Manfrin et al. 2019).

Conclusion

Finally, it could be summarized that marbled crayfish is able to dominate over size-matched calico crayfish and could replace the invasive calico crayfish from its region of establishment. Over-invasion/replacement in a similar niche with other invaders may be modified by life history traits, behavioural plasticity (Reisinger et al. 2017), adaptability to environmental and anthropogenic factors, and predator–prey relationships. The competitiveness of marbled crayfish in agonistic interactions (Jimenez and Faulkes 2011; Linzmaier et al. 2018; Hossain et al. 2019b) and its life history traits confer an ability to coexist with or displace existing invasive crayfish species as well as native in over-invaded ecosystems. Further research is needed to understand complex mechanisms in invaded areas at community and ecosystem levels.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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CHAPTER 6

MARBLED CRAYFISH IS DOMINANT OVER SPINY-CHEEK CRAYFISH: EVIDENCE FROM DIRECT INTERACTIONS AND COMPETITION FOR SHELTER

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Marbled crayfish is dominant over spiny-cheek crayfish: evidence from direct interactions and competition for shelter

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Abstract

Biological invasions pose a serious threat to native biota and ecosystem functioning. Life histories, niche preference, and adaptation strategies of invasive species may be similar, but interactions among them remain unexplored. The co-occurrence of spiny-cheek crayfish *F. limosus* and parthenogenetic marbled crayfish *P. virginalis* has been reported in Europe, and the number of such localities is expected to increase as the latter species expands its range. The present study compared the interaction patterns of weight-matched spiny-cheek and marbled crayfish *P. virginalis* in the presence and absence of a strategic resource (shelter) in a 15 min interaction. We found that the percentage of marbled vs. spiny-cheek agonistic interactions that resulted in established dominance was significantly higher than that of spiny-cheek crayfish *F. limosus*. Marbled crayfish *P. virginalis* were significantly more likely to be dominant in interactions with female spiny-cheek crayfish *F. limosus* but not with males. However, shelter occupancy dominance did not differ in marbled and spiny-cheek crayfish *F. limosus*, regardless of spiny-cheek sex. Marbled crayfish *P. virginalis* won a significantly higher number of fights than spiny-cheek crayfish *F. limosus* both with and without availability of shelter. The total number of fights, mean fight duration, and cumulative fight duration did not significantly vary among the groups in both treatments. Marbled crayfish *P. virginalis* appear dominant over size-matched spiny-cheek crayfish *F. limosus*, with sex and shelter availability affecting their interaction. Further research is needed to elucidate mechanisms of marbled crayfish *P. virginalis* invasion at community and ecosystem levels.

Key words: *behavioural interaction, biological invasion, co-occurrence, dominance, Procambarus virginalis, Faxonius limosus*

INTRODUCTION

Freshwater biodiversity is threatened globally (Strayer and Dudgeon, 2010), with biological invasions being critical (Catford et al., 2012; Richman et al., 2015; Strayer, 2010). In addition to spread of disease such as crayfish plague (Edgerton, 2002; Lodge et al., 2000), aggressive interactions of crayfish species are a mechanism of species replacement (Klocker and Strayer, 2004). An invader may force a less competitive crayfish to leave refuge, making it vulnerable to predation (Holdich et al., 1999a; Pecor and Hazlett, 2003; Underwood, 2015). Growth and maturation patterns are key components of species success (Garvey et al., 1994; Hill and Lodge, 1994; Söderbäck, 1994). European indigenous crayfish species (ICS), being keystone organisms of freshwater ecosystems, are severely affected. Entire populations have been lost, and many more substantially reduced, due to direct and indirect effects of non-indigenous crayfish species (NICS). Invaders impose strong competitive pressure on native counterparts, as documented in spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817) (Buřič et al., 2009a; Buřič et al., 2009b; Petrusek et al., 2006), signal crayfish *Pacifastacus leniusculus* (Dana, 1852) (Hudina et al., 2016), and red swamp crayfish *Procambarus clarkii* (Girard, 1852) (Hossain et al., 2019c; Jackson et al., 2016). These species from pre-1975 introduction are referred to as "Old NICS" (Holdich et al., 2009) and are particularly widespread on the European continent (Kouba et al., 2014). Devastating effects of Old NICS on ICS have been well documented (Holdich et al., 1999b; Kouba et al., 2014). However, more recent crayfish invasions, so called "New NICS," are increasing (Hossain et al., 2018; Kouba et al., 2014).

Studies assessing the interactions between NICS are appearing but remain limited (Chucholl et al., 2008; James et al., 2016). The marbled crayfish *Procambarus virginalis* Lyko, 2017 is an emerging candidate for such research. The species was first discovered in the German aquarium trade in the mid-1990s (Scholtz et al., 2003). It is the only known crayfish to reproduce via obligate apomictic parthenogenesis, producing genetically uniform offspring (Martin et al., 2010). It is also characterised by early maturation and high fecundity (Jimenez and Faulkes, 2011; Lipták et al., 2017; Seitz et al., 2005), reproducing throughout the year under favourable conditions (Seitz et al., 2005; Vogt et al., 2004). Its high competitiveness has been documented (Hossain et al., 2019a; Hossain et al., 2019c; Jimenez and Faulkes, 2011), and survival ability at low temperatures shown in laboratory and field conditions (Haubrock et al., 2019b; Lipták et al., 2016; Lipták and Vitázková, 2015). It has been listed, together with all Old NICS and *Faxonius virilis* (Hagen, 1870), among the European Union invasive alien species of concern according to recent legislation (EU Regulation No. 1143/2014 and Commission Implementing Regulation No. 2016/1141).

Its current distribution provides growing evidence that newly emerging marbled crayfish will compete with the already established spiny-cheek crayfish (Chucholl, 2015; Hossain et al., 2018; Lipták et al., 2017; Patoka et al., 2016). Linzmaier et al. (2018) found that marbled crayfish were generally more aggressive than carapace length matched spiny-cheek crayfish in terms of agonistic encounters even against larger opponents. Outcome of competition of weight-matched marbled and spiny-cheek crayfish and direct competition of these species for shelter has not been conducted.

The goal of the present study was to determine differences in aggression of *P. virginalis* and *F. limosus* during interspecific interactions under laboratory conditions by characterising their direct agonistic interactions and competition for shelter. Differences in aggression may potentially impact their interactions under natural conditions.

MATERIALS AND METHODS

Experimental animals

Marbled crayfish originated from the laboratory culture at the Faculty of Fisheries and Protection of Waters USB in Vodňany. Spiny-cheek crayfish were captured in the Elbe River, Ústí nad Labem, Czech Republic in early July 2018. Only adult crayfish with fully developed claws and without eggs were selected. For fifteen days prior to experimentation, specimens were held individually in floating 580L×400W×120H mm perforated plastic boxes with eight individual chambers (180×130×100 mm) in troughs with circulating water at ~20 °C. Crayfish were fed dry commercial feed for aquarium fish enriched with algae (Sera Granugreen, Sera GmbH, Germany), slices of fresh carrot, and pieces of common carp *Cyprinus carpio* L. 1758. Troughs were cleaned daily.

Experimental design

Following the acclimation period, interspecific pairs of size-matched (weight difference < 5%) specimens were selected. Given the differing body proportions among crayfish species and sex, weight is considered the most suitable characteristic for size matching in inter- and intra-specific comparisons (Hossain et al., 2019a). Selected specimens were held individually in clear 250×190×70 mm plastic boxes containing ~1.0 L aged-tap water covered with a lid for seven days to eliminate any previously established social hierarchy. The animals were fed commercial feed with 5-7 pellets of the mentioned dry feed. Boxes were cleaned daily during water exchange. The water temperature was 19.3 ± 0.4 °C. Animals that moulted or spawned during this period were removed from the experiment.

The crayfish pairs (Table 1) were placed in oval 600L×400W×250H mm plastic arenas containing 12L aged tap water at ~20 °C and 750 g (dry weight) fine aquarist sand. For an experimental group with shelter, half a ceramic plant pot, 94 mm deep with a 60 mm diameter entrance, was placed at the centre of one long side of the arena. Prior to observation, crayfish were placed under transparent perforated plastic cups on opposite sides of the arena with the equal distance to the shelter. After 5 min acclimatization, the cups were removed to enable crayfish to move freely and interact for a 15 min period that was recorded using a digital video camera (Sony HDR-CX240, Sony, Japan) mounted under the ceiling.

An ethogram was generated using methods modified by Hossain et al. (2019a) and Fořt et al. (2018) to count the number of contact initiations, the number of fights, fight duration, and avoidance behaviours as well as to identify the fight initiator and winner (Fořt et al., 2018; Hossain et al., 2019c) (Supplementary Materials Table 1). Dominance establishment was determined as the time at which an agonistic event led to persistent avoidance of fighting, contact, or remaining within approximately one body length of the other crayfish. Shelter occupancy dominance was allocated to the crayfish inside the shelter at the termination of the experiment. To support this determination, the number of fights initiated and won was also taken into account.

Data analysis

The weight of crayfish pairs was compared using Student matched-pairs test. Chi-squared ($\chi 2$) tests were used to analyse the values of dominant status percentages with a value of 50% being no difference). Mann-U Whitney test was used to detect differences in number of fights, total fight duration, and mean fight duration of male/female and female/female pairs in trials with and without shelter. The null hypothesis was rejected at $p < 0.05$ in all tests. Statistical analyses were performed using IBM SPSS Statistics v. 22.0.

Results

In total, 80.0% (66.7% + 13.3%) and 41.2 % (35.3% + 5.9%) of pairs developed dominant status in groups without shelter and with shelter, respectively (Table 2). Marbled crayfish were consistently dominant over spiny-cheek crayfish in both conditions ($\chi 2$ test without shelter = 10.667; $p = 0.001$; $\chi 2$ test with shelter = 7.143; $p = 0.008$). Pairs without dominance status were observed in both conditions (Table 2). Marbled crayfish were significantly more likely to exhibit dominance over female spiny-cheek crayfish without shelter ($\chi 2$ test = 9.000, $p = 0.003$) and with shelter ($\chi 2$ test = 8.000, $p = 0.005$), but not over male spiny-cheek crayfish ($\chi 2$ test = 2.000, $p = 0.157$; $\chi 2$ test = 0.607, $p = 0.414$ without and with shelter, respectively) (Table 2).

In the shelter condition, 82.3 % (52.9%+29.4%) of pairs developed dominance status (Table 3). No significant difference was found between marbled crayfish and spiny-cheek crayfish in shelter occupancy dominance, although the percentage of marbled was consistently higher than spiny-cheek crayfish (χ2 test = 2.286, p = 0.131) (Table 3). Pairs without defined shelter occupancy dominance were also observed between marbled crayfish with female and with male spiny-cheek crayfish.

The marbled crayfish initiated a significantly higher number of fights (t without shelter = 6.268, df = 14, p < 0.001; t with shelter = 2.083, df = 16, p = 0.047) and won significantly more fights (t without shelter = 6.796, df = 14, p < 0.001; t with shelter = 2.063, df = 16, p =0.049) against spiny-cheek crayfish in both absence and presence of shelter (Table 4). There were no significant differences in contact initiation (t = 0.780, df = 14, p = 0.449) and avoidance (t = -1.140, df = 14, p = 0.273) in the absence of shelter or in contact initiation (t = 1.731, df = 16, p = 0. 03) with shelter. The incidence of avoidance behaviours was significantly lower in marbled crayfish than in spiny-cheek (t = -2.850, df = 16, p = 0.012) (Table 4).

Number of fights, mean duration of fights, and cumulative fight duration per pair, did not significantly vary with shelter condition with respect to sex of spiny-cheek crayfish (Table 5). Number of fights, mean duration of fights, and cumulative fight duration per pair without shelter were significantly higher than observed with shelter (Z = -2.842, p = 0.005; Z = -2.267, p = 0.028; Z = -2.416, p = 0.019, respectively). The number of fights between females without shelter was significantly higher than seen in female pairs with shelter available (Z = -2.537 p = 0.015) (Table 5).

Discussion

Understanding displacement mechanisms of invasive species can predict their range expansion. In crayfish, aggressive behaviour during interspecific interactions is a major trait associated with their invasion potential (Capelli and Munjal, 1982; Usio et al., 2001) that can be used to estimate the ecological consequences of interaction with species sharing a similar niche (Church et al., 2017). We found marbled crayfish to dominate size-matched spiny-cheek crayfish in agonistic encounters and to exhibit a tendency toward shelter occupancy dominance. Studies have shown that marbled crayfish dominate size-matched red swamp (Hossain et al., 2019c; Jimenez and Faulkes, 2011) and calico crayfish *Faxonius immunis* Hagen, 1870 (Hossain et al., 2019a). The higher activity and aggressiveness of marbled crayfish could facilitate displacement of other NIC species either by direct interaction or through competition for resources such as shelter and food. However, the marbled crayfish has been reported to be less successful in agonistic interactions with similar-sized signal crayfish *Pacifastacus leniusculus*, Dana, 1852 and the common yabby *Cherax destructor* Clark, 1936 (Fořt et al., 2019), suggesting species-specific outcomes in interspecific encounters.

Laboratory results might not accurately reflect behaviour under natural conditions, and differences from field conditions have been reported. Linzmaier et al. (2018) found that wild marbled crayfish were less active than aquarium-reared during agonistic interactions with spiny-cheek crayfish. Still, results of agonistic interactions between crayfish in laboratory trials have been found to be consistent with combat dynamics and replacement patterns observed in the field (Davis and Huber, 2007; Vorburger and Ribi, 1999).

We found considerable influence of sex on the crayfish behaviour. Marbled crayfish showed significantly lower levels of avoidance behaviour during interactions in the presence of shelter than observed in female spiny-cheek crayfish. The marbled crayfish also initiated significantly more fights than female spiny-cheek crayfish in the absence of shelter. Marbled crayfish established significantly greater dominance over female spiny-cheek crayfish in both shelter conditions, but that was not true of encounters with males. Lack of sexual dimorphism (Buřič et al., 2010a; Buřič et al., 2010b) in marbled crayfish most likely contributed to the more equal combat between sexes. This is also demonstrated by the longer duration of combat in marbled crayfish vs. male spiny-cheek crayfish encounters. Linzmaier et al. (2018), however, concluded that the absence of sexual dimorphism in the marbled crayfish does not constrain its ability to win agonistic encounters against another species. In accordance to our results and above-mentioned studies we can even state that marbled crayfish as only female stock are comparable in fights with males of other species, but dominate over their females.

The ability to occupy shelters or refuges is a critical trait of freshwater crayfish (Davis and Huber, 2007; Kouba et al., 2016; Nakata and Goshima, 2003). Eviction from shelter or refuge during aggressive interactions makes a crayfish vulnerable to predation (Garvey et al., 1994; Hill and Lodge, 1994; Söderbäck, 1994). Although no significant difference in shelter occupancy was found between these size-matched specimens, the rate of marbled crayfish shelter occupancy dominance was higher than that of spiny-cheek crayfish. This suggests that essential resources such as shelter will be more frequently appropriated by marbled crayfish when these species co-occur (Linzmaier et al., 2018). It was also found the calico crayfish dominant over the spiny-cheek crayfish in competition for shelter, contributing to the displacement of the latter (Chucholl et al., 2008). In the present study, the crayfish inside the shelter was consistently forced out by the size-matched opponent, suggesting that the availability of shelter is critical to both species. The marbled crayfish success in competition for shelter against the spiny-cheek might lead to higher mortality of the latter in natural conditions due to predation. The spiny-cheek crayfish excavate simple shallow burrows in the shoreline of lakes and ponds and along river banks (Hamr, 2002; Holdich and Black, 2007; Statzner et al., 2000). Marbled crayfish have been shown to construct larger and deeper burrows than do spiny-cheek crayfish when faced with drought (Kouba et al., 2016). Hence, the marbled crayfish could be a strong competitor against the spiny-cheek crayfish for shelter utilization and burrow construction in natural conditions.

The distribution of marbled crayfish in natural water bodies may expand rapidly as showed by the continuous new records in Europe (Andriantsoa et al., 2019). Apart from direct aggressive interactions, factors possibly affecting displacement include different response to predation (Hazlett, 2003; Söderbäck, 1994), interference with mating (Butler and Stein, 1985), hybridization (Perry et al., 2001), and favourable life history traits (Hamr, 2002).

Conclusions

Invaded ecosystems demand increasing attention (Haubrock et al., 2019a; Hossain et al., 2019a). Behaviour plasticity of crayfish is a key factor behind co-existence with, or replacement of, invaders by native species sharing overlapping niches (Fields, 20018; Glon et al., 2018; Hanshew and Garcia, 2012; Reisinger et al., 2017; Saura-Mas and Benejam, 2019). The marbled crayfish is able to dominate size-matched spiny-cheek crayfish, suggesting the possibility of its replacement in the wild. The competitiveness of marbled crayfish in agonistic interactions with other species (Hossain et al., 2019a; Jimenez and Faulkes, 2011; Linzmaier et al., 2018) and its life history traits favour expanding dispersal. Further research is needed to elucidate mechanisms of marbled crayfish invasion at community and ecosystem levels.

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Conflicts of interest

The authors have no conflict of interest to declare.

Author contributions

Conceived and designed the experiments: A.K., M.B., and W.G.; Performed the experiments: W.G., M.S.H., M.M.,and J.K.; Analysed the data: W.G. and M.S.H.; Wrote the first draft: W.G. All authors contributed to and edited the manuscript.

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Table 1. *Number of pairs (n), carapace length (CL), weight (W), and mean percent weight difference (D) of size-matched pairs of marbled crayfish (MC) P. virginalis and spiny-cheek crayfish (SC) F. limosus used in interactions without and with shelter. Data are shown by mean ± SD; Pairs t-test, p < 0.05.*

Experimental Conditions	**Species**	**n**	**CL (mm)**	**W (g)**	**D (%)**	*p*
Without shelter	MC	5	29.2 ± 2.6	8.5 ± 2.9	0.5 ± 0.9	*t* =0.598; *p* = 0.576
	SC (male)		27.8 ± 7.7	8.5 ± 2.7		
	MC	10	28.5 ± 2.6	6.9 ± 1.8	1.5 ± 1.6	*t* < 0.0001; *p* = 1.000
	SC (female)		28.3 ± 3.9	6.9 ± 1.8		
	MC (combined)	15	29.0 ± 2.6	7.5 ± 2.4	1.1 ± 1.5	*t* = 0.355; *p* = 0.728
	SC (combined)		28.1 ± 5.4	7.5 ± 2.3		
With shelter	MC	7	29.3 ± 4.3	7.9 ± 3.7	2.2 ± 1.9	*t*= -0.510; *p* = 0.629
	SC (male)		29.7 ± 3.3	7.9 ± 3.5		
	MC	10	29.3 ± 3.0	7.3 ± 2.1	0.6 ± 0.8	*t* = -1.000; *p* = 0.343
	SC (female)		30.3 ± 2.9	7.3 ± 2.1		
	MC (combined)	17	29.3 ± 3.5	7.5 ± 2.7	1.3 ± 1.6	T = -0.837; *p* = 0.416
	SC (combined)		30.1 ± 3.0	7.5 ± 2.7		

Table 2. *Dominance establishment in marbled crayfish (MC) P. virginalis and spiny-cheek crayfish (SC) F. limosus during interactions with and without shelter. Different superscripts within group indicate significant differences; Chi-squared (χ2), p < 0.05.*

Group	**Crayfish**	**Established dominance (%), (number of pairs)**	**Not established (%)**	**Percentage of establishment (%)**	**χ2**
Without shelter	MC	70.0 (7)	20.0 (2)	87.5^b	χ2 = 9.000, *p* = 0.003
	SC (female)	10.0 (1)		12.5^a	
	MC	60.0 (3)	20.0 (1)	75.0^a	χ2 = 2.000, *p* = 0.157
	SC (male)	20.0 (1)		25.0^a	
	MC (combined)	66.7(10)	20.0 (3)	83.4^b	χ2 = 10.667; *p* = 0.001
	SC (combined)	13.3 (2)		16.6^a	
With shelter	MC	40.0 (4)	60.0 (6)	100^b	χ2 = 8.000, *p* = 0.005
	SC (female)	0.0 (0)		0^a	
	MC	28.6 (2)	57.1 (4)	66.7^a	χ2 = 0.607, *p* = 0.414
	SC (male)	14.3 (1)		33.3^a	
	MC (combined)	35.3 (6)	58.8 (10)	85.7^b	χ2 = 7.143; *p* = 0.008
	SC (combined)	5.9 (1)		14.3^a	

Table 3. *Dominance over shelter of marbled crayfish (MC) P. virginalis vs. spiny-cheek crayfish (SC) F. limosus. No significant differences were detected in tested groups; Chi-squared (χ2) test, p < 0.05.*

Crayfish	Established dominance in % (number of pairs)	Not established (%)	Percentage of establishment	χ2
MC	60.0 (6)	10.0 (1)	66.7	χ2 test = 2.000; *p* = 0.157
SC (female)	30.0 (3)		33.3	
MC	42.9 (3)	28.6 (2)	60.1	χ2 test = 0.400; *p* = 0.527
SC (male)	28.6 (2)		39.9	
MC (combined)	52.9 (9)	17.6 (3)	64.2	χ2 test = 2.286, *p* = 0.131
SC (combined)	29.4 (5)		35.8	

Table 4. *Agonistic bouts of marbled crayfish (MC) P. virginalis vs. spiny-cheek crayfish (SC) F. limosus during interactions with and without shelter. Different superscripts within group indicate significant differences at p < 0.05. Data are mean ± SD; Wilcoxon matched pairs test, p < 0.05).*

Group	Crayfish	Contact initiator (%)	Avoidance (%)	Fight initiator (%)	Winner (%)
Without shelter	MC	51.7 ± 35.7	46.8 ± 39.1	67.7 ± 27.4^{a}	59.3 ± 37.9
	SC (male)	48.3 ± 35.7	53.1 ± 39.1	32.3 ± 27.4^{a}	40.6 ± 37.9
	MC	61.0 ± 32.8	31.1 ± 37.6	67.1 ± 42.6^{b}	62.5 ± 42.9
	SC (female)	38.9 ± 32.8	68.9 ± 37.6	32.8 ± 42.6^{a}	37.5 ± 42.9
	MC (combined)	55.9 ± 29.5	39.7 ± 35.0	75.7 ± 15.6^{b}	71.2 ± 12.1^{b}
	SC (combined)	44.1 ± 29.5	60.3 ± 35.0	24.3 ± 15.6^{a}	28.8 ± 12.1^{a}
With shelter	MC	62.1 ± 38.9	21.4 ± 35.7	70.2 ± 41.9^{a}	71.4 ± 48.8
	SC (male)	37.9 ± 38.9	78.6 ± 35.7	29.7 ± 41.9^{a}	28.6 ± 48.8
	MC	56.2 ± 48.7	5.5 ± 17.3^{a}	50.0 ± 52.7^{a}	40.0 ± 51.6
	SC (female)	43.8 ± 48.7	94.5 ± 17.3^{b}	50.0 ± 52.7^{a}	60.0 ± 51.6
	MC (combined)	69.0 ± 36.9	18.6 ± 31.6^{b}	78.7 ± 37.1^{b}	75.0 ± 46.3^{b}
	SC (combined)	31.0 ± 36.9	81.4 ± 31.6^{a}	21.3 ± 37.1^{a}	25.0 ± 46.3^{a}

Table 5. *Mean number of fights, mean fight duration, and mean cumulative fight duration in marbled crayfish (MC) P. virginalis vs. spiny-cheek crayfish (SC) F. limosus during interactions between the without and with shelter treatments. Different superscripts within group indicate significant differences at $p < 0.05$, by sex and by combined data of the parameters between the two groups. Data are mean ± SD, $^{*, **}$ for the combined number of fights, cumulative fight duration, and mean fight duration in a column. $^{a, b}$ for number of fights in female vs female pairs in a column. (Mann-U Whitney test to detect a) differences between male vs. female pairs in with and without shelter treatment in a column; b) the difference of the data of same sex (by male and by female) and combined data between with and without shelter treatment in a column).*

Group	Crayfish	Number of fights	Cumulative fight duration (s)	Mean duration/ fight (s)
Without shelter	MC x SC (male)	4.6 ± 2.1	304.4 ± 215.5	71.5 ± 48.6
	MC x SC (female)	2.5 ± 2.2^{b}	160.7 ± 183.6	57.2 ± 24.2
	MC x SC (combined)	3.2 ± 2.3**	195.6 ± 199.8**	68.7 ± 34.4*
With shelter	MC x SC (male)	1.6 ± 2.4	118.1 ± 236.1	63.1 ± 38.5
	MC x SC (female)	0.4 ± 0.5^{a}	40.9 ± 46.8	102.3 ± 65.2
	MC x SC (combined)	0.9 ± 1.7*	72.7 ± 157.5*	85.5 ± 55.3**

Supplementary Materials Table 1. *Definitions of the events described in present study.*

Event	Definition
Contact	Touch with claws, body (carapace, abdomen) rather than antennae and antennules
Avoidance	Retreat from an approaching crayfish that has not exhibited threatening behaviour
Threat	Approach with signs of warning-mainly raising, waving and shaking claws
Winner	The individual that did not retreat from the fight or that left the opponent that assumed a body down posture or remined motionless. In the very few cases when both individuals retreated simultaneously no winner was scored
Tail flip avoidance	Avoidance with bending tail and rapid backward

CHAPTER 7

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGEMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING THE STUDY
CURRICULUM VITAE

General discussion

The effects of drought on crayfish reproduction

Crayfish have evolved to construct or use burrows not only to survive under fluctuating aquatic conditions, but possibly to reproduce when water is scarce. However, given the nature of burrowing crayfish and the challenges of conducting observations of naturally constructed and sealed burrows, little is known about how early life history of crayfish in burrows is influenced by environmental factors. My research (**Chapter 2**) clearly demonstrated that high air humidity alone is sufficient during late phases of embryogenesis, including the hatching period, in marbled crayfish. Importantly, the finding that juveniles could remain at Stage 2 for 20 days under humid conditions, and resume normal development when re-immersed, greatly increases our understanding of species resilience in habitats with variable hydrology. I speculate that such adaptation may be a characteristic of other crayfish species that have evolved in seasonal aquatic ecosystems.

The adaptations of crustaceans to avoid desiccation are diverse. Crayfish females, especially primary and secondary burrowers, often use burrows for egg incubation (Hobbs, 1942; Riek, 1972). Females of most species tend to construct deeper burrows compared to males and frequently occupy the deepest parts of the burrow during egg incubation (Hazlett et al., 1974; Payne, 1972). Seasonal fluctuations in groundwater can influence burrow depth (Helms et al., 2013; Stoeckel et al., 2011). The water table may be lower than burrows in dry localities, as in seen those of *P. clarkii*, which frequently reproduce under dryconditions (McClain and Romaire, 2004; Souty-Grosset et al., 2014). Crayfish ability to excavate into a simulated water table under laboratory conditions is species-specific (Dyer et al., 2015). Crayfish of the genus Engaeus, which excavate burrows or chambers on slopes, often do not reach the water table (Horwitz and Richardson, 1986; Suter and Richardson, 1977), and water in the burrows is most likely present at the time of their excavation, or perhaps periodically refreshed from seepage or percolation from rainfall.

Chimney-like structures built by many species of the genera *Cambarus, Engaeus*, and *Geocharax* (Grow, 1981; Grow and Merchant, 1980; Horwitz et al., 1985; Noro et al., 2007; Suter and Richardson, 1977) and closing of the burrow entrance by a mud plug, particularly seen in females (Barbaresi and Gherardi, 2006; Correia and Ferreira, 1995) and observed in my research (**Chapters 3 and 4**), helps maintain suitable living conditions. Estivation chambers to maintain humidity during drying of streambeds are built by adult Geocharax sp. (Chester and Robson, 2011; Strachan et al., 2014). The non-burrowing crayfish *Gramastacus insolitus* Riek, 1972 is dependent on burrows of larger crayfish species to survive seasonal drought (Johnston and Robson, 2009). Such behavioural adaptations highlight the importance of maintaining humidity to crayfish survival.

Findings of late embryonic and early postembryonic crayfish development in the absence of free-standing water are particularly noteworthy for marbled crayfish, as it is a unique species with a high invasion potential due to its parthenogenetic mode of reproduction (Gutekunst et al., 2018; Scholtz et al., 2003a). Crayfish males and females are sometimes found sharing a burrow in sexually reproducing species (Horwitz et al., 1985; Johnston and Robson, 2009). However, the presence of a male in the burrow is not a necessary prerequisite for reproduction in burrows during drought, given that spermatophores are attached to the ventral area of females (in Astacidae and Parastacidae) or deposited inside the annulus ventralis (in Cambaridae and Cambaroididae) prior to ovulation (Albaugh, 1973; Buřič et al., 2013). It is probable that results of my studies utilizing marbled crayfish females with attached and developing eggs, are valid for sexually reproducing species. Our findings suggest that successful development under drought-like conditions for marbled crayfish is primarily related to the tolerance of the eggs to periods without free-standing water, similar to adaptations of other drought-tolerant crayfish species, such as those constructing aestivation burrows (Chester and Robson, 2011; Strachan et al., 2014), co-opting burrows of other crayfish (Johnston and Robson, 2009), and digging burrows below the water level during dry seasons (Horwitz et al., 1985). Resistance to drought is critical to inhabiting ecosystems with high fluctuations in water level in crayfish.

The effects of water contamination on crayfish burrowing behaviour

The quality of surface waters plays an essential role in maintaining ecosystem biodiversity and function (Bakker, 2012). I chose two PhACs, methamphetamine and tramadol and two commonly used herbicides, terbuthylazine and metazachlor, at environmental concentrations to explore their possible effects on non-target organisms by studying burrowing behaviour of the red swamp crayfish *P. clarkii*. I reported for the first time that depth and volume of burrows constructed by methamphetamine-exposed females was significantly lower than of unexposed controls. Tramadol-exposed females consistently exhibited a tendency to excavate smaller burrows compared with controls, although this was not significant (**Chapter 3**). Impact of herbicides on burrowing behaviour was less clearly manifested, although male crayfish exposed to META constructed a significantly higher number of burrows than did other groups (**Chapter 4**).

The effect of the investigated contaminants may be devastating to crayfish, as the need for shelter is an essential life history trait (Kouba et al., 2016). Female primary and secondary burrowers (Hobbs, 1942; Riek, 1972) use burrows for oviposition and/or incubation. Most likely to maintain contact with water or remain in an environment sufficiently humid for successful reproduction (**Chapter 2**), in general, females tend to construct deeper burrows than do males and frequently occupy the deepest parts of the burrow during egg incubation (Hazlett et al., 1974; Kouba et al., 2016; Payne, 1972). This was observed in the control group in research reported in **Chapter 3** but not in any exposure group. It was also not seen in the herbicide study controls, suggesting that effects of herbicide exposure might remain hidden due to a low number of observations (**Chapter 4**). The volume and depth of female red swamp crayfish *P. clarkii* burrows relative to body size was significantly lower in the METH group compared with controls, but this was not observed in the TRA-exposed group (**Chapter 3**). However, metazachlor-treated males excavated a higher number of burrows than did other groups (**Chapter 4**). This may be an anxiety behaviour (Buřič et al., 2013) related to stress resulting from the exposure (Ložek et al., 2019; Matozzo et al., 2004). In the wild, the availability of shelter and resources such as suitably moist conditions is critical to crayfish (Guo et al., 2019; Kouba et al., 2016), as the survival of an individual does not depend only on health status but also is the indirect consequence of behaviour patterns involving reproduction, social interaction, and decision making (Kubec et al., 2019). Considering its greater resistance to desiccation and superior burrowing abilities, the impact of the selected contaminants under severe drought on the red swamp crayfish may be more pronounced than on the native European noble crayfish *Astacus astacus* (L., 1758), narrow-claw crayfish *Pontastacus leptodactylus* s.l. (Eschscholtz, 1823), and stone crayfish *Austropotamobius torrentium* (von Paula Schrank, 1803), and on the invasive yabby *Cherax destructor* Clark, 1936, spiny-cheek crayfish, and marbled crayfish (Kouba et al., 2016).

In an earlier experiment, I found marbled crayfish to exhibit significantly lower velocity and move shorter distances than controls after exposure to tramadol at environmentally relevant concentrations (Buřič et al., 2018). Stara et al. (2016) recorded gill and hepatopancreas pathology, alterations in biochemical parameters of haemolymph, oxidative damage to hepatopancreas, and changes in antioxidant biomarkers in muscle and hepatopancreas of red swamp crayfish after 14 days exposure to terbuthylazine-desethyl, a terbuthylazine degradation product, at the environmental concentration of 2.9 µg/L (Stara et al., 2016). Differences in behaviour, including significantly lower speed and velocity compared with controls, as well as gill and hepatopancreas pathology, were observed in marbled crayfish after a 28-day exposure to S-metolachlor and its degradation product metolachlor OA at the environmentally relevant concentration of 4.2 µg/L (Stara et al., 2019). Significantly lower growth and delayed development were detected in marbled crayfish larvae exposed to environmentally relevant concentrations of metazachlor and metazachlor OA (Velisek et al., 2020). Lower velocity and reduced utilization of shelter or burrows often leads to higher losses to predation in the wild (Holdich et al., 1999; Pecor and Hazlett, 2003; Underwood, 2015), and unstable weather conditions associated with climate change may compound these effects on crayfish in the future (Cruz-McDonnell and Wolf, 2016; Kouba et al., 2016).

More than 60 PhACs have been reported in tissue of aquatic invertebrates and in some riparian spiders in Australia (Richmond et al., 2018). Eleven selected PhACs, including tramadol, were detected in liver and kidney of fish inhabiting a contaminated stream in the Czech Republic (Grabicova et al., 2017). Behaviour changes, including differences in velocity and distance moved, in shrimp, crayfish, killifish, and European perch exposed to PhACs have recently been reported at exposures to low and environmentally relevant concentrations (Barry, 2013; Brodin et al., 2013; Buřič et al., 2018; González-Ortegón et al., 2016; Hossain et al., 2019b). The hepatopancreas is the main organ of detoxification of xenobiotics in crustaceans (Vogt, 2002), and swimmerets on the crayfish abdomen functions in forward swimming and other behaviours (Mulloney and Hall, 2000). In **Chapter 4**, the concentration of selected herbicides found in hepatopancreas and abdomen of both control and exposed crayfish were below the limits of quantification. Reports of analyses of herbicide accumulation in hepatopancreas and abdomen of crayfish are scarce; however, a significant increase in heart rate was observed in signal crayfish *Pacifastacus leniusculus* Dana, 1852 after a three-week exposure to an environmentally relevant (1 µg/L) concentration of tramadol (Ložek et al., 2019).

The predatory European perch *Perca fluviatilis* (L., 1758) became more active after preying on the invertebrate Coenagrion hastulatum (Charpentier, 1825) contaminated with the psychoactive drug, benzodiazepine (Brodin et al., 2014).

Emerging contaminants and micro-pollutants comprise a vast and expanding array of anthropogenic compounds that are commonly present in water but have only recently been identified as significant water pollutants (Gomes et al., 2018). These contaminants contain a variety of synthetic compounds extensively used in cosmetics, pesticides, pharmaceuticals, and personal care products (Luo et al., 2014) that are widely considered indispensable to modern society. Environmental concentrations may increase during drought because of reduced dilution of treated waste waters (K'Oreje et al., 2018) or sorption into sediment (Aljeboree and Alshirifi, 2018), resulting in severe impact on non-target organisms.

Except for the effects of water comtaminations of the burrrowing behaviour in freshwater crayfish, their agonsitic behaviour may be also affected by comtaminants like herbiciedes. For example, metolachlor may be interfering with the ability of crayfish to receive or respond to social signals and thus affect certain agonistic behaviors (Cook and Moore, 2008). In crayfish, these social signals are important in establishing dominance, which in turn has an impact on an individual's ability to find and use mates, food, and habitat space like shelter, which also reflected in **Chapter 5** and **Chapter 6**.

Competition for shelter in crayfish

Crayfish need to leave shelter for foraging, migration, and mating. Understanding displacement mechanisms of invasive species can predict their range expansion. Along with investigation into ecological strategies and adaptability (Holdich et al., 2009; Lindqvist and Huner, 1999), the potential success of an invader may be assessed through agonistic behaviour during interspecific interactions (Gherardi, 2002; Hudina et al., 2016). In crayfish, aggressive behaviour against heterospecifics is a major trait influencing their invasion potential (Capelli and Munjal, 1982; Usio et al., 2001) that can be used to estimate the ecological consequences of interactions of species sharing a similar niche (Church et al., 2017). My research found marbled crayfish to dominate size-matched calico crayfish (**Chapter 5**) and spiny-cheek crayfish (**Chapter 6**) in agonistic encounters. The higher activity and aggressiveness of marbled crayfish could facilitate displacement of other non-indigenous crayfish species either by direct interaction or through competition for resources such as shelter and food. The marbled crayfish has been reported to be less successful in agonistic interactions with similar-sized signal crayfish and the common yabby (Fořt et al., 2019), suggesting species-specific dynamics at play in the encounters. In spite of its calm disposition during interactions with conspecifics (Kawai et al., 2016), marbled crayfish showed competitiveness against wild spiny-cheek crayfish (Linzmaier et al., 2018) as well as against red swamp crayfish, the most successful introduced crayfish species worldwide (Hossain et al., 2019c; Jimenez and Faulkes, 2011).

Aggressive behaviour has ecological consequences, even when two phylogenetically unrelated species interact in a functionally similar niche (Church et al., 2017). Current dispersion of marbled crayfish in natural waters worldwide amplifies the importance of assessing its invasiveness and competitiveness with respect to established widespread invaders (Hossain et al., 2018; Kawai et al., 2016). Eviction from refuge during aggressive interactions makes a crayfish vulnerable to predation (Garvey et al., 1994; Hill and Lodge, 1994; Söderbäck, 1994). Although no significant difference in shelter occupancy was found between size-matched specimens, the rate of marbled crayfish shelter occupancy dominance was higher than that of spiny-cheek crayfish (**Chapter 6**). This suggests that essential resources such as shelter will be more frequently appropriated by marbled crayfish when these species co-occur (Linzmaier et al., 2018). The calico crayfish has been demonstrated to be dominant over the spiny-cheek crayfish in competition for shelter, contributing to the displacement of the latter (Chucholl et al., 2008). In a shelter-behaviour study of marbled and spiny cheek crayfish (**Chapter 6**) any crayfish within a shelter was invariably forced out by a size-matched opponent, suggesting that the availability of shelter is critical to both species.

The marbled crayfish success in competition for shelter against the spiny-cheek might lead to higher mortality of the latter in natural conditions due to predation. Spiny-cheek crayfish excavate simple shallow burrows at the shoreline of lakes and ponds and along river banks (Hamr, 2002; Statzner et al., 2000). Marbled crayfish are reported to construct larger and deeper burrows than spiny-cheek crayfish when faced with drought (Kouba et al., 2016) and, hence, could be a strong competitor against the spiny-cheek crayfish under natural conditions. Laboratory results might not accurately reflect behaviour under natural conditions, and differences from field conditions have been reported: Linzmaier et al. (2018) found that wild marbled crayfish were less active than aquarium-reared during agonistic interactions with spiny-cheek crayfish. Nevertheless, results of agonistic interactions between crayfish in laboratory trials have been found to be consistent with combat dynamics and replacement patterns observed in the field (Davis and Huber, 2007; Vorburger and Ribi, 1999).

Considerable influence of sex on crayfish behaviour was also found (**Chapter 6**). Marbled crayfish showed significantly lower levels of avoidance behaviour during interactions in the presence of shelter than observed in female spiny-cheek crayfish. The marbled crayfish also initiated a significantly greater number of fights than female spiny-cheek crayfish in the absence of shelter. Marbled crayfish established significantly greater dominance over female spiny-cheek crayfish regardless of shelter availability, but that was not true in encounters with males. Lack of sexual dimorphism (Buřič et al., 2010a; Buřič et al., 2010b) of marbled crayfish *P. virginalis* most likely contributed to the more equal combat between sexes and this was also reflected in the longer duration of combat in marbled crayfish vs. male spiny-cheek crayfish encounters. Linzmaier et al. (2018) concluded that the female-only characteristic of marbled crayfish does not constrain its ability to prevail in agonistic encounters against another species. Our results and the cited studies demonstrate that marbled crayfish are competitive in fights with males of other species, and dominate over their females.

The distribution of marbled crayfish in natural waters may expand rapidly (Andriantsoa et al., 2019). In addition to direct aggressive interactions, factors potentially affecting displacement include responses to predation (Hazlett, 2003; Söderbäck, 1994), interference with mating (Butler and Stein, 1985), hybridization (Perry et al., 2001), and life history traits (Hamr, 2002). The marbled crayfish is a unique species with a high invasion potential, especially due to its parthenogenetic mode of reproduction (Gutekunst et al., 2018; Scholtz et al., 2003b), high fecundity (Lipták et al., 2017), early maturation (Hossain et al., 2019a; Seitz et al., 2005), potential for year-round reproduction (Seitz et al., 2005; Vogt et al., 2004), and tolerance to a broad range of food sources and habitats (Lipták et al., 2019). Our results suggest that marbled crayfish would be successful in agonistic encounters with calico crayfish (**Chapter 5**), spiny-cheek crayfish (**Chapter 6**), and red swamp crayfish (Hossain et al., 2019c; Jimenez and Faulkes, 2011), but the situation may be modified by life history traits, behavioural plasticity (Reisinger et al., 2017), and adaptability to environmental and anthropogenic factors. Therefore, more research is needed to understand the complex mechanisms operating in invaded regions at community and ecosystem levels.

Conclusions

This thesis incorporates results of five studies investigating egg incubation and early postembryonic development in the marbled crayfish in high air humidity; the effects of selected psychoactive compounds and herbicides in water at environmentally relevant concentrations on the burrowing behaviour of the red swamp crayfish; and the direct interactions and competition for shelter of marbled crayfish vs. spiny-cheek and calico crayfish.

The main conclusions drawn from these studies are:

1. Marbled crayfish can complete final phases of embryogenesis, including hatching, as well as early postembryonic development in high air humidity alone. Juveniles can remain at stage 2 for as long as 20 days on average under humid conditions, resuming development when re-immersed. This trait enables marbled crayfish to occupy a wide range of habitats that experience water scarcity and might be unsuitable for other, less adapted crayfish species as well as other aquatic taxa.
2. Exposure to PhACs at environmentally relevant concentrations is associated with atypical burrowing behaviour in red swamp crayfish, with methamphetamine-exposed females excavating burrows reduced in both depth and volume. Tramadol exposure of females may also result in their digging smaller burrows. Burrow size can be critical to survival of the individual and of the community through consequences to reproduction.

3. Exposure to the herbicides terbuthylazine and metazachlor at environmentally relevant concentrations does not appear to exert a major influence on the burrowing of red swamp crayfish, although it may be associated with stress-induced hyper-activity.
4. Sex and resource availability may influence agonistic behaviour in crayfish. Marbled crayfish are equal to or superior in competition with the calico crayfish, which is, in turn, dominant over the serious invader, the spiny-cheek crayfish. This suggests that the marbled crayfish may out-compete incumbent NICS for food, shelter, and other resources in natural habitats.
5. The predisposition to compete for shelter does not differ in marbled and spiny-cheek crayfish irrespective of spiny-cheek sex, but marbled crayfish are generally dominant in interactions with female spiny-cheek crayfish, suggesting the potential for their replacement in the wild.

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English summary

Burrowing behaviour in crayfish

Wei Guo

A key survival trait of crayfish is the ability to find shelter or excavate a burrow to provide protection against predators and refuge from environmental extremes. They also use these refuges as a source of water or cover during moulting, oviposition, and egg incubation, as well as protection of offspring. Shelters or burrows are therefore critical to crayfish survival, and their unavailability is the principle bottleneck in crayfish population.

This study revealed the ability of marbled crayfish to undergo final phases of embryogenesis, including hatching, and early postembryonic development in high air humidity alone. Importantly, our finding that juveniles were able to remain at Stage 2 for a prolonged duration under humid conditions, and resume normal development when re-immersed, greatly increases our knowledge of this species' resilience in habitats with variable hydrology. A similar ability to tolerate and reproduce during drought is expected to occur in other crayfish species classified as primary burrowers and some secondary burrowers, especially the highly invasive red swamp crayfish Procambarus clarkii. This unprecedented life history trait in crayfish is likely crucial for inhabiting ecosystems with highly fluctuating water levels or periods of drought. In the context of biological invasions, the adaptations to drought will potentially contribute to species displacement, favouring species that are better adapted to such environmental conditions (**Chapter 2**).

In addition to extreme climate conditions, water pollution is considered among the most important drivers of global biodiversity decline, with frequent incidents of contamination and deterioration of water quality impacting aquatic ecosystems. Atypical burrowing behaviour of crayfish exposed to pharmaceutically active compounds (PhAC) at an environmentally relevant concentration was documented for the first time, results that might be applicable to other aquatic organisms. Notably, the depth and volume of burrows constructed by methamphetamine-exposed females were significantly lower compared to controls. Tramadol-exposed females also consistently exhibited a tendency to dig smaller burrows although this was not significant. The role of surface waters for human use in agriculture, aquaculture, and domestic water is critical, and the potential impact of PhACs on freshwater biodiversity should not be ignored. The effects of environmentally relevant concentrations of selected PhACs were evident when tested as single compounds (**Chapter 3**). Selected herbicides (**Chapter 4**), terbuthylazine and metazachlor, at environmentally relevant concentrations did not show a demonstrable effect on crayfish, with the exception of the significantly higher number of burrows excavated by metazachlor-exposed males compared to other groups. A similar stress response to aquatic contamination might be expected in other hyporheic-dwelling aquatic biota such as unionid mussels, clams, and a wide range of aquatic insects. Based on results of these studies (**Chapters 2**, **3**, and **4**), we suggest that sublethal effect of PhACs, herbicides, and other aquatic contaminants including chemical fertilizers, detergents, petrochemicals, and plastics on non-target organisms should be investigated. The potential adverse effects of herbicides at environmental concentrations calls for further research, and should be an obligatory part of the herbicide registration process.

In addition to water pollution and extreme weather conditions, freshwater biodiversity is threatened globally with biological invasions, and increasing attention is focused on invaded ecosystems. Behaviour plasticity of crayfish is a key factor behind co-existence with, or replacement of, native species by invaders sharing overlapping niches. The marbled crayfish

P. virginalis is able to dominate size-matched established non-native calico crayfish *Faxonius immunis* (**Chapter 5**), spiny-cheek crayfish *Faxonius limosus* (**Chapter 6**), and red swamp crayfish *P. clarkii*, suggesting the potential of marbled crayfish to replace these species in the wild. Further research is needed to elucidate mechanisms of marbled crayfish invasion at community and ecosystem levels.

These data represent basic scientific information valuable for practical application. The outcomes of interactions among crayfish under laboratory conditions can be critical to evaluating over-invaded communities and ecosystems harbouring invasive species that share similar life histories, niche preference, and adaptation strategies.

Czech summary

Norovací chování u raků

Wei Guo

Pro sladkovodní druhy raků je schopnost tvorby nor nebo nalezení vhodného úkrytu jedním z nejdůležitějších faktorů zajišťujících jejich přežití. Nory a úkryty poskytují řadu dalších funkcí, jako je ochrana před predátory a ochrana před extrémními podmínkami prostředí. Tato útočiště také používají jako zdroj vody nebo úkryt při svlékání, kladení a inkubaci vajíček, stejně tak i při ochraně potomků. Úkryty nebo nory jsou proto často důležité pro přežití raků a jejich dostupnost patří k limitujícím faktorům račích populací.

Naše studie odhalila schopnost raků mramorovaných *P. virginalis* podstoupit terminální fáze embryogeneze, včetně líhnutí a také raný postembryonální vývoj pouze při vysoké vlhkosti vzduchu. Důležité je naše zjištění, že juvenilové ve 2. vývojovém stadiu byli ve vlhku schopni dlouhodobě přežívat a při vysazení zpět do vodního prostředí normálně pokračovali ve svém vývoji. Tyto skutečnosti výrazně zvyšují naše chápání odolnosti tohoto druhu v lokalitách s proměnlivými hydrologickými podmínkami. Podobné schopnosti tolerance a reprodukce během sucha můžeme očekávat i u jiných druhů raků, jako jsou primárně a sekundárně norující druhy, ale třeba i vysoce invazivní rak červený *P. clarkii*. Tato bezprecedentní vlastnost sladkovodních raků je pravděpodobně klíčovým faktorem pro obývání ekosystémů s vysokými výkyvy hladiny vody a během období extrémního sucha. V kontextu biologických invazí bude úroveň těchto adaptací pravděpodobně také přispívat k nahrazování některých druhů a zvýhodnění těch, které jsou lépe přizpůsobeny takovým podmínkám prostředí (kapitola 2).

Kromě extrémních výkyvů počasí, jako jsou dlouhodobá období sucha, je znečištění vody považováno za jeden z nejdůležitějších faktorů poklesu globální biodiverzity, s častými incidenty znečištění a zhoršení kvality vody s dopady na vodní ekosystémy. Poprvé jsme popsali rozdíly v chování při norování sladkovodních raků vystavených působení farmaceuticky aktivních sloučenin (PhACs) v koncentracích relevantních pro životní prostředí, což je aplikovatelné i pro jiné vodní organizmy. Zejména relativní hloubka a objem nor vybudovaných samicemi vystavenými methamphetaminu byla ve srovnání s kontrolami průkazně nižší. Samice exponované v tramadolu konzistentně vykazovaly tendenci k menším norám, rozdíl však nebyl průkazný. Vzhledem k rozhodující úloze povrchových vod pro lidské použití v zemědělství, akvakultuře a domácímu použití by se neměl ignorovat potenciální dopad PhACs na sladkovodní biodiverzitu. Účinky vybraných PhACs v relevantních koncentracích pro životní prostředí jsou evidentní při testování jednotlivých sloučenin (kapitola 3). Účinek vybraných herbicidů terbuthylazinu a metazachloru v environmentálně relevantních koncentracích se u sladkovodních raků projevoval méně (kapitola 4). Zjistili jsme však, že počet nor vybudovaných samci exponovaných v metazachloru byl signifikantně vyšší než v jiných skupinách. V případě studií norovacího chování u raků je rozhodující dostatečný počet opakování s ohledem na značnou variabilitu individuálních odpovědí. Na základě modelové skupiny raků lze podobný způsob stresové reakce na kontaminaci vodního prostředí očekávat i u jiných vodních organizmů potenciálně využívajících i hlubší vrstvy dna (hyporeál), jako jsou mlži a mnoho druhů vodního hmyzu. Na základě výsledků v této a dalších studiích (kapitola 2) navrhujeme, aby bylo subletálním účinkům PhAC, herbicidů a dalších kontamintů na necílové vodní organizmy věnováno více pozornosti. Potenciální nepříznivý účinek herbicidů v environmentálně relevantních koncentracích vyžaduje další výzkum a také jejich odpovědné vypoužívání. Hodnocení těchto účinků by mělo být součástí jejich registračního procesu.

Kromě znečištění vody a extrémních výkyvů počasí je globálně ohrožena sladkovodní biodiverzita také biologickými invazemi, což zvyšuje pozornost věnovanou invadovaným ekosystémům. Plastičnost chování raků je klíčovým faktorem koexistence nebo nahrazení původních druhů druhy invazivními, jež sdílejí ekologické niky. Rak mramorovaný je schopen vytlačit stejně velké raky kalikové *F. immunis* (kapitola 5), raky pruhované *F. limosus* (kapitola 6) a raky červené, což naznačuje možnost druhových změn ve volné přírodě. Další výzkum je nutný k pochopení mechanizmů invaze raka mramorovaného jak na úrovni komunity, tak i celých ekosystémů.

Prezentovaná data představují zajímavý pohled jak z vědeckého hlediska, tak i pro praktické využití. Například výsledky interakcí mezi zmiňovanými druhy raků v laboratorních podmínkách mohou být použity například jako pontenciální důkaz při hodnocení napadených komunit a ekosystémů invazivními druhy sdílejícími podobné životní historie, preference ekologické niky a adaptační strategie.

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List of publications

Peer-reviewed journals with IF

Guo, W., Hossain, M.S., Kubec, J., Grabicová, K., Randák, T, Buřič, M., Kouba, A., 2020. Psychoactive compounds at environmental concentration alter burrowing behavior in the freshwater crayfish. Science of the Total Environment 711: 135138. (IF 2019 = 6.551)

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Abstracts and conference proceedings

Guo, W., Hossain, M.S., Kubec, J., Grabicová, K., Randák, T., Buřič, M., Kouba, A., 2019. Psychoactive compounds at environmental concentration alter burrowing behavior in the freshwater crayfish. SETAC Latin America 13th Biennial Meeting, 15–18 September 2019 in Cartagena, Colombia (oral presentation)

Hossain, M.S., **Guo, W.**, Kubec, J., Roje, S., Grabicová, K., Randák, T., Kouba, A., Buřič, M., 2019. Cocktail of pharmaceutically active compounds alter the behavior of clonal marbled crayfish. SETAC Latin America 13th Biennial Meeting, 15–18 September 2019 in Cartagena, Colombia. (oral presentation)

Guo, W., Buřič, M., Jiří Patoka J., Kouba, A., 2017. Red swamp crayfish *Procambarus clarkii*: economic importance but also environmental threat. The 3rd International Congress on Biological Invasions, 19–24 September 2017 in Hangzhou, China. (oral presentation)

Projects I got in Czech Republic

GAJU-the Grant Agency of the University of South Bohemia (No. 102/2018/Z), Principal Investigator (129,000 CZK in total) – Wei Guo, Title: Interaction, growth and maturation racing: comparison of two prominent crayfish invaders

GAJU-the Grant Agency of the University of South Bohemia (No. 078/2019/Z), Principal Investigator (149,000 CZK in total) – Wei Guo, Title: Boiling crayfish? A comparison of burrowing behaviour under conditions of simulated drought as influenced by herbicide pollution

Deans award for the best students publication of 2019

Guo, W., Kubec, J., Veselý, L., Hossain, M.S., Buřič, M., McClain, R., Kouba, A., 2019. High air humidity is sufficient for successful egg incubation and early post-embryonic development in the marbled crayfish (*Procambarus virginalis*). Freshwater Biology 64: 1603–1612.

Training and supervision plan during study

Name	Wei Guo
Research department	2016–2020 – Laboratory of Freshwater Ecosystems
Supervisor	Antonín Kouba, Ph.D.
Period	17th October 2016 until 17th September 2020

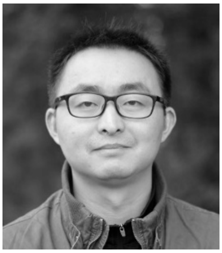
Ph.D. courses	**Year**
Basic of scientific communication	2017
Pond aquaculture	2017
Applied hydrobiology	2017
Ichthyology and fish taxonomy	2017
English language	2018

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2017 2018 2019 2020

International conferences	**Year**
Guo, W., Buřič, M., Jiří Patoka J., Kouba, A., 2017. Red swamp crayfish *Procambarus clarkii*: economic importance but also environmental threat. The 3rd International Congress on Biological Invasions, 19–24 September 2017 in Hangzhou, China. (oral presentation)	2017
Guo, W., Hossain, M.S., Kubec, J., Grabicová, K., Randák, T., Buřič, M., Kouba, A., 2019. Psychoactive compounds at environmental concentration alter burrowing behavior in the freshwater crayfish. SETAC Latin America 13th Biennial Meeting, 15–18 September 2019 in Cartagena, Colombia (oral presentation)	2019
Hossain, M.S., Guo, W., Kubec, J., Roje, S., Grabicová, K., Randák, T., Kouba, A., Buřič, M., 2019. Cocktail of pharmaceutically active compounds alter the behavior of clonal marbled crayfish. SETAC Latin America 13th Biennial Meeting, 15–18 September 2019 in Cartagena, Colombia. (oral presentation)	2019
Guo, W., Weiperth, A., Hossain, M.S., Kubec, J., Grabicová, K., Ložek, F., Velíšek, J., Veselý, L., Bláha, M., Buřič, M., Kouba, A., 2020. The effect of selected-herbicides compounds at environmental concentrations on burrowing behaviour in the freshwater crayfish. 23 Symposium of the International Association of Astacology, Hluboká nad Vltavou, June 29–July 3, 2020, Czech Republic. (poster)	2020

Curriculum vitae

PERSONAL INFORMATION



Name:	Wei
Surname:	Guo
Title:	M.Sc.
Born:	12th June 1989, Henan, China
Nationality:	Chinese
Languages:	English (B2 level – IELTS Certificate), Mandarin
Contact:	wguo@frov.jcu.cz; guowei612@foxmail.com

EDUCATION

2016–present	Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, Czech Republic
2013–2016	M.Sc., Institute of Hydrobiology, University of Chinese Academy of Sciences
2010–2013	B.Sc., College of Business Administration, Zhongnan University of Economics and Law
2009–2013	B.Sc., College of Fisheries, Huazhong Agricultural University

COMPLETED COURSES

01/04–07/04 2019	Stable Isotopes in Environmental Sciences, Ecology and Physiology (Technische Universität München and University of South Bohemia)
19/06–23/06 2017	Biological Specimens in Electron Microscopes – Biology Centre, Academy of Sciences Laboratory of Electron Microscopy, České Budějovice, Czech Republic
27/02–03/03 2017	Basics of scientific communication, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Vodňany, Czech Republic

RESEARCH STAY AND COLLABORATIONS

14/04–01/07 2018	Department of Biology, University of Florence, Italy (Dr. Elena Tricarico); The impacts of burrowing behaviour of red swamp crayfish *Procambarus clarkii* to the riverbanks and utilizing the stable isotope analysis approach, using invasive channel catfish *Ictalurus punctatus* as a model organism.