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The marbled crayfish: parthenogenetic invasive species
as an applicable biological model

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Mramorovaný rak: parthenogenetický invazivní druh jako použitelný biologický model

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CHAPTER 1

GENERAL INTRODUCTION

1.1. General overview and objectives

1.2. Hossain, M.S., Patoka, J., Kouba, A., Buřič, M., 2018. Clonal crayfish as biological model: a review on marbled crayfish. Biologia 73, 841–855.

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1.1. General overview and objectives

General overview of marbled crayfish

Crayfish belong to the order Decapoda, and are the only mobile crawling freshwater invertebrates with large chelae (Scholtz and Richter, 1995). Crayfish are increasingly recognized as ecologically important benthic, polytrophic omnivorous macroinvertebrates, and as keystone species in both standing and running waters (Momot, 1995; Nyström, 2002). They function as an ecosystem engineers by impacting the physical structure of the environment and biological interactions (Kouba et al., 2014).

The two recognized superfamilies of crayfish are the Astacoidea, the Northern Hemisphere crayfish and the Parastacoidea, Southern Hemisphere crayfish. There are three families in Astacoidea, the Astacidae, the Cambaridae, and Cambaroididae. Parastacoidea have only one family, the Parastacidae (Crandall and De Grave, 2017). The family Astacidae dominates on the European and western Asian continent west of the Ural Mountains and western North America. The family Cambaridae is dominant on the whole of North America (including the island of Cuba), except for the Pacific slope of the western United States and Canada. The family Cambaroididae occurs in East Asia and Japan (Scholtz, 2002). The family Parastacidae, prevails on the islands of Australia, New Guinea, New Zealand and Madagascar and in southern South America (Fig. 1). Crayfish are markedly absent from the vast interior of central Asia and continental Africa (Hobbs et al., 1989). Among the total 692 crayfish species (Crandall and De Grave, 2017) the distribution is far from uniform, with approximately 77% of the world's crayfish species and subspecies in North America, 20% in Australia, 1.5% in South America and only 1.5% in Europe/Asia (Fig. 1). European native astacofauna is represented only by genus *Astacus, Austropotamobius* and *Pontastacus* which were recently divided into fifteen species, instead of the five species traditionally recognized in the past (Crandall and De Grave, 2017).

European freshwater crayfish populations have faced major instabilities due to habitat destruction, water quality modifications through anthropogenic activities, and extreme commercial overexploitation of aquatic resources (Souty-Grosset et al., 2006). However, the main driver of decline in native crayfish populations is the introduction of the crayfish plague pathogen, *Aphanomyces astaci* (Filipova et al., 2013). Crayfish plague appeared following the introduction of an unknown crayfish species in the second half of the 19th century causing mass mortality across the Europe. The issue of non-native crayfish introduction continues with many potential introductions (mainly of North American origin) through the pet trade on the European continent (Kouba et al., 2014). Introductions of North American non-indigenous crayfish species (NICS) (Table 1) (Faulkes, 2015a) progressed through the 20th century, by dispersing from one water body to another (Souty-Grosset et al., 2006). These NICS are carriers of the crayfish plague pathogen which increases their devastating effects not only on native astacofauna but even entire ecosystems. Currently, the aquarium trade is the dominant source of new NICS into Europe (Peay, 2009).

The parthenogenic marbled crayfish, *Procambarus virginalis* was first discovered in the German aquarium trade in the mid-1990s (Martin et al., 2010; Martin et al., 2016; Scholtz et al., 2003). Due to their characteristic and conspicuous color pattern, these crayfish quickly became known as `Marmorkrebs´ (German, which translates into English as 'marbled crayfish'). This non-native species established became established in the wild of different European countries through intentional or unintentional release from overpopulated aquaria (Hossain et al., 2018). As a fast growing, highly fecund, and frequent breeding species, marbled crayfish (Seitz et al., 2005) have the potential to be a serious invasive threat to freshwater ecosystems

(Veselý et al., 2015) due to its minimal habitat requirements, (Jimenez and Faulkes, 2010), ability to withstand low winter temperatures and desiccation in the summer (Guo et al., 2019; Kouba et al., 2016). However, these same characteristics along with its reproductive mode which produces genetically uniform clones (Hossain et al., 2018; Vogt, 2008, 2010, 2011) gives the marbled crayfish great potential to become a model organism in biological science research.

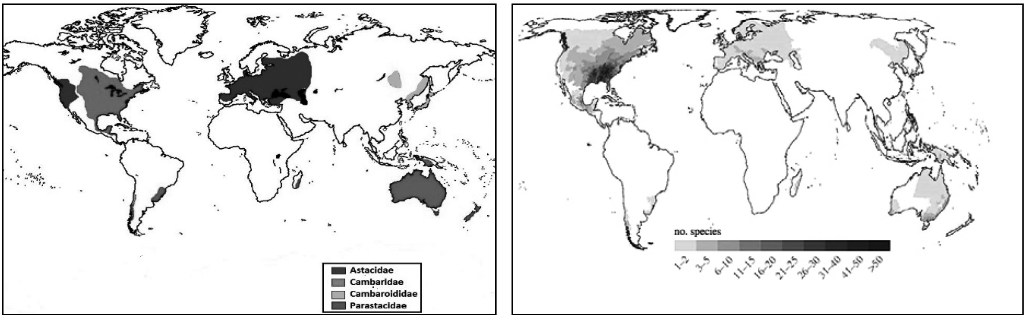

Figure 1. *The distribution of the crayfish families (Hobbs et al., 1989) and their species richness across the world. Modified from Hobbs et al. (1989); Richman et al. (2015) and Crandall and De Grave (2017).*

Table 1. *List of established NICS species in Europe including their origin, probable introduction pathway and year of first record according to Holdich et al. (2009), Chucholl (2013) and Kouba et al. (2014).*

Species	**Origin**	**Introduction pathway**	**Year of first record and location in Europe**
Signal crayfish *Pacifastacus leniusculus*	Western North America	DS, AQ, AR	Late 1970s, Finland
Red swamp crayfish *Procambarus clarkii*	North America	DS, AQ, AR	1973, Spain
White river crayfish *Procambarus* cf. *acutus*	North America	Unknown	2005, Netherlands
Marbled crayfish *Procambarus virginalis*	North America#	AT	2003, Germany
Spiny-cheek crayfish *Faxonius limosus*	North America	DS, AR	1956, Poland
Calico crayfish *Faxonius immunis*	North America	FB	1993, Southern Germany
Kentucky River crayfish *Faxonius juvenilis*	North America	Unknown	2005, Eastern France
Virile crayfish *Faxonius virilis*	North America	AT	2004, Netherlands
Yabby *Cherax destructor*	Australia	DL, AQ	1983, Northern Spain
Redclaw *Cherax quadricarinatus*	Australia	AT, AQ	Early 1990s, Italy
Florida crayfish *Procambarus alleni*	North America	AT	1993th, France
Narrow-clawed crayfish *Pontastacus leptodactylus**	Eastern Europe	DS	Late 19th century, Central Europe

AQ – aquacultural purposes, AT – Aquarium Trade, DS – Deliberate Stocking, AR – Active range expansion from adjacent territories, FB – Fishing Bait, * Native to eastern Europe (Ponto-Caspian region), # no native range known, ‡ only single specimen seen in the wild, establishment not confirmed

Marbled crayfish as a model species in biological sciences

In science, a model is a simplified system that is accessible and easily handled. A model organism is a non-human species that is extensively studied to understand particular biological phenomena, usually because it is easy to maintain and breed in a laboratory setting and has particular experimental advantages. The fruit fly *Drosophila melanogaster* comes to light in the early 1900 century by the zoologist Thomas Hunt Morgan as a maiden model organism to test some of Charles Darwin's evolutionary theories (Singer, 2016). Over time, model organisms such as the nematode *Caenorhabditis elegans*, thale cress *Arabidopsis thaliana*, zebrafish *Danio rerio*, frogs *Xenopus* spp. and house mice *Mus musculus* etc. have become irreplaceable tools of fundamental biological and clinical research, and help scientists to amass an enormous amount of knowledge about general biology, physiology, cell biology, neuroscience, behavior, etc. of plants and animals. (Hunter, 2008).

Vertebrate models have the benefit of bearing closer biological resemblance to humans whereas invertebrates have the advantages of easier culture, lower costs and fewer restrictions by animal protection and welfare acts (Vogt, 2010). Among the invertebrates, decapod crustaceans (including crayfish) have been used as model creatures for laboratory-based investigation in many arenas of biology, which highlight the prospects for marbled crayfish as an emerging model organism (Faulkes, 2015b). The marbled crayfish fulfills the basic requirements as a general laboratory animal. They are easy to culture, of suitable size, show individual variation, are highly tolerant to handling stress, are highly fertile, have relatively short generation times and are adaptable to a wide range of environmental and nutritional conditions. Other attractive features include various morphological characters that are easy to evaluate (Vogt et al., 2008), a lack of resting stages, alternation of growth and reproductive phases, indeterminate growth and the associated lifelong functionally diverse stem cell systems (Vogt, 2010), a broad behavioral repertoire, high regeneration capacity and DNA methylation throughout the lifetime (Vogt et al., 2008). Perhaps the most important advantage of marbled crayfish is that their parthenogenetic offspring are genetically identical. The mother, offspring, and even all batch-mates are clones (Gutekunst et al., 2018; Martin et al., 2007; Vogt et al., 2008).

Marbled crayfish in aggressive interactions

Biotic factors including predation risk (Light, 2003; Magoulick, 2004; Usio and Townsend, 2000) and competitive interactions (Garvey et al., 1994; Hill and Lodge, 1999) play a role in changing crayfish distributions. Both interspecific and intraspecific competition are important factors affecting crayfish habitat preference and distribution patterns (Bovbjerg, 1970; Garvey et al., 1994). The development of dominance hierarchies in crayfish is settled through dyadic social and often aggressive interactions, whereby winners obtain access to a resource e.g. food, shelter or mates (Zulandt et al., 2008). Aggressive behavior is characteristic for all life stages of crayfish (Gherardi, 2002). Marbled crayfish display the same agonistic behaviors known from other decapods such as lobsters and several crayfish species (Farca Luna et al., 2009; Vogt et al., 2008). They have the capability to fight with other species on the same level as *P. clarkii*, which is a successful introduced species around the world (Jimenez and Faulkes, 2011). The marbled crayfish has been detected in the wild in 11 countries of Europe (Hossain et al., 2018). However, there is no data about their interactions with other aquatic organisms and non-native crayfish species.

Marbled crayfish as model species in toxicological reseach

Aquatic environments are continuously polluted by various anthropogenic activities (Walker et al., 2012). Therefore, aquatic organisms have to face different inorganic (sulphur dioxide, fertilizers and heavy metals) and organic pollutants (pesticides and pharmaceutically active compounds, PhACs) (Brooks et al., 2005). The release of pharmaceuticals and human personal care products has recently captured the attention of researchers (Boxall et al., 2012) largely because conventional sewage treatment techniques have limited abilities to remove these chemicals from sewage waters before discharging their effluent back into the environment (Golovko et al., 2014; Heberer, 2002). PhACs are emerging pollutants in the aquatic world which produce non-lethal effects on organisms at low concentrations and have the potential for bioaccumulation in the tissues of aquatic organisms (Grabicova et al., 2015; Huerta et al., 2012; Hughes et al., 2013). The presence of these compounds in the aquatic ecosystem can influence the behavior of exposed animals, which alters predator-prey relationships, social traits, reproduction, and migration strategies etc. (Brodin et al., 2014). These non-lethal effects are likely to have ecological implications. The marbled crayfish is already known as a useful model species for toxicology or ecotoxicology studies (Velíšek et al., 2014, 2017; Vogt, 2007), and it is expected that its use will increase as the model organism becomes known to more scientists.

Objectives

The primary goal of this study was to define the growth and reproductive patterns, competitiveness of marbled crayfish observed through aggressive interactions and support their suitability in eco-toxicological studies as a model organism. To achieve this goal, the study addressed the following objectives-

1. To determine morphometric and reproductive relationships for better exploitation of marbled crayfish as a model species.
2. To assess their competitiveness in novel environments for strategic resources with other established non-native invasive crayfish species found in Europe.
3. To determine the effects of PhACs on clonal marbled crayfish behavior and physiology.

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CHAPTER 1.2.

CLONAL CRAYFISH AS BIOLOGICAL MODEL: A REVIEW ON MARBLED CRAYFISH

Hossain, M.S., Patoka, J., Kouba, A., Buřič, M., 2018. Clonal crayfish as biological model: a review on marbled crayfish. Biologia 73, 841–855.

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REVIEW

Clonal crayfish as biological model: a review on marbled crayfish

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Abstract
Since the mid-twentieth century, numerous vertebrates and invertebrates have been used as model organisms and become indispensable tools for exploring a broad range of biological and ecological processes. Crayfish seem to be adequate models which resulted in their involvement in research. In the two decades since its discovery, ongoing research has confirmed that the marbled crayfish (*Procambarus virginalis* Lyko, 2017) is an ideal taxon in this regard, especially due to its almost continuous asexual reproduction providing a source of genetically identical offspring. This review provides an overview of the occurrence, biology, ecology, ethology, and human exploitation of marbled crayfish with primary focus on its use as a laboratory model organism as well as potential risks to native biota in case of its introduction. Genetic uniformity, ease of culture, and a broad behaviour repertoire fosters the use of marbled crayfish in epigenetics and developmental biology, as well as physiological, ecotoxicological, and ethological research. Marbled crayfish could be further exploited for basic and applied fields of science such as evolutionary biology and clonal tumour evolution. However, due to its high invasive potential in freshwater environments security measures must be taken to prevent its escape into the wild.

Keywords Model species · Epigenetics · Developmental biology · *Procambarus virginalis* · Biological invasion

Introduction

The marbled crayfish (*Procambarus virginalis* Lyko, 2017) is an enigmatic species with unknown origin and primary population range (Martin et al. 2010a; Lyko 2017). For almost a decade after its first observation, its phylogenetic position and species status have remained unclear (Scholtz 2015), being considered a possible hybrid of the slough crayfish *Procambarus fallax* (Hagen, 1870) and another species of the same genus or possibly of an autopolyploid (Martin et al. 2016). It was usually regarded as *P. fallax* f. *virginalis* (Martin et al. 2007, 2010a), but Vogt et al. (2015); Lyko (2017) suggested its elevation to species status.

The marbled crayfish is unique among decapods by reproduction via obligatory apomictic parthenogenesis, and males are unknown (Martin et al. 2007; Vogt 2007; Vogt et al. 2008; Martin and Scholtz 2012). It was discovered in the mid-1990s in the German aquarium trade (Lukhaup 2001; Scholtz et al. 2003), and, due to its characteristic conspicuous colour pattern it quickly became known as Marmorkrebs, which literally translates to English as 'marbled crayfish'. This species has gradually shown its adaptability and invasive potential, which is higher than was originally expected (Veselý et al. 2015; Kawai et al. 2016). Its popularity in the pet trade (Chucholl et al. 2012; Patoka et al. 2014; Faulkes 2015) along with rapid and frequent reproduction and high stress resistance, facilitates its present occurrence in several European countries with further spread predicted (Lőkkös et al. 2016; Patoka et al. 2016b; Lipták et al. 2017; Pârvulescu et al. 2017). This makes the marbled crayfish an ecologically important species. It is also valuable as a model organism (Vogt 2011), due to its biological characteristics and production of genetically identical offspring (Martin et al. 2007). Despite this genetic uniformity, substantial variability in growth rate, size, age at maturation, fecundity, frequency of spawning, colour patterns (Vogt et al. 2008), and behaviour patterns exist (Farca Luna et al. 2009). These variations have not been adequately

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explored and represent a wide field for research and its use as a model species (Vogt 2008c, 2010, 2011). On the other hand, it must be mentioned that European Commission Regulations (EU Regulation No. 1143/2014 and Commission Implementing Regulation No. 2016/1141), among others, currently prohibits the import, trade, keeping and breeding of marbled crayfish in the European Union (EU). Keeping for research is allowed but requires permission. Therefore, propagation for research or commercial use should follow these regulations to avoid possible escapes into the natural environment. The aim of this review is to summarize the available information on marbled crayfish distribution, growth, maturation, reproduction, behaviour, and responses to chemical, physical, and ecological factors to document its impact on natural ecosystems. We also discuss its potential as a biological model in scientific research.

Probable origin and current distribution

As mentioned above, the marbled crayfish was originally discovered in the German aquarium trade in the mid-1990s (Scholtz et al. 2003), and indigenous populations have never been reported (Taylor et al. 1996; Martin et al. 2016). Martin et al. (2010a) suggested that marbled crayfish originated directly from *P. fallax* (Decapoda: Cambaridae), native to Florida and southern Georgia) without hybridization. Martin et al. (2016) detected a large subtelocentric chromosome, which appears once in haploid and twice in diploid cells of sexually-reproducing individuals of *P. fallax* and three times in the parthenogenetic marbled crayfish. Its triploid AA B homologous genotype with heterozygosity at third copy advocated its origin from the autopolyploidization of a *P. fallax* eggs followed by fertilization by sperm of a distantly related *P. fallax* individual (Gutekunst et al. 2018). One way or another, marbled crayfish is now considered as an independent species (Lyko 2017).

The marbled crayfish is frequently sold in pet shops and online (Chucholl 2013; Faulkes 2013; Lipták and Vitázková 2015; Mrugała et al. 2015). Due to its asexual mode of reproduction and prolific breeding, home aquaria can be rapidly overpopulated, a situation that often leads to sale or disposal of redundant individuals into the wild, including in garden ponds (Peay 2009; Patoka et al. 2014, 2016a). Such releases led to numerous marbled crayfish populations in natural ecosystems (Chucholl 2011; Faulkes et al. 2012; Patoka et al. 2016b). Risks of crayfish introduction, including marbled crayfish, from the pet trade, has been studied worldwide (Faulkes 2015; Chucholl and Wendler 2017; Uderbayev et al. 2017).

The first marbled crayfish captured from the wild was reported in southwestern Germany in late 2003 (Marten et al. 2004) and was followed by reports from other areas of Germany (Chucholl and Pfeiffer 2010; Martin et al. 2010b). Chucholl and Pfeiffer (2010) suggested that the published records from Germany represent merely the tip of the iceberg, a statement that applies to other regions and countries as well. Since the German discovery, there have been numerous reports of the presence of marbled crayfish in European countries. In 2003, approximately 30 individuals of marbled crayfish were introduced into a small waterbody near Dordrecht in the Netherlands (Souty-Grosset et al. 2006) but, similar to other cases, their population status is unknown (Chucholl et al. 2012; Kouba et al. 2014). A single individual of marbled crayfish was found in a red swamp crayfish (*Procambarus clarkii* Girard, 1852) population in a flowing canal in Tuscany, Italy in 2008 (Nonnis-Marzano et al. 2009), and an individual was found near Venice, Italy in 2009 (Vojkovská et al. 2014). Well-established populations were reported in Germany (Chucholl and Pfeiffer 2010) and Slovakia (Janský and Mutkovič 2010) in 2010, followed by other records in the basin of the Danube and Rhine rivers (Chucholl et al. 2012; Weiperth et al. 2015; Lipták et al. 2016, 2017). In December 2012, 13 individuals of marbled crayfish were found in the Märstaån River in Sweden (Bohman et al. 2013). Marbled crayfish was also detected in southern Sweden, but the established population was not confirmed (Bohman et al. 2013). Its presence was also reported in Croatia (Samardžić et al. 2014), Ukraine (Novitsky and Son 2016), Hungary (Lőkkös et al. 2016), Romania (Pârvulescu et al. 2017), Estonia (Estonian Research Council 2018) and the Czech Republic (Patoka et al. 2016b).

Outside Europe, the marbled crayfish has established abundant populations in Madagascar, where it was probably introduced in 2003 (Jones et al. 2009), and in the recent decade the dispersal area increased by 100 times from 10^3 km^2 to >10^5 km^2 (Gutekunst et al. 2018). Individuals were captured in Japan near Sapporo City, Hokkaido in 2006 (Kawai and Takahata 2010; Faulkes et al. 2012), and 2017 (Usio et al. 2017). Currently, there are no confirmed introductions in the United States, where it is a popular ornamental animal (Faulkes 2010, 2015). The reported occurrence of marbled crayfish worldwide is summarized in Table 1. One can assume a high probability of future marbled crayfish establishment in natural waterbodies through intentional or unintentional releases or escapes, basically anywhere in the world. Therefore, it is necessary to continuously monitor wild stocks and waterbodies and to educate the public with respect to risks of marbled crayfish invasions and related impacts.

Life cycle

Based on life history, we can classify crayfish as *K*-selected or *r*-selected (Holdich 2002). Marbled crayfish clearly belong to

Table 1 The records of marbled crayfish *Procambarus virginalis* in natural habitats with first record (F) and year of the report of first established population (E). NE = not established

Country	Recorded year F / E	Reference
Croatia	2013 / 2013	(Cvitanić 2017)
Czech Republic	2015 / 2016	(Patoka et al. 2016b)
Estonia	2018 / 2018	(Estonian Research Council 2018)
Germany	2003 / 2010	(Marten et al. 2004; Chucholl and Pfeiffer 2010)
Hungary	2013 / 2014	(Weiperth et al. 2015; Lőkkös et al. 2016)
Italy	2008 / NE	(Nonnis-Marzano et al. 2009)
Japan	2006 / NE	(Kawai and Takahata 2010)
Madagascar	2005 / 2007	(Jones et al. 2009)
Netherlands	2003 / NE	(Soes and Koese 2010)
Slovakia	2010 / 2010	(Janský and Mutkovič 2010)
Sweden	2012 / NE	(Bohman et al. 2013)
Ukraine	2015 / 2015	(Novitsky and Son 2016)
Romania	2016 / 2016	(Pârvulescu et al. 2017)

the latter, with rapid growth, early maturation, high fecundity, short embryogenesis, and frequent spawning (Seitz et al. 2005). Crayfish growth is typically isometric in juveniles and allometric in adults (Rhodes and Holdich 1979; Holdich 2002). Growth of marbled crayfish is not isometric (Seitz et al. 2005) resembling other species such as the noble crayfish *Astacus astacus* (Linnaeus, 1758), signal crayfish *Pacifastacus leniusculus* (Dana, 1852) (Abrahamsson 1971), and spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817) (Buřič et al. 2010).

In a favourable environment, marbled crayfish can grow to ~12 cm in total body length. Its optimal thermal range is from 18 to 25 °C, with highest growth rate observed at 25 °C and maximum survival rate (80%, at maturity) at 20 °C (Seitz et al. 2005; Vogt 2008b). It was originally supposed that the species could thrive at temperatures <8 °C and >30 °C for many weeks, but with increased mortality and limited reproduction, which ceased at temperatures ≤ 15 °C (Seitz et al. 2005). Pfeiffer (2005) stated that marbled crayfish can survive under ice during winter season, though Martin et al. (2010b) assumed low winter temperature as a main obstacle against successful long-term establishment of wild populations in Central Europe. However, in accordance with outdoor experiments realized by Kaldre et al. (2016), marbled crayfish might be able to survive even North European winters and establish there permanent populations. Moreover, it was recently confirmed (in experimental as well as in field conditions) that marbled crayfish can survive (Veselý et al. 2015) and reproduce (Chucholl and Pfeiffer 2010) in moderate climate winter conditions (Lipták et al. 2016). Moulting usually ceases at 10 °C (Seitz et al. 2005), but successful moulting has been reported during gradual temperature decrease to lower values (5.1–9.5 °C) (Kaldre et al. 2016). Water temperature also affects gonad development and reproduction in crayfish (Carmona-Osalde et al. 2004; Vogt 2013; Kaldre et al. 2016) and is the most important factor influencing biological and physiological processes, including behaviour (Gherardi 2002; Reynolds 2002). Under favourable conditions, marbled crayfish reproduces year-round with intervals of only 8–9 weeks between spawnings (Vogt et al. 2004), but with peaks in spring and autumn under laboratory conditions (Vogt 2015). Reproducing females can be found throughout the whole year (Vogt 2015; Vogt et al. 2015), but reproduction timing in free-living populations remains poorly studied (Lipták et al. 2016, 2017). Jones et al. (2009) reported that ovigerous females have been observed in warm conditions of Madagascar frequently during the year leading to fast overpopulation and disturbance of environmental stability. In moderate climates, ovigerous females were frequently found from early June to mid-October (Chucholl and Pfeiffer 2010; Lipták et al. 2016), but females with developed oocytes and well developed glair glands have been reported also in March (Patoka et al. 2016b). Recently, Cvitanić (2017) observed continuous growth and reproduction in a Croatian population throughout the warm season and with peaks of reproductive activity in September, October, and November. Therefore, in tropical climate marbled crayfish has a higher chance to become widespread and a sustainable invasive species within a very short period of time.

The maximum reported lifespan of marbled crayfish is 1610 days, while it is typically 2–3 years. Under laboratory conditions, marbled crayfish can complete up to seven reproductive cycles within that lifespan (Vogt 2010). Marbled crayfish begin to reproduce when younger than 6 months, at total body length of c. 40 mm (Vogt 2008c), which is early in comparison with other crayfish species (Table 2). Seitz et al. (2005) reported first spawning at 141–255 days (cephalothorax length of 14.0 to 21.5 mm, weight 0.63–2.0 g) at a water temperature of 20–25 °C. However, the onset of maturity in crayfish can be postponed or interrupted by low hierarchical

Table 2 The maximum life span, age at sexual maturity, total body length at sexual maturity (TL), fecundity, and incubation period

Species	Maximum life span (yr)	Age at sexual maturity (yr)	TL at maturity (mm)	Fecundity (no./female)	Incubation period (days)	Reference
Austropotamobius torrentium (Schrank, 1803)	> 10	3–5	50–65	40–70	240–270	(Kozák et al. 2015)
Astacus astacus (Linnaeus, 1758)	> 20	3–5	70–90	80–200	240–270	(Abrahamsson 1971; Kozák et al. 2015)
Pacifastacus leniusculus (Dana, 1852)	> 20	2–3	70–90	200–400	166–280	(Guan and Wiles 1999; Capurro et al. 2015; Kozák et al. 2015)
Faxonius limosus (Rafinesque, 1817)	≤ 4	1–2	40–60	200–300	37–56	(Kozák et al. 2006)
Procambarus clarkii (Girard, 1852)	≤ 2 (4–5)*	< 1	45–90	< 900	21–30	(Alcorlo et al. 2008)
Procambarus virginalis Lyko, 2017	> 3	< 0.5	32–40	50–700	20–42	(Seitz et al. 2005; Vogt et al. 2008; Kozák et al. 2015; Cvitanić 2017)

*in colder habitats (e.g. Holdich et al. 2006)

status or unfavourable conditions (Reynolds et al. 2002). In laboratory condition, moulting was identified as the main cause of death for marbled crayfish. Specimens with lowest life span showed fast growth, early onset of reproduction and short time intervals between reproduction cycles. Females reproduced until high age indicating negligible reproductive senescence and active stem cells in different organs at all ages. Regeneration of appendages did not negatively affect longevity. In contrast, social stress shortened life expectancy considerably (Vogt 2012). Therefore, Vogt (2012) emphasizes the suitability of marbled crayfish for general biogerontology, particularly the investigation of non-genetic aspects of ageing.

Marbled crayfish reproduction is less complex than in other crayfish species, since all individuals are females. They reproduce via obligatory apomictic parthenogenesis, producing genetically identical offspring, which are clones of the mother (Martin et al. 2007; Martin 2015). Parthenogenesis is fairly common in the animal kingdom (Suomalainen et al. 1987; Simon et al. 2011) but animal groups differ considerably with respect to the frequency of parthenogenetic forms (Vogt et al. 2004). Out of nearly 15,000 decapod crustacean species, the marbled crayfish is alone in reproducing via obligatory parthenogenesis (Scholtz et al. 2003; Martin et al. 2007; Vogt et al. 2008). Facultative parthenogenesis has been detected (Buřič et al. 2011, 2013) or suggested (Yue et al. 2008) in other cambarids that generally show typical sexual reproduction. In those cases, asexual reproduction is suggested to be an adaptation to non-optimal or extreme conditions, but the induction stimulus is not confirmed (Buřič et al. 2013). Marbled crayfish spawning is, in other respects, similar to other crayfish species, so the only important difference is the absence of egg fertilization. However, it is necessary to explore the mechanisms and genes responsible for obligate parthenogenesis in marbled crayfish and the physiological, molecular and genetic mechanisms underlying spawning in marbled crayfish compared to other freshwater crayfish.

Marbled crayfish is morphologically similar to its sexual ancestor, *P. fallax*, but marbled crayfish reproductive efficiency (e.g. clutch size, frequency of reproduction) is considerably larger due to parthenogenetic mode of reproduction. Conversely, facultative parthenogenesis in spiny-cheek crayfish lead to opposed patterns (Buřič et al. 2013). In marbled crayfish, clutch size increases with female size (Vogt 2011), as is typical for other sexual crayfish species (Reynolds 2002). The maximum values reported in laboratory culture and free-living populations ranged from 416 to 731 eggs (Seitz et al. 2005; Jones et al. 2009; Chucholl and Pfeiffer 2010; Vogt et al. 2015; Lipták et al. 2017). Due to the apomictic parthenogenetic production of up to 400 offspring per female and clutch, its suitability as a laboratory model for research was first suggested by Scholtz et al. (2003).

Marbled crayfish incubation and embryo development extend over 17–28 days depending on water temperature (Vogt

2010; Kozák et al. 2015). Commonly, hatched juveniles remain at developmental stage I for 4–5 days and stage II and III for 6–7 days and 10–26 days, respectively. During this period, juveniles undergo two moulting events while attached to female abdominal appendages (pleopods) (Vogt and Tolley 2004; Kozák et al. 2015). After the second moult, juveniles are fully developed (stage III, carapace length 2.7 mm and TL 5.7–6 mm) and are free-living individuals that actively move and forage. The juveniles often rest on the maternal pleopods and stay near their mothers for several weeks (Seitz 2001; Vogt and Tolley 2004; Vogt 2008b). The marbled crayfish embryonic development is almost identical to that of other crayfish species (Seitz et al. 2005) which also contributed to its use as a model organism for crustacean developmental biology.

Ecology and adaptability

All animals must act within their environment and modify their behaviour based on sensory information obtained through various stimuli (Bierbower 2010). They have evolved physiological adaptations to survive in changing conditions (Holdich 2002). Globally, we presently see an increasing numbers of non-indigenous invasive species, including crayfish. These species are assumed to be drivers of changes in energy flow, decreasing biodiversity richness and altering food webs (Holdich 2002; Larson et al. 2016).

The marbled crayfish is a prominent recent and potential future invader characterized by low requirements for living conditions (Jimenez and Faulkes 2010; Kawai et al. 2016). It can survive in dry periods by burrowing (Kouba et al. 2016) and withstand low winter temperature (Veselý et al. 2015). In addition to high environmental adaptability (Vogt 2017), it shows competitiveness against the highly invasive red swamp crayfish when size matched individuals are faced to each other (Jimenez and Faulkes 2011). This high competitiveness might represent a serious threat to freshwater ecosystems when they meet with similarly sized individuals of already prevalent crayfish species. The marbled crayfish is also a known vector of the crayfish plague pathogen *Aphanomyces astaci* Schikora (Keller et al. 2014; Mrugała et al. 2015), which is highly harmful to crayfish of non-North-American origin (Feria and Faulkes 2011; Kawai et al. 2016; Svoboda et al. 2017).

For marbled crayfish, as well as for other species, establishment in a new environment requires suitable conditions after the initial introduction (Catford et al. 2009). New incomers usually face unfamiliar physiological and behaviour challenges in the new environments (Chung et al. 2012). Generally, the “tens rule” is valid for invasive species: only about 10% of species introduced to new regions become established, and ~10% of them become pests (Williamson and Fitter 1996; Jeschke and Strayer 2005). Highly invasive cambarid crayfish (including marbled crayfish and red swamp crayfish) defy the tens rule and are more successful than previously expected (Buřič et al. 2011). Although marbled crayfish is genetically uniform its heterozygous triploid chromosome pattern (Gutekunst et al. 2018) make it more adaptable to variable environmental conditions, making it a super invasive crayfish in aquatic environments (Vogt 2017). In freshwaters of Madagascar marbled crayfish was identified as a particularly dangerous invader (Gutekunst et al. 2018).

In aquatic crustaceans, the environmental acclimation response and physiological and behavioural adaptations are highly influenced by water temperature (Espina and Herrera 1993; Hill et al. 2004). The ability to withstand short temperature extremes or long-term changes in water temperature varies significantly among crayfish species (Holdich 2002) and is associated with oxygen saturation and the crayfish’s physiological state (Reynolds 2002). The occurrence of marbled crayfish in temperate as well as tropical waters (Jones et al. 2009; Martin et al. 2010a; Novitsky and Son 2016; Patoka et al. 2016b) shows its tolerance to a wide range of temperatures. However, crayfish, including marbled crayfish, may avoid or prefer some temperatures when they have a choice in experimental situations, or under natural conditions such as the proximity of thermal springs (Payette and McGaw 2003; Lőkkös et al. 2016).

Crayfish species differ greatly in the ability to survive out of water during drought conditions (Reynolds et al. 2012). The survival of crayfish in dry periods depends on burrowing (Bravo et al. 1994; Correia and Ferreira 1995) and active dispersal capacities on the terrestrial environment (Correia and Ferreira 1995; Cruz and Rebelo 2007). Indigenous European crayfish species are primarily predestined to a life in permanent water bodies (Holdich 2002; Kozák et al. 2015; Kouba et al. 2016). In contrast, invasive crayfish are usually able to move over land (Peay et al. 2010; Ramalho 2012; Puky 2014; Herrmann et al. 2018) to withstand long drought conditions (Holdich et al. 1995; Banha and Anastácio 2014), and to excavate burrows connected to the water table or at least sufficiently humid for survival (Kouba et al. 2016; Souty-Grosset et al. 2016; Stebbing 2016). The marbled crayfish can be considered as a highly resistant species due to its origin from *P. fallax*, triploidy, and overall adaptability, along with its ability to excavate vertical burrows (Chucholl 2014; Kawai et al. 2016; Kouba et al. 2016; Vogt 2017). Marbled crayfish have been frequently reported on land during migration or found dead in places distant from waterbodies (Chucholl et al. 2012). It can probably react to unfavourable conditions by active overland dispersal.

Aquatic environments are increasingly polluted due to human activity (Walker et al. 2012) as well as by atmospheric deposition (Holdich 2002). The release of pharmaceuticals and personal care products into water bodies has been increasing (Boxall et al. 2012; Ebele et al. 2017) and can outweigh the adverse effects of the above mentioned sources of pollution (Brodin et al. 2014). Exposure to these substances at environmentally relevant concentrations in aquatic ecosystems can result in modifications of behaviours associated with predator-prey relationships, social traits, reproduction, and migration strategies (Valenti Jr et al. 2012; Brodin et al. 2013). Crayfish are susceptible to the presence of external substances in water that can affect behaviour patterns (Lahman et al. 2015; Neal and Moore 2017). Jackson (2016) reported that marbled crayfish locomotion can be significantly affected by drug exposure and suggested marbled crayfish as suitable model for the investigation of mechanisms of behavioural epigenetics and drug addiction. Information on the sensitivity of marbled crayfish to chemicals is still scarce, but it seems to be a suitable model organism for the evaluation of ecological, behavioural, and physiological responses to pollutants, due to its availability and genetic uniformity (Vogt 2011; Buřič et al. 2018). The marbled crayfish has already been used in toxicological and ecotoxicological studies (Vogt 2007; Velíšek et al. 2018; Marenkov et al. 2017; Císař et al. 2018), and it can be expected that its exploitation for this purpose will increase.

Behaviour

Crayfish are able to detect the presence of predators via olfactory organs and avoid predators by seeking shelter (Blake and Hart 1993; Shave et al. 1994). Visual and mechanical stimuli provoke escape through rapid backward movement by repeated flexing of the abdomen (tail-flips) in response to a predatory attack (Wine and Krasne 1972; Herberholz et al. 2004). This escape behaviour is costly and tends to reduce growth, due to reduced feeding opportunities (Resetarits 1991; Hill and Lodge 1999). However, marbled crayfish seem to be generally more calm in stressful conditions (Kawai et al. 2016) but its predator-prey responses remain poorly understood (Veselý et al. 2017).

Apart from basic responses to impending danger or unfavourable conditions, crayfish exhibit complex social and reproductive behaviour patterns. The dominance hierarchies in crayfish are settled through dyadic social, and often aggressive, interactions, in which winners obtain better access to resources including food, shelter, and mates (Zulandt et al. 2008). The winners are also more territorial, with greater territory assigned to dominant individuals (Delgado-Morales et al. 2004; Fero and Moore 2014). Crayfish are able to recognize their home range and repeatedly return to the same system of shelters (Merkle 1969; Guan and Wiles 1997; Buřič et al. 2009) through visual cues under light and tactile cues at dark conditions (Takahashi and Nagayama 2016). Defence of the territory, individual shelters, and other resources are mainly the reasons of aggressive agonistic interactions (Gherardi 2002). Crayfish movement and encounters are rhythmic behaviours which are regulated by light-entrained endogenous pacemaker (Farca Luna et al. 2009).

Crayfish primarily use chemical or visual communication signals as an alternative to physical combat to avoid related higher energy costs (Breithaupt and Thiel 2011) and are able to recognize the dominance status of conspecifics (Schneider et al. 1999). Agonistic behaviour can be provoked by availability, quality, and quantity of shelters and the distance to shelter rather than past social experience and current social status (Chibucos et al. 2015). These behaviour patterns are confirmed also for marbled crayfish (Vogt et al. 2008). The marbled crayfish displays agonistic behaviour similar to that observed in crabs (Sneddon et al. 2000), lobsters (Kravitz 2000) and other crayfish species (Farca Luna et al. 2009). It establishes dominance hierarchies similar to sexual species (Herberholz et al. 2007), and, despite its placid nature (Vogt et al. 2008; Jimenez and Faulkes 2011; Kawai et al. 2016), is successful in combat with similarly sized highly aggressive red swamp crayfish, the most successful introduced crayfish species worldwide (Jimenez and Faulkes 2011). In a situation of multiple invaded ecosystems it is worth to be explored how marbled crayfish acts, and how influences other taxa, including both, natives and non-natives. The level and intensity of aggressive interactions can be increased, reduced or compensated by means of other survival techniques, but there is still lack of information i.e. broad space for future research.

Increased aggressiveness is reported in ovigerous female crustaceans, mainly against conspecifics approaching their shelters or burrows (Gherardi 2002; Thiel 2007). Ovigerous and juvenile-carrying *P. clarkii* females have been reported to win a significantly higher proportion of fights than the non-maternal individuals (Figler et al. 2001). It is confirmed that serotonin is closely associated with aggressive or dominant behaviour in crayfish and indirectly with the synthesis of vitellogenin (Fossat et al. 2014; Fossat et al. 2015). Several researchers proved that 5-Hydroxytryptamine (5 HT, serotonin), an important biogenic amine present in the central nervous system (Laxmyr 1984; Fingerman et al. 1994), stimulates the release of neurohormones such as vitellogenesis-inhibiting hormone (Bomirski et al. 1981; Quackenbush and Keeley 1988; Quackenbush 1989), vitellogenesis-stimulating ovarian hormone (Takayanagi et al. 1986), vitellogenesis-stimulating hormone (Eastman-Reks and Fingerman 1985), and methyl farnesoate (Laufer et al. 1993), which are involved in the formation of vitellogenin in crustaceans (Vaca and Alfaro 2000; Alfaro et al. 2004; Kuo et al. 2009). Relatively little is known about the role of different vitellogenin and serotonin levels in mediating female behaviour e.g. aggression in pre-maternal or egg-carrying females.

Reproductive behaviour of animals usually comprises mate selection, pair formation, mating, and offspring care (Holdich 2002). In marbled crayfish, with obligatory asexual reproduction, only maternal care is relevant (Vogt 2008b). Maternal care is complex and critical to egg incubation as well as to early development of the offspring (Gherardi 2002; Vogt 2013). Crayfish females engage in extended care to protect hatchlings and juveniles from predation and cannibalism (Mathews 2011). Ovigerous females dramatically reduce activity, and eggs and juveniles are fanned at regular intervals by quivering movements of the pleopods (Vogt 2013). Vogt (2013) reported that the ovigerous marbled crayfish leaves the water for a period of time to ventilate the brood in the air during unfavourable conditions. Females also regularly groom the eggs and juveniles with the pereopods and discard and eat the dead juveniles as well as decaying eggs (Thiel 2007; Aquiloni and Gherardi 2008). Hatchlings attach to the maternal pleopods by innate reflexes and remain attached by special hooks on their chelipeds (Vogt 2008b). Independent marbled crayfish juveniles, at stage III, occasionally leave the mother to move freely and forage in the vicinity (Vogt and Tolley 2004). Juveniles return to the mother's abdomen after short excursions or if disturbed (Ameyaw-Akumfi 1976; Figler et al. 1997) with the help of species-specific brood pheromones (Little 1975) released by the female. Vogt (2013) reported that red swamp and marbled crayfish juveniles return to the mother pleopods also in response to visual maternal signals. Laboratory experiments with marbled crayfish demonstrated that stage III juveniles preferred maternal feces compared to other food (Vogt 2008b). Crayfish juveniles are able to differentiate their own mother from unfamiliar maternal females more than 10 days after separation and prefer to stay close to their own mother when offered a choice (Mathews 2011). Premature isolation from the mother then have a negative effect on juvenile growth as reported in red swamp crayfish (Patoka et al. 2015). With respect to marbled crayfish, it is not known whether the clonal offspring can differentiate between the own mother and another genetically uniform mother with the identical reproduction status.

Crayfish plague transmission

The fungal-like organism *Aphanomyces astaci* (Oomycetes) is considered among the world's one-hundred most invasive species (Lowe et al. 2000), causing crayfish plague, which has destroyed a great proportion of the native crayfish populations in Europe (Unestam 1972; Edgerton et al. 2004; Dieguez-Uribeondo et al. 2006) but also susceptible crayfish species worldwide (Hsieh et al. 2016). Currently, five genotypes (A, B, C, D, and E) of *A. astaci* have been recognized using random amplified polymorphic DNA (RAPD) (Svoboda et al. 2017). These genotypes exhibit close association with particular crayfish species and vary in virulence (Makkonen et al. 2012; Viljamaa-Dirks et al. 2013; Becking et al. 2015) as well as climate requirements (Diéguez-Uribeondo et al. 1995; Rezinciuc et al. 2014). Strains from genotype groups A, B, D and E have been identified in crayfish plague epidemics throughout Europe and Japan (Viljamaa-Dirks et al. 2013; Kozubíková-Balcarová et al. 2014; Rezinciuc et al. 2014).

Generally, crayfish of North American origin are resistant to the pathogen (Cerenius et al. 2003) and serve as its vectors in the invaded ecosystems (Kozubíková et al. 2009). The marbled crayfish has been confirmed as a host of the *A. astaci* strain from genotype group D, the group originally isolated from the red swamp crayfish (Keller et al. 2014). New data on the presence of *A. astaci* in the aquarium trade (Mrugała et al. 2015) suggest that *A. astaci* strains can be horizontally transmitted among North American crayfish species. We speculate that the marbled crayfish may soon also host other strains of *A. astaci*, due to its increasing occurrence in natural ecosystems that may be populated by other crayfish species and other *A. astaci* strains (James et al. 2017). This might have devastating consequences for extended East European crayfish stocks (Kotovska et al. 2016; Vodovsky et al. 2017). Evidence of a strain of genotype group E originating from spiny-cheek crayfish (Svoboda et al. 2017) can be expected in marbled crayfish as a result from future co-occurrence of these species (Chucholl et al. 2012; Lipták et al. 2016; Patoka et al. 2016b). Besides the crayfish plague, detection of rickettsial and coccidian-like organisms in the ovary and further organs of marbled crayfish make fears also real for native European species by transmission of pathogens once escaped into the wild (Vogt et al. 2004). In addition, crayfish could be an important vector in the spread of chytrid fungus, *Batrachochytrium dendrobatidis* pathogenic for amphibians (Brannelly et al. 2015). These disease transfers can increase the potential impact of marbled crayfish in the wild.

The marbled crayfish as a biological model

In science, a model organism is an animal, plant or microbe that can be used to study certain biological processes. Model organisms generally grow quickly, are commonly available, and the work with them is relatively simple and inexpensive. The first model organism, small fruit fly *Drosophila*, was introduced in the early nineteenth century by the Columbia University zoologist Thomas Hunt Morgan as an organism to test Darwin's hypotheses (Singer 2016). Over time, model organisms (protists, fungi, plants, invertebrates, and vertebrates) became the irreplaceable tools of fundamental biological and clinical research and helped scientists to amass an enormous amount of knowledge (Hunter 2008). Among invertebrates, decapod crustaceans have been used as models for laboratory-based investigation in many areas of biology, highlighting the prospects for marbled crayfish as a valuable model organism (Faulkes 2015).

Crayfish show complex morphology, development, and behaviour, including elaborate social interactions (Gherardi 2002; Alwes and Scholtz 2006; Vilpoux et al. 2006). Hence, marbled crayfish is ideally suited for physiological, ecological, genetic, developmental, and evolutionary studies representting decapods and other invertebrates. Due to elevated heterozygous ploidy (Gutekunst et al. 2018), parthenogenetic lineages can significantly mitigate the disadvantages of lethal alleles by masking deleterious mutations through the presence of multiple alleles of an affected locus and by increasing functional heterozygosity (Suomalainen et al. 1987; Otto and Whitton 2000; Comai 2005). Therefore, marbled crayfish would be suitable to spell out the association between genetic and epigenetic dissimilarities, the transgenerational inheritance of epigenetic signatures, and the contribution of epigenetic phenotype variation to the establishment of social hierarchies, environmental adaptation and speciation (Vogt 2018a, b).

The marbled crayfish satisfies the basic requirements of a general laboratory animal: ease of culture, suitable size, individual traits, tolerance to handling, high fertility, relatively short generation time, and adaptability to a wide spectrum of environmental and nutritional conditions (Kawai et al. 2016). In addition, they have the particular advantage of genetic identity of mother and all offspring, which is important in investigation of epigenetic aspects of phenotypes (Martin et al. 2007; Vogt 2010, 2018a). Further benefits are direct development, short germ cell development, and stereotyped cell lineage in early development (Seitz et al. 2005; Alwes and Scholtz 2006), allowing precise experimental interventions in developmental studies. Other advantages are morphological characters that are easy to evaluate (Vogt et al. 2008), absence of free-swimming larvae, lack of resting stages, alternation of growth and reproduction phases, indeterminate growth and associated lifelong functionally diverse stem cell systems (Vogt 2010), a broad behaviour repertoire, high regeneration capacity (Vogt et al. 2008), and tissue-invariant housekeeping gene methylation throughout life (Falckenhayn 2017).

The marbled crayfish may be a suitable model intermediate between small short-lived and larger long-lived organisms, since it is small enough to be mass-cultured and sufficiently large to allow individual physiological, biochemical, and behavioural analyses (Vogt 2008a, b, c; Kouba et al. 2010; Kawai et al. 2016). It allows individual longitudinal studies via collection of haemolymph samples or biopsies or by analysing the exuviae of subsequent life stages, to provide a valuable archive of morphological traits inclusive of external sense organs. This character is particularly appropriate for examining fluctuating asymmetry, the right-to-left difference in a trait (Vogt 2010).

The marbled crayfish have advanced circulatory, hormone, and immune systems (Vogt 2002; Gherardi et al. 2010), which, in mammals, are primary targets of age-related alterations. The increased prevalence of cancer seen in mammals due to senescence is rare in crustaceans, even in later stages of life (Vogt 2008a). Tumour formation has not been observed in marbled crayfish (Vogt 2011) so far. Therefore, it is suggested that crustaceans such as marbled crayfish may be appropriate models for studying mechanisms to prevent environmentally induced cancers. The life-span of marbled crayfish is adequate to complete time-consuming processes such as regeneration of damaged body parts, investigation of the effects of chronic doses of toxicants, and alteration of the epigenetic code with age (Vogt 2010).

The marbled crayfish have been exploited to investigate many research goals in e.g. developmental biology (Seitz et al. 2005; Alwes and Scholtz 2006; Jirikowski et al. 2010), neurobiology (Vilpoux et al. 2006; Fabritius-Vilpoux et al. 2008; Rieger and Harzsch 2008), epigenetics (Vogt 2008c; Vogt et al. 2008, 2009), stem cell biology (Vogt 2010), ethology (Vogt et al. 2008; Farca Luna et al. 2009; Jimenez and Faulkes 2010), biogerontology (Vogt 2010), toxicology (Lidova et al. 2016; Koutnik et al. 2017; Velíšek et al. 2018), ecology (Jones et al. 2009; Chucholl and Pfeiffer 2010), or evolutionary biology (Sintoni et al. 2007). Challenges to wider use of marbled crayfish as a model organism remain. Their generation time is relatively long (about 210 days, Seitz et al. (2005) compared to other model species (180 days in *Danio rerio* (Hamilton, 1822), Singleman and Holtzman 2014, and 7–9 days in *Drosophila*, Ashburner and Thompson Jr 1976). Secondly, the mortality rate of the juvenile stage is relatively high (40–50%) (Jimenez and Faulkes 2010) even when grouped with similar-sized individuals at low density (Vogt 2010). Thirdly, the egg contains a large amount of yolk, making it opaque. The long generation time, compared e.g. with fruit flies, may be advantageous for aging and cancer research. The yolk problem can be resolved by dissecting the yolk from embryos prior to fluorescent microscopy, which is highly valuable in studying the dynamics of cell movement and gene expression during development (Fabritius-Vilpoux et al. 2008; Rieger and Harzsch 2008; Jirikowski et al. 2010). The recent exploration of the whole genome sequence of marbled crayfish (Gutekunst et al. 2018) would help to overcome some issues and open a promising new door to study evolutionary biology as well as establish marbled crayfish as a super model organism. Recently published annotated bibliography on marbled crayfish also will help for its broader use (Vogt 2018b).

Conclusions

Within the past two decades, the marbled crayfish has become a promising model organism for laboratory research and at the same time represented an increasing risk as a non-indigenous invasive species. The potential of the unique aspects of its biology needs to be more intensively investigated. It is also important to identify factors triggering variation in growth, behaviour, and reproductive parameters among clones of the same mother. There is a good potential for using marbled crayfish to investigate the physiological, behavioural, and functional responses to stimuli or to assess effects of chemical compounds. In addition, marbled crayfish offers the advantage of using genetically identical organisms for various research topics.

The marbled crayfish is also a highly invasive species. A single female is theoretically sufficient to establish a new population. The European Commission implementing Regulation 2016/1141, among others, prohibits the trade and breeding of marbled crayfish. It allows research with marbled crayfish in secure in-door environments. Hence, the future use of marbled crayfish for research purposes should follow these recommendations for laboratory studies to avoid the possibility of release or escape into the natural environment.

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Compliance with ethical standards

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

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CHAPTER 2

MORPHOMETRY, SIZE AT MATURITY, AND FECUNDITY OF MARBLED CRAYFISH (*PROCAMBARUS VIRGINALIS*)

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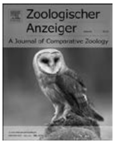

Research paper

Morphometry, size at maturity, and fecundity of marbled crayfish (*Procambarus virginalis*)

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ABSTRACT

Morphometric ratios, relationships and condition factors provide information regarding growth patterns, population dynamics and well-being of individuals in particular environments. Ecology of any species is then better understandable through life history traits and reproductive potential. The present study is aimed at exploring the morphometric and reproductive patterns of the parthenogenetic marbled crayfish, *Procambarus virginalis*. Marbled crayfish exhibited positive allometric growth and statistically significant common length–weight relationships. Abdomen width, claw length, width, and height increase significantly faster than the chosen most robust body size parameter, postorbital carapace length (POCL). Conversely, total length, carapace length, carapace width, and abdomen length grow proportionally slower than POCL. The condition factor was higher than the threshold value of ~1 indicating the well-being of the crayfish used. Marbled crayfish, at their first reproduction, were recorded as long as 42.8 ± 10.8 (31.5–73.5) mm total length with a mean fecundity of 89.72 ± 56.9 (22–349) eggs. The relative fecundity (per 1 mm of POCL) of females reproducing for the first time was significantly lower compared to those reproducing repeatedly. Fecundity and relative fecundity exhibited a significant linear relationship with the carapace length and weight of mothers. As a tool for total fecundity estimation, the number of eggs on the third pair of pleopods was counted and plotted against size of mothers, and total fecundity. The number of eggs attached to the third pair of pleopods very closely correlates with the carapace length, weight and total fecundity. Based on our results, it can be concluded that abdomen width and claw growth is allometric compared to POCL which confirms the importance of these body parts. Fecundity parameters were confirmed to have a tight relationship to marbled crayfish size. Clutch size estimation can be used in future research based on the close correlation of egg counts on third pleopods and total fecundity.

1. Introduction

The length–weight relationship (LWR) is a useful tool for environmental monitoring programs especially for calculating weight at a certain length (Lindqvist & Lahti 1983) as well as providing information on the condition, growth patterns, ontogenetic changes and population dynamics (Oscoz et al. 2005; Simon et al. 2009; Simon & Stewart 2014). It helps to calculate a condition factor which indicates the well-being of the organisms in their habitat where its higher value signals better conditions for an animal (MacGregor 1959). The condition factor of aquatic organisms can be modulated by stress, food availability or habitat quality (Khallaf et al. 2003). LWR and condition factor are regarded as suitable not only for fish, but also crustaceans and are reported in relation to different habitat variables or aquaculture conditions (Acosta & Perry 2000; Mazlum et al. 2007; Weya et al. 2017). Morphometric characters in relation to specific length parameters are not so frequently evaluated in crayfish, while they are among important characters in fish identification. Morphometric traits can be used for evaluation of different importance and use of different body parts during the whole animal life or even during yearly changing conditions (Buřič et al. 2010b). In crayfish, larger claws are beneficial in agonistic encounters, especially during the mating season in males. Females also benefit from larger claws during the reproductive period for defense of the egg clutch or rejection of an

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unwanted male suitor (Bovbjerg 1956; Stein 1976; Buřič et al. 2010b). Beside growth related indices, reproductive patterns relate to some highly important aspects of animal life. Information regarding fecundity is important in understanding the recruitment strategy of a population (Rhodes & Holdich 1979) and also essential for evaluating the commercial potential, life history, practical culture, invasiveness and management of stocks (Gupta & Srivastava 2001; Wang et al. 2011; Hossain 2014). There is a lack of information on basic life history data for most crayfish species (Moore et al. 2013) and morphometric patterns and relationships are often limited (Stein 1976; Romaire et al. 1977; Rhodes & Holdich 1984; Garvey & Stein 1993) except for commercial species (Mazlum et al. 2007; Wang et al. 2011).

The marbled crayfish, *Procambarus virginalis* Lyko, is an enigmatic obligatory apomictic parthenogenic species possessing exclusively female individuals (Martin et al. 2007; Vogt et al. 2008; Martin et al. 2010), first explored from the German aquarium trade in the mid-1990s (Scholtz et al. 2003). It is now reported in many waterbodies, not only in Europe (Patoka et al. 2016; Gutekunst et al. 2018). Fast growth, early maturation, high fecundity, short embryogenesis and frequent spawning (Seitz et al. 2005), together with an ability to withstand drought conditions (Kouba et al. 2016) and low winter temperatures (Veselý et al. 2015), point to its low demands for living conditions (Jimenez & Faulkes 2010). It is also capable of successfully competing with other species (Jimenez & Faulkes 2010; Hossain et al. 2019) and can transmit the crayfish plague pathogen (Keller et al. 2014). All these features define the species as a serious threat to native crayfish but also for other representatives of the freshwater biota (Feria & Faulkes 2011; Faulkes et al. 2012; Kawai et al. 2016). Aside from the possible threats which marbled crayfish entail for freshwater ecosystems around the world, it has great potential to become a model organism (Hossain et al. 2018) due to the production of clones of individuals (Vogt 2011; Vogt 2018). If marbled crayfish would be used more broadly as model species in the future, there is a need to recognize all basic biological patterns of this species (Gutekunst et al. 2018). On the other hand, the European Commission Implementing Regulation 2016/1141, among others, must be mentioned, currently prohibiting the trade and breeding of this crayfish. Therefore, propagation for research or commercial use must be carefully regulated, to avoid possible releases or escapes into the natural environment.

There are still gaps in knowledge about morphometric and reproductive patterns of this species. For example, the fecundity of marbled crayfish has been observed by several authors from laboratory culture and free-living populations (Vogt et al. 2004; Seitz et al. 2005; Jones et al. 2009; Chucholl & Pfeiffer 2010; Vogt 2010) but knowledge remains limited on the relationships between fecundity and body size or weight. Therefore, there is an opportunity to investigate the basic phenomenon of growth and fecundity in this species, prior to its wider use as a model species. Aims of this study were to investigate the correlations between different morphometric patterns, fecundity related to body size and weight of marbled crayfish at first and repeated spawning.

2. Material and methods

2.1. Experimental animals

Marbled crayfish specimens originated from our own laboratory culture held at the Research Institute of Fish Culture and Hydrobiology in Vodňany, FFPW USB, Czech Republic. Animals were maintained in homogenous environmental conditions (light regime of 12L: 12D, water temperature ~20 °C, fed in excess) in culture tanks until morphometric measurements and fecundity observations were conducted.

2.2. Measurements of animals

Crayfish (n = 132) were placed on a tissue paper to remove excess water prior to measurements and then weighed using a digital balance (AND HR 200) with an accuracy of 0.1 mg. Crayfish were sacrificed in a freezer at −20 °C and stored there for several days. Thawed specimens were photographed with a fixed scale and measured by picture analysis using the software QuickPHOTO 2.3 (Olympus, Lens, France). Visibly deformed individuals and individuals with missing and regenerating body appendages were not included. According to Sint et al. (2005) and Buřič et al. (2010a), the following indices were measured: abdomen length, abdomen width, width at the hind edges of the carapace, width at the cervical groove, claw height, claw length, claw width, carapace height, carapace width, head width, telson length, carapace length, postorbital carapace length, and total length (Fig. 1), to determine some relationships among the measurements.

The ratios of all measured length and width parameters (ABL, ABW, CEW, CGW, CL, CLH, CLL, CLW, CPH, CPW, HEW, POCL, TEL, and TL) were calculated in respect to post-orbital carapace length (POCL). POCL is considered as the least variable and most rigid size parameter which is suitable for this analysis in crayfish (Buřič et al. 2010a; Buřič et al. 2010b). Ratios of CLW/CLL, CPW/CL, and ABW/ABL were also calculated to determine the robustness of the body parts. The ratios were then plotted against the POCL to estimate the possible allometric growth of different body parts, following Buřič et al. (2010a). Similarly, length and weight data were also fitted in the exponential equation of Le Cren (1951) after log transfer to find a linear relationship and indicator of growth pattern, b value (Riedel et al. 2007).

The condition factor ‘K’ refers to the robustness, or the total wellbeing of the crayfish. Condition factor was determined by the following formula:

$$K = 100W/TL^3$$

where W = weight (mg), and TL = total length (mm) (Ricker 1975).

2.2.1. Fecundity

The fecundity was observed in randomly collected ovigerous specimens of marbled crayfish at the first week of incubation. In total, 241 females were evaluated, among them 107 females

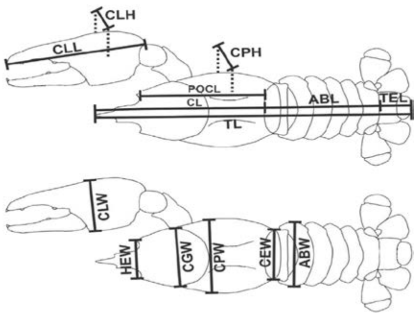

Fig. 1. Outline of morphometric measurement of experimental animals. ABL – abdomen length, ABW – abdomen width, CEW – width at the hind edges of the carapace, CGW – width at the cervical groove, CL – carapace length, CLH – claw height, CLL – claw length, CLW – claw width, CPH – carapace height, CPW – carapace width, HEW – head width, POCL – postorbital carapace length, TEL – telson length, TL – total length. Adapted from Sint et al. (2005) and Buřič et al. (2010b).

reproducing for the first time, and the rest, 134 females, who had reproduced repeatedly. The number of eggs on the 3rd pair of pleopods and the total pleopodal fecundity was recorded to find a relationship between total fecundity and the number of eggs on the 3rd pleopods. In these females, wet weight (W, to the nearest 0.1 g) and carapace length (CL, to the nearest 0.1 mm) were determined by an electronic balance (Kern & Sohn GmbH, Balingen, Germany) and vernier caliper, respectively. The number of eggs per 1 mm of CL was calculated in each crayfish in order to gain information about the relative fecundity (i.e. egg carrying capacity of females at different sizes).

2.3. Statistical analysis

Statistical analyses were performed using Statistica 13.2 software (StatSoft Inc., Tulsa, USA) and Microsoft Excel. Normality was tested for the data set. The biometric and fecundity parameters were evaluated through linear regression analysis by plotting dependent and independent variables against each other. The relative fecundity of the first and following spawners was compared using student's *t*-test. The null hypotheses were tested at $\alpha < 0.05$ in all tests of this study. Data were presented as mean ± SD.

3. Results

3.1. Morphometric ratios

The ratios between POCL and other morphometric parameters of marbled crayfish had significant positive or negative ($P < 0.05$) relationships (Fig. 2) which interpret the different growth of particular body parts with increasing body size, i.e. allometry. The ABW/POCL ($F = 32.8$; $P < 10^{-4}$), CLL/POCL ($F = 62.4$; $P < 10^{-4}$), CLW/POCL ($F = 198.9$; $P < 10^{-5}$), CLH/POCL ($F = 57.2$; $P < 10^{-3}$), HEW/POCL ($F = 20.6$; $P < 10^{-4}$), CGW/POCL ($F = 3.9$; $P = 0.048$), and ABW/ABL ($F = 177.8$; $P < 10^{-5}$) are positively correlated with increasing POCL, which means that these body parts grew faster than POCL. TL/POCL ($F = 85.09$; $P < 10^{-3}$), CL/POCL ($F = 12.8$; $P < 10^{-3}$), TEL/POCL ($F = 26.7$; $P < 10^{-4}$), HEL/POCL ($F = 4.9$; $P = 0.03$), ABL/POCL ($F = 46.7$; $P < 10^{-3}$), CPW/POCL ($F = 5.1$; $P = 0.03$), CPH/POCL ($F = 61.5$; $P < 10^{-4}$), and CEW/POCL ($F = 6.8$;

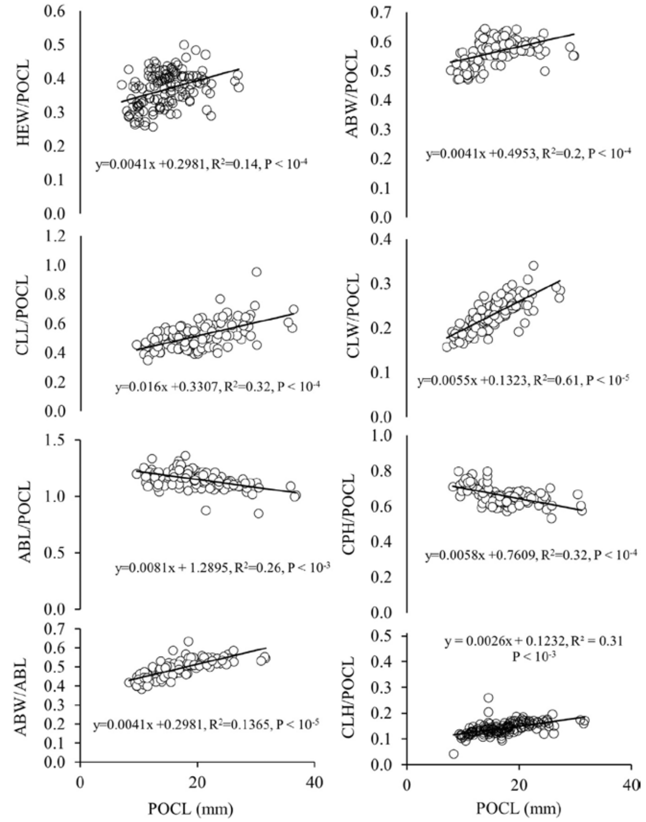

Fig. 2. Relationships of marbled crayfish body ratios to postorbital carapace length (POCL). CLL, claw length; CLW, claw width; ABL, abdomen length; ABW, abdomen width; HEW, head width; CPH, carapace height; CLH, claw height. Coefficients of determination and P-values are shown in the figure. The number of crayfish was 132.

$P = 0.01$) have negative relationships with increasing POCL. Eight ratios are selected to show the growth trends in strategic body parts of crayfish (claws, abdomen, carapace and head width) (Fig. 2). The mean values with standard deviation and range are described in detail in supplementary Table S1.

3.2. *Length–weight relationship and condition factor*

Length (TL) and weight (W) of 132 specimens, in average 50.2 ± 12.8 mm and 3230 ± 2580 mg respectively, were log transformed to plot regression lines. The length-weight regressions were found to be highly significant ($F = 2002.1$, $P < 10^{-6}$) (Fig. 3). The 95% confidence limits of ‘b’ values ranged between 2.70 and 2.95. The condition factor (K) value ranged from 1.36 to 3.91 with a mean of 2.14 ± 0.33.

3.3. *Fecundity*

The mean 3rd pleopodal and total fecundity of maiden marbled crayfish vary significantly (t-test = −8.15, $P < 10^{-6}$; t-test = −7.45, $P < 10^{-6}$, respectively) from the parameters of repeatedly reproducing marbled crayfish, while the percentage of egg on 3rd pleopods did not differ significantly (t-test = 0.96, $P = 0.33$) between the first and repeated spawners. Marbled crayfish first spawned at a weight of 0.73 g (2.19 ± 1.38) when carapace length was 14 mm (19.7 ± 3.7) and total length 31.5 mm (42.8 ± 10.8). The average and highest fecundity of marbled crayfish at first clutch were 89.7 ± 56.9 and 349 eggs, respectively. Average and highest fecundity in repeated spawners were 155.3 ± 75.6 and 524, respectively.

Fecundity was also recalculated to get relative fecundity, expressed as the number of eggs per 1 mm of CL for females at their 1st spawning and females at repeated spawning. This relative fecundity varies significantly (t-test = −7.49, $P < 10^{-6}$) between groups (Fig. 4). Statistically significant positive correlations were observed between the fecundity per 1 mm of CL with increasing weight (F-test = 310.6, $P < 10^{-5}$) and CL (F-test = 341.8, $P < 10^{-5}$ (Fig. 5).

Statistically significant positive linear relationships were observed when fecundity (F) was plotted against weight (W) ($P < 10^{-5}$; $R^2 = 0.75$) and carapace length (CL) ($P < 10^{-6}$; $R^2 = 0.74$) of marbled crayfish (Fig. 6).

A very strong and statistically significant positive linear relationship between the total fecundity and the number of eggs on the 3rd pair of pleopods (F3P) was observed. Relative F3P (%) significantly decreased with increasing total fecundity (Fig. 7).

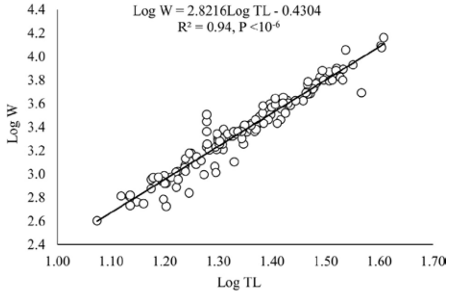

Fig. 3. Linear relationship between total length (TL) and body weight (W) of marbled crayfish. Coefficients of determination, P-value and linear equation are shown in figure. W, weight; TL, total length.

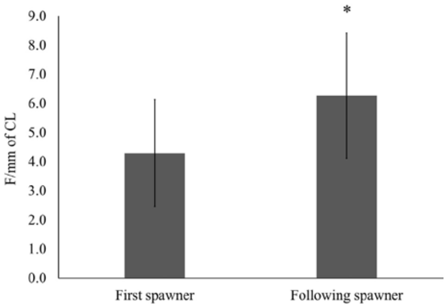

Fig. 4. Relative number of eggs (F) per 1 mm of carapace length (CL) in maiden females (first spawner) and repeatedly spawned (ongoing spawner) females of marbled crayfish. F, fecundity; CL, carapace length. Values are presented as mean ± SD. Asterisk symbol indicates the statistically significant differences at $\alpha < 0.05$.

4. Discussion

The importance of marbled crayfish is increasing due to its easy availability in the aquarium trade, currently spreading in natural habitats worldwide and its invasive characteristics (Kawai et al. 2016; Patoka et al. 2016). Thanks to low requirements of this species, its culture is very simple enabling fast establishment among hobbyists as well as in natural habitats worldwide (Jimenez & Faulkes 2010; Faulkes 2015). In aquarium culture, the marbled crayfish is reported to grow best at 20 °C and 12L:12D photoperiod conditions, with several reproductions per year (Seitz et al. 2005; Vogt et al. 2015). Moreover, it is able to withstand winter temperatures in the temperate zone and successfully reproduce there (Veselý et al. 2015; Lipták et al. 2017).

Body weight and total length, carapace length and carapace width are the most frequently used dimensions in studies of crustaceans (Atar & Seçer 2003), while postorbital carapace length (POCL) is considered as the least variable body size related parameter which can be therefore used to compare with other body parts (Buřič et al. 2010a). The ratios of body parts related to POCL provide information concerning the robustness and relative growth of different body parts. Different growth of particular body parts is related to different importance of these body parts during ontogenesis of an animal (Stein 1976; Buřič et al. 2010b). Anderson & Simon (2015) showed that body morphometry also varies in terms of native and invaded ranges of individuals which are related to the invasive potential of crayfish. In our study, the growth of the abdomen in all female crayfish increases in width, while abdomen length shows a negative relationship with increasing POCL. Generally, all females have wider abdomens than the males of the same species (Hamr & Berrill 1985; Buřič et al. 2010b), which tightly correlates with the need to provide more space for their upcoming eggs and offspring. A broad abdomen is an effective shield providing protection for attached eggs (Holdich 2002a). In ovigerous females, widening of the abdomen (ABW) reflects a sexually active female that is correlated with either swollen or white glair glands, dependent offspring, or remnants of egg stalks attached to pleopods (Wetzel 2002). Besides reproductive benefits (Smith 1981), a broad abdomen could aid in aggressive behaviour during fighting (maintaining stability during aggressive

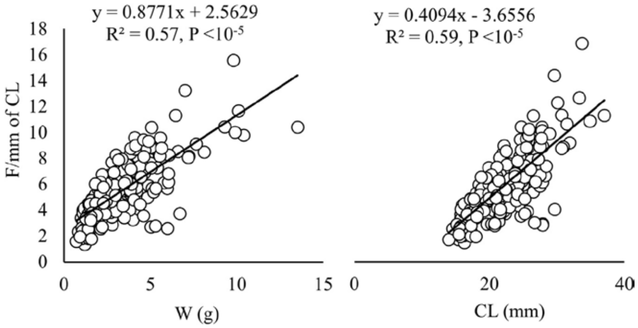

Fig. 5. Regression equation between the fecundity (F) per 1 mm of carapace length (CL) and weight (left) and carapace length (right) of the marbled crayfish. Coefficients of determination, equations of linear relationship and P-values are shown in the figures.

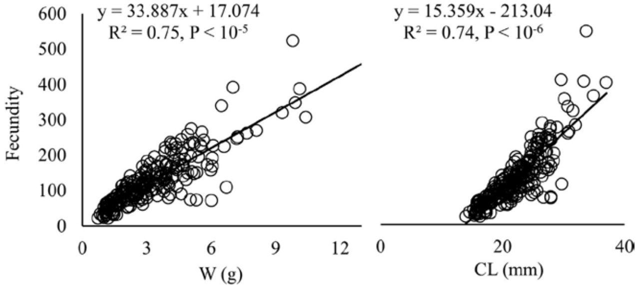

Fig. 6. Linear relationships of total pleopodal fecundity on weight (W) and carapace length (CL) in the marbled crayfish. Coefficients of determination and linear equation are shown in the figures.

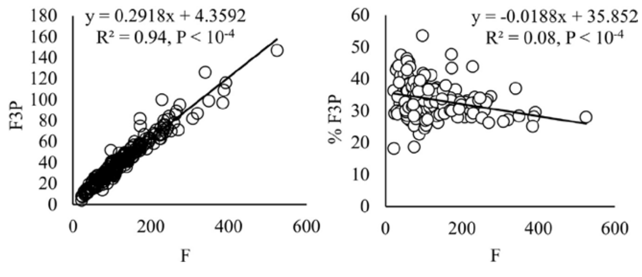

Fig. 7. Linear relationship between the total pleopodal fecundity (F) and the number of eggs on the 3rd pair of pleopods (F3P) (left), and relationship of percentage of eggs on 3rd pleopods and total fecundity (right) of marbled crayfish. Coefficients of determination, linear equations, and p-values and are shown in the figures.

interactions). Nevertheless, abdomen growth is not as important as overall growth of body size in this case.

Claws of decapods are excellent models for morphologic studies due to their unique structure and a variety of functions (Lee 1995). In fiddler crabs (*Uca pugnax*) the major chelipeds are related to social behaviour and mating while minor ones are associated with feeding (Rosenberg 1997). As in crabs, dimorphism of crayfish claws may be linked with prey capture and manipulation, burrowing, defence against predators, inter- and intra-specific interactions, and reproduction (Holdich 2002b; Buřič et al. 2010a; Kouba et al. 2016). In the present study claw length, width, and height were observed to grow significantly faster than size of crayfish, i.e. claws becomes bigger compared to body size. In crayfish *Austropotamobius pallipes*, the claws show positive allometric growth, not isometric (Rhodes & Holdich 1979), which coincides with our results. Larger claws then allow better competitiveness for basic resources, in predation, agonistic encounters, and reproduction (Garvey & Stein 1993) as well as aid in chemoreception (Belanger et al. 2008). In marbled crayfish, robust claws at a larger size might be mostly associated with interspecific or intra-specific competition for basic resources (shelter, food etc.). Nakata & Goshima (2003) confirmed that larger claws are helpful to overpower opponents in aggressive interactions under restricted resources conditions. Total length, carapace length, telson length, abdomen length and carapace width also did not increase proportionately with the POCL (Fig. 2). This might be related to the length of the rostrum, because rostrum length decreases proportionately with the larger size of individuals (Buřič et al. 2010a).

The LWR relationships demonstrate the positive allometric growth pattern in marbled crayfish. Similarly, Anderson & Simon (2015) observed positive allometric growth in both sexes and all male forms including juveniles of rusty crayfish (*Faxonius rusticus*) in respect to carapace length and weight. Holdich (2002a) described allometric growth in adult crayfish only, not in juveniles. Nevertheless, some authors also recorded both isometric and allometric growth in cases of crayfish in different regions and habitats of the world (Wang et al. 2011; Qoraychy et al. 2015). These differences may reflect the cumulative effect of different factors including photoperiod, population density, food abundance, water level fluctuations, water temperature, and water quality (Huner & Barr 1991; Lindqvist & Huner 1999). The condition factor of individuals fluctuates with some variation but in all cases exceeded the value 1.36, where condition factor value > 1 indicates the wellbeing of crayfish in their environment (Weya et al. 2017). It means that our culture conditions can be described as favorable for marbled crayfish growth and values obtained can be used for comparison with animals living in natural conditions where crayfish are faced with numerous environmental factors and stressors. Crayfish in natural conditions can therefore be limited in their growth and development, but the situation can also be opposite in favorable conditions (Acosta & Perry 2000).

More important than growth patterns is the ability to successfully reproduce. Fecundity of crayfish is mostly estimated in terms of number of pleopodal eggs (Tropea et al. 2012), but can be assessed also as the number of oocytes, number of freshly spawned eggs, number of eggs attached in different stages of development or as the number of juveniles in particular developmental stages (Kozák et al. 2006). In the present study, total fecundity is the sum of all eggs attached to the female's pleopods in the first week of their incubation, to rule out the effect of possible eggs losses during incubation. In the marbled crayfish first reproduction is reported at a total length of about 40 mm in lab culture and wild condition, with the maiden fecundity ranging between 10 and 416 (Vogt et al. 2004; Seitz et al. 2005; Kozák et al. 2015; Cvitanić et al. 2016). In the present work, the smallest spawning female was reported at 31.5 mm TL. However, size at first reproduction varied widely from 31.5 to 73.5 mm of TL (weight 0.7–9.9 g), with the fecundity ranging between 22 and 349 eggs. Age and size at first reproduction is a tremendously significant life-history attribute, influenced by several nutritional and environmental factors (e.g. temperature, food, season) (Baker et al. 2003). The size differences of animals at the first spawning is worth exploring when taking into account that crayfish were reared in largely comparable conditions including light regime, temperature, rearing densities and feeding. In addition, all marbled crayfish are genetically identical, so the detected variability can be explained by different life strategies of individuals or their different positions in stock hierarchy. Besides the size at maturity, clutch size is larger in larger and older crayfish (Abrahamsson 1971; Seitz et al. 2005; Alcorlo et al. 2008). Also in this study, maiden females have significantly lower relative fecundity (fecundity recalculated to 1 mm of CL) than that observed in repeatedly reproducing females. The explanation should be increased experiences of females which reproduced repeatedly, or incomplete development in first spawning females. However, the general pattern that larger crayfish have more eggs than smaller ones (Harlioğlu & Türkgülü 2000) was confirmed. Several authors suggested that pleopodal egg number increases with female length and weight (Nakata & Goshima 2004; Tropea et al. 2012) and has significant correlation between absolute fecundity and total body length, cephalothorax length, and body weight (Chybowski 2013). These relationships are most often defined by either linear or power equations (Nakata & Goshima 2004; Kozák et al. 2006; Chybowski 2007). To enable a precise fecundity estimation without destroying the whole clutch we calculated the relationship of the number of eggs on 3rd pair of pleopods and total fecundity. It shows a very tight linear relationship regardless of fecundity variability, suggesting that fecundity of similarly sized females, for example, with a hypothetical fecundity of 50 and 100 eggs can be estimated very precisely by the equation provided. This estimation method is therefore also usable with a female that had partially lost its clutch. It can therefore be a good tool for fecundity estimation in studies using marbled crayfish as a model species or in studying its population dynamics.

The results reached can support the use of marbled crayfish as a model species in various research disciplines (Hossain et al. 2018). Parameters evaluated in the present study can be used e.g. for detection of effects of environmental pollution on such basic manifestations of life as growth, development and reproduction.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcz.2019.06.005.

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CHAPTER 3

INTERACTIONS OF MARBLED CRAYFISH WITH OTHER CRAYFISH INVADERS IN EUROPE

3.1. Hossain, M.S., Kubec, J., Kouba, A., Kozák, P., Buřič, M., 2019. Still waters run deep: marbled crayfish dominate over red-swamp crayfish in agonistic interactions. Aquatic Ecology 53, 97–107.

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

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Still waters run deep: marbled crayfish dominates over red swamp crayfish in agonistic interactions

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Abstract Intra- and interspecific interactions contribute to the successful establishment and consequent spreading of species in the environment, which became particularly apparent in the context of ongoing biological invasions. The parthenogenetic marbled crayfish, *Procambarus virginalis*, Lyko 2017 is recently recognized as an emerging invader due to its high adaptability, fast growth, early maturation, and high fecundity. The present study explored the interaction patterns of size-matched (including 15 body parts morphometry evaluation) pairs of marbled crayfish and red swamp crayfish *Procambarus clarkii*, a well-known highly aggressive and widely distributed invader of freshwater ecosystems. Despite this, marbled crayfish won significantly more fights and establish dominancy in more cases in both premature and mature experimental trials. Premature red swamp crayfish pairs were more active in contact and fight initiation than mature. In mature, the dominance over female red swamp crayfish was 100%, in males it reached 60%. Premature marbled crayfish dominated in more than 75% pairs. Agonistic behaviour and intensity of fights significantly dropped after establishment of dominance in particular (size and sex) pairs. Therefore, we confirmed that sex and age (size) have effects on agonistic behaviour in crayfish as well as the dominance of marbled crayfish within similarly sized specimens. Despite described behavioural patterns, we can expect that the situation in the potential sympatric occurrence of both species will not be as clear as found in experimental conditions due to greater maximal size of red swamp crayfish.

Keywords Competition · Dominance · Interaction · *Procambarus virginalis* · *Procambarus clarkii*

Introduction

Agonistic interactions (i.e. antagonism and aggression) are the key aspects of animal behaviour (Parker 1974; Manning and Dawkins 2012) contributing to a success of species in the environment. Aggressive behaviour occurs during the entire life span of crayfish, and is associated with an invasion success (Sih et al. 2012). Their importance often arise up when animals forage in overlapping ecological niches and

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use identical resources, e.g. food and shelter (Wilson 2000; Huntingford 2013). Agonistic behaviour is primarily demonstrated by increased aggressiveness between participants of conflict (Gherardi 2002) and boosts success in coping with novel predators and competitors (Duckworth 2008; Hudina and Hock 2012). The non-native species that persist, establish, and become invasive can cause negative effects on economy, native biota and ecosystem functioning (Kolar and Lodge 2001).

The mysterious, obligate apomictic parthenogenetic marbled crayfish *Procambarus virginalis*, Lyko 2017 which has no male individuals (Martin et al. 2007; Vogt et al. 2008) and native range (Taylor et al. 1996; Martin et al. 2016) were firstly discovered in the German aquarist trade in the mid-1990s (Scholtz et al. 2003). Their fast growth, early maturation, high fecundity and frequent spawning (Seitz et al. 2005), tolerance to various environmental conditions facilitate their spread over many localities over the Europe during the last decade through human translocation (Hossain et al. 2018). Due to unintentional or intentional releases by hobbyists marbled crayfish have been reported from natural conditions in the Netherlands (Souty-Grosset et al. 2006), Germany (Chucholl et al. 2012; Chucholl 2015), Italy (Nonnis-Marzano et al. 2009; Vojkovská et al. 2014), Czech Republic (Patoka et al. 2016), Slovakia (Janský and Mutkovič 2010; Lipták et al. 2016), Hungary (Lőkkös et al. 2016), Sweden (Bohman et al. 2013), Madagascar (Jones et al. 2009), Japan (Kawai and Takahata 2010), Croatia (Samardžić et al. 2014), Ukraine (Novitsky and Son 2016), Hungary (Lőkkös et al. 2016), Estonia (Estonian Research Council 2018) and Romania (Pârvulescu et al. 2017).

Marbled crayfish have higher potential to be invasive than expected in the past (Kawai et al. 2016) being able to withstand desiccation by burrowing (Kouba et al. 2016) and winter temperatures of temperate climate (Veselý et al. 2015), which could pose a great threat for native crayfish and other representatives of freshwater biota (Faulkes et al. 2012; Kawai et al. 2016). However, ecological impacts are hypothesized rather than demonstrated. It is only assumed that marbled crayfish can be a perfect invader for its high reproductive performance (Jones et al. 2009) and ecological adaptability (Kawai et al. 2016). Marbled crayfish engaged in intraspecific fight (Farca Luna et al. 2009), in groups or in resourceless environment, and establish dominance hierarchies like other decapods of sexual species (Bovbjerg 1953; Huber and Kravitz 1995; Issa et al. 1999; Herberholz et al. 2007) although exhibit less aggressiveness than other crayfish (Vogt et al. 2004).

Recently, Jimenez and Faulkes (2011) showed that the marbled crayfish is able to compete with the red swamp crayfish *Procambarus clarkii,* the utmost successful crayfish invader around the world (Souty-Grosset et al. 2016). Nevertheless, the authors examined nearly adult marbled crayfish (in accordance with size used) interaction with only intermolt premature group of red swamp crayfish. Moreover, the pairs used were only roughly size-matched where difference was about 10–20% in size. Hence, this study aims on the revision of observed patterns together with gaining results from two size groups of interacting pairs (using size-matched individuals with detailed morphometry of used animals) and evaluation of more behavioural characteristics. The present study is therefore designed to clear discover the interaction patterns of the emerging invasive crayfish with highly aggressive and widely established invader.

Materials and methods

Experimental animals and measurements

Marbled crayfish and red swamp crayfish originated from our own laboratory culture reared at the same environment, having the similar social experience from their monospecific culture tanks (temperature was maintained at 20 °C and crayfish were fed daily in excess by commercial dry feed for aquarium fish enriched with algae). For experimental work, we selected two size groups representing premature (yet non-reproducing and without developed glair glands) and mature (reproducing) of both species in January 2017. In juveniles or premature animals are the morphological and behavioural differences between sexes determined commonly as minimal or unimportant (Gherardi 2002; Reynolds 2002). Only mature red swamp crayfish were therefore paired considering also the sex since it has significant effect on dominant status in adults (Bovbjerg 1956; Pavey and Fielder 1996). The size of individuals used in interaction pairs was matched prior experiment to be at lower difference than 5% of wet weight (Aquiloni and Gherardi

2008; Momohara et al. 2013) despite the fact that some previous studies were less strict in this regard (Hudina et al. 2011; Jimenez and Faulkes 2011). The carapace length (CL) and weight of live crayfish used in experiments are shown in Table 1. The values did not differ in both, premature (*t* test, $t_{CL} = 0.53$, $P_{CL} = 0.60$; $t_W = -0.12$, $P_W = 0.91$) and mature animals (*t* test, $t_{CL} = 1.09$, $P_{CL} = 0.28$; $t_W = -0.03$, $P_W = 0.98$).

Acclimation and experimental set-up

In order to avoid the effects of previous hierarchical status in the source stocks, all selected specimens were kept individually in separated plastic boxes for 7 days (individuals cannot see each other during this acclimatization period) prior the experiment. During this period, the water temperature was maintained at 20 °C, and crayfish were fed daily in excess by commercial dry feed for aquarium fish enriched with algae (Sera Granugreen, Sera GmbH, and Germany).

After acclimation period, crayfish pairs were stocked into the rectangular experimental arenas (Steele et al. 2018). Smaller arena for premature animals (265 × 185 × 70 mm in size) was filled by 2 l of aged tap water (~ 20 °C) and 0.2 l (376 g of dry weight) of fine sand (particle size < 1 mm). Arena for mature animals (400 × 250 × 200 mm in size) was filled by 6 l of aged tap water (~ 20 °C) and 0.4 l (752 g of dry weight) of fine sand. Crayfish were stocked under transparent perforated plastic cups to acclimate to experimental water conditions for 5 min (Buřič et al. 2018). Then perforated cups were removed to enable crayfish to move and interact freely with each other in experimental arena. The experimental period lasted for 15 min (Steele et al. 2018). Crayfish in experimental arenas were recorded using digital video camera (Sony HDR-CX240, Sony, Japan). In total, 10 and 17 size-matched pairs were recorded for premature and mature animals, respectively. In mature animals, 9 males and 8 females of red swamp crayfish were used.

Data were evaluated later using both visual and automatic analysis of obtained recordings. Visual evaluation was carried out to evaluate the number of contacts, threats, the number of fights, fight duration, to recognize fight initiator, fight winner, avoidance behaviour, and tail flips (Table 2). The dominance establishment was determined as the time where the last underwent agonistic event lead to further

Table 1 Carapace length (CL) and weight (*W*) of premature and mature *P. virginalis* and *P. clarkii* used in interaction pairs

Experimental group	Species	CL (mm)	*W* (g)	*D* (%)
Premature	Marbled crayfish	16.07 ± 0.53	0.96 ± 0.09	2.65 ± 0.57
		(13.65–18.13)	(0.54–1.35)	(0–4.65)
	Red swamp crayfish	16.45 ± 0.50	0.94 ± 0.08	
		(14.38–19.25)	(0.55–1.27)	
Mature	Marbled crayfish	27.37 ± 1.36	4.81 ± 0.94	2.13 ± 0.49
		(22.32–34.47)	(2.28–11.38)	(0.24–4.60)
	Red swamp crayfish—males	29.01 ± 1.59	4.84 ± 0.95	
		(23.78–38.35)	(2.39–11.6)	
	Marbled crayfish	30.68 ± 1.11	5.66 ± 0.53	2.45 ± 0.73
		(24.93–35.57)	(3.94–7.93)	(0.19–4.68)
	Red swamp crayfish—females	32.03 ± 0.91	5.67 ± 0.53	
		(27.94–35.93)	(4.12–7.90)	
	Marbled crayfish	28.93 ± 0.95	5.41 ± 0.74	2.28 ± 0.42
		(22.32–35.57)	(2.28–11.38)	(0.19–4.68)
	Red swamp crayfish—in total	30.43 ± 0.99	5.39 ± 0.73	
		(23.78–38.35)	(2.39–11.60)	

Last column shows the average percent weight difference (*D*) between individuals in particular pairs. Data are presented as mean ± SE and as the range (minimum–maximum) in parentheses

Table 2 Definition of agonistic interaction pattern of *P. virginalis* and *P. clarkii.* Modified from Sato and Nagayama (2012), Aquiloni et al. (2008) and (Fort et al. 2019)

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 remained motionless. In the very few cases when both individuals retreated simultaneously no winner was scored
Fight	Crayfish make contact with pushing or slight pinching with claws or direct contact and biting with clear intent to inflict injury
Fight initiator	Crayfish which one start first the fight activity recognize as a fight initiator
Tail flip	Rapid abdominal flexions results powerful backward swimming stroke

persistent avoidance of one opponent to fight, contact or stay in the proximity (one body length) of the dominant crayfish. To support this determination, the number of fights won and fights initiated were also taken into account.

Ethical statement

The study did not involve endangered or protected species. No specific permissions were required for the locations and activities involved in this study. It must be mentioned that both species used are involved to a species list of European Commission Regulations (EU Regulation No. 1143/2014 and Commission Implementing Regulation No. 2016/1141). That means that the import, trade, keeping and breeding of marbled crayfish is prohibited in the European Union (EU). Keeping for research is allowed but requires permission and should be done in proper way to avoid possible escapes into the natural environment (Hossain et al. 2018). However, Czech Republic still not has the national implementation of regulations mentioned above, and there are no permissions needed for research organisations yet. All experimental manipulations (rearing, capture and measurements) were conducted according to the principles of the Ethical Committee for the Protection of Animals in Research of the University of South Bohemia, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, Vodňany, based on the EU harmonized animal welfare act of Czech Republic. The above-named Institutional Animal Care and Use Committee (IACUC) specifically approved this study. The principles of laboratory animal care and the national laws 246/1992 and regulations on animal welfare were followed (Ref. No. 22761/2009-17210).

Statistical analysis

Statistical analyses were performed using Statistica 13.2 software (StatSoft Inc., Tulsa, USA). Chi-square (χ^2) test was used to test expected frequencies of interactions outcomes with observed values (contact initiator, fight initiator, avoidance, winner and dominance establishment) according to hypotheses of experiment. Fight number, duration and latency to first fight were analysed using student *t* test. Tailflip and retreat were analysed through nonparametric Mann–Whitney U test. The null hypothesis was rejected at $\alpha < 0.05$ in all tests of this study. Data were presented as mean ± SE.

Results

Dominance establishment

There were no significant differences were observed among the measured parameters in the species compared except the abdomen width (Table S1). In premature animals, the dominance was clearly established in 90% of pairs observed. In this group, marbled crayfish dominated over red swamp crayfish significantly (χ^2 test = 30.91; $P < 10^{-3}$). In mature animals group, 52.9% of pairs showed no defined dominancy over each other. While among the rest (i.e. 8 pairs where dominance was established) in mature animals, marbled crayfish governed a dominating role in greater percentage than red swamp crayfish (χ^2 test = 25.0; $P < 10^{-4}$). When the sex of red swamp crayfish is taken into account, marbled crayfish dominate in all female–female pairs (χ^2 test = 100.0; $P < 10^{-4}$). In the case of male–female pairs, the situation was more balanced, but the overweight of marbled crayfish was still evident (χ^2 test = 4.0; $P = 0.045$). Data are described in more details in Table 3.

Latency to start first fight was similar among experimental groups (t test = 0.52; $P = 0.600$), though mature pairs tend to initiate fight slightly earlier than prematures. However, total fights duration (t test = -2.85; $P = 0.010$) and number of fights per pair (t test = -2.46; $P = 0.020$) differed significantly between premature and mature animals. Though the mean fight duration showed large variation between the groups, statistically there was no significant variation (t test = -1.82; $P = 0.08$) (Table 4). The difference in crayfish behaviour was detected in the pairs after the dominance establishment (Table 5) with the number of fights significantly dropped down in both premature (t test = 9.44; $P < 10^{-3}$) and mature animals (t test = 3.71; $P = 0.003$). The mean fight duration was significantly lower after dominance establishment in premature (t test = 3.04; $P = 0.010$) and mature animals (t test = 2.36; $P = 0.040$).

Agonistic encounters and contacts

In the premature animals, red swamp crayfish was significantly more active in contact initialization (χ^2 test = 4.796; $P = 0.029$), while in fight initialization, it also showed higher number while not significant (χ^2 test = 0.34; $P = 0.560$). On the other hand, marbled crayfish won significantly more fights over red swamp crayfish (χ^2 test = 20.04; $P < 10^{-3}$); thus the avoidance to meet the opponent was higher in the latter (χ^2 test = 6.44; $P = 0.010$).

In mature animals, the situation was different in the case of contact (χ^2 test = 0.52; $P = 0.470$) and fight initialization (χ^2 test = 5.29; $P = 0.020$), where marbled crayfish were significantly more active. Similarly as in premature, marbled crayfish were more successful in the fights won (χ^2 test = 30.78; $P < 10^{-4}$), with significantly more avoidance events in the mature red swamp crayfish (χ^2 test = 9.85; $P = 0.002$). In addition, mature marbled crayfish also won significantly more fight, when grouped and analysed with considering the male and female of red swamp crayfish (χ^2 $\text{test}_{\text{Male}}$ = 232.79; $P < 10^{-4}$ and χ^2 $\text{test}_{\text{Female-}}$ = 208.84; $P < 10^{-4}$). These differences are shown in Figs. 1, 2 for premature and mature group (sex together) and for mature group when sexes are considered, respectively. Tailflip (premature: 1.7 ± 0.3; adult: 1.1 ± 0.1) (U = 13.5, $P = 0.2$) and retreat (premature: 2.0 ± 0.6; adult: 2.6 ± 0.7) (U = 16.0, $P = 0.6$) in interacting pair were not significantly vary among premature and adult groups.

Table 3 Percentage of premature and mature *P. virginalis* and *P. clarkii* pairs with established and not defined dominance, and percentage of dominant specimens of each species in pairs where dominance was established

Group	Species	Established dominance (%, *n*)	Not defined (%, *n*)	Dominant from established (%)
Premature	Marbled crayfish	70.0 (7)	10.0 (1)	77.8^{a}
	Red swamp crayfish	20.0 (2)		22.2^{b}
Mature	Marbled crayfish	33.3 (3)	44.5 (4)	60.0^{a}
	Red swamp crayfish—males	22.2 (2)		40.0^{b}
	Marbled crayfish	37.5 (3)	62.5 (5)	100.0^{a}
	Red swamp crayfish—females	0.00 (0)		0.0^{b}
	Marbled crayfish	35.3 (6)	52.9 (9)	75.0^{a}
	Red swamp crayfish—in total	11.8 (2)		25.0^{b}

Number in parentheses indicates the no. of crayfish pairs. Different superscripts in the same column shows the statistically significant differences at $\alpha < 0.05$ between used species within the groups

Table 4 Total number of fights per pair, time to start first fight, total fights duration per pair and mean fight duration in premature and mature *P. virginalis* and *P. clarkii*

Group	Total no. of fights	Time to start fight (s)	Total fights duration (s)	Mean fight duration (s)
Premature	3.4 ± 0.7^{b}	257.9 ± 65.0^{a}	57.4 ± 15.5^{b}	16.9 ± 3.9^{a}
Mature (male–female)	5.1 ± 0.7^{a}	228.1 ± 56.1^{a}	163.0 ± 39.6^{a}	31 ± 6.6^{a}
Mature (female–female)	6.0 ± 0.9^{a}	218.4 ± 39.5^{a}	272.8 ± 74.3^{a}	53.6 ± 20.3^{a}
Mature (total)	5.6 ± 0.5^{a}	223.3 ± 32.2^{a}	217.9 ± 41.8^{a}	42.3 ± 10.4^{a}

Values are presented as mean ± SE. Different superscripts in the same column highlight the statistically significant differences at $\alpha < 0.05$

Table 5 Percentage of fights and mean fight duration till the dominant/subordinate status of crayfish was established and after that in interacting premature and mature pairs of *P. virginalis* and *P. clarkii*

Group	Percentage of fights		Mean fight duration (s)	
	Till dominance establishment	After dominance establishment	Till dominance establishment	After dominance establishment
Premature	88.2 ± 5.7^{a}	11.8 ± 5.4^{b}	19.8 ± 5.6^{a}	1.6 ± 0.7^{b}
Mature (male–female)	70.4 ± 7.2^{a}	29.6 ± 7.7^{b}	36.8 ± 9.2^{a}	12.1 ± 3.9^{b}
Mature (female–female)	81.0 ± 11.7^{a}	19.0 ± 11.7^{b}	140.4 ± 32.2^{a}	8.3 ± 5.1^{b}
Mature (total)	74.9 ± 9.5^{a}	25.1 ± 6.1^{b}	81.2 ± 19.1^{a}	10.5 ± 2.8^{b}

Values are presented as mean ± SE. Different superscripts in the same row highlight the statistically significant differences at $\alpha < 0.05$ between the same parameters

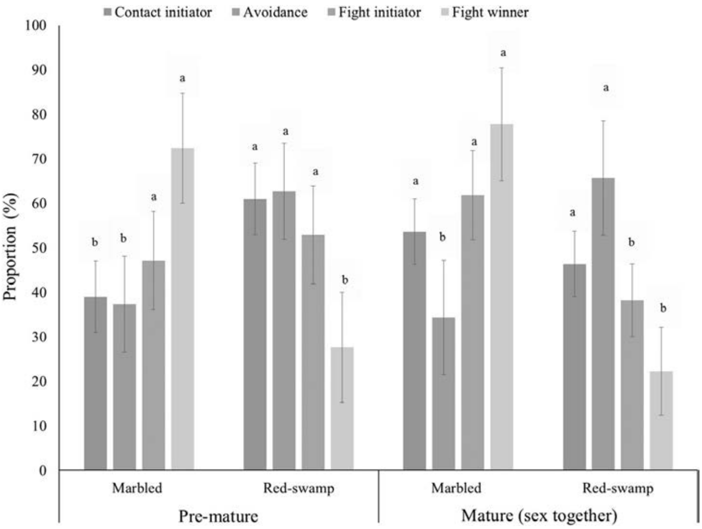

Fig. 1 Comparison of percentage of contact initialization, avoidance events, fight initialization and fight wins between premature and mature *P. virginalis* and *P. clarkii*. Values are presented as mean ± SE. Different superscripts within the column colures (groups apart) highlight the statistically significant differences at $\alpha < 0.05$. (Color figure online)

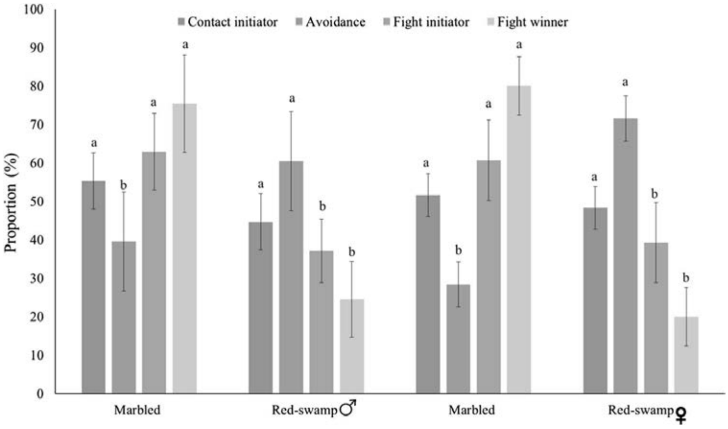

Fig. 2 Comparison of percentage of contact initialization, avoidance events, fight initialization and fight wins between mature *P. virginalis* and *P. clarkii* in respect to sex of *P. clarkii*. Values are presented as mean ± SE. Different superscripts within the column colours (groups apart) indicates the statistically significant differences at $\alpha < 0.05$. (Color figure online)

Discussion

Successful crayfish invaders are basically determined, aside from ecological strategies and adaptability (Lindqvist and Huner 1999; Holdich et al. 2009) through agonistic behaviour during interspecific interactions (Gherardi 2002; Hudina et al. 2016). The importance of marbled crayfish is increasing due to its current spreading in natural habitats worldwide (Kawai et al. 2016; Patoka et al. 2016). The information about the competitiveness of marbled crayfish with other species, including other already established aliens, is scarce (Jimenez and Faulkes 2010; Kawai et al. 2016). Generally, marbled crayfish is considered more placid in stress conditions (Kawai et al. 2016) often stimulated by mechanical stimuli only (Kasuya and Nagayama 2016). Despite its reputation for a calm disposition, it has the previously dominance over size-matched spiny cheek crayfish caught from wild populations in Germany (Linzmaier et al. 2018) and very active and aggressive red swamp crayfish, which is probably an utmost successful introduced species worldwide (Jimenez and Faulkes 2011). In some previous research, some (Jimenez and Faulkes 2011) trials were not performed on size-matched individuals, which resulted in potential bias in results since size is one of the major determinants of dominance in agonistic interactions in crayfish. In addition, from the size of marbled and red swamp crayfish can be supposed that nearly adult marbled crayfish, especially when 8–10 months old (Seitz et al. 2005), fought with juveniles of red swamp crayfish. However, this stock was assessed as premature but unfortunately without evidence of glair glands development consideration (Faulkes, personal communication). We therefore decided to revise these results to a greater extent using two size groups tightly matched for weight and with detailed morphometric evaluation of specimens used. Moreover, two observation methods (including visual and automatic software evaluation) were used to see deeply to the patterns accompanied with dominance establishment.

Our results indicate that marbled crayfish might be a potent challenger in interspecific competition even with highly aggressive and widely distributed alien crayfish. Marbled crayfish significantly more successfully established dominance in interspecific pairs with red swamp crayfish in both premature and mature group. In addition, the status of hierarchy remained not defined in about 50% pairs in mature group, whereas in premature, it was only 10%. The mature group seems to require more time to establish dominancy. More time to establish dominance could be due to prolonged sequential assessment of the opponent due to pair matching. Moreover, the pattern of more rapid formation of social hierarchy in small size crayfish than in larger has been already reported (Sato and Nagayama 2012). In the mature group, marbled crayfish dominated in female–female pairs. In the female–male pairs, the dominance of marbled crayfish was less pronounced (60% with male and 100% with female) probably due to more aggressive nature of males (Gherardi 2002). Males are usually also advantaged with resource-holding potential (RHP) over females (Parker 1974; Shine 1989; Wilson 1992) because males primarily compete for access to female (Draud et al. 2004), while female spent most time and energy in reproduction (including vitellogenesis) and carrying young (Trivers 1972). Male and female crayfish also use different strategies during agonistic interactions (Wofford 2013). Mature crayfish males are more aggressive than females, but in premature animals, their agonistic features are not pronounced by sex (Bovbjerg 1956) though dominant–subordinate relationship is formed between premature as early as the third developmental stage (Issa et al. 1999).

The morphometric comparison did not reveal any significant differences in strategic body parts, as chelae and carapace dimensions, abdomen or total length (Bovbjerg 1953; Stein 1976; Gherardi 2002), and the differences of body dimensions therefore probably did not play important role in our study. The only evident difference was significantly wider abdomen of red swamp crayfish in premature group and conversely wider abdomen of marbled crayfish in mature group, which can hypothetically be at the utmost the precursor of better stability of marbled crayfish during fights (Buřič et al. 2010; Hossain, Buřič, unpublished work). However, other variables such as plasma protein level and exoskeleton calcium concentrations (Vye et al. 1997) should be considered in future studies being physiological and biochemical status has effect on aggressive motivation (Kravitz 1988).

Size and developmental stage are important factors related to agonistic behaviour of crayfish (Bovbjerg 1956) with an elevated level of aggression and more fighting events occurred as juvenile (premature) crayfish became larger (Sato and Nagayama 2012). In our experiment, we found that total fight number per pair, fight duration and mean fight duration was significantly higher in mature group. Schroeder and Huber (2001) also showed that duration of fights (fights escalated more rapidly) is shorter and resolved generally at lower intensities in small crayfish. Premature red swamp crayfish was more active in contact and fight initiation than marbled crayfish. On the other hand, their attacks (contact and fights initialization) was in the majority of cases unsuccessful which led to more established dominant marbled crayfish in experimental pairs. Therefore, lower rate of success of red swamp in fights might be due to continuous locomotion in experimental period compared to less active marbled crayfish.

Crayfish aggressive behaviour is mediated by visual, tactile and chemical communication (Breithaupt and Eger 2002; Bergman et al. 2005; Bergman and Moore 2005) with body vital statistics as possible predictors of dominance (Pavey and Fielder 1996). During direct interaction or conflict, fighters of many animal taxa are thought to evaluate an opponent's relative fighting ability, comparing it with their own (Parker 1974; Whitehouse 1997). The subordinate one avoids the dominant one and also the activity of contact initiation drops down (Rubenstein and Hazlett 1974; Höjesjö et al. 1998; Issa et al. 1999). Chemical signals are usually responsible for this social recognition, and crayfish have therefore the ability to recognize even unfamiliar dominant and subordinate individuals through chemical cues (Schneider et al. 1999). We therefore performed a prolonged 7-day lasting acclimation where crayfish were reared individually to reset their previous social status in original communal stocks. In crayfish, use of antennules as chemosensory organs has been well documented in agonistic interaction (Rutherford et al. 1996; Smith and Dunham 1996). In our experiment, in most of cases, both crayfish avoided each other or engaged in fight before communicated with their antennules. It is also noticeable that the subordinate crayfish also avoided the dominant one after seeing raised claws at a distance. This behaviour is therefore result of visual and chemical communication. Subordinate crayfish often avoid dominate individuals from possible contact through tailflip or retreat. In present study, tailflips frequency were higher value in premature animals, whereas retreat frequency was elevated in mature group which was also true for American lobster *Homarus americanus* with the increasing size escape behaviour through tailflip replaced by a defensive strategy (Lang et al. 1977). Sato and Nagayama (2012) also observed that with increasing body size, the probability of tailflips decreased, while retreats increased as the submissive behavioural act of subordinate animals. This contrast in the repertoire of submissive behavioural acts between smaller and larger crayfish could be due to a different level of activation of the neural circuitry underlying tailflip (Edwards et al. 1994a, b).

Conclusion

Finally, it can be the assumed that the marbled crayfish is able to dominate to similarly sized premature, mature females as well as mature males of red swamp crayfish despite its less activity in initialization of contact and fights in premature animals. Marbled crayfish seems to be thrifty in terms of energy expenditure, but is able to effectively react of the challenges. When dominance is established, both crayfish species lost the interest to initialize other contacts and fights. However, the interaction pattern would not be always straightforward, because in natural conditions, the population structure is not balanced as in experimental conditions, and crayfish usually have to face to different-sized specimens in more complex habitat and predation risk. In addition, red swamp crayfish have faster growth than marbled crayfish and disproportionate claw size in large male adults (Holdich et al. 2006). On the other hand, marbled crayfish being all female specimens have moderately sized claw also in larger specimens (Seitz et al. 2005). These would limit the success of marbled crayfish in natural habitats. Either way, marbled crayfish was confirmed as successful competitor in interactions with the probably most aggressive alien crayfish species (Gherardi and Cioni 2004; Gherardi and Daniels 2004; Jimenez and Faulkes 2011). Moreover, the successful invader is not determined only by aggressive behaviour and dominancy in the fights but also in other specifics like reproduction strategy and outputs together with an ability to adapt in different conditions (Lindqvist and Huner 1999). In this view, marbled crayfish is recently known as highly adaptable, early maturating and fecund species which can reproduce more than once per year via apomictic parthenogenesis (Martin et al. 2007; Kawai et al. 2016; Kouba et al. 2016). Moreover, marbled crayfish seems to have an ability to spare the energy and be more active only in the inevitable conditions. Hence, together with the found abilities to directly outcompete even such aggressive species as red swamp crayfish, marbled crayfish pose a substantial threat to native ecosystems and biodiversity of aquatic environment which can establish not only in crayfish free habitats, but is able to compete with resident species. One way or another, future studies are needed for example to evaluate the competition for strategic resources like food or shelter in basic as well as complex experimental conditions and in established populations.

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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 ~ 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 (~ 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

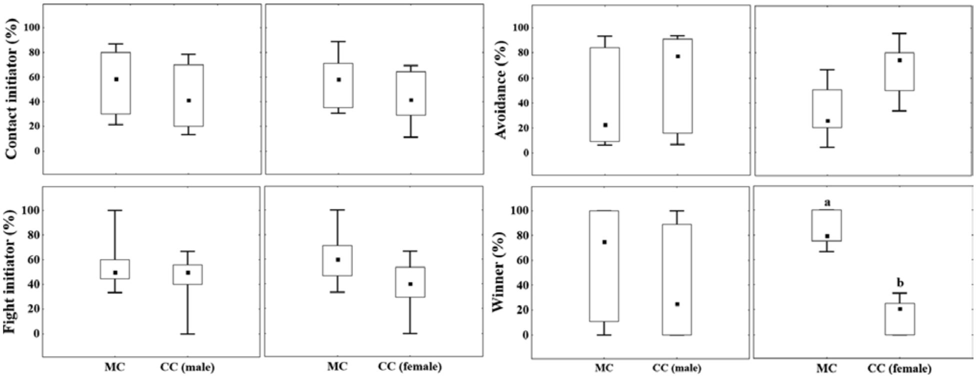

Fig. 1 Comparison of agnostic interactions (%) of MC, marbled crayfish and CC, calico crayfish males and females in the absence of shelter. Values are presented as median (small square), percentiles (large box, 25–75%) and min–max (error bar). Different superscripts indicate significant differences at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

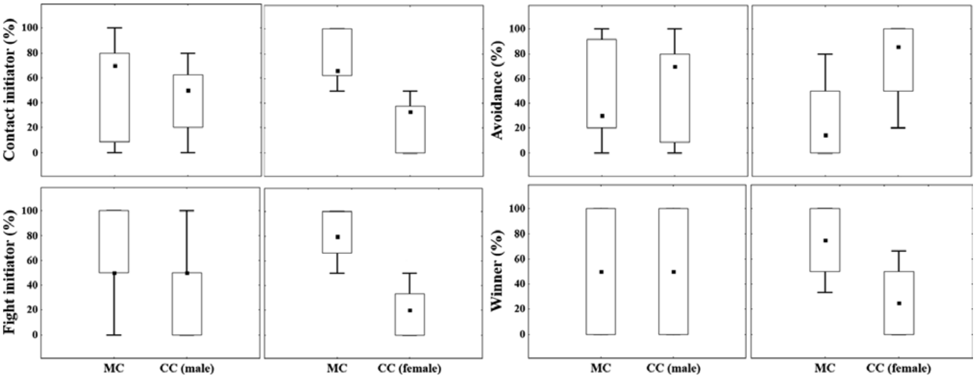

Fig. 2 Comparison of agnostic interactions (%) of MC, marbled crayfish and CC, calico crayfish. Male and female in the presence of shelter. Values are presented as median (small square), percentiles (large box, 25–75%), and min–max (error bar). Different superscripts indicate significant differences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

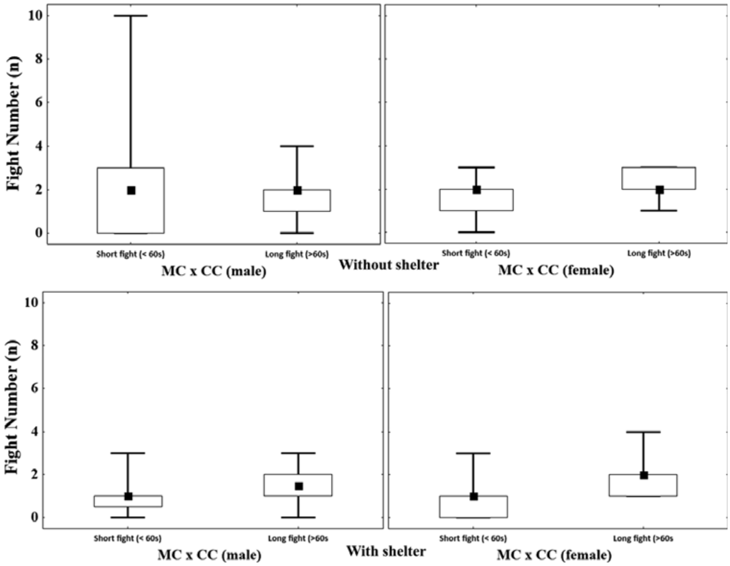

Fig. 3 Comparison of fight types (%) of MC, marbled crayfish and CC, calico crayfish in the presence and absence of shelter with considering the sex of calico crayfish. Values are presented as median (small black square), percentiles (large box, 25–75%), and min–max (error bar)

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 4

EFFECT OF PHARMACEUTICALS ON MARBLED CRAYFISH

4.1. Hossain, M.S., Kubec, J., Grabicová, K., Grabic, R., Randák, T., Guo, W., Kouba, A., Buřič, M., 2019. Environmentally relevant concentrations of methamphetamine and sertraline modify the behavior and life history traits of an aquatic invertebrate. Aquatic Toxicology 213, 105222.

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Environmentally relevant concentrations of methamphetamine and sertraline modify the behavior and life history traits of an aquatic invertebrate

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ABSTRACT

Pharmaceutically active compounds are major contaminants of aquatic environments that show direct and indirect effects on aquatic organisms even at low concentrations. The aim of this study was to assess the effects of the illicit drug methamphetamine and the antidepressant sertraline on clonal marbled crayfish *Procambarus virginalis*. Crayfish exposed to the environmentally relevant concentrations of methamphetamine of ~1 μg L^{-1} did not exhibit significant differences from unexposed controls in distance moved, velocity, and activity level with or without available shelter. Sertraline-exposed (~1 μg L^{-1}) crayfish were significantly more active, regardless of available shelter, and moved greater distances when shelter was available, compared to control crayfish. Crayfish exposed to methamphetamine and sertraline spent significantly more time outside the shelters compared to controls. Sertraline-exposed crayfish spawned more frequently and showed higher mortality than controls. The results suggest that the low environmental concentrations of the tested compounds could alter the behavior and life history traits of crayfish, resulting in higher reproductive effort and mortality.

1. Introduction

The presence of pharmaceutically active compounds (PhAC) in aquatic environments, particularly in surface waters, has created environmental concern (Burkina et al., 2015) as emerging contaminants (Ebele et al., 2017). Pharmaceutically active compounds arising from human and animal excretions, hospital wastes, and improper disposal (Chang et al., 2007; Collado et al., 2014; Loos et al., 2013) find their way to surface waters via effluents from sewage treatment plants which at best may only partially remove them from sewage (Blair et al., 2013; Golovko et al., 2014; Heberer, 2002). In surface waters and sediments, different PhACs are reported at concentrations from ng to μg L^{-1} (Loos et al., 2013; Silva et al., 2012). These residues may affect non-target organisms and whole ecosystems (Boxall et al., 2012; Buřič et al., 2018), as they are designed to be effective in target organisms at low concentrations (Huerta et al., 2012; Kaushik et al., 2016). Most past research focused on antibiotics and hypertension drugs (Grenni et al., 2018; Parrott and Balakrishnan, 2017; Zhou et al., 2018), which occur in the environment at higher frequency and greater concentrations (Marti et al., 2018; Stankiewicz et al., 2015) than do antidepressants (Fong and Ford, 2014) and other psychotropic substances (Yadav et al., 2017) that are present at lower concentrations in surface waters (Schulz et al., 2012).

Sertraline and methamphetamine affect brain function by flooding with the neurotransmitter serotonin (5-HT) (Hyttel, 1994; Kish, 2008), which alters the physiology and behavior of vertebrates and invertebrates (Fingerman et al., 1994; Fong and Ford, 2014; Frazer and Hensler, 1999) through the regulation of hormonal and neural signals (Santos et al., 2010). Some invertebrates possess receptors for psychotropic compounds similar to those of mammals (Rosi-Marshall et al., 2015), and the potential for bioaccumulation of some antidepressants is present in tissues and brain of fish, crayfish, and other benthic invertebrates (Fong and Ford, 2014; Grabicova et al., 2015, 2017). This increases the likelihood of observable effects on these animals at low concentrations of the above-mentioned PhAC (Meredith-Williams et al., 2012).

Selective serotonin re-uptake inhibitors are highly bioactive and persist for an extended time in sediments of surface waters (Klaminder et al., 2015; Shen et al., 2011). Methamphetamine half-life in soil is up to 1.5 years (Pal et al., 2011). Their persistence in nature leads to

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transfer to the food-web (Lagesson et al., 2016) and possible behavior modifications in aquatic organisms (Brodin et al., 2017). However, the effects of these psychotropic compounds in behavior (Brodin et al., 2014; Pal et al., 2013; Rosi-Marshall et al., 2015) are less studied than are their physical effects (eco-toxicity) (Pal et al., 2013). Impact on behavior is important from an ecological standpoint for short-term survival of an individual as well as long-term sustainability of a population (Nielsen et al., 2018). Similar to fish and aquatic and semi-aquatic insects (Barry, 2013; Jonsson et al., 2014), crayfish have been shown to exhibit behavior changes induced by PhAC in water (Buřič et al., 2018; Stropnicky, 2017; Tierney, 2016).

Crayfish are a suitable model species to assess the effect of PhAC through simple and rapid behavior tests as described by Buřič et al. (2018) and Hossain et al. (2018). Crayfish are also keystone species in ecosystems (Creed and Reed, 2004), and their altered behavior may have both direct and indirect effects on the ecosystems they inhabit. As a single species model, the obligate parthenogenetic marbled crayfish *Procambarus virginalis* Lyko 2017 is an ideal model organism (Hossain et al., 2018; Vogt, 2011) because of eliminated variability related to genotype (Martin et al., 2007; Vogt et al., 2008).

The primary goal of this study was to investigate effects of environmentally relevant water concentrations of sertraline and methamphetamine on the clonal marbled crayfish behavior (exploratory behavior and shelter use behavior), post-exposure and molting, mortality, and spawning during exposure.

2. Materials and methods

2.1. Chemicals

Methamphetamine and sertraline were obtained from Lipomed (Cambridge, MA, USA) and AK Scientific (Union City, CA, USA), respectively. Individual stock solutions were prepared at 10 mg L^{-1} ultra-pure water (AquaMax Basic 360 Series and Ultra 370 Series, Young Lin Instruments, Korea) and stored at 4 °C. The exposure solutions of 1 μg L^{-1} were prepared by dilution of the stock solution with aged tap water.

Isotopically labelled standard of methamphetamine (D5-MEA) and sertraline (D3-SER) were purchased from Lipomed and Cerilliant Corporation (Round Rock TX, USA), respectively, and used as internal standards for liquid chromatography with tandem mass spectrometry (LC-MS/MS) analyses of water samples. Ultra-pure water and acetonitrile (LC/MS grade, Merck), both acidified with formic acid (Sigma-Aldrich, Germany), were used as mobile phases in LC.

2.2. Experimental animals

Marbled crayfish (carapace length 16–22 mm) were randomly selected from our laboratory cultured population. At the conclusion of the behavior trial, the presence of glair glands was recorded, and carapace length was measured to the nearest 0.1 mm using Vernier calipers and whole animal weighed to the nearest 0.1 g with an electronic balance (Kern & Sohn GmbH, Balingen, Germany). The mean length and weight (Table 1) did not differ between control and exposed groups.

2.3. Experimental design

Crayfish were exposed at environmentally relevant concentrations of ~1 μg L^{-1} (Fedorova et al., 2014; Grabicova et al., 2015; Sehonova et al., 2018; Thomas et al., 2014) of pure methamphetamine (n = 73) or sertraline (n = 75) for 7 and 21 days, respectively. The exposure durations were based on the mechanism of action of the selected compounds. Methamphetamine acts immediately (Freye, 2009), in contrast to sertraline in which a steady-state plasma concentration is achieved in 2–3 weeks (Warrington, 1991). An equal number of crayfish maintained in aged tap water were used as controls and handled in the same way as exposed animals. Crayfish were held individually in clear 1.25 L (190 × 140 x 75 mm) plastic boxes containing 0.5 L of exposure solution or aged tap water alone. Water quality parameters of tap water were as follows: ammonium (NH_4^+) < 0.01 mg L^{-1}, nitrite (NO_2-) < 0.01 mg L^{-1}, nitrate (NO_3-) 4.2 mg L^{-1}, fluoride (Fl) 0.6 mg L^{-1}, chloride (Cl-) 8.9 mg L^{-1}, iron (Fe) 0.093 mg L^{-1}, calcium (Ca) 32.0 mg L^{-1}, magnesium (Mg) 8.5 mg L^{-1}, manganese (Mn) < 0.02 mg L^{-1}, conductivity 24.1 mS m^{-1}, hardness 1.16 mmol L^{-1}, and turbidity 0.65 FNU (provided by supplier of tap water).

During the exposure, crayfish were fed *ad libitum* with commercial feed (Sera Granugreen, Sera, Heinsberg, Germany) and boxes were cleaned during the exchange of all exposure solution/water every 24 h (sertraline) or 48 h (methamphetamine). To avoid contamination with tested compounds, the control group was always handled first. Crayfish that molted or spawned before ten days of the video-tracking trial were omitted from the experiment. Water temperature did not differ ($P > 0.05$) among exposed and control groups and reached values of 20.4 ± 1.2 °C and 20.3 ± 0.9 °C, respectively. During the experiment exposure period a natural light: dark cycle used was 15:9 h.

Concentrations of methamphetamine and sertraline in control, to confirm the absence of tested chemicals, and exposed treatments were analyzed by LC-MS/MS three times (methamphetamine) and four times (sertraline) during the experiment. Water was sampled by 05 mL plastic syringe (Braun, purchased from Labicom, CR) before (time 0) and at 24 h (sertraline) or 48 h (methamphetamine) after exchange. Samples were filtered through 0.20 μm regenerated cellulose (Labicom, CR) in a 10 mL glass vials for LC-MS (LaPhaPak, purchased from Labicom, CR) and stored at −20 °C until analysis. After thawing and addition of internal standards, the samples were measured within 10 min using a Hypersil Gold aQ column (50 x 2.1 mm; 5 mm particles) coupled with an Accela 1250 LC with TSQ Quantum Ultra Mass Spectrometer (Thermo Fisher Scientific) (Table S1).

2.4. Behavior observations and data acquisition

After the exposure period, crayfish were placed individually in 280 mm diameter plastic tanks containing 2 L aged tap water and 200 mL fine sand (< 1 mm). For methamphetamine and sertraline 120

Table 1

Carapace length (CL) and whole animal weight (W) of marbled crayfish *Procambarus virginalis* specimens in experimental groups. n, number of tested individuals. *crayfish that molted during video tracking were omitted from count and measurement. Data are presented as mean ± standard deviation.

Tested compound	Group (n)*	Shelter available	CL (mm)	*t*-test	*P*	W (g)	*t*-test	*P*
Methamphetamine	Exposed (30)	no	19.4 ± 1.7	1.23	0.223	2.0 ± 0.5	1.78	0.082
	Control (30)	no	18.8 ± 1.6			1.8 ± 0.4		
	Exposed (28)	yes	18.7 ± 1.9	0.24	0.809	1.9 ± 0.6	−0.12	0.902
	Control (30)	yes	18.8 ± 2.0			1.9 ± 0.6		
Sertraline	Exposed (27)	no	20.0 ± 1.8	1.37	0.175	2.3 ± 0.7	1.90	0.060
	Control (27)	no	19.3 ± 2.1			2.0 ± 0.5		
	Exposed (24)	yes	19.0 ± 1.8	0.44	0.660	2.1 ± 0.6	1.06	0.294
	Control (25)	yes	18.8 ± 1.5			1.9 ± 0.4		

and 104 crayfish were video-recorded, respectively. The video-recording trial was repeated six times for each compound with each trial comprising 20 crayfish (10 exposed and 10 control) with the exception of the sixth trial of sertraline, which comprised two crayfish in each group. Half of the trials for each compound were conducted without shelter and half with a shelter consisting of half a ceramic plant pot, 50 mm depth with 60 mm diameter entry attached to the bottom of the tank. Following exposure, each day two trials were conducted between 7:00 AM to 05:00 PM. The remaining crayfish were maintained in the same way as mentioned in section 2.3 until used in next video trails.

Crayfish were video-recorded for 4 h using a digital video camera (Sony HDR-CX240, Sony, Japan) attached above arenas. Permanent indirect illumination was provided by fluorescent tubes (daylight, 2310 lm). Video-recordings were evaluated by an EthoVision® XT 13.0 software (Noldus Information Technology, Wageningen, Netherlands) using a multiple-arena module to detect individual patterns of crayfish movement. Distance moved (cm), activity (percentage of time that crayfish locomotion was detected), and velocity (cm s^{-1}) were evaluated. The software also detected the percentage of time spent outside the shelter when shelters were present. Glair gland presence was recorded. Crayfish molting or spawning during video recording were omitted from behavior analysis. For further details see also Buřič et al. (2018) and Kubec et al. (2019).

2.5. Statistical analysis

Statistical analyses used Statistica 12.0 (StatSoft, Tulsa, OK, USA). The concentrations of the tested compound at time 0 and time 24/48 were compared through paired *t*-tests. The *t*-test for independent samples was used to compare the length and weight of exposed and control crayfish. Distance moved, velocity, activity, and time spent outside the shelter (replicate groups as a random factor, exposure as a fixed factor) were analyzed by factorial ANOVA followed by multiple comparisons of means as a post hoc test (Fisher's exact test). The null hypothesis was rejected at $\alpha = 0.05$.

3. Results

3.1. Analysis of water samples

The concentrations of tested compounds in water samples from exposure boxes at time 0 and 24/48 h of exposure did not differ. Water from the control group showed concentrations below limits of detection (Table 2) and details of sample data are presented in Supplementary Table (S2).

3.2. Methamphetamine

In the systems without shelter, there were no significant differences among observed parameters between exposed and control crayfish or among replicate groups within trials.

In the systems with shelter, exposed crayfish spent (54.2 ± 31.5%) significantly more time outside the shelter ($F_{1,\ 50} = 5.41$, $P = 0.02$) than did controls (37.3 ± 26.1%). There were no significant differences in distance moved, velocity, or activity between exposed and control crayfish. No significant differences among trials were detected.

Table 2
Concentration of methamphetamine and sertraline in water at time 0 (control, exposed), and after 24 h/48 h of exposure (control, exposed) ($P = 0.05$). Data are presented as mean ± standard deviation.

Tested compound	Group	n	Time 0 (µg L^{-1})	Time 24/48 (µg L^{-1})	Paired *t*-test	*P*
Methamphetamine	exposed	3	1.3 ± 0.3	1.1 ± 0.1	2.219	0.08
	control	3	< 0.01	< 0.01	—	—
Sertraline	exposed	4	0.8 ± 0.3	0.9 ± 0.5	−0.731	0.26
	control	4	< 0.05	< 0.02	—	—

3.3. Sertraline

In the group without available shelter, crayfish exposed to sertraline moved longer distances at higher velocity than did control animals with the difference marginally significant ($F_{1,\ 36} = 3.65$, $P = 0.06$; $F_{1,\ 36} = 3.20$, $P = 0.08$, respectively). Activity of exposed animals was significantly higher ($F_{1,\ 36} = 10.60$, $P = 0.002$) than that of the control crayfish (Fig. 1). No differences among trials were detected.

In the system with shelter available, the distance moved differed significantly between exposed and control animals ($F_{1,\ 43} = 6.28$, $P = 0.02$). The difference in velocity of exposed and control animals was not significant ($F_{1,\ 43} = 3.01$, $P = 0.09$). Activity level of exposed crayfish differed significantly ($F_{1,\ 43} = 4.99$, $P = 0.03$) from the control. Exposed animals spent a significantly higher proportion of time outside the shelter ($F_{1,\ 43} = 5.22$, $P = 0.03$) than did controls (Fig. 2). No significant differences were detected among trials.

3.4. Life history traits

In systems with shelter, both control and methamphetamine exposed crayfish with visible glair glands showed significantly lower activity ($F_{1,\ 52} = 5.12$, $P = 0.03$) and spent less time outside the shelter ($F_{1,\ 52} = 4.59$, $P = 0.04$) than those without glands (Fig. 3). No significant differences were observed in distance moved ($F_{1,\ 52} = 3.06$, $P = 0.09$) or velocity ($F_{1,\ 52} = 0.05$, $P = 0.82$) compared to crayfish without glands. No differences were observed between crayfish with and without visible glair glands in the trials without shelter.

Crayfish with glands also moved significantly shorter distances ($F_{1,\ 48} = 8.66$, $P = 0.005$) but at higher velocity ($F_{1,\ 48} = 8.61$, $P = 0.005$) and showed lower activity ($F_{1,\ 48} = 15.17$, $P < 10^{-3}$) compared to crayfish without glands in absence of shelter in both sertraline exposed and control groups (Fig. 4).

In presence of shelter, crayfish with and without glands did not show significant difference in distance moved ($F_{1,\ 45} = 0.50$, $P = 0.48$), velocity ($F_{1,\ 45} = 0.06$, $P = 0.81$), activity ($F_{1,\ 45} = 0.07$, $P = 0.80$), or time spent outside the shelter ($F_{1,\ 45} = 0.02$, $P = 0.65$).

During the exposure period, the number of molted crayfish did not significantly differ from controls in either methamphetamine ($\chi^2 = 0.25$, $P = 0.62$) or sertraline ($\chi^2 = 1.32$, $P = 0.25$) exposure. The number of crayfish that spawned during the exposure period was significantly higher ($\chi^2 = 4.45$, $P = 0.03$) in the sertraline exposed group than in control. The sertraline exposed group showed significantly higher mortality ($\chi^2 = 8.00$, $P = 0.005$) than seen in controls. No spawned or dead crayfish were observed in the methamphetamine group (Table 3).

4. Discussion

Crayfish exposed to methamphetamine at ~1 µg L^{-1} with or without shelter did not significantly differ in total distanced moved, velocity, or activity from unexposed control. This is in contrast to previous research demonstrating that a single, repeated, or chronic exposure to cocaine, morphine, or methamphetamine increased locomotor activity in crayfish (Imeh-Nathaniel et al., 2016, 2017) and mammals (Arencibia-Albite et al., 2017; Pang et al., 2016). These results were obtained using injections of higher doses than were applied in our study, and therefore only confirm that methamphetamine can affect invertebrates. Our exposure of crayfish to a low concentration showed a significant effect only in the longer time spent outside the shelter. This did not extend to increased exploring behavior outside the shelter which was passive. This can have a detrimental effect on crayfish, as sheltering is an essential need of crayfish throughout life,

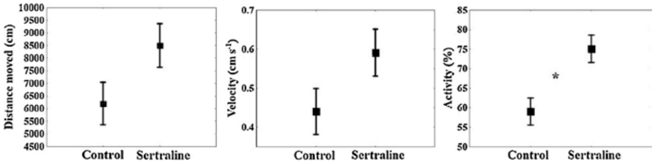

Fig. 1. Total distance moved (cm), velocity (cm s^{-1}), and activity (%) of *Procambarus virginalis* exposed to an environmentally relevant concentration (~1 μg L^{-1}) of sertraline and control without shelter available. The number of replicates in both groups was 27. * = significant differences (α = 0.05) between exposed and control crayfish. Data are presented as mean ± standard error of mean.

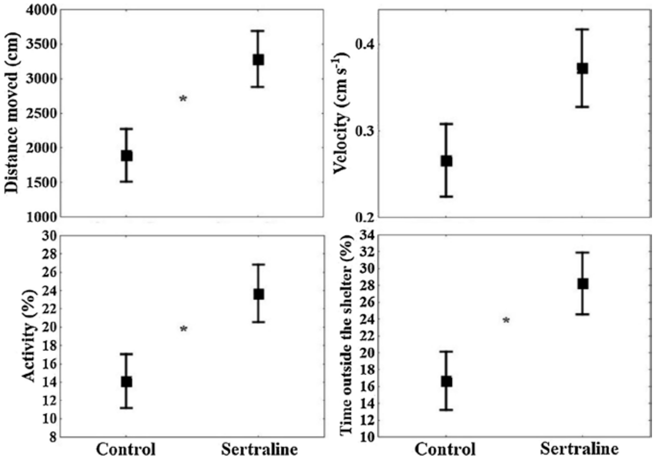

Fig. 2. Total distance moved (cm), velocity (cm s^{-1}), activity (%), and time spent outside the shelter (%) of *Procambarus virginalis* exposed to environmentally relevant concentration (~1 μg L^{-1}) of sertraline and control with available shelter. The number of replicates was 25 and 24 for control and exposed group respectively.* = significant differences (α = 0.05) between exposed and control crayfish. Data are presented as mean ± standard error of mean.

especially in daylight (Gherardi, 2002; Kubec et al., 2018; Lozán, 2000).

There is a question of whether longer exposure can have more severe effects. Methamphetamine acts acutely on the nerve terminals to stimulate initial euphoric action and, with long-term exposure, can contribute to cognitive impairment leading to loss of memory, attention, and decision-making skills (Marshall and O'Dell, 2012; Vollm et al., 2004). Our seven-day exposure produced some visible effects, and it could be assumed that continuous exposure at environmental concentrations might damage the monoamine nerve terminals of the brain (Amanda et al., 2016). These effects have been reported at thousand-fold our tested concentrations (Valente et al., 2016; Yamamoto et al., 2010).

We found crayfish exposed to an environmental concentration of sertraline to exhibit significantly higher activity with and without available shelter, move significantly greater distances in presence of shelter, and approach significance in velocity and distance moved in conditions without shelter. Research has revealed that antidepressants such as SSRIs reduce locomotor activity in fish through reducing anxiety, similar to their action in humans (Giacomini et al., 2016; Kohlert et al., 2012; Nielsen et al., 2018; Simmons et al., 2017). They have also been shown to alter behavior and life history traits in species including crustaceans, mollusks, and amphibians (Bossus et al., 2014; Buřič et al., 2018; Carfagno and Fong, 2014; Di Poi et al., 2014). Crayfish and lobsters are reported to show reduced locomotion due to upsurge of serotonin levels in synapses after SSRI binding to the serotonin transporter (Fossat et al., 2014; Peeke et al., 2000).

The effects of antidepressants on aquatic organisms vary with concentration and exposure duration. Tierney (2016) and Buřič et al. (2018) observed reduced locomotion in crayfish exposed to fluoxetine and citalopram, respectively, at ~1 μg L^{-1}. Other studies have shown that fluoxetine at 20 μg L^{-1} and 40–345 μg L^{-1} affected movement and burrowing behavior of freshwater mussels (Hazelton et al., 2014) and retarded locomotion in marine snails through foot detachment (Fong

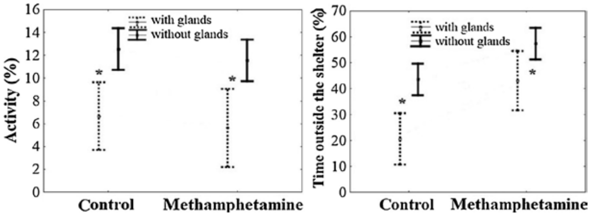

Fig. 3. Activity (%) and time spent outside the shelter (%) in *Procambarus virginalis* exposed to an environmentally relevant concentration (~1 μg L^{-1}) of methamphetamine and control in systems with shelter with respect to glair gland status. The number of replicates was 30 (9 with gland and 21 without gland) and 28 (7 with gland and 21 without gland) for control and exposed group respectively.* = significant differences (α = 0.05) between exposed and control crayfish. Data are presented as mean ± standard error of mean.

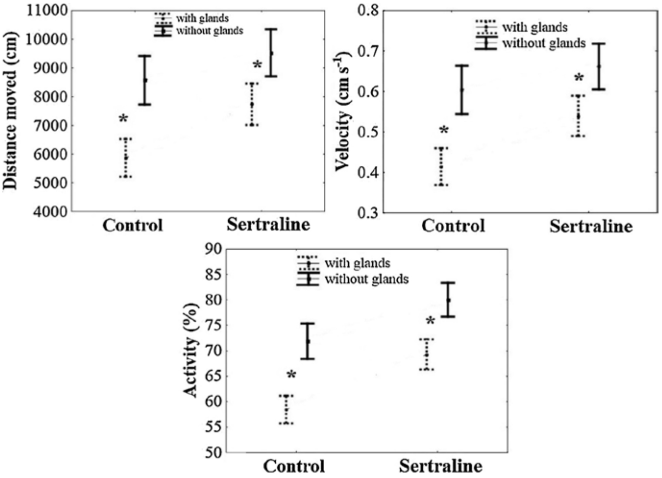

Fig. 4. Total distance moved (cm), velocity (cm s^{-1}) and activity (%) of *Procambarus virginalis*, with and without glair glands, exposed to an environmentally relevant concentration (~1 µg L^{-1}) of sertraline and control in systems without available shelter. The number of replicates was 27 for both groups (17 with gland and 10 without gland in control and 15 with gland and 12 without gland in exposed group) * = Significant differences (α = 0.05) between exposed and control crayfish. Data are presented as mean ± standard error of mean.

Table 3

Number of molted, spawned, and dead crayfish in control and exposed groups during the exposure to sertraline and methamphetamine. Different superscripts indicate significant differences ($P =$ 0.05) between exposed and control groups.

Pharmaceutical	Group (n)	Molted (n)	Spawned (n)	Mortality (n)
Methamphetamine	Control (73)	7	0	0
	Exposed (73)	9	0	0
Sertraline	Control (75)	15	2^b	0^b
	Exposed (75)	22	9^a	8^a

et al., 2015; Fong and Molnar, 2013). Arabian killifish *Aphanius dispar* Ruppel 1829 and Siamese fighting fish *Betta splendens* Regan 1910 decreased activity after exposure to an environmental concentration of fluoxetine (Barry, 2013; Kohlert et al., 2012). In contrast, Fong et al. (2015) found venlafaxine to be associated with increased crawling speed in both *Lithopoma americanum* Gmelin 1791 and *Urosalpinx cinerea* Say 1822 at higher concentrations, and Mesquita et al. (2011) observed increased walking speed of European green crab *Carcinus maenas* L. 1758 at 120 and 750 µg L^{-1} fluoxetine. Lower concentrations of fluoxetine and sertraline (0.01 µg L^{-1}) than used in the present study have been reported to increase swimming speed in an amphipod *Echinogammarus marinus* Leach 1815 (Bossus et al., 2014). This inconsistency in SSRI effect might be due to variation in experimental design or to species differences (Backhaus, 2014; Fong and Ford, 2014), which in turn affect serotonin level in exposed organisms (Lillesaar, 2011). In Crustacea, the increase in serotonin level could influence other hormones, *e.g.* crustacean hyperglycemic hormone, to increase locomotor activity (McPhee and Wilkens, 1989) and exploration behavior. The duration of exposure may also modify serotonin secretion; chronic exposure to fluoxetine leads to gradual decrease in serotonin level in hybrid striped bass (Gaworecki and Klaine, 2008) and rats (Amodeo et al., 2015). Bossus et al. (2014) observed elevated swimming velocity of *E. marinus* after one-hour and one-day exposures to fluoxetine and sertraline at environmental concentrations but not after 8 days of exposure. Contrary to this, but similar to our study, Guler and Ford (2010) found significant differences in phototaxis behavior of *E. marinus* male after three weeks exposure to fluoxetine at 0.1 µg L^{-1}. Most previous studies have focused on acute effects of PhACs, while we evaluated outcomes with chronic exposure to low concentration. Neurotransmitter concentrations in the circulatory system or brain of crayfish or other invertebrates after exposure may be a topic for future research.

With available shelter, crayfish exposed to both sertraline and methamphetamine spent significantly more time outside the shelter than did unexposed controls. Similarly, shelter seeking behavior was reduced in flathead minnow *Pimephales promelas* Rafinesque 1820 after a four-week exposure to sertraline at 3–30 µg L^{-1} (Valenti et al., 2012). Juvenile Piauçu fish *Leporinus microcephalus* Garavello & Britski 1988 did not respond to alarm signals from conspecifics after fluoxetine administration at 10 µg g^{-1} body weight (Barbosa Junior et al., 2012) and zebrafish boldness increased after exposure to escitalopram (Nielsen et al., 2018). Our current observations, along with previous results (Buřič et al., 2018) suggests an increase in boldness and activity of crayfish after SSRI or methamphetamine exposure. Boldness are associated with aggressiveness (Huntingford, 2013), but lead to higher risk of predation. Boldness or reduction in shelter seeking behavior is also linked to increased anxiety through elevated brain serotonin levels (Fossat et al., 2015; Fossat et al., 2014). Anxiety, high aggressiveness, and higher vulnerability to predators has repercussions for ecosystems functioning, and there is a need to investigate further details with alarm or predator cue.

In addition to behavior patterns, the long exposure period enabled us to evaluate effects on the crayfish life cycle such as molting, reproduction, and mortality. Maturation, reproduction, and molting are regulated by neurohormones, the secretion of which is directly or indirectly regulated by serotonin and dopamine (Fingerman et al., 1994; Fong and Ford, 2014; Huber et al., 1997). Serotonin influences vitellogenesis in ovarian development (Alfaro et al., 2004; Kuo et al., 2009; Wongprasert et al., 2006), and dopamine inhibits ovarian and testicular maturation (Chen et al., 2003; Sarojini et al., 1995a,b). Therefore, it can be expected that molting and spawning can be affected in a similar manner as spawning behavior.

We also studied crayfish behavior in control and exposed groups with respect to glair gland development, an indication of maturity that plays a key role during spawning. Only two crayfish exposed to methamphetamine without shelter exhibited glair glands, and no behavior effects were observed. With available shelter, the presence of glands was significantly associated with reduced activity and increased shelter-seeking behavior of 35methamphetamine-exposed crayfish and controls compared to those without glair glands. Conversely, without shelter, sertraline-exposed and control crayfish with glair glands demonstrated significantly reduced activity and velocity and shorter distance moved compared to those without glair glands. This behavior in marbled crayfish with glair glands might be related to maternal characteristics. Crayfish females during reproduction are much less active than non-reproducing females (Gherardi, 2002; Reynolds and Holdich, 2002). However, glair gland presence affected behavior in all groups. Both chemicals were associated with increase in certain activity (time spent outside the shelter with methamphetamine and activity, velocity and distance moved with sertraline) of crayfish with glands to the level of controls without glands (Fig. 4). This implies that the tested chemicals diminished maternal characteristics and maternal aggression (Figler et al., 1995) to defend offspring from conspecifics (Brain et al., 1992) and predators, for resource acquisition, defining territory, rank in social hierarchies, and mate choice (Cavigelli and Pereira, 2000; Figler et al., 1995; Martin and Moore, 2010).

No differences were detected in the methamphetamine-exposed crayfish with respect to molting, spawning, and mortality. Crayfish exposed to sertraline for 21 days demonstrated significantly higher spawning and mortality than seen in control crayfish. The number of molting events to be higher, but not significantly (Table 3). The elevated spawning frequency might be attributed to serotonin-driven influence of the reproductive neuroendocrine, axis as mentioned earlier. Fong (1998) reported that fluoxetine at 34 and 340 μg L^{-1} induced spawning in male and female zebra mussel *Dreissena polymorpha* Pallas 1771 in less than 1 h. Lazzara et al. (2012) assumed that spawning could be induced in the same species even at lower concentrations with several days of exposure. Escitalopram has been found to disrupt egg production in zebrafish (Nielsen et al., 2018), and, in goldfish, fluoxetine decreased estradiol circulation with increasing mRNA levels of genes encoding LH and ovarian aromatase (Mennigen et al., 2017). Serotonin modulates the molt hormone in crustaceans (Fanjul-Moles, 2006) which might result in higher molting in sertraline-exposed crayfish. Higher mortality of sertraline-exposed marbled crayfish could be due to influence on the immune system or to molting abnormalities. It was reported that antidepressant SSRIs impaired the bone healing process (Abu Nada et al., 2018) and act as immunosuppressants (Gobin et al., 2014).

The effect of methamphetamine and sertraline at environmentally relevant concentration was evident. The clonal marbled crayfish presented altered behavior and life history traits, which may have importance in assessing the ecological effect of PhACs across species. Disturbance in behavior or a physiological process leads to the disruption of other related functions/system and breakdown of ecosystem processes (Brodin et al., 2014). The effect of sertraline was more pronounced than methamphetamine at the applied concentration, possibly due to the higher toxicity of sertraline to crustaceans (Christensen et al., 2007). Nevertheless, behavior endpoints show potential to assess the risk of ecological effects of PhACs (Backhaus, 2014; Brodin et al., 2014; Klaminder et al., 2014). Previous results, together with our contribution, show wide variability in type, strength, and direction of PhAC effects. These effects can be considered dependent on species tested, concentration used, exposure time, exposure technique as well as experimental conditions and observed endpoints.

5. Conclusions

The effects of environmentally relevant concentrations of selected PhACs are evident when tested as single compounds. The effect of a cocktail of PhAC substances needs study, as aquatic organisms are subjected to a mixture of PhACs in their environment. Due to the large number of compounds in surface waters and their differing modes of action, there is a need to explore their effects for better understanding of their real impact on ecosystems. Further study is also needed to investigate mechanisms of action on life history traits and reproduction as well as to determine persistence of the detected effects.

Author contribution

MSH, KG, RG, TR, AK, MB designed the experiment. MSH, JK, WG, MB conducted the experiment. MSH, JK, MB analyzed the data. MSH drafted the manuscript. All authors revised the final version of manuscript.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2019.105222.

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Combination of six psychoactive pharmaceuticals at environmental concentrations alter the locomotory behavior of clonal marbled crayfish

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Highlights

- The PhAC exposure has no significant effect on molting, mortality and spawning.
- The mixture of PhAC at environmental concentration altered the crayfish behaviors.
- Hemolymph glucose level showed no significant relationship with observed behaviors.

ABSTRACT

Pharmaceutically active compounds (PhAC) are ubiquitous in the aquatic environment worldwide and considered emerging contaminants. Their effects on growth, behavior, and physiological processes of aquatic organisms have been identified even at very low concentrations. Ecotoxicological investigations have primarily focused on single compound exposure, generally at a range of concentrations. In the natural environment, pollutants seldom occur in isolation, but little is known about the effects and risks of combinations of chemicals. The aim of this study was to investigate the effects of concurrent exposure to six psychoactive PhACs on locomotory behavior of clonal marbled crayfish *Procambarus virginalis*. Crayfish were exposed to ~1 µg L^{-1} of the antidepressants sertraline, citalopram, and venlafaxine; the anxiolytic oxazepam; the opioid tramadol; and the widely abused psychostimulant methamphetamine. In the absence of shelter, exposed crayfish moved significantly shorter distances and at lower velocity and showed significantly less activity compared with controls. With available shelter, exposed crayfish moved significantly greater distance, showed higher activity, and spent a significantly higher proportion of time outside the shelter than did controls. The molting, mortality and spawning frequency did not vary significantly between the groups. Hemolymph glucose level did not vary among groups and was not correlated with observed behaviors. Results suggest that environmental concentrations of the tested compounds in combination can alter the behavior of non-target aquatic organisms, which may lead to disruption of ecosystem processes due to their reduced caution in stressful conditions. Further research is needed using varied chemical mixtures, exposure systems, and habitats, taking into consideration molecular and physiological processes connected to behavior alterations.

Keywords: *antidepressant, ethology, emerging contaminant, opioid, Procambarus virginalis*

1. Introduction

Currently, thousands of pharmaceutically active compounds (PhAC) and their metabolites are present in aquatic environments worldwide and are considered emerging contaminants (Ebele et al., 2017). The source is largely municipal and hospital wastewater via sewage treatment plants as well as direct input from human or animal excretions and improper waste disposal (Blair et al., 2013; Chang et al., 2007; Golovko et al., 2014; Heberer 2002). Sewage treatment plants are not commonly designed to remove PhACs (Kolpin et al., 2002), and steroidal hormones, synthetic hormones, ingredients of personal care products, antibiotics, hypertension drugs, beta blockers, illicit drugs, opioids, antidepressants, and others are reported in the surface waters and sediments of Europe and North America at concentrations from ng to μg L^{-1} (Benotti and Brownawell, 2007; Loos et al., 2013; Silva et al., 2012). Although these substances are found in the environment at concentrations lower than their therapeutic doses, they show an impact on non-target organisms (Boxall et al., 2012; Huerta et al., 2012), as they are designed to act at very low concentrations (Huerta et al., 2012; Kaushik et al., 2016). Pharmaceutically active compounds also have been shown to bioaccumulate in muscle and brain of aquatic organisms (Grabicova et al., 2014). Therefore, low concentrations *per se* do not represent a margin of safety for non-target organisms or the environment.

In recent decades, research has shown detrimental effects of PhACs on growth, physiology, reproduction, and behavior of aquatic organisms even at environmentally relevant concentrations (Hossain et al., 2019; Kubec et al., 2019). Those investigations were primarily focused on determining effects of a single chemical at various concentrations through static (Brodin et al., 2017; Brodin et al., 2014; Fent et al., 2006; Fong and Ford 2014; Hossain et al., 2019) or dynamic (Hossain et al., 2020; Neal and Moore, 2017; Steele et al., 2018) exposure. In general, pollutants persist in aquatic environments as mixtures (Boxall et al., 2012; Heberer, 2002), and toxicity of chemicals in combination may be higher than that of single compounds (Backhaus, 2016; Smith et al., 2013). Vital questions regarding the effect of PhACs in combination on aquatic organisms remain largely unresolved (Rudd et al., 2014).

Among the classes of PhACs, antidepressants, selective serotonin re-uptake inhibitors (SSRI), anxiolytics, opioids, and several illicit drugs have received attention by toxicologist, as they are commonly used (Cipriani et al., 2009; Kolpin et al., 2002; Schultz et al., 2010) and frequently recorded in raw and treated wastewater, and effluent, and surface waters (Brooks et al., 2005; Mole and Brooks, 2019). They show bioaccumulation potential (Grabicova et al., 2017), variation in mode of action, incomplete removal by waste water treatment, and lack of eco-toxicological data (Boxall et al., 2012).

Acute and chronic exposure to pharmaceutical mixtures show effects on algae (DeLorenzo and Fleming, 2008), crustaceans (Dietrich et al., 2010; Godoy et al., 2019), bivalves (Franzellitti et al., 2015; Gonzalez-Rey et al., 2014), and fish (Ding et al., 2016). A quaternary mixture of acetaminophen, carbamazepine, gemfibrozil, and venlafaxine was reported to impact reproduction and induce histological changes in zebrafish *Danio rerio* after a six week exposure period similar to those seen with their individual exposures (Galus et al., 2013). A combination of 19 pharmaceuticals including antibiotics, beta blockers, lipid regulators, psychiatric drugs, and non-steroidal anti-inflammatory drugs was associated with higher mortality of *Cyprinus carpio* compared to treatment with any of the compound individually (Li and Lin, 2015). Ding et al. (2016) confirmed that interaction of fluoxetine, roxithromycin, and propanol led to enhanced bioaccumulation and disturbance of biochemical responses in Crucian carp *Carassius auratus*. Yu et al. (2017) found that a mixture of bensulfuron-methyl and acetochlor affected behavior, morphology, and histopathology of *Procambraus clarkii* juveniles at higher concentrations. Di Poi et al. (2018) tested the biocides methylparaben and triclosan, a pesticide degradation product, as well as the pharmaceuticals venlafaxine and carbamazepine on three aquatic organisms and reported species-specific effects. However, these studies were primarily focused on acute toxicity of chemicals at higher concentrations than seen in the natural environment.

The goal of this study was to investigate effects of a mixture of pharmaceuticals at environmentally relevant concentrations on the clonal marbled crayfish *Procambarus virginalis*, Lyko, 2017. We selected six pharmaceuticals that pose high risks for environmental contamination: sertraline, venlafaxine, citalopram, oxazepam, tramadol, and methamphetamine (Boxall et al., 2012). Tramadol (~1.4 μg L^{-1} in the Czech Republic), venlafaxine (65–170 ng L^{-1} in the Czech Republic), and citalopram (0.4–76 μg L^{-1} in India) have been detected in waters in higher concentrations than sertraline (4–100 ng L^{-1} in the Czech Republic), while sertraline shows bioaccumulation potential (Fick et al., 2009; Grabicova et al., 2015; Grabicova et al., 2017; Grabicova et al., 2014). The anxiolytic drug oxazepam has been most often detected in combination with other PhACs in sewage effluent and water bodies in Europe (Loos et al., 2013). The widely abused amphetamine psychostimulant methamphetamine is frequently detected in the aquatic environment of Europe, USA, UK, Oceania, and Asia (Kasprzyk-Hordern et al., 2009; Wang et al., 2019). Methamphetamine use is high in the Czech Republic, and its concentration in waste water is the highest of detected PhACs (http://www.emcdda.europa.eu/publications/edr/trends-developments/2017). The effects of these individual chemicals on aquatic species has been assessed (Brodin et al., 2017; Estévez-Calvar et al., 2017; Fong et al., 2015; Grabicova et al., 2017; Simmons et al., 2017; Wang et al., 2019); however, no reports are available with respect to their combined impact.

Our objectives were to determine the effects of exposure to the combined six pharmaceuticals on exploration and sheltering behavior as well as on molting, mortality, and spawning. We also measured hemolymph glucose to assess potential correlation with behavior, as it has been shown positively correlated with stress in crayfish (Fossat et al., 2014).

2. Materials and methods

2.1. Experimental animals

The unique reproductive characteristics and clonal genetics (Vogt et al., 2008) makes the obligate parthenogenetic marbled crayfish *Procambarus virginalis*, Lyko, 2017 an ideal model organism for various disciplines of science (Hossain et al., 2018; Vogt, 2011), including toxicology. Marbled crayfish of carapace length 15–23 mm were randomly selected from our laboratory culture.

2.2. Chemicals and stock solution

Individual stock solutions of citalopram hydrobromide (AK Scientific, Inc., USA), methamphetamine (Sigma-Aldrich, USA), oxazepam (Lipomed, USA), sertraline (AK Scientific), tramadol hydrochloride (Sigma-Aldrich), and venlafaxine hydrochloride (AK Scientific) at concentrations of ~1000 mg L^{-1} (40 mg L^{-1} for oxazepam) were prepared in ultra-pure water (AquaMax Basic 360 Series and Ultra 370 Series, Young Lin Instruments, Korea). The stock solutions were combined to obtain a mixture at concentration of 12 mg L^{-1} of each compound, and 4.6 mL of the stock mixture was added to 55 L of fresh tap water to obtain an exposure bath at concentrations of 1 μg L^{-1} of each compound.

Isotopically labeled compounds of citalopram (D6, Toronto Research Chemicals, Canada (TRC)), methamphetamine (D5, Chiron, Norway), oxazepam (D5, Chiron), sertraline (D3, Cerriliant, USA), tramadol (D3, Lipomed), and venlafaxine (D6, Chiron) were used as internal standards for quantification of tested compounds in water samples from exposed and control groups.

Acetonitrile (Merck, Germany) and ultra-pure water, both acidified with formic acid (Sigma Aldrich, Germany), were used as mobile phases for liquid chromatography.

2.3. Experimental design

Marbled crayfish (n = 120) were exposed to a combination of the PhACs at ~1 μg L^{-1} of each compound. The exposure duration of 21 days was selected based on the mechanism of action of the selected compounds. Sertraline, citalopram, and venlafaxine require 14–21 days exposure to achieve a steady-state plasma concentration (Courtney, 2004; Warrington, 1991), while oxazepam, tramadol, and methamphetamine show immediate effects (Freye, 2009). Control crayfish (n = 120) were maintained in aged tap water and managed in the same way as exposed animals. Each crayfish was held individually in a clear 1.25 L (190 x 140 x 75 mm) plastic box with 0.5 L of aged tap water or exposure solution.

During the experimental period, crayfish were fed *ad libitum* with commercial feed (Sera Granugreen, Sera, Heinsberg, Germany), and boxes were cleaned during the exchange of exposure solution/water daily. Water temperature was recorded routinely with alcohol thermometer to the nearest 0.1 °C and did not differ ($P > 0.05$) between exposed and control groups (20.4 ± 0.5 °C and 20.7 ± 0.8 °C, respectively). Crayfish that molted or spawned during the exposure period were removed from the experiment. A natural 15L:9D photoperiod was maintained during the exposure period.

The real concentration of tested chemicals in exposure treatment and control were analyzed by liquid chromatography with a tandem mass spectrometer TSQ Quantum (LC-MS/MS, Thermo Fisher Scientific, USA) four times during the trial. Freshly prepared solution before exchange (0 h) and exposure water was sampled using a 5 mL plastic syringe (Braun, from Labicom, CR) 24 h after exchange, filtered through 0.20 μm regenerated cellulose filter (Labicom, CR). The filtered water was stored at -20 °C until analysis. Thawed samples were prepared and analyzed according to method of Hossain et al. (2019).

2.4. Behavior observations and data acquisition

At the end of the exposure period, crayfish were placed individually in 280 mm diameter plastic tanks containing 2 L aged tap water and 200 mL fine sand (<1 mm). Sixty control and fifty-four exposed crayfish were video-recorded. The recording trial was conducted six times with each trial including 20 crayfish (10 exposed and 10 control) with the exception of the sixth trial, which comprised four crayfish in the exposed group without available shelter. Three trials were conducted without shelter and three with shelter, which consisted of a half of ceramic plant pot (50 mm depth with 60 mm diameter entry) attached to the bottom of the tank. Following exposure, each day, two trials were conducted between 08.00 and 18.00. The crayfish were maintained in exposure solution as described in section 2.3 until used in video trials.

The activity of crayfish was recorded for 4 h using a digital video camera (Sony HDR-CX240, Sony, Japan) attached ~180 cm above arenas. Constant indirect illumination was provided by fluorescent tubes (daylight, 2310 lm). Individual crayfish movement patterns were analyzed by EthoVision® XT 13.0 software (Noldus Information Technology, Wageningen, Netherlands) using a multiple-arena module. Distance moved (cm), activity (percentage of time that crayfish locomotion was detected), velocity (cm s-1), and percentage of time spent outside the shelter were evaluated. Crayfish that molted or spawned during recording were omitted from behavior analysis. For further details see Buřič et al. (2018).

At the end of the behavior trial, the carapace length was measured to the nearest 0.1 mm using Vernier calipers, and animals were weighed to the nearest 0.1 g with an electronic balance (Kern & Sohn GmbH, Balingen, Germany). The presence of glair glands was recorded (Hossain et al., 2019). There was no significant difference between control and exposed groups in crayfish mean length and weight (Table 1).

Table 1. *Carapace length (CL) and weight (W) of marbled crayfish Procambarus virginalis, Lyko 2017 specimens in exposed and control groups. Crayfish were exposed to a combination of sertraline, oxazepam, citalopram, venlafaxine, tramadol, and methamphetamine at 1 µg L^{-1}. The control group was held in aged tap water. Trial lasted 21 days. Data are presented as mean ± standard deviation.*

Group	Shelter available	CL (mm)	t-test	P	W (g)	t-test	P
Exposed	no	19.1±1.6	1.143	0.258	1.98±0.4	1.284	0.205
Control	no	19.7±1.8			2.17±0.6		
Exposed	yes	19.3±2.2	0.203	0.839	2.08±0.6	0.815	0.418
Control	yes	19.4±1.7			2.21±0.5		

2.5. Hemolymph collection and detection of glucose level

Immediately after the behavior experiment, a 100 µL aliquot hemolymph sample was obtained from the ventral abdomen at the space between myosepta using an insulin syringe and placed in 100 µL of 6% perchloric acid. The extract was centrifuged at 12,000 x g for 2 mins and stored at -20 °C until further analysis. From thawed centrifuged samples, about 50 µL of the supernatant was placed in 100 µL reactive solution [Glucose (GO) Assay Kit, Sigma Aldrich, Germany] and incubated at 37 °C for 30 min. The reaction was stopped by adding 100 µL of 12 N H_2SO_4 into each tube and mixing thoroughly. The optical density was measured with a spectrophotometer (Infinite M200, Tecan, Switzerland) at 540 nm according to the procedures modified from the glucose assay kit and Fossat et al. (2014).

2.6. Statistical analysis

Analysis was conducted using Statistica 12.0 (StatSoft, Tulsa, OK, USA). The water temperature of experimental groups was compared through paired t-tests. The t-test for independent samples was used to compare length and weight of exposed and control crayfish. Distance moved, velocity, activity, and time spent outside the shelter (replicate groups as a random factor, exposure as a fixed factor) were analyzed by factorial ANOVA followed by multiple comparisons of means as a post hoc test (Fisher's exact test). Hemolymph glucose level of groups and interactions between glair gland presence and groups were compared by general linear modeling. Relationships of hemolymph glucose level to observed behavior parameters were analyzed by linear regression analysis. The null hypothesis was rejected at $\alpha \le 0.05$.

3. Results

3.1. Concentration of psychoactive PhACs in water

The concentration of individual PhACs in water of exposed and control groups is shown in Table 2. The concentrations did not differ between 0 h and 24 h. In the case of sertraline, the concentration was ~20% of nominal (expected) value.

Table 2. *Concentration of sertraline, oxazepam, citalopram, venlafaxine, tramadol, and methamphetamine in water samples at time 0 and after 24 h of exposure. Data are presented as mean ± standard deviation and range.*

Group	**Tested compound**	**n**	**Time 0 ($\mu g\ L^{-1}$)**	**Time 24 ($\mu g\ L^{-1}$)**
Exposed	Sertraline	4	0.23±0.10 (0.15–0.38)	0.18±0.04 (0.15–0.24)
	Oxazepam	4	0.74±0.03 (0.69–0.77)	0.70±0.08 (0.63–0.77)
	Citalopram	4	0.77±0.07 (0.69–0.84)	0.70±0.06 (0.64–0.78)
	Venlafaxine	4	0.94±0.08 (0.84–1.0)	0.92±0.07 (0.83–1.0)
	Tramadol	4	0.79±0.04 (0.75–0.83)	0.73±0.07 (0.64–0.80)
	Methamphetamine	4	0.76±0.03 (0.72–0.78)	0.68±0.06 (0.60–0.74)
Control	Sertraline	4	< 0.02	< 0.02
	Oxazepam	4	< 0.01	< 0.01
	Citalopram	4	< 0.01	< 0.01
	Venlafaxine	4	< 0.01	< 0.01
	Tramadol	4	< 0.03	< 0.04
	Methamphetamine	4	< 0.01	< 0.01

3.2. Behavior patterns

Crayfish without available shelter exposed to the combined PhACs moved significantly shorter distances ($F_{1,50}$ = 8.64, P = 0.005) at lower velocity ($F_{1,50}$ = 8.23, P = 0.006) than did control animals. Activity of exposed animals was significantly lower ($F_{1,50}$ = 6.64, P = 0.013) than that of the control crayfish (Fig. 1, right part).

The exposed crayfish with shelter available moved significantly greater distances ($F_{1,56}$ = 9.22, P = 0.004) and exhibited higher activity ($F_{1,56}$ = 10.93, P = 0.002) than control animals. The difference in velocity of exposed and control animals was not significant ($F_{1,56}$ = 3.01, P = 0.723). Exposed animals spent a significantly higher proportion of time outside the shelter ($F_{1,56}$ =24.17, P < 10-5) than did controls (Fig. 1, left part).

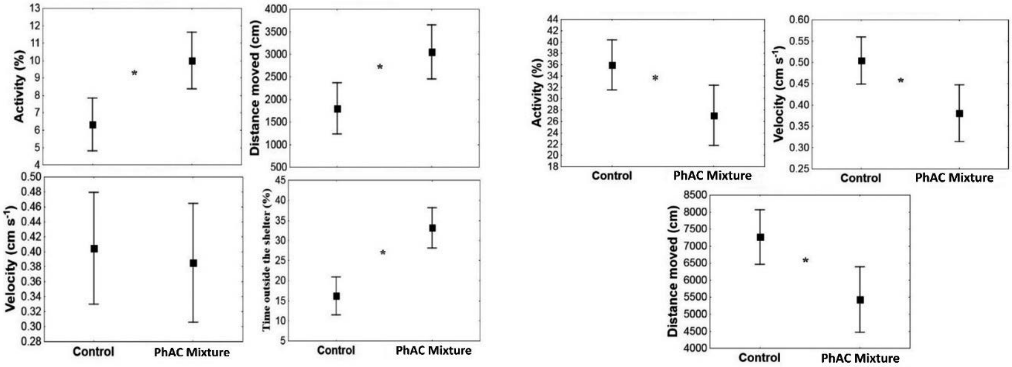

Figure 1. *Activity (% time) of marbled crayfish Procambarus virginalis, Lyko, 2017 exposed to a mixture of sertraline, oxazepam, citalopram, venlafaxine, tramadol, and methamphetamine at ~1 µg L^{-1} of each compound and of control, with (left) and without available shelter (right). The number of crayfish, n = 30 in both groups (with shelter) and without shelter in exposed and control was n = 30 and 24, respectively. * = significant difference (α ≤ 0.05) between exposed and control crayfish. Data are presented as mean ± standard error of mean.*

The development of glair glands had no significant effect on the distance moved, velocity, or activity level of crayfish in control and exposed group or with respect to availability of shelter. Glair gland presence had no significant association with time spent outside the shelter in either control or exposed crayfish. Data are shown in Table 3.

Table 3. *Values of distance moved, velocity, activity, and time outside the shelter in crayfish exposed to a combination of sertraline, oxazepam, citalopram, venlafaxine, tramadol, and methamphetamine at ~1 µg L^{-1} for each compound and in a control group relative to presence of glair glands with and without available shelter. Data are shown as mean ± standard deviation.*

Group (n)	Shelter available	Glair glands	Distance moved (cm)	Velocity (cm s^{-1})	Activity (%)	Time outside shelter (%)
Exposed (24)	no	yes (7)	6372±2193	0.45±0.15	33.0±14.7	---
		no (17)	4494±1871	0.32±0.13	21.2±10.6	---
Control (30)		yes (12)	7388±1922	0.51±0.13	36.8±9.8	---
		no (18)	7148±2466	0.50±0.17	35.1±12.9	---
Exposed (30)	Yes	yes (8)	3279±1524	0.37±0.11	11.31±4.1	37.1±17.6
		no (22)	2833±1880	0.41±0.17	8.7±4.5	29.3±15.5
Control (30)		yes (10)	1918±748	0.43±0.25	6.9±3.5	16.4±4.6
		no (20)	1687±1114	0.38±0.21	5.8±3.3	16.2±6.8

3.3. Life history traits

During the exposure period, the number of crayfish that molted (χ^2 = 0.00, P = 1.00), spawned (χ^2 = 0.16, P = 0.689), and died (χ^2 = 0.00, P = 1.00) did not differ significantly between the control and PhAC exposed groups (Table 4).

3.4. Glucose level and possible relationship to behavior

The hemolymph glucose level did not vary significantly among groups ($F_{1, 76}$ = 0.3002; P = 0.59), and interaction between glair gland presence and group was also not significant ($F_{1, 76}$= 3.4503; P = 0.07). There was no significant correlation between glucose level and observed behavior of exposed or control groups. The control group behaviors showed non-significant positive correlation, and the exposed group showed non-significant negative correlation with hemolymph glucose level (Fig. 2).

Table 4. *Number of molted, spawned, and dead crayfish during exposure to a combination of sertraline, oxazepam, citalopram, venlafaxine, tramadol, and methamphetamine at ~1 µg L^{-1} for each compound and aged tap water controls. Chi-square (χ^2) test were done at $\alpha \leq 0.05$.*

Group (n)	Molted (n)	Spawned (n)	Died (n)
Control (120)	56	22	1
Exposed (120)	56	20	1

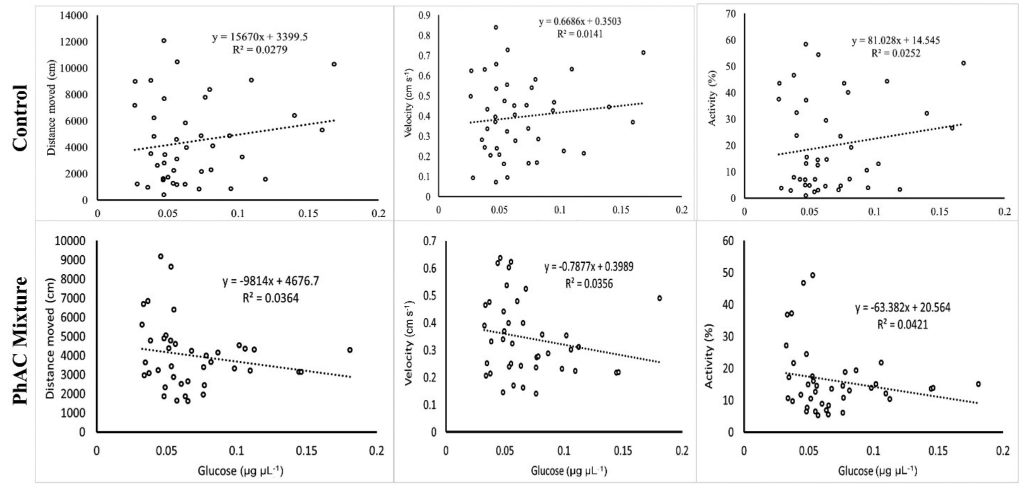

Figure 2. *Relationship between hemolymph glucose level and distance moved (cm), velocity (cm s^{-1}), and activity (% time) of marbled crayfish. n = 40 in both groups.*

4. Discussion

In the absence of shelter, crayfish exposed to a combination of environmentally relevant levels of sertraline, venlafaxine, citalopram, oxazepam, tramadol, and methamphetamine for 21 days moved significantly shorter distances at lower velocity and showed significantly less activity than unexposed crayfish. Hence, in absence of shelter, the exploring behavior and activity of crayfish was reduced. Exposed crayfish seemed to experience less stress, as indicated by reduced escape response in novel conditions, compared to controls. In the presence of shelter, the exposed crayfish spent significantly more time outside the shelter than did controls. As a result, exposed crayfish moved significantly longer distances and showed higher activity levels than observed in the control crayfish in the presence of shelter, again showing reduced reaction to normally stressful situations.

Individual pharmaceuticals (SSRIs, anxiolytes, illicit drugs, and opioids) are reported to alter locomotion behavior, increase boldness, and reduced anxiety in fish and other organisms, including invertebrates, similar to human responses (Giacomini et al., 2016; Kohlert et al., 2012; Nielsen et al., 2018; Simmons et al., 2017). Oxazepam has been reported to significantly increase marbled crayfish activity and motility (Kubec et al., 2019); whereas citalopram, tramadol, and fluoxetine reduced locomotion of marbled crayfish (Buřič et al., 2018; Tierney, 2016). Escitalopram reduced swimming speed of zebrafish Danio rerio (Nielsen et al., 2018). Sertraline-exposed crayfish showed significantly higher activity, travelling greater distance at higher velocity (Hossain et al., 2019). Crayfish exposed to sertraline and methamphetamine alone have been found to spend significantly more time outside of shelter than did unexposed crayfish (Hossain et al., 2019), similar to the results seen with the combination exposure in the current experiment. Reduced shelter seeking behavior has also been observed in flathead minnows after a four-week exposure to sertraline at 3–30 μg L^{-1} (Valenti et al., 2012). Zebrafish showed increased boldness after exposure to 1.50 μg L^{-1} escitalopram (Nielsen et al., 2018).

Pharmaceutically active compounds have been shown to alter behavior and life history traits in crustaceans, mollusks, and amphibians (Bossus et al., 2014; Buřič et al., 2018; Carfagno and Fong, 2014; Di Poi et al., 2014). In the present study, the development of the glair gland showed no significant association with any tested parameter in control or exposed crayfish regardless of available shelter. The number of molted, spawned, and dead crayfish did not differ significantly between groups. Similar findings have been reported with methamphetamine, oxazepam, and venlafaxine solo exposure at environmental concentrations (Hossain et al., 2019; Kubec et al., 2019). Sertraline-exposed marbled crayfish spawned and died in significantly higher numbers than unexposed controls during a 21 day exposure period at environmental concentrations (Hossain et al., 2019; Kubec et al., 2019). The combined effect in this experiment was not as straightforward as the individual effect of the tested compounds observed by Buřič et al. (2018); Hossain et al. (2019) and Kubec et al. (2019). Differences in effects of combined and individual compounds have been previously reported: A combination of nineteen pharmaceuticals including antibiotics, beta blockers, lipid regulators, psychiatric drugs, and non-steroidal anti-inflammatory drugs was demonstrated to increase mortality of *Cyprinus carpio* compared to exposure to the pharmaceuticals individually (Li and Lin, 2015). On the other hand, a quaternary mixture of acetaminophen, carbamazepine, gemfibrozil, and venlafaxine impacted zebrafish reproduction and induced histological changes after six weeks of exposure similar to those observed with their single compound exposure (Galus et al., 2013). The combined effect on aquatic organisms may be the result of additive effects of multiple chemicals or be antagonistic or synergistic in nature (Stenersen, 2004). In the present study, we used an equal concentration of each chemical, but, in some cases, found differences in concentrations before and after exposure. This might be due to interaction of the chemicals or to the detection variability of apparatus used (15–20%). The measured concentration of sertraline was about 20% of that expected. This might the result of the high adsorption potential of sertraline. This has also been observed in fish studies with a combination of pharmaceuticals, including sertraline (personal communication, Pavel Horky).

Animal behavior is the result of underlying neural and physiological processes as well as external stimuli and previous experience (Kubec et al., 2018). Chemicals alter the regulation of hormonal and neural signals (Santos et al., 2010), implying that behavior should also change. We measured the hemolymph glucose level of exposed and control crayfish, since stress shows a significant positive correlation with glucose level (Fossat et al., 2014). Although behavior of exposed and control crayfish differed, we found PhAC exposure and the interaction of group and glair gland presence to show no significant effect on hemolymph glucose levels, and there was no significant correlation between hemolymph glucose level and observed behaviors. This might be due to the collection technique and time of collection. The hemolymph was collected after four hours of video recording with crayfish held in aged tap water, which may have eliminated the chemical exposure effect. We did observe nonsignificant positive and negative relationship patterns between hemolymph glucose level and behavior of control and exposed crayfish, respectively. We can cautiously assume that tested compounds reduced crayfish stress and increased boldness resulting in significantly more time spent outside the shelter and reduced activity in the set-up without shelter. This can have negative consequences in a natural environment, as sheltering is an important need of crayfish throughout life, especially in daylight and in a novel environment in which crayfish generally explore the available strategic resources (Gherardi, 2002; Kubec et al., 2018; Lozán, 2000). Increased boldness and reduction in shelter-seeking behavior is also linked to elevated brain serotonin levels (Fossat et al., 2015; Fossat et al., 2014). Along with reduced locomotion, this could lead to higher vulnerability to predation (Hossain et al., 2019). Crayfish are ecosystem engineers and play an important role in structuring stream communities. Changes in their behavior repertoire can have profound effects at the ecosystem, leading to breakdown of ecosystem processes and the disruption of related functions/system (Brodin et al., 2014; Ludington and Moore, 2017).

5. Conclusion

The results demonstrated that the combination of PhACs induced behavior changes in marbled crayfish at concentrations that are considered environmentally safe and that are present in aquatic systems (Kostich et al., 2014). Obtained research data can vary depending on species tested (Di Poi et al., 2018), concentrations used, exposure time, and exposure technique as well as experimental conditions and observed endpoints (Hossain et al., 2019, 2020). The outcomes could also depend on the mode of action of tested chemicals. Combinations of PhACs have been reported to limit the effects of single compounds (Rede et al., 2019), but the effect of mixtures may be the converse, due to interaction of compounds (Franzellitti et al., 2015; Riva et al., 2019). Results of behavior assessments are thus far inconclusive. Future investigation is warranted and should include a focus on different combinations of chemicals and their interactions, and be interpreted with respect to habitat structure, species tested, and effects on physiological processes including at the molecular level.

Author contributions

MSH, KG, TR, AK, MB designed the experiment. MSH, JK, WG, SR conducted the experiment. MSH, JK analyzed the data. MSH drafted the manuscript. All authors revised the final version of manuscript.

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CHAPTER 5

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

General discusion

The parthenogenetic marbled crayfish, *Procambarus virginalis* has been in the spotlight since its discovery in the German pet trade in mid-1990s. It has received extra attention by biologists, ecologists or other scientists due to its peculiar mode of reproduction, short generation time, fast growth and marbled body color. Research revealed that external morphological changes during embryonic development and behavior features of different life stages in marbled crayfish correspond to those described in other, sexually reproducing, freshwater crayfish although the marbled crayfish possesses an unusual mode of reproduction (Seitz et al., 2005; Vogt, 2008, 2013; Vogt et al., 2004). Along with this similarity, rapid growth, high fecundity, frequent breeding, suitable size, and genetic uniformity of individuals makes this crayfish an ideal candidate for laboratory research in different research disciplines. In the last decade, marbled crayfish have been used for diverse range of scientific research, e.g. morphology, ecology, neurobiology, epigenetics, behavior, bio-chronology and evolutionary biology which also strongly favored it as a good contender for model organism (Hossain et al., 2018; Vogt, 2010, 2018; Vogt et al., 2015). However, there are still some gaps in knowledge which need to be considered to support this declaration. Thus, marbled crayfish morphometrics and reproductive relationships, interaction patterns with established invasive species, and behavioral and physiological effects after pharmaceutical exposure were investigated to explore the crayfish's role in the ecosystem as well as to strengthen its use as a model species in behavioral and toxicological research.

Morphometry, growth, size and maturity

Arthropods and crustaceans do not grow continuously due to their jointed protective exoskeletons. Increases in size are only possible after shedding the rigid exoskeleton, termed a molt event (Holdich, 2002a). Crayfish growth may be either isometric (typically seen in juveniles) or allometric, where some parts of body grow disproportionately to the rest (Rhodes and Holdich, 1979). In the present study, marbled crayfish exhibited allometric growth in several body parts (abdomen width, claw length, claw width, claw height, total length, carapace length, carapace width, and abdomen length). Seitz et al. (2005) also found that marbled crayfish growth is not isometric similar to other species of crayfish like *Astacus astacus, Pacifastacus leniusculus* (Abrahamsson, 1971), *Faxonius limosus* (Buřič et al., 2010a, b) or *Faxonius rusticus* (Anderson and Simon, 2015). The condition factor in our study was estimated as >1 which indicates that our culture conditions for marbled crayfish were satisfactory. The values obtained from animals in a laboratory culture can be used for comparison with wild crayfish where which are exposed to various environmental factors and stressors. In this study, we provided evidence concerning the robustness and relative growth of different body parts. Some of morphometric parameters increased significantly faster than the body size, while others grow proportionally slower. For example, the growth of the abdomen in all female crayfish (males are not present in the species) increases in width, while abdomen length shows a negative relationship with increasing body size. The wider abdomen of females tightly correlates with the need to provide sufficient space for their upcoming eggs and offspring to attach to the pleopods (Buřič et al., 2010b; Hamr and Berrill, 1985). Marbled crayfish chelae also grew faster than the body size as observed in other crayfish, e.g. *Austropotamobius pallipes* (Rhodes and Holdich, 1979). Large chelae might be important in intra- and inter-specific competition for basic resources (Nakata and Goshima, 2003) or in antipredator behavior, agonistic encounters, and reproduction (Garvey and Stein, 1993) as well as aid in general communication through chemoreception (Belanger et al., 2008).

Beyond growth and morphometry, the sustainability of any species depends on its reproductive efficiency and fecundity. In the present study, total fecundity was defined as the sum of eggs attached to the female's pleopods in the first week of their incubation. We also measured the size of marbled crayfish at first reproduction which varied widely from 31.5 to 73.5 mm of total length (weight 0.7–9.9 g), with the fecundity ranging between 22 and 349 eggs per female. Previously, length at first maturity was recorded at 40 mm in lab cultures and wild conditions, with the initial fecundity ranging between 10–416 eggs per female (Cvitanić et al., 2016; Kozák et al., 2015; Seitz et al., 2005; Vogt et al., 2004). The difference in size at maturity can be influenced by several nutritional and environmental factors (e.g. temperature, food, season) (Baker et al., 2003) and could be also clarified on the basis of rearing condition, and the position of the animal with the social hierarchy. The variability described above is not known, because of similar rearing conditions for all observed individuals. We hypothesize that the possible drivers of this variation could be the social position or personality of individuals. In addition, clutch size is greater in larger and older (to some extent) crayfish (Abrahamsson, 1971; Alcorlo et al., 2008; Seitz et al., 2005). The highest fecundity observed (528 eggs) was recorded in an individual that reproduced multiple times. Therefore, maiden females have significantly lower relative fecundity (fecundity recalculated to 1 mm of CL) than that observed in repeatedly reproducing females. The pleopodal egg number increases with female length and weight (Nakata and Goshima, 2004; Tropea et al., 2012) and is significantly correlated with absolute fecundity and total body length, cephalothorax length, and body weight (Chybowski, 2013). We also described a tight linear relationship between the number of eggs on the 3rd pair of pleopods and total fecundity, regardless of fecundity variability and the size of the females. This finding provides a useful tool for fecundity estimation in studies using marbled crayfish as a model species or in studies of its population dynamics in natural populations and even in investigations of the fecundity of females which partially lost their clutch.

Interactions of marbled crayfish with other crayfish invaders in Europe

Agonistic behavior is common in all crayfish species and depends on multiple factors, including direct competition for limited resources or for the establishment of a hierarchy (Gherardi, 2002; Krebs and Davies, 1997). Crayfish use chemical or visual communication signals to threaten their opponent during direct interactions (Breithaupt, 2010). Successful invaders often exhibit greater aggression during interactions than the native species they displace (Gherardi, 2002). The success of any invader could be best elucidated by comparative analysis of competitive interactions (Van Kleunen et al., 2010) when organisms share identical ecological niches and resources (food, shelter, space) (Wilson, 1992, 2000). Freshwater ecosystems are frequently invaded by non-indigenous species that interact with one another as well as with native biota (Hudina et al., 2011). Among these non-native species, marbled crayfish have spread to wild habitats worldwide, posing a competitive threat to other species, despite the marbled crayfish's reputation for a calm disposition (Faulkes, 2015). In this chapter we presented evidence that the marbled crayfish is strong enough to create a threat to already established alien species with higher levels of activity and aggression. As the first opponent was used well-known invader of freshwater ecosystems worldwide, the red swamp crayfish, *Procambarus clarkii*. It shows higher activity and aggression towards other species as well as conspecifics (Jimenez and Faulkes, 2011). The other species was an invader of the Rhine River, the calico crayfish, *Faxonius immunis* which is displacing the spiny-cheek crayfish, *Faxonius limosus* from within their concurrent range (Chucholl et al., 2008). Both species are described in the literature as very aggressive (Jimenez and Faulkes, 2011; Chucholl et al., 2008), compared to more docile nature of the marbled crayfish.

However, marbled crayfish were able to dominate over similarly sized red swamp crayfish in both juvenile and adult individuals. Marbled crayfish were less active in initialization of contacts and fights in juvenile animals but, the dominance establishment was more successful in the juvenile group than in the adults. Mature crayfish needed more time to establish dominance due to prolongation of cognition and assessment of the size-matched opponent. The tendency of rapid formation of social hierarchies in small crayfish has been reported also by Sato and Nagayama (2012). In mature animals, dominance of marbled crayfish was more visible in female-female pairs than female-male pairs (100% with female-female pairs and 60% with female-male pairs). This might be related to the more aggressive nature of males (Gherardi, 2002), use of different strategies by either sex during agonistic interactions (Wofford, 2013), and the superior resource holding potential of males over females (Wilson, 1992). Patterns of agonistic behavior are also dependent on the size and age of the crayfish (Bovbjerg, 1956). Mature individuals showed significant differences in fight statistics (total number of fights and their duration) compared to the premature group. Schroeder and Huber (2001) described that small crayfish escalate fights more rapidly and also resolve them quickly. The higher activity of premature red swamp crayfish, and thus higher energy loss for locomotion, might give the less active marbled crayfish an energetic advantage in the formation of dominance hierarchies.

In the second experiment, we found that marbled crayfish also compete with calico crayfish. Marbled crayfish was dominant over similarly sized animals and won significantly more fights with females of the calico crayfish in the absence of shelter. In the presence of shelter marbled crayfish were more active in contact and fight initiation with female calico crayfish. With male calico crayfish, all of the observed parameters were comparable between species (no significant difference) in the presence and absence of shelter. This difference might be related to male aggression or fighting strategies as described by Gherardi (2002) and Wofford (2013). The dominance pattern of marbled crayfish was same as we described in our previous experiment (Hossain et al., 2019b), but the proportion of the undefined hierarchy was higher than in the previous study in both the absence (25%) and presence of shelter (50%). The marbled crayfish formed hierarchies more rapidly with calico crayfish males than with females. From their dominant status it is ruled out that marbled crayfish shelter occupancy was equal to that of male calico crayfish and higher than that of females. This could be also possible in nature as aggressive encounters in laboratory conditions are similar to combat dynamics of crayfish in the wild (Bergman et al., 2003). The elevated activity and aggressiveness[1] we observed in marbled crayfish towards calico and red swamp crayfish could speed dispersal in the wild during interactions for space and resources.

The intra- and interspecific interactions involving marbled crayfish provide a wide spectrum of new knowledge towards our understanding of behavioral patterns and could inform estimates of the impacts of invasive species on native populations, or for comparison with other invasive species. The parthenogenetic, highly fecund marbled crayfish has the potential to overpower known aggressive species, and has other, previously mentioned abilities, which make the marbled crayfish a substantial threat to native communities and the biodiversity of freshwater ecosystems. However, a comparison of size distribution of natural population in relation to fight outcome would have been recommended as a future study to evaluate the ecological impact.

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[1] According to thesis reviewer suggestions when the establish dominance results (which crayfish become more dominant after fight) of the experiments are evaluated through binominal test, the marbled crayfish were not significantly more successful than the other tested species (marbled or red swamp crayfish) in any combination of groups

Effect of pharmaceuticals on marbled crayfish

The lives of aquatic organisms and therefore, whole freshwater ecosystems, are heavily influenced by changes in habitat and water quality. Aquatic ecosystems continuously receive pollutants from a variety of anthropogenic sources. Pharmaceutically active compounds (PhAC) can have sub-lethal impacts on organisms and their ecology even at very low concentrations which are available in surface waters (ng-μgL^{-1}) (Grabicova et al., 2015; Grabicova et al., 2017). These active compounds influence the behavior of aquatic organisms without causing actual pathological damage (Brodin et al., 2013) due to their mode of action. Therefore, we focused on behavioral endpoints at environmentally relevant concentration of PhACs using clonal marbled crayfish as a model organism. Behavioral studies were suggested as a good criterion for pharmacological risk assessment (Brodin et al., 2014; Klaminder et al., 2014).

In our experiment we found that environmentally relevant concentrations (~1 μgL^{-1}) of methamphetamine and sertraline had effects on the behavior and life processes of the marbled crayfish, such as molting, mortality and spawning. Methamphetamine exposure did not change crayfish locomotion activity but significantly influenced the time spent outside the shelter compared to unexposed crayfish which are naturally shy (Gherardi, 2002). Crayfish exposed to sertraline significantly increased both their locomotion activity and time spent outside the shelter. These effects might be due to the increase of serotonin level in nervous tissue of an exposed organism through these psychotropic compounds (SSRI, Selective Serotonin Reuptake Inhibitor) (Lillesaar, 2011). McPhee and Wilkens (1989) showed that increased serotonin levels influenced the crustacean hyperglycemic hormone, and consequently increased their locomotor activity and exploration behavior. Psychotropic compounds have variable effects on organisms, on the basis of concentration, exposure duration and even the species being studied. Tierney (2016) and Buřič et al. (2018) observed, contrary to our findings after sertraline exposure, reduced locomotion in crayfish exposed to fluoxetine and citalopram at environmentally relevant concentrations. Again, oxazepam at environmentally relevant concentration increased locomotion activity in marbled crayfish, while the antidepressant venlafaxine had no such effect (Kubec et al., 2019). However, Fong et al. (2015) found reduced locomotion in two species of marine snails at higher concentrations of venlafaxine (31.3 μgL^{-1}).

Many pollutants or pharmaceuticals are not available as a single compound in aquatic environment. They are usually present as a mixture of different types of chemicals, PhACs or their different groups with different modes of action. Therefore, the effects on aquatic organisms might be not as straight forward as observed in a single compound exposure system. In this regard, we made a mixture of PhACs (sertraline, venlafaxine, citalopram, oxazepam, tramadol, and methamphetamine) at environmentally relevant concentrations and exposed marbled crayfish to the mixture for 21 days. Exposed crayfish demonstrated significant behavioral effects such as reduced locomotion activity and reduced shelter seeking behavior. Previous experiments with these compounds as single chemical exposures have documented similar reduced shelter seeking behavior (Buřič et al., 2018; Hossain et al., 2019a; Kubec et al., 2019). The reductions in locomotor activity resulting from exposure to the mixture were different from the results of single compound exposures of other PhACs. The mixture of pharmaceuticals had no detectable effect on glair gland development, molting, spawning or mortality rate during the exposure period though sertraline had a positive influence on the frequency of molting, spawning and mortality. We found that the effects of a PhACs mixture are not like single exposure studies as have been observed by other researchers at higher concentrations rather than environmentally relevant concentrations (Ding et al., 2016; Galus et al., 2013; Li and Lin, 2015). Consequently, the mixture effect may be an additive or antagonistic or synergistic contribution from each chemical (Stenersen, 2004). This effect also might be depend on the model species being tested (Di Poi et al., 2018). Other effects of mixtures (e.g. gametogenesis, embryonal development etc.) cannot be excluded.

The glucose level has a positive relationship with crayfish stress and a negative relationship with brain serotonin level (Fossat et al., 2015; Fossat et al., 2014). In this experiment, we did not find any significant difference in hemolymph glucose levels between exposed and unexposed crayfish. Meanwhile, there were insignificant positive and negative relationship patterns between hemolymph glucose levels and the observed behaviors of control and PhACs exposed crayfish respectively. Thus, we assume that the chemicals made the crayfish less stressed and increased their boldness. Ultimately, exposed crayfish spent significantly more time outside of the shelter. This could have negative consequences for the crayfish, as sheltering is an important behavior for crayfish throughout their life, especially in daylight and in a novel environment where crayfish explore the available strategic resources (shelters) first (Gherardi, 2002; Kubec et al., 2018; Lozán, 2000). This behavior and reduced locomotion could lead to higher mortality due to predation (Hossain et al., 2019a). Anxiety, high aggression, and higher vulnerability to predators could lead to the disruption of larger ecosystem-level processes (Brodin et al., 2014) as crayfish are important keystone species in the freshwater ecosystem (Holdich, 2002b). Further studies are needed which focus on variable mixtures of pollutants, their mode of action in dilution, and the possible behavioral effects and molecular relationships.

Conclusion

The small size at maturity, high fecundity, short generation time, captive culture suitability, similar growth pattern as like other crustacean and genetic uniformity suggest that marbled crayfish are desirable as a model organism. The clonal marbled crayfish fits as an ideal model organism for behavioral studies in response to pharmaceuticals at environmental concentrations. The interaction experiments showed marbled crayfish's aggressiveness and competitiveness with other invasive species. Therefore, it could be also used in mesocosm as a model animal to know the invasion process and ecosystem function through non-native invasive species. All studies included in this thesis confirmed that the marbled crayfish is feasible to be used as a model organism in biological and ecological research. However, the marbled crayfish is also an invasive species and is listed in the European Commission Regulations (EU Regulation No. 1143/2014 and Commission Implementing Regulation No. 2016/1141). Among other species, this regulation currently prohibits the import, trade, keeping and breeding of marbled crayfish in the European Union (EU). Keeping for research is allowed but requires permission. Therefore, propagation for research or commercial use should follow these regulations to avoid possible intentional or unintentional releases into the natural environment.

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English summary

The marbled crayfish: parthenogenetic invasive species as an applicable biological model

Md Shakhawate Hossain

In this thesis, I provide a review on clonal marbled crayfish and examples of its use as a model organism and the fortitude of possible threats it poses for the ecosystem. Behavioral observations offer a quick look into changes in the organisms or ecosystem due to anthropogenic factors before lethal effects occur in organisms or the ecosystem becomes irreversibly altered. Crustaceans, and especially crayfish, represent practical model organisms for ethological investigation in aquatic ecosystems. Crayfish have benefits over other invertebrates given their complex social interactions, exclusive eco-ethological behavior in a variety of feeding habits, activity cycles, habitat selection, as well as resource utilization at the level of species, sex, and age in both field and laboratory conditions. Crayfish play prominent roles in the ecosystem and are often considered keystone species and ecosystem engineers. Therefore, pollution and invader's which effect the native crayfish stock can results in ecosystem alterations.

In chapter two, marbled crayfish general growth pattern was investigated through length-weight relationships and the use of a condition factor. Crayfish have allometric growth and attain good health under lab culture conditions. The abdomen width and claw growth was allometric compared to post orbital carapace length which confirms the importance of these body parts. The first reproduction events were recorded at the smallest total length of 31.5 (range 31.5–73.5) mm with a fecundity range from 22–349. Fecundity parameters were confirmed to have a fitted linear relationship to marbled crayfish length and weight. The tight correlation of egg counts on the third pleopods and total fecundity have been observed which could be used in clutch size estimation of females with partially lost clutches. This results could also be used in crayfish conservation research, captive breeding, and recovery programs as well as in the field of aquaculture.

In chapter three, experimental interaction patterns of marbled crayfish with two prominent invasive crayfish species were studied. Ecosystems increasingly face concurrent invasions by multiple species, but knowledge about interaction relationships amongst invasive species is understudied. The marbled crayfish have been engaged in interactions with well-known invaders like the red-swamp crayfish and the Rhine River established invasive species, calico crayfish which displaced the invasive spiny-cheek crayfish within areas of co-occurrence. With both species, the marbled crayfish have shown competency to dominate. We found that the interactions success and intensity depends on size, sex and available resources, including shelter. After the establishment of dominance, fight intensities and aggressiveness decreased in general. Our animals were size-matched, so the observed patterns could be different in natural conditions due to the greater maximal size of red swamp crayfish and calico crayfish. Nevertheless, aggression and social hierarchies are not only factors determining invader success. Other factors, such as reproductive strategy and adaptability/plasticity are also important. This parthenogenetic species poses a substantial threat to native ecosystems and biodiversity of aquatic environments, since it could be established not only in crayfish free habitats, but also capable of outcompeting native species and at least some aliens.

Ubiquitous pharmaceutically active compounds are a class of emerging pollutants that affect aquatic organisms. The effects of pharmaceuticals on clonal marbled crayfish have been presented in the fourth chapter using simple behavioral assays along with observations of life history and physiological changes. The results showed that pharmaceuticals at environmentally

relevant concentration alter the behavior and life history attributes of crayfish. The effects of these chemicals showed variability in single chemical exposures. These variations were due to exposure durations, chemical concentrations, exposure methods and even species used, as evident from our study and the literature. The effects of compound mixtures at environmentally relevant concentration had significant effects on crayfish behavior but were not as straight forward to interpret as single exposure effects. The mixture effect depends upon the additive, synergistic or antagonistic nature of chemicals involved. However, both single and mixture exposures significantly altered the behavior of marbled crayfish which could lead to disruption of natural ecosystem processes.

In conclusion, these studies convey information regarding marbled crayfish general biology, potency of invasiveness and possible use in toxicological research as a model organism highlighting behavioral assays. It suggests that observations of behavior is a useful applied approach not only for investigating basic biology, or intra- and inter-specific interactions, but also for calculating the risks associated with micro-pollutants.

Czech summary

Mramorovaný rak: parthenogenetický invazivní druh jako použitelný biologický model

Md Shakhawate Hossain

V této disertační práci předkládáme v první kapitole přehledovou práci o klonálním raku mramorovaném a příklady jeho použití jako modelového druhu a zároveň představení možných hrozeb, které může znamenat pro invadovaný ekosystém. Behaviorální sledování umožňuje rychlý náhled do změn na úrovni organizmů či ekosystémů, způsobených antropogenními faktory ještě předtím, než tyto faktory mají letální efekt nebo dojde k nevratným změnám v ekosystému. Korýši, a zejména raci, představují praktické modelové organizmy pro etologické studie ve vodních ekosystémech. Raci mají výhody v jejich komplexních sociálních interakcích, různé potravní návyky, cykly aktivity, preference habitatů, stejně jako využití zdrojů na úrovni druhů, pohlaví či věku, a to jak v přírodních, tak i v laboratorních podmínkách. Raci hrají důležitou roli v ekosystému a jsou často považováni za klíčové druhy a ekosystémové inženýry. Proto invazní druhy raků ve spojitosti se znečištěním prostředí můžou vést ke značným změnám ekosystémů.

V druhé kapitole byly popsány základní morfometrické zákonitosti raka mramorovaného analýzou délko-hmotnostních vztahů a kondičního faktoru. Rak mramorovaný vykazoval v laboratorních podmínkách alometrický růst a dobrou kondici. Šířka abdomenu a velikost klepet rostla prokazatelně rychleji než velikost těla, což poukazuje na důležitost těchto částí těla. První reprodukce byla zaznamenána při nejmenší celkové délce těla 31,5 mm (ale pohybovala se v rozmezí 31,5–73,5 mm) a plodnost při první reprodukci se pohybovala mezi 22–349 vajíčky. Plodnost byla úzce závislá na délce a hmotnosti samic. Nejužší závislost celkové plodnosti byla ale zjevná oproti počtu vajíček na třetím páru pleopodů, což může být dobrým nástrojem pro odhad celkové snůšky vajíček i u samic, které části snůšky ztratily. Výsledky mohou pomoci v experimentálních chovech a ve využití druhu pro výzkumné účely.

Ve třetí kapitole byly sledovány interakce raka mramorovaného s dalšími dvěma invazními druhy raků. Ekosystémy totiž v současnosti čelí mnohonásobným invazím, ale vědomosti o interakcích těchto druhů stále nejsou dostatečné. Proto byl rak mramorovaný studován v interakcích s dvěma velmi dobře známými invazními a agresivními druhy, rakem červeným a rakem kalikovým, který vytlačuje raka pruhovaného v řece Rýn. Rak mramorovaný byl schopen dominovat nad velikostně srovnatelnými jedinci obou druhů. Úspěšnost v interakcích a jejich intenzita zároveň korelovala s velikostí a pohlavím oponentů a přítomností zdrojů (úkrytu). Po ustanovení dominance intenzita soubojů a agresivity klesala. Sledované výsledky mohou být v reálných podmínkách jiné zejména vzhledem k větší maximální velikosti raka červeného, nicméně agrese a sociální hierarchie nejsou jediné faktory určující úspěšnost invazního druhu. Dalšími důležitými faktory jsou zejména reprodukční strategie a adaptabilita druhu. Partenogenetický rak mramorovaný představuje značnou hrozbu pro invadované ekosystémy a původní biodiverzitu, a to nejen pro původní druhy raků, ale i pro ostatní organizmy. Rak mramorovaný je zároveň prokazatelně schopen být minimálně rovnocenným či dokonce schopnějším kompetitorem jiným invazním druhům.

Stranou biologických invazí stojí další faktor ovlivňující vodní organizmy a ekosystémy – chemické znečištění, ve které farmaceuticky aktivní látky hrají recentně velmi výraznou roli. Efekt farmak na raka mramorovaného použitého jako modelový organizmus je náplní čtvrté kapitoly disertační práce. Efekt environmentálně relevantních koncentrací vybraných farmak byl sledován s použitím relativně jednoduchého sledování změn v chování, životním cyklu a fyziologii sledovaných jedinců. Výsledky ukazují, že i takto nízké koncentrace polutantů jsou schopny změnit životní projevy raků. Při testování jednotlivých farmak byla ale sledována značná variabilita v jejich účinku. Tato variabilita i mezi terapeuticky podobnými substancemi může být zapříčiněna délkou expozice, koncentrací látek, metodami expozice, ale i použitým druhem, jak je patrné zejména ze známé literatury i z naší práce. Efekt směsí polutantů na úrovni environmentálně relevantních koncentrací má viditelný přímý efekt na chování raků, ale není snadné jej tak jednoduše interpretovat jako jednotlivě testované látky. Efekt těchto směsí záleží na aditivním, synergickém nebo antagonistickém vztahu jednotlivých použitých látek. Tak či onak, naše práce poukazují, že jak jednotlivé látky, tak jejich směsi signifikantně mění chování sledovaných organizmů, což může ve finále znamenat narušení přirozených procesů ve vodních ekosystémech.

Prezentovaná práce obecně sděluje informace o základní biologii raka mramorovaného, jeho invazním potenciálu a zároveň o jeho potenciální využitelnosti nejen v ekotoxikologickém výzkumu. Práce rovněž vyzdvihuje sledování chování raků jako užitečnou metodu nejen pro základní biologický výzkum, ale i pro rizika spojená s výskytem mikro-polutantů.

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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: 135–138. (IF 2018 = 5.589)

Hossain, M.S., Buřič, M., Moore, P.A., 2020. Exposure paradigm of fluoxetine impacted the *Faxonius virilis* agonistic behavior differently. Science of the Total Environment 699: 134300. (IF 2018 = 5.589)

Hossain, M.S., Guo, W., Martens, A., Adámek, Z., Kouba, A., Buřič, M., 2020. Potential of marbled crayfish *Procambarus virginalis* to supplant invasive *Faxonius immunis*. Aquatic Ecology 54: 45–56. (IF 2018 = 2.505)

Hossain, M.S., Kubec, J., Guo, W., Roje, S., Grabicová, K., Randák, T., Kouba, A., Buřič, M., 2020. Combination of six psychoactive pharmaceuticals at environmental concentrations alter the locomotory behavior of clonal marbled crayfish. Manuscript.

Ethin, R., **Hossain, M.S.**, Roy, A., Rutegwa, M., 2019. Stock identification of minor carp, *Cirrhinus reba*, Hamilton 1822 through landmark-based morphometric and meristic variations. Fisheries and Aquatic Sciences 22: 12. (IF 2018 = 0.71)

Fořt, M., **Hossain, M.S.**, Kouba, A., Buřič, M., Kozák, P., 2019. Agonistic interactions and dominance establishment in three crayfish species non-native to Europe. Limnologica 74: 73–79. (IF 2018 = 2.051)

Guo, W., Kubec, J., **Hossain, M.S.**, Buřič, M., McClain, R., Kouba, A., 2019. High air humidity is sufficient for successful egg incubation and early postembryonic development in the marbled crayfish. Freshwater Biology 64: 1603–1612. (IF 2018 = 3.404)

Hossain, M.S., Kubec, J., Grabicová, K., Grabic, R., Randák, T., Guo, W., Kouba, A., Buřič, M., 2019. Environmentally relevant concentrations of methamphetamine and sertraline modify the behavior and life history traits of an aquatic invertebrate. Aquatic Toxicology 213: 105222. (IF 2018 = 3.794)

Hossain, M.S., Kubec, J., Kouba, A., Kozák, P., Buřič, M., 2019. Still water run deep: marbled crayfish dominate over red-swamp crayfish in agonistic interactions. Aquatic Ecology 53: 97–107. (IF 2018 = 2.505)

Kubec, J., **Hossain, M.S.,** Grabicová, K., Randák, T., Kouba, A., Grabic, R., Roje, S., Buřič, M., 2019. Oxazepam alters the behavior of crayfish at diluted concentrations, Venlafaxine does not. Water 11: 196. (IF 2018 = 2.524)

Rashid, I., **Hossain, M.S**., Salam, M.A., Rafiquzzaman, S.M., 2019. Evaluation of seminal plasma composition and spermatozoa quality parameters of silver barb, *Barbonymus gonionotus* Bleeker, 1850. Fish Physiology and Biochemistry 45: 105–114. (IF 2018 = 1.729)

Hossain, M.S., Kouba, A., Buřič, M., 2019. Morphometry, size at maturity and fecundity of marbled crayfish (*Procambarus virginalis*). Zoologischer Anzeiger 281: 68–75. (IF 2018 = 1.601)

Hossain, M.S., Patoka, J., Kouba, A., Buřič, M., 2018. Clonal crayfish as biological model: a review on marbled crayfish. Biologia 73: 841–855. (IF 2018 = 0.728)

Rahman, M.L., Salam, M.A., Ahsan, M.E., **Hossain, M.S.,** Hossain, M.A., 2017. Protein-sparing ability of carbohydrates from different sources in diets for fry of stinging catfish *Heteropneustes fossilis*. Sains Malaysiana 46: 239–244. (IF 2018 = 0.540)

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, Cartagena, Colombia.

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, Cartagena, Colombia.

Hossain, M.S., Kubec, J., Grabicová, K., Randák, T., Guo, W., Kouba, A., Buřič, M., 2019. Environmentally relevant concentrations of psychotropic drugs modify the behavioral patterns of an aquatic invertebrate. 25th ISCB International Conference, Trends in Chemical and Biological Sciences: Impact on Health and Environment, 12–14 January 2019, Lucknow, India.

Guo, W., **Hossain, M.S**., Buřič, M., Kouba, A., 2018. Marbled crayfish is able to rule over calico crayfish in agonistic interactions. In: NEOBIOTA 2018, 10th International Conference on Biological Invasions: New Directions in Invasion Biology, 3–7 September 2018, Dún Laoghaire, Dublin, Ireland, p. 100.

Hossain, M.S., Guo, W., Kubec, J., Buřič, M., Kouba, A., 2018. High air humidity is sufficient for successful incubation and early postembryonic development of invasive freshwater crayfish. In: NEOBIOTA 2018, 10th International Conference on Biological Invasions: New Directions in Invasion Biology, 3–7 September 2018, Dún Laoghaire, Dublin, Ireland, p. 77.

Hossain, M.S., Kubec, J., Kouba, A., Kozák, P., Buřič, M., 2017. Placid but powerful: unexpected success of marbled crayfish over red-swamp crayfish. European Crayfish Conference 2017: The IAA Cruise 2017, Helsinki (Finland) and Stockholm (Sweden), 16–18 August 2017.

Training and supervision plan during study

Name	Md Shakhawate Hossain
Research department	2016–2020, Laboratory of Freshwater Ecosystems of FFPW
Supervisor	Milos Buřič, Ph.D.
Period	26th October 2016 until 22nd June 2020

Ph.D. courses	**Year**
Pond aquaculture	2017
Applied hydrobiology	2017
Basic of scientific communication	2017
Ichthyology and fish taxonomy	2017
English language	2016

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

International conferences	**Year**
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, Cartagena, Colombia.	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, Cartagena, Colombia.	2019
Hossain, M.S., Kubec, J., Grabicová, K., Randák, T., Guo, W., Kouba, A., Buřič, M., 2019. Environmentally relevant concentrations of psychotropic drugs modify the behavioral patterns of an aquatic invertebrate. 25th ISCB International Conference, Trends in Chemical and Biological Sciences: Impact on Health and Environment, 12–14 January 2019, Lucknow, India.	2019
Guo, W., **Hossain, M.S.**, Buřič, M., Kouba, A., 2018. Marbled crayfish is able to rule over calico crayfish in agonistic interactions. In: NEOBIOTA 2018, 10th International Conference on Biological Invasions: New Directions in Invasion Biology, 3–7 September 2018, Dún Laoghaire, Dublin, Ireland, p. 100.	2018
Hossain, M.S., Guo, W., Kubec, J., Buřič, M., Kouba, A., 2018. High air humidity is sufficient for successful incubation and early postembryonic development of invasive freshwater crayfish. In: NEOBIOTA 2018, 10th International Conference on Biological Invasions: New Directions in Invasion Biology, 3–7 September 2018, Dún Laoghaire, Dublin, Ireland, p. 77.	2018
Hossain, M.S., Kubec, J., Kouba, A., Kozák, P., Buřič, M., 2017. Placid but powerful: unexpected success of marbled crayfish over red-swamp crayfish. European Crayfish Conference 2017: The IAA Cruise 2017, Helsinki (Finland) and Stockholm (Sweden), 16–18 August 2017.	2017

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Internship at Bowling Green State University and University of Michigan Biological Station, USA	2018
Internship at University of Hull, UK	2020

Pedagogical activities	**Year**
Delivered lecture on “Bangladesh” in MEVPIS with grammar school student	2017
Consultant of B.Sc. student Josef Mareš (successfully defence in 5/2019)	2019
Consultant of M.Sc. student Milan Man (successfully defence in 5/2019)	2019
Consultant of M.Sc. student Miroslav Krejčí (to be defended in 5/2020)	2020

Curriculum vitae

PERSONAL INFORMATION

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Nationality:	Bangladeshi
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EDUCATION

2016 – present	Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic
2007–2008	M.S. in Fisheries Biology and Genetics, Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh, Bangladesh
2002–2006	B.Sc. (Hons) in Fisheries, Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh, Bangladesh
1997–2001	SSC, HSC; Dhaka Residential Model College, Dhaka, Bangladesh

Workshops / Training Schools

12/11–15/11 2018	Exploring Biological Sequence. European Bioinformatics Institute (EMBL-EBI), Wellcome Genome. Cambridge, UK

RESEARCH STAY AND COLLABORATIONS

12/06–20/08 2018	Professor Paul A. Moore. Bowling Green State University, Ohio, USA
15/01–16/03 2020	Associate Professor Thomas Breithaupt, University of Hull, Hull, United Kingdom