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Some reproductive and physiological aspects of invasive crayfish

Některé reprodukční a fyziologické aspekty invasivních raků

Buket Yazıcıoğlu

Czech Republic, Vodňany, 2017

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Reproduction strategies in Crayfish

1.2. Yazicioglu B., Reynolds, J., Kozák, P., 2016. Different aspects of reproduction strategies in crayfish: A review. Knowl. Manag. Aquat. Ecosyst. 417, 33.

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1.1. Reproduction strategies in crayfish

Freshwater crayfish (Crustacea: Decapoda) are highly diverse animals currently comprising 3 families, 33 genera, and over 640 known species (Crandall and Buhay, 2007). They are commercially and ecologically important animals and considered keystone species with considerable biological impacts on the environment (Kozak et al., 2011; Kawai et al., 2015). Many non-native crayfish species have been introduced outside of their endemic ranges and caused serious problems for resident species (Kouba et al., 2014). Study of reproductive biology in non-native crayfish may contribute to a better understanding of their spreading mechanism so that some approaches for controlling of their negative effects could be developed. Crayfish possess unusual traits in different aspects of their reproduction including mode of reproduction, gamete biology and reproductive behavior. In this thesis, we tried to carry out some experiments to further clarify unusual characteristics of reproduction in these aquatic animals.

Mode of reproduction

The normal crayfish reproduction strategy is called gonochorism and each individual has only one separate sex as male or female. However, some other modes of reproduction such parthenogenesis and hermaphroditism have been reported in some freshwater crayfish (Harlıoğlu and Farhadi, 2017).

Mode of reproduction in crayfish has recently been extended by the discovery of the parthenogenesis in the marbled crayfish *Procambarus fallax* as the only obligate parthenogenetic decapod known to date (Scholtz et al., 2003). Also, it is worthy to mention that intersex individuals showing male and female characters have been regularly observed in low frequencies in some crayfish species, particularly in the Parastacidae. However, functional sequential hermaphroditism is very rare among crayfish (Rudolph, 1995). In addition, some cases of hybridization have been reported in different crayfish species (e.g. Maguire et al., 2013). We have addressed these issues in a review article as the Chapter 1.2 of the present thesis. In addition, a paper dealing with intersex in signal crayfish forms second chapter of this thesis.

Gamete biology

Crayfish gametes possess unusual morphology and mechanism of action. Spermatozoa of crayfish like other decapods and most crustaceans are immotile (Tudge, 2009). They are packed in multi-layered package so called spermatophore when they are passing through vasa deferantia (Vogt, 2002). Despite their simple structure, morphological features and biometrical data have been used to distinguish different species of crayfish (Niksirat et al., 2013a, b; Kouba et al., 2015). As third chapter of this thesis, we have used morphological features and biometrical data from eleven species of crayfish for phylogenetic studies.

The reproductive behavior

One of the most outstanding features of crayfish reproductive behavior is post-mating spermatophore storage in females. It has been shown that male gametes undergoes some morphological and molecular modification to gain the ability of fertilization during post-mating storage period (Niksirat et al., 2014, 2015). We carried out a comparative study to determine timing of mating and duration of post-mating spermatophore storage in noble and signal crayfish as the last chapter of this thesis.

To address above mentioned issues regarding unusual reproductive traits in crayfish, we carried out several experiments aimed to:

1) Clarify different unusual reproductive modes in crayfish including the first report of intersex in signal crayfish.
2) Distinguish different species using morphological study of spermatozoa in eleven crayfish species.
3) Investigate any differences in post-mating spermatophore storage strategies in signal and noble crayfish.

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1.2. Different Aspects of Reproduction Strategies in Crayfish: A review

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REVIEW PAPER OPEN ACCESS

Different aspects of reproduction strategies in crayfish: A review

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Abstract – Study of the reproductive strategy of crayfish species is of great importance in the current astacological world. Crayfish are among the largest freshwater invertebrates, and as keystone species, they are able to regulate the structure of the benthic fauna in the freshwaters, demonstrating different ecological strategies and life spans ranging up to 20+ years. In order to bring together the various pieces of information related to this issue, this overview of published scientific reports was conducted. The majority of crayfish species studied show sexual dimorphism, with approximately equal numbers of males and females. However, over some decades numerous observations have been made for a few species that may have different modes of reproduction, such as hermaphroditism or intersex (*e.g. Cherax quadricarinatus*, *Samastacus spinifrons, Parastacus virilastacus* and *Pacifastacus leniusculus*) and parthenogenesis (only *Procambarus fallax* f. *virginalis*). A recent study showed a new case of parthenogenesis as apomictic parthenogenesis (only *Orconectes limosus*). In addition, there are many investigations into the reproduction biology of crayfish, including using eyestalk ablation or androgenic gland ablation under various lab conditions and hybridization under natural conditions (*e.g. Astacus astacus* X *Astacus leptodactylus*, *Orconectes rusticus* X *Orconectes propinquus*). There are also some chemical factors which could possibly affect the reproduction system of crayfish in the wild.

Key-words: crustacea / parthenogenesis / intersex / hybridization

Résumé – Différents aspects des stratégies de reproduction des écrevisses : une revue. L'étude de la stratégie de reproduction des espèces d'écrevisses est d'une grande importance dans le monde astacicole actuel. Les écrevisses sont parmi les plus grands invertébrés d'eau douce, et comme des espèces clés, elles sont capables de réguler la structure de la faune benthique dans les eaux douces, présentant différentes stratégies écologiques et des durées de vie allant jusqu'à 20 ans. Afin de réunir les différents éléments d'information relatifs à cette question, une revue des publications scientifiques a été conduite. La majorité des espèces d'écrevisses étudiées montrent un dimorphisme sexuel, avec un nombre approximativement égal de mâles et de femelles. Cependant, depuis quelques décennies, de nombreuses observations ont été faites pour quelques espèces qui peuvent avoir différents modes de reproduction, tels que l'hermaphrodisme ou l'intersexe (par exemple *Cherax quadricarinatus*, *Samastacus spinifrons*, *Parastacus virilastacus* et *Pacifastacus leniusculus*) et la parthénogenèse (seulement *Procambarus fallax f. virginalis*). Une étude récente a montré un nouveau cas de parthénogenèse, une parthénogenèse apomictique (seulement *Orconectes limosus*). En outre, il y a beaucoup de recherches sur la biologie de la reproduction de l'écrevisse, y compris celle de l'ablation du pédoncule oculaire ou ablation de la glande androgène dans diverses conditions de laboratoire et de l'hybridation dans des conditions naturelles (par exemple *Astacus astacus* X *Astacus leptodactylus*, *Orconectes rusticus* X *Orconectes propinquus*). Il y a aussi des facteurs chimiques qui pourraient éventuellement avoir une incidence sur le système de reproduction des écrevisses à l'état sauvage.

Mots-clés : crustacés / parthénogénèse / intersexualité / hybridation

1 Introduction

Reproductive patterns in crustaceans are relatively diverse with most species exhibiting separate sexes (Chang and Sagi, 2008; Parnes *et al*., 2008; Nagaraju, 2011). Crustacea encompass approximately 233 families that contain 2725 genera and with fossil and extant species 17 635 species (Grave *et al*., 2009; González-Tizón *et al*., 2013). The normal mode of reproduction for the Decapoda, the largest and most diversified group of the malacostracan Crustacea, is with separate sexes (Charniaux-Cotton, 1975). However, there are numerous deviations from this basic principle either for the entire species

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(Bauer and Holt, 1998), for certain populations of a species (Rudolph, 2002), or for some individuals (Sagi *et al*., 2002).

Freshwater crayfish of the order Decapoda (McLay and López Greco, 2011) are keystone species and due to their trophic activities they may have a considerable biological impact on the environment. Crayfish are currently divided into 4 superfamilies, 5 families, 44 genera and 653 species (Bracken-Grissom *et al*., 2014) with diversity being highest in North America and Australia (Holdich, 2002). All families (Astacidae, Parastacidae) are monophyletic except the Cambaridae (Bracken-Grissom *et al*., 2014). However, the numbers of crayfish species are disputable because several new species are described every year (*e.g. Cherax (Cherax) subterigneus*, *Cambarus (Hiaticambarus) longirostris*) (Patoka *et al*., 2015; Jones, 2016).

This report attempts to summarize the more unusual crayfish reproductive patterns varying from gonochorism such as parthenogenesis, intersex and hermaphroditism and describing some factors that could affect their genesis. In addition, there are hybridizations and manipulations (e.g. ablation of eyestalk and androgenic gland) which directly affect crayfish reproductive behaviour.

2 Sexual reproduction in the crayfish

The normal crayfish reproduction strategy is gonochorism where each individual has only one sex: male or female. The gonads, testis or ovary, whose size and appearance depend on the age and reproductive cycle of the individuals, lie dorsally in the thorax between the layer of the pericardial sinus and the hindgut. During the breeding season the internal reproductive organs enlarge significantly. The *vas deferens* takes on a milky-white colour due to the production of sperm masses while the ovary becomes full of eggs, whose colour can be yellowish-brown or dark blue (Ďuriš *et al*., 2015).

The testis, the site of spermatogenesis and its final stage, spermiogenesis, is covered with a cortex of connective tissue and is composed of seminiferous tubules. The spermatocytes and spermatids develop gradually during the first and second meiotic division from spermatogonia (Ďuriš *et al*., 2015). A highly convoluted *vas deferens* arises from each side of the testis and opens at the gonopore on the basis of the fifth pereopod. The *vas deferens* is responsible for packaging the spermatozoa into spermatophores and for conducting the spermatophores to the gonopods (Vogt, 2002). The ovary is trilobed and has one straight oviduct on each side that opens to the outside on the bases of the third pereopods. In the ovary oogenesis takes place that finally produces large, centrolecithal eggs. The oviducts lead the ova down to the gonopores. Crayfish have external fertilization (Hamr, 2002). As is well known, there are differences between the crayfish families in their genital morphology (*e.g.* summarized in Reynolds and Souty-Grosset, 2012).

In astacids and parastacids the spermatophores are deposited either on the ventral surface of the female, or into the *annulus ventralis* which is known as a sperm storage chamber in cambarids that is located between the fourth and fifth pereiopods (Andrews, 1906a; Hobbs *et al.*, 1977), during the mating period (Hamr, 2002). Cambarid (males and females) (Hobbs, 1989; Wetzel, 2002; Buřič *et al*., 2010) and astacids (males and females) (Buřič *et al*., 2015) are cyclically dimorphic. The mating period is characterized by increased activity when sexually mature adult individuals actively seek their partners (Buřič *et al*., 2009). The behaviour is hormonally controlled and influenced by stimuli which are, above all, water temperature and photoperiod (Dubé and Portelance, 1992; Reynolds, 2002).

Egg laying from the oviduct of the female is stimulated by the mating itself as well as by other effects such as a decrease in water temperature or creating short daylight by photoperiod (Skurdal and Taugbol, 2002). The time period between copulation and ovulation varies, ranging from days to weeks (Vogt, 2002) or even several months (Buřič *et al*., 2013). A dense secretion is released from glair glands that dissolves spermatophore wall and releases spermatozoa across the abdominal part of the female body. These spermatozoa subsequently fertilize eggs which are released through the gonopores. The eggs a attach to the pleopods of female for brooding (Hamr, 2002; Niksirat *et al.*, 2014b, 2015a).

3 Biology of gametes in the crayfish

The spermatozoon is produced in the process of spermatogenesis inside the testis that consists of two and three lobes in parastacids and astacids, respectively (Rudolph, 1995a,b). After completion of spermatogenesis, spermatozoa arrive into the two convoluted *vasa* deferentia, originating from each side of the testis and opening at the gonopore on the basis of the fifth pereopod (Vogt, 2002). Although the *vasa deferentia* are mainly known to be responsible for packaging the spermatozoa into spermatophores, it has been reported that in red swamp crayfish *Procambarus clarkii* the acrosomal spike on the anterior part of the spermatozoon is developed in the *vas deferens* (Niksirat *et al*., 2013a).

Spermatozoal cell ultrastructure has been successfully studied in taxonomic and phylogenetic studies across many animal taxa, including crustaceans (Felgenhauer, 1991; Medina, 1994; Tudge, 1995; Jamieson *et al*., 1995a, b; Jamieson and Tudge, 2000; Martin and Davis, 2001; Tirelli *et al*., 2008; Tudge, 2009; Niksirat *et al*., 2013b). Apart from its taxonomic and phylogenetic importance, knowledge of spermatozoal morphology can contribute to understanding the complex mechanisms of acrosome reaction and gamete fertilization (Simeo *et al*., 2010). The acrosome complex and nucleus are located at the anterior and posterior of the crayfish spermatozoon, respectively. A comparison with the acrosome dimensions of studied crayfish species shows that representatives of Parastacidae have a smaller acrosome compared to Cambaridae. The representatives of Astacidae show the largest acrosome within the three families of crayfish (Jamieson and Tudge, 2000; Niksirat *et al*., 2013a, b; Kouba *et al*., 2015).

The acrosome complex organelle is divided into two main parts: the main body of the acrosome that is a dense inverted cup-shaped structure organized into three layers of differing electron densities and extended parallel filaments, and the sub-acrosome zone occupying the central part of the acrosome complex, divided into two electron dense areas. An acrosome spike in the spermatozoon, also called the

horn-like process or anterior acrosomal process, has been reported in spermatozoa of *Cambaroides japonicus* (Yasuzumi and Lee, 1966), *Cambarus* sp. (Anderson and Ellis, 1967), *Procambarus leonensis*, (Felgenhauer and Abele, 1991) and *Procambarus clarkii* (Niksirat *et al*., 2013a). Microtubular radial arms are visible on each side of the acrosome or nucleus in sagittal view and wrap around the spermatozoon and are extended into the nucleus (Niksirat *et al*., 2013a, b). Radial arms are present in the Astacidae and Cambaridae but are absent in the parastacids *Cherax tenuimanus*, *C. albidus,* red claw *C. quadricarinatus* and the yabby *C. destructor* (Beach and Talbot, 1987; Kouba *et al*., 2015). All organelles of the crayfish spermatozoon are tightly enclosed in an extracellular capsule. It has been suggested that the capsule confines the radial arms and permits tighter packaging of the sperm in the spermatophores (Dudenhausen and Talbot, 1983); this is supported by the absence of such capsules in *Cherax* species where radial arms are lacking (Beach and Talbot, 1987; Vogt, 2002). The decapod spermatozoon does not have a true flagellum as in other animals and is non-motile (Jamieson and Tudge, 2000; Tudge, 2009). The microtubules within each arm are not arranged in the 9 + 2 axonemal pattern typical of the flagellated tails of spermatozoa of other animals. Therefore, they are not responsible for sperm movement (Tudge, 2009; Poljaroen *et al*., 2010). One hundred and fifty proteins from nine different categories have been identified in the signal crayfish *P. leniusculus* spermatophore, the most diverse categories in the protein profile being cytoskeleton proteins including actin and tubulin (Niksirat *et al*., 2014a).

The spermatophore in astacids consists of a spermatophore wall with three different layers, which covers a central sperm mass (Dudenhausen and Talbot, 1983; Vogt, 2002; Niksirat *et al*., 2015b). A sticky layer covers the surface of freshly ejaculated spermatophore and helps attachment of the spermatophore to the body of a female until fertilization (Dudenhausen and Talbot, 1983; Niksirat *et al*., 2014b). The freshly ejaculated spermatophore is soft in decapods, but after mating it becomes hardened to protect the sperm mass (Malek and Bawab, 1971; Uma and Subramoniam, 1979). Post-mating spermatophore storage in crayfish and other decapods is accompanied by morphological and molecular changes that are necessary for the spermatozoon to develop fertilizing ability (Alfaro *et al*., 2003; Vanichviriyakit *et al*., 2004; Alfaro *et al*., 2007; López Greco and Lo Nostro, 2008; Aungsuchawan *et al*., 2011; Braga *et al*., 2014; Niksirat *et al*., 2014b, 2015b).

Extensive morphological changes associated with post-mating storage have been observed in the spermatophore of the crayfish, including changes in the morphology of spermatophore wall components and spermatozoon organelles such as plasma membrane and subacrosome zone (Dudenhausen and Talbot, 1983; Niksirat *et al*., 2014b). These changes occur after release of the spermatozoon from the capsule, especially the formation of the filament/droplet structure, may contribute to the mechanism of egg-spermatozoon binding in the narrow-clawed crayfish *A. leptodactylus* (Niksirat *et al*., 2014b). Subcellular localization of calcium in the noble crayfish spermatophore using oxalate-pyroantimonate techniques showed that calcium plays important roles in the post-mating spermatophore hardening and spermatozoon capacitation. The post-mating changes in the calcium distribution may happen because of some secretions from the end part of the *vas deferens* which are added to the spermatophore just before ejaculation, or through contact with ambient water (Niksirat and Kouba, 2016). Extensive proteomic changes occur during post mating storage of noble crayfish *A. astacus*, on the body surface of the female. The concentration of several proteins in the protein profile of male gametes changes significantly during storage on the body surface of the female, indicating post-mating final maturation of the spermatozoon (Niksirat *et al*., 2015b).

As egg laying approaches, the female lies back and secretes glair from glands located on the abdomen surface of the body. These secretions dissolve the spermatophore wall and scatter spermatozoa across the ventral abdomen a few hours before egg release. This high viscosity secretion prevents spermatozoa from being washed away until the end of fertilization. The female forms a brood chamber by curling the abdomen to keep the spermatozoa and eggs floating in the glair secretions (Andrews, 1906b; Niksirat *et al*., 2014b).

Different types of vesicles, including some that are highly or moderately electron-dense, occur in the cortex of the noble crayfish oocyte. One hour post-spawning, the first envelope, especially its inner layer, is condensed. The highly and moderately dense vesicles discharge their contents into the perivitelline space, where they combine and form a second envelope around the egg. Twenty-four hours post-ovulation, a second envelope is visible in the perivitelline space and the outer part of the egg cortex (Niksirat *et al*., 2015a). In crayfish, fertilized eggs are attached to the pleopods of females until hatching (Andrews, 1906b). The egg attachment stalk in the noble crayfish is derived from the first envelope (Niksirat *et al*., 2015a).

4 Unusual reproduction strategies

4.1 Parthenogenesis

In the Crustacea, parthenogenesis is frequent among entomostracan groups such as Anostraca, Cladocera, Conchostraca and Ostracoda (Bell, 1982; Suomalainen *et al*., 1987; Vogt *et al*., 2004); Sassaman (1995) reviewed sex determination and evolution of unisexuality in the Conchostraca. Malacostracan crustaceans are primarily gonochoristic with sex that is genetically determined (Charniaux-Cotton, 1975). Exceptions are some parasitic and free-living isopods and amphipods whose sex is determined environmentally (Adams *et al*., 1987; Vogt *et al*., 2004). For parthenogenesis in malacostracans confirmed examples are limited (Suomalainen, 1950; Bell, 1982; Suomalainen *et al*., 1987; Gruner, 1993). In addition, the occurrence of parthenogenesis is suspected in some tanaidaceans and amphipods, *e.g.*, *Corophium bonelli*, as males have never been found (Gruner, 1993).

Sexuality in crayfish has recently been extended by the discovery of the first case of parthenogenesis in the marbled crayfish (Scholtz *et al*., 2003), which was initially suspected to be a hermaphrodite. Marbled crayfish *Procambarus fallax* (Hagen, 1870) f. *virginalis* (Martin *et al*., 2010) is the only obligate parthenogenetic decapod known to date (Scholtz *et al*., 2003).

It first appeared in the German aquarium trade in the mid-nineties, and since then it has become popular and widespread due to rapid reproduction and easy handling which resulted in its releases to open waters in several countries particularly in Europe (Kouba *et al*., 2014). The first identification of their previous morphological and genetic data argue for an assignment of the marbled crayfish to the North American Cambaridae, and more specifically, to the large and diverse genus *Procambarus* (Scholtz *et al*., 2003). Of the cambarid species included in their genetic analysis relationship the closest species was the similar-looking *Procambarus fallax*. Difficulties in the identification of the marbled crayfish are basically related to the fact that cambarid crayfish are largely determined on the basis of the male gonopods (Hobbs, 1972, 1989), lacking in exclusively all-female marbled crayfish stocks (Vogt *et al*., 2004). Investigation of the development of the external female characters in the marbled crayfish was first performed using a scanning electron microscope, covering life stages from hatchling to repeatedly spawned adult. The first external female character unambiguously recognized is the *annulus ventralis*, which emerges in stage 4 juveniles, and both *annulus ventralis* and gonopores appear completely sculptured in adolescents of ~2 cm TL, being only slightly transformed thereafter (Vogt *et al*., 2004). The observed increase in a number of acuminate and pappose setae of putative mechanoreceptive function (Hobbs, 1972; 1989) around the gonopores and the annulus during maturation had not been described before in decapods (Vogt *et al*., 2004). Martin *et al*. (2015) recently showed that the marbled crayfish is a triploid, showing a triple amount of the haploid number of chromosomes. In addition, they detected a huge amount of a subtelocentric chromosome which became clear firstly in haploid and secondly in diploid cells of sexual individuals of the *P. fallax* complex. In the parthenogenetic marbled crayfish, this featured chromosome occurs thrice (Martin *et al*., 2015). For the marbled crayfish this origin of parthenogenesis is remote, however, no pre-existing parthenogenetic forms are known in the Astacidae (Vogt *et al*., 2004). Additionally, Vogt (2007) showed that exposure of crayfish eggs to 17α-methyl testosterone did not change female reproduction organs and secondary sex characters, also there were no discernible effects on early ovarian development.

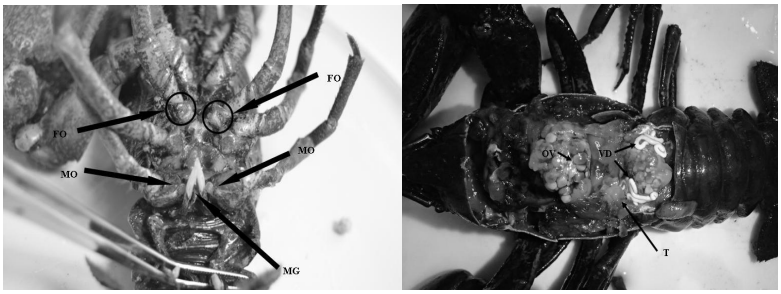
In a study of cross-breeding and parentage analysis, Vogt *et al*. (2015) showed that marbled crayfish and *P. fallax* are different cases of the reproduction strategy. *Procambarus fallax* (Hagen, 1870) f. *virginalis* could be a new species but they originate from *P. fallax* by triploidisation and concomitant epigenetic alterations, evidence from using morphological, behavioural, genetic and epigenetic studies. Marbled crayfish is morphologically very similar to the parent species but has superior fitness traits. Genetic data suggest an instantaneous speciation by autopolyploidisation and parallel change of the mode of reproduction from gonochorism to parthenogenesis. Consequently, the *P. fallax*-marbled crayfish pair provides an interesting new model system to study asexual speciation and saltational evolution in animals and to determine how much genetic and epigenetic change is necessary to create a new species.

With the exception of the marbled crayfish, with its obligatory reproduction by apomictic parthenogenesis (Martin *et al*., 2007), the only other decapod species for which a potential for asexual reproduction has been suggested are the red swamp crayfish *P. clarkii* (Yue *et al*., 2008) and spiny-cheek crayfish *O. limosus* (Buřič *et al*., 2011). For the explanation of facultative parthenogenesis in *O. limosus*, these authors provided experimental evidence that females of the spiny-cheek crayfish are capable of facultative parthenogenesis. Such a reproductive mode has never before been recognized in decapods. Analyses with seven microsatellite loci showed that crayfish females kept physically separate from males, produced genetically homogeneous offspring identical with maternal individuals; this suggests they reproduced by apomixis, unlike those females which mated with males and had a diverse offspring. Also, according to Buřič *et al*. (2011) studies complemented by the genetic analysis of variable nuclear markers, prove that at least one species of cambarid crayfish is capable of facultative parthenogenesis. However, the possibility exists that this reproduction behaviour mode is more widespread among cambarids, and may contribute to the success of this group when colonizing new habitats and territories. Asexual generations may also have contributed to observations of supposed *P. clarkii* clones by Yue *et al*. (2008), and possibly to significant heterozygote deficiencies observed in Chinese populations of *P. clarkii* (Yue *et al*., 2010) as well as in some recent studies of existing invasive populations of *O. limosus* in the Czech Republic (Filipova *et al*., 2009), up to now explained by founder effects or assortative mating (Buřič *et al*., 2011, 2013).

4.2 Intersexuality

Intersexuality is characterized by the presence of male and female sexual characteristics in the same individual, in gonochoric or hermaphroditic species, and may be limited to the internal morphology or may extend to gonadal morphological differentiation (Sagi *et al*., 1996; Vogt, 2002; Noro *et al*., 2008). Variation from normal development of the androgenic gland (AG) frequently causes the development of some male properties. According to Ďuriš *et al*. (2001) a part of or almost the full complement of male gonopods was recorded in adult females of *A. leptodactylus* in mud lagoons in the Karviná District (Ďuriš *et al*., 2015), while in South American crayfish of the genera *Parastacus* and *Samastacus*, the changes in development and activity of this gland occur probably in the background of intersex phenomena, *i.e.*, sex changes in crayfish individuals (Rudolph and Almeida, 2000). Experimental insertion of fresh androgenic gland into a female crayfish body caused not only masculinization but also a change in her behaviour towards a male (Karplus *et al*., 2003).

From the literature on freshwater crayfish (Astacida), the most common deviation from separate sexes is intersexuality in all three families, the Astacidae, Cambaridae, and Parastacidae. At least 12 of the 29 crayfish genera (Vogt, 2002) are reported to include intersexuality. Among the Parastacidae, intersexuality has been determined in species from Oceania and South America. In the genus *Parastacus* of southern South America, simultaneous presence of male and



(a) (b)

Fig. 1. *Pasifastacus leniusculus*. (a) Both male and female genital openings, as well as male gonopods, FO, female opening; MO, male opening; MG, male gonopods; (b) *vasa deferentia* (VD), ova (OV), testes (T) (Yazicioglu *et al*., 2014).

female gonopores is a common characteristic (Rudolph and Almeida, 2000). Although intersexuality is common among parastacids and is a strong indication of the occurrence of hermaphroditism, functional hermaphroditism is rare among crayfish and is of the protandric type, as in *Samastacus spinifrons* and *Parastacus nikoleti* (Rudolph, 1995a; Noro *et al*., 2008). With some individuals having gonopores of both sexes the Chilean species *P. nicoleti* is an exception (Rudolph, 1995a). Intersexuality is also observed in species of Parastacidae, namely of *Parastacus pilimanus* and *P. brasiliensis* (Rudolph and Almedia, 2000). For the other two South American genera, namely *Samastacus* and *Virilastacus*, the literature points to the presence of only one pair of gonopores in each individual. In Oceania, intersexuality is also seen in the Australian genera *Engaewa*, *Engaeus*, *Euastacus* and *Cherax* (Horwitz, 1988; Sokol, 1988; Medley, 1993; Sagi *et al*., 1996).

Although the frequency of intersex individuals showing external male and female characters is often less than 1%, in some species, *e.g. C. quadricarinatus*, higher percentages, up to 14%, have been recorded (Sagi *et al*., 2002). Medley *et al*. (1994) report a normal testis and a previtellogenic ovary in a single *C. quadricarinatus* intersex individual and describe it on the basis of histological examinations as a case of "true hermaphroditism". They report one intersex individual that had a right-side male genital opening and two female genital openings and functioned as a male, siring a batch of offspring. However, Sagi *et al*. (1996) mention that an ovary exists in *C. quadricarinatus* only when a female genital opening is present in the absence of a male genital opening on the same side. Rudolph (2002) collected intersexed specimens of *S. spinifrons* from rivers in Chile and in the description of a new species, *Virilastacus rucapihulensis*, Rudolph and Crandall (2005) note the presence of specimens with extra gonopores.

Yazicioglu *et al*. (2014) report the first evidence of intersex in the astacid signal crayfish, *P. leniusculus* (Fig. 1). An intersexual specimen's morphology shows both male and female genital openings, as well as male gonopods. Also, histology reveals both spermatocytes and oocytes. Among the cambarids, Kozák *et al*. (2007) reported that during the mating season of *O. limosus* the male spermatophores were predominantly filled with spermatozoa and sperm was also noted in the *vas deferens*. However, two out of 15 males sampled during the winter were found to be intersex, in which atretic oogonia and oocytes were present at the periphery of testicular tissue and occupied less than 15% of testicular tissue. The evidence of intersex strongly suggested transitional stages of a gradual change of sex, which may be qualified as partial hermaphroditism.

4.3 Hermaphroditisim

Hermaphrodites are individuals that have a functional male and female reproductive system at some time during their lives (Ghiselin, 1969). There are two major types of hermaphroditism, which have received considerable study (Michiels, 1998).

(a) Simultaneous hermaphroditism: both sexes are present simultaneously.
(b) Sequential hermaphroditism: sex change during life; a change from male to female is called protandry (*e.g.* Amphiprioninae, some sea anemones and certain freshwater limpets) and sex change from female to male is protogyny (*e.g.* certain fish species in the families Serranidae and Sparidae, also some isopods and tanaid crustaceans).

As was mentioned above, intersex individuals showing male and female characters occur regularly in low frequencies in a number of crayfish species, particularly in the Parastacidae, however, functional sequential hermaphroditism (Rudolph, 1995a) is very rare among crayfish and is protandric (Rudolph, 1995a, Table 1). On the other hand, the intersex individuals in natural populations of decapods may also be associated with non-functional hermaphroditism (Sagi *et al*., 1996). Functional protandric hermaphroditism is only known for *P. nicoleti* and *S. spinifrons* (Rudolph and Almeida, 2000; Rudolph, 2002). Interestingly, only the fluvial populations of *S. spinifrons* included protandric hermaphrodites, but not the lake populations. An extensive analysis of 10 populations of *S. spinifrons* revealed that of the 1114 specimens sampled 597

Table 1. Overview of unusual reproduction strategies in some crayfish species.

Family	Species	Reproduction strategy	References
Astacidae	*Pacifastacus leniusculus*	Intersexuality	Yazicioglu *et al*., 2014
Cambaridae	*Procambarus fallax* f. *virginalis (Marbled crayfish)*	Parthenogenesis	Scholtz *et al*., 2003
	Procambarus clarkii	Intersexuality	Taketomi *et al*., 1996
	Orconectes limosus	Facultative parthenogenesis, Intersexuality	Buřič *et al*., 2011; Kozák *et al*., 2007
Parastacidae	*Samastacus spinifrons*	Intersexuality, Partial protandric hermaphroditism	Rudolph, 1995b; Rudolph and Almedia, 2000
	Parastacus pugnax	Intersexuality	Rudolph and Almedia, 2000
	Parastacus varicosus	Intersexuality	Rudolph and Almedia, 2000
	Parastacus pilimanus	Intersexuality	Rudolph and Almedia, 2000
	Parastacus defosus	Intersexuality	Rudolph and Almedia, 2000
	Parastacus saffordi	Intersexuality	Rudolph and Almedia, 2000
	Cherax quadricarinatus	Hermaphroditism, Intersexuality	Sagi *et al*., 2002; Parnes *et al*., 2003
	Virilastacus araucanius	Intersexuality	Rudolph and Rivas, 1988; Martinez *et al*., 1994
	Virilastacus rucapihuelensis	Partial protandric hermaphroditism	Rudolph and Crandall, 2005
	Parastacus brasiliensis	Hermaphroditism	Almedia and Buckup, 2000
	Parastacus nicoleti	Partial protandric hermaphroditism	Rudolph, 1995a ; Rudolph and Almedia, 2000

were males, 476 females, and 41 intersexes. All of the intersexes had gonoducts of both sexes but 30 specimens had only testes, 4 had only ovaries, and 7 had ovotestes.

5 Laboratory manipulations: effect of eyestalk ablation and androgenic gland ablation on reproductive behaviour of crayfish

The androgenic gland (AG) is present in embryos and juvenile crayfish of both sexes, but it is specially developed in males. It is found in the male's *vas deferens* as an important glandular structure. The androgenic gland hormones control the so-called masculinization of the body, *i.e.*, growth and development of the body in male proportions, but it also supports the production of spermatozoa in the testes. In females, it causes inhibition of differentiation of their body (Sagi and Khalaila, 2001; Ďuriš *et al*., 2015).

Male differentiation and primary and secondary characteristics in crustaceans are regulated by the AG. In gonochoristic crustaceans, the AG is also linked to intersexuality. Whereas the co-occurrence of various male and female characteristics is demonstrated in intersex crustaceans, there are only a few studies on sexually dimorphic behaviour patterns in such individuals (Barki *et al*., 2006) Sexual differentiation in crustaceans is hormonally controlled by the androgenic hormone(s) (AGH) secreted by the AG (Charniaux-Cotton and Payen, 1985). During early development, AG primordia are expressed in genetic males and they determine the development of masculine gametogenic (testes) and endocrine (AG) organs – distinct organs in crustaceans. Consequently, the endocrine function of the AG regulates the development of male phenotypic characteristics (Charniaux-Cotton and Payen, 1988; Katakura, 1989; Payen, 1990; Sagi *et al*., 1997). The AG is also implicated in the mediation of intersexuality, although causative factors vary among crustacean species and are unclear in some cases (Barki *et al*., 2006).

In isopods, on the other hand, it is argued that intersex individuals, functional males, are the product of a delayed expression/action of AGH in genetic males (Azzouna *et al*., 2004). It is also known that endocrine-disrupting pollutants cause de-masculinization and intersexuality in amphipods, probably by interfering with the function of the AG (Ford *et al*., 2004). Therefore, it is clear that AG, which is responsible for the control of male primary and secondary sexual characteristics (including behaviour), is also a key factor in the formation of intersex individuals and sexual plasticity in crustaceans. As intersex individuals can be plastic in terms of sexually dimorphic behaviours and their responsiveness to sex-related hormonal manipulations, they provide a useful model for inducible sexual plasticity for the analysis of hormonal control of sexually dimorphic behaviours in Crustacea (Sagi *et al*., 1996, 2002; Barki *et al*., 2006).

Neurohormones are part of the sinus gland-Y-organ endocrine axis and the sinus gland-mandibular organ endocrine axis, respectively (Fingerman, 1995; Aguilar *et al*., 1996; Liu and Laufer, 1996; Terauchi *et al*., 1996; Wainwright *et al*., 1996; Keller *et al*., 1999; Boecking *et al*., 2002). While the inhibitory effect of the eyestalk on the male reproductive system has been reported in some species, the identity and mode

of action of the factor actually responsible for this inhibition are not yet known. It is concluded that the secretory product(s) of the AG is proteinaceous in nature. Early support for this proposition may be found in the considerable amount of proteins in the cytoplasmic secretory vesicles of the AG of the crab *Pachygrapsus crassipes* (King, 1964). The ultrastructure of the AG of *P. clarkii* also supports the possibility of a proteinaceous secretion (Miyawaki and Taketomi, 1978; Taketomi, 1986). Some histological evidence in the Giant river prawn *Macrobrachium rosenbergii* supports the idea of a proteinaceous androgenic hormone (Awari and Dube, 1999). As is the case for all peptide hormones, the target tissue is expected to have specific cell-surface receptors. Therefore, a presumably proteinaceous hormone such as AG should initiate multiple signal transduction pathways that change the activation of kinases or phosphatases on specific proteins in its target organ, namely the testis.

In decapod crustaceans, both male and female, the X-organ-sinus gland complex in the eyestalk produces the neurohormones that regulate various physiological processes (Tan-Fermin, 1991; Keller, 1992; Wilder *et al*., 1994; Sagi *et al*., 1997). The effect of such neurohormones on male reproduction is less known than that of the female. On the other hand, it is well-known that while regulation of the male reproductive system is controlled by AG (Charniaux-Cotton and Payen, 1988; Sagi *et al*., 1997; Sagi and Khalaila, 2001), the initiation, completion, and intensity of spermatogenic activity are regulated by circulating AG hormone (Charniaux-Cotton and Payen, 1988). In some decapod species, spermatogenesis initiates only after the AGs are fully developed (Taketomi *et al*., 1996). In the male prawn, *M. rosenbergii* (Nagamine *et al*., 1980), and in intersex individuals of the Australian red claw crayfish, *Cherax quadricarinatus,* the removal of AG leads to cessation or regression of spermatogenesis (Khalaila *et al*., 2002).

According to Sagi *et al*. (2002) studies reveal that eyestalk ablation in the mature *C. quadricarinatus* male leads to hypertrophy of AG. Hypertrophy of AG is determined by increases in its size and weight and by the enrichment of the polypeptide profile of the AG. These results are consistent with the literature in a number of decapod crustaceans of hypertrophy of AG combined with hyperactivity and increase in RNA synthesis after eyestalk ablation (Hoffman, 1968; Foulks and Hoffman, 1974; Adiyodi, 1984; Kulkarni *et al*., 1984). Several polypeptides that are expressed in AGs of eyestalk-ablated *C. quadricarinatus* males necessitate further studies based on previous findings regarding the identified glycosylated AG hormone found in the isopod *Armadillidium vulgare* (Okuno *et al*., 1997; Martin *et al*., 1999; Okuno *et al*., 1999; Sagi and Khalaila, 2001) as in addition to the general effect of eyestalk ablation on protein synthesis, it can cause overexpression of specific polypeptides representing androgenic factors. Eyestalk ablation also causes dynamic changes in the reproductive system of mature *C. quadricarinatus* males. As far as is known, descriptions of similar dynamic processes on crustaceans are missing in the literature (Sagi *et al*., 2002). After eyestalk ablation, there is a gradual and significant increase in the weight of the sperm duct due to accumulation in the sperm duct of spermatophores ready for ejaculation. This accumulation is an indicator of the potency of the spermiation process following eyestalk removal. Further evidence for the enhancement of spermiation is provided by the decrease in the amount of DNA in the testes from the second week onward and by the significantly higher number of empty spermatogenic lobules found in testicular sections of eyestalk-ablated males' testes versus those of intact animals. It is probable that the enhanced spermiation process leads to a decrease in the weight of the testes in the third and fourth weeks after eyestalk ablation that is parallel with previous findings in *C. quadricarinatus* (Khalaila *et al*., 2002). The decrease in testis weight is also consistent with previous findings in the prawn *Parapenaeopsis hardwickii* (Kulkarni *et al*., 1984; Manor *et al*., 2004).

Recently, for the first time direct evidence for masculinization effects of AG on agonistic and mating behaviour was found in decapod crustaceans (Karplus *et al*., 2003; Barki *et al*., 2006), in addition to its morphological, anatomical and physiological effects (Khalaila *et al*., 2002; Manor *et al*., 2004; Barki *et al*., 2006). While previous studies explored the masculinization effects on behaviour of implanting AGs into *C. quadricarinatus* females, current studies used the intersex model for investigating the de-masculinization effects of AG ablation (Barki *et al*., 2006).

6 Factors possibly affecting the genesis of unusual reproduction in crayfish: hybridization

In crayfish, males may copulate not only with conspecific females but also with females of congeners (Reynolds, 2002). Therefore, the existence of fertile crayfish hybrids in the wild is not surprising (Hogger, 1988). In the literature the first hybridization instance between indigenous and invading crayfish was between two crayfish of European origin, the astacid crayfish *A. astacus* and *A. leptodactylus* (Cukerzis, 1968). Furrer *et al*. (1999) obtained hybrid offspring between *A. astacus* males and *A. leptodactylus* females. In the opposite case (*A. astacus* females X *A. leptodactylus* males), *A. astacus* females lost eggs after interbreeding. As reported in Mlinarec *et al*. (2011), no hybrid population between *A. astacus* and *A. leptodactylus* are known in nature. Despite earlier reports, a potential hybrid between two native crayfish species, *A. astacus* and *A. leptodactylus*, was proved using a morphological approach in Mrežnica River, Croatia (Maguire et al., 2013). In addition, Jelić *et al*. (2013) proposed that mixed populations of noble (*A. astacus*) and narrow-clawed crayfish (*A. leptodactylus*) could be come across in Europe. They tested the possibility of hybridisation in two interspecific mating combinations under lab condition twice, in two mating seasons. According to their first results, mating was successful, but the majority of females had lost their eggs or died. At the end of their experiment, hybrids with *A. leptodactylus* females had successful interspecific mating, but there was no success with conspecifically mated *A. leptodactylus* (Jelić *et al*., 2013). The same types of experiments were also done by our group but without success in obtaining hybrid juveniles. Only egg laying and maybe partly developing embryos were observed in some cases (P. Kozák, unpublished).

Generally, hybridization in cambarid crayfish has relatively rarely been observed, exceptions being hybridization between *O. rusticus* and *O. limosus* in a small stream of N-central Massachusetts (Smith, 1981), and hybridization between *Cambarus robustus* and *C. bartonii* in New York (Crocker, 1957; Smith, 1979), also hybridization between the native species *O. propinquus* and the introduced species *O. obscurus* and *O. rusticus* in New York and Ontario (Crocker, 1957; Crocker and Barr, 1968). The evidence of morphological features of hybridization between the *O. juvenilis* and *O. cristavarius* in the Kentucky River basin was reported by MacDonald *et al*. (2006). A more recent study on unique genetic documentation is provided by Perry *et al*. (2001, 2002) for hybridization between native and invasive *Orconectes* species (Cambaridae). They compared crayfish from allopatric and sympatric populations of the invasive *O. rusticus* and of the indigenous *O. propinquus* (Girard) and *O. virilis* (Hagen) in Wisconsin (USA) by utilizing diagnostic nuclear and mitochondrial DNA markers along with morphological data (Gherardi, 2007). In sympatric sites hybridization occurs between *O. rusticus* and *O. propinquus* while no hybridization occurs between *O. virilis* and either of these species. A detailed study on the dynamics of hybridization conducted in Trout Lake, Country revealed that over 6% of crayfish were F1 hybrids, 4% were F2 individuals (hybrid × hybrid origin), and 13% were backcrosses (product of hybrid × parental mating) (Perry *et al*., 2001). The majority of F1 hybrids (95%) were the result of *O. rusticus* females mating with *O. propinquus* males; only 1% of the total crayfish population was the product of F1 hybrids backcrossing to *O. propinquus*, whereas 13% represented backcrosses to *O. rusticus*. Hence F1 hybrids appeared to mate disproportionately with pure *O. rusticus* that cause greater genetic introgression of nuclear DNA form *O. propinquus* to *O. rusticus* than in the reverse direction. The result is the gradual elimination of *O. propinquus* genes from the population (Gherardi, 2007). There are also another hybridization report between native (*O. sanbornii*) and invasive (*O. rusticus*) species in the Huron River in north-central Ohio based on a combination of molecular markers such as nuclear DNA, mitochondrial DNA and allozymes (Zuber *et al*., 2012). Their results showed that while there are no differences between the species at nuclear DNA loci, mtDNA and allozyme loci confirmed the presence of individuals of hybrid ancestry. On the other hand, they also found preliminary evidence of possible mitochondrial recombination and biparental inheritance and examination of morphological features of both species in sympatry and allopatry and confirmed species-diagnostic morphological features including gonopod traits and shape of the *annulus ventralis*.

Lawrence *et al*. (2000) have obtained hybrids between the parastacids *Cherax rotundus* females and *C. albidus* males that constantly produced only male progeny. This unanticipated finding underlines the resilience of crayfish sexuality. Hence it is possible that the parthenogenetic marbled crayfish might be the result of hybridization of two unidentified cambarids. Hybridization may have occurred both in nature and in the aquarium. Parthenogenesis of a contagious origin, that is the hybridization of females from a pre-existing parthenogenetic lineage with males of either the same or a closely related species, is common in brine shrimp and ostracods and generally results in polyploid unisexuals (Simon *et al*., 2003). The critically endangered hairy marron, *Cherax tenuimanus*, an endemic species in the Margaret River, Western Australia, is being rapidly replaced by the invasive smooth marron, *C. cainii* (Bunn *et al*., 2008). According to Imgrund (1998) the hairy and smooth marron in the Margaret River mate and their hybrids also have the distinctive setation of the hairy Margaret River marron. Additionally, Lawrence (2002) showed, based on the fertilization of hybrids, that it is possible to determine hybrid fertility, and mentioned that identification of hybrids is possible using microsatellites but not by morphological methods.

All these findings led to wide morphological evidence of putative hybrids among crayfish species (Perry *et al*., 2002). The implication of the study of Perry *et al*. (2002) is clear that hybridization and introgression pose a substantial threat to the conservation of crayfish biodiversity and that further research is required into the potential for hybridization among resident and invasive species. Such studies would have the potential to predict species at risk of losing their genetic identity (Gherardi, 2007).

7 Chemical factors

There is still no consensus on whether genetic (population or individual) or phenotypic (environmental) factors are more important in the control of fecundity and successful reproduction. Current knowledge suggests that phenotypic variation is considerable, yet the selection of breeding stock should not be ruled out. The timing of breeding may be related in part to water temperature, but the initiation of mating and spawning may occur at different temperatures and dates, suggesting that a timing mechanism seems to be involved, which can be reset to some extent by continued high temperatures (Reynolds, 2002).

The phenotype of an organism is determined by the genes, the environment and by stochastic developmental events (Vogt *et al*., 2008). Vogt *et al*. (2008) stated that, even in the same environment, individual genotypes can produce different phenotypes because of developmental variation, resulted in generating variability among clone-mates and individuality in a parthenogenetic species. This developmental variation, an apparently ubiquitous phenomenon in living beings, can introduce elements of randomness into life histories, altering individual fitness and subsequently population dynamics.

Many aspects of crayfish reproduction are still unknown, such as the endocrine control of reproduction in astacid crayfish. Pheromones may be crucial in attracting mates and stimulating mating. Other hormones are no doubt significant in controlling brooding behaviour, including inhibiting maternal cannibalism, and related knowledge may be crucial in astaciculture (Reynolds, 2002). Sex is determined and irreversibly fixed during embryonic or larval development in response to particular environmental cues in invertebrates (Adams *et al*., 1987); in crayfish unusual reproduction strategies may also have occurred. Future studies should address the possible mechanisms triggering abnormal reproductive biology in crayfish, including whether environmental pollution

Table 2. Some examples of unusual reproduction strategies in freshwater vertebrates and invertebrates.

	Family	Species	Reproduction strategy	References
Vertebrate	Cyprinidae	*Rutilus rutilus*	Intersexuality	Jobling *et al*., 1998
	Gasterosteidae	*Gasterosteus aculeatus*	Intersexuality	Borg and Van den Hurk, 1983
	Acipenseridae	*Scaphirhynchus platorynchus*	Intersexuality	Harshbarger *et al*., 2000
Invertebrate	Margelopsidae	*Margelopsis haeckeli*	Parthenogenetic	Werner, 1955
	Alcyoniidae	*Alcyonium hiberniculum*	Parthenogenetic	Hartnoll, 1977
	Lasaeidae	*Lasaea australis*	Hermaphrodite	O'Foighil, 1988
	Thiaridae Pachychilidae	*Aylacostoma* sp., *Melanoides* sp., *Tarebia* sp., *Thiara* sp., and *Sulcospira* sp.	Parthenogenetic	Morrison, 1954
	Paludomidae	*Bathanalia* sp., *Tanganyicia* sp. and *Tiphobia* sp.	Parthenogenetic	Moore, 1898
	Tateidae	*Potamopyrgus* sp.	Parthenogenetic	Winterbourn, 1970
	Hydrobiidae	*Potamopyrgus antipodarum*	Parthenogenetic	Jokela *et al*., 1997
	Viviparidae	*Campeloma decisum*	Parthenogenetic	Johnson, 1992
	Sphaeromatidae	*Gnorimosphaeroma naktongense*	Protogynous hermaphroditism	Abe and Fukuhara, 1996
	Sphaeromatidae	*Gnorimosphaeroma oregonense*	Protandry hermaphroditism	Brook *et al*., 1994
	Cyprididae	*Cyprinotus incongruens*	Unisexual	Turgeon and Hebert, 1994
	Daphniidae	*Daphnia pulex*	Parthenogenetic	Hebert *et al*., 1983
	Littorinidae	*Littorina littorea*	Intersex	Schulte-Oehlmann *et al*., 1998
	Artemiidae	*Artemia salina* and *A. parthenogenetica*	Parthenogenetic	Browne, 1992; Golubev *et al*., 2001
	Atyidae	*Paratya curvirostris*	Protandry hermaphroditism	Carpenter *et al*., 1978

may be responsible for it in some instances. Researches reveal that some DDTs (dichloro diphenyl trichloroethanes) and brominated flame retardants can disrupt the endocrine system in aquatic animals (Hajslova *et al*., 2007; Havelková *et al*., 2007). Although crayfish become more widely used in toxicological studies (Kouba *et al*., 2012), the impacts on sex determination and reproduction systems remain largely unstudied. Although biochemical changes may be expected in testes or ovaries, the exposure is too short to see impacts on reproduction (Velisek *et al*., 2013).

8 Conclusions

Unusual reproductive strategies, such as hermaphrodites and parthenogenesis in crustaceans, especially in crayfish, have been demonstrated. In most of the studies in the literature, intersexuality in crayfish is a non-functional hermaphroditism supposedly induced by damage to or disturbances of the androgenic gland that determine the male sex via hormones. Although such individuals often have ovotestes, they act as males since the ovarian portion of the gonad remains arrested in a previtellogenic state.

Only the marble crayfish is known as a parthenogenetic species up to now, however spiny-cheek crayfish and some other species can under some specific conditions reproduce partenogenetically as well. On one hand, hybridization could be an effective tool in aquaculture production and it is well demonstrated in several crayfish species. On the other hand, the development of new parthenogenetic species by interspecific hybridization is well established by molecular evidence for gastropods ostracods and reptiles (see Table 2). Such lineages, mostly polyploid and due to heterosis effects, often have a broad environmental tolerance. As mentioned by Parker *et al*. (1999) and Gherardi (2007), invader species, such as the American crayfish species now occurring in Europe, may cause an indirect genetic effect on native species, subsequently resulting in transformed patterns of natural selection or gene flow within these species. Invaders may change selection regimes or disrupt gene flow due to their fragmentation of indigenous species populations, leading them to risky bottlenecks. Hybridization between a non-indigenous and an indigenous species may cause a direct effect that may have three possible results: (1) the emergence of a new invader genotype; (2) the production of sterile hybrids with a resulting waste of gametes and resource competition with indigenous species; and (3) the hybrid may become dominant in natural habitats, leading to a virtual extinction of indigenous taxa through "genetic pollution". In the last point, there is a possibility that chemical factors may influence the release of different reproductive strategies in crayfish.

Published and unpublished data show that it is possible to come across abnormalities in reproductive patterns in crayfish, even in Crustacea, either by chance in the wild or under laboratory conditions through experimentation. Additionally, many factors such as laboratory manipulations, hybridization or pollution may lead to changes not only in the reproduction system but also other systems of crayfish. Studies are needed to help

further evaluation and understanding of triggering factors of unusual reproduction strategies.

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CHAPTER 2

FIRST REPORT OF INTERSEX IN THE SIGNAL CRAYFISH *PACIFASTACUS LENIUSCULUS* (DANA, 1852)

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CRUSTACEANA

FIRST REPORT OF INTERSEX IN THE SIGNAL CRAYFISH *PACIFASTACUS LENIUSCULUS* (DANA, 1852)

BY

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ABSTRACT

The reproductive strategies of species of non-indigenous crayfish make an important issue in astacological research. Although crayfish reproduction has been well studied, there is little information available on the occurrence of intersexuality. We report the first evidence of intersex in the signal crayfish, *Pacifastacus leniusculus* (Dana, 1852). An intersexual specimen was found among five tested signal crayfish. Morphology revealed both male and female genital openings, as well as male gonopods. The specimen superficially appeared male but contained both testes and ovaries, with an atypical dispersed structure. Sperm was obtained from this crayfish. Histology showed both spermatocytes and oocytes. The gonadosomatic index (GSI = 3.79) was considered as a morphometric parameter of the intersexual crayfish. The ova present in the intersex male raised its GSI to the three-fold of that of normal males.

RÉSUMÉ

Les stratégies de reproduction des espèces d'écrevisses non-indigènes constituent une question importante en recherche astacologique. Bien que la reproduction des écrevisses ait été bien étudiée, il y a peu d'information disponible sur la fréquence de l'intersexualité. Nous rapportons la première preuve d'un intersexué chez l'écrevisse *Pacifastacus leniusculus* (Dana, 1852). Un spécimen intersexué a été trouvé parmi les cinq écrevisses testées. La morphologie révèle les deux ouvertures génitales mâles et femelles, ainsi que des gonopodes de mâle. Le spécimen apparaît superficiellement mâle mais contient à la fois testicules et ovaires, avec une structure dispersée atypique. Du sperme a été obtenu de cette écrevisse. L'histologie montre à la fois des spermatocytes et des ovocytes. L'index gonadosomatique (GSI = 3,79) a été considéré comme un paramètre morphométrique de l'écrevisse intersexuée. Les ovules présents chez le mâle intersexué ont multiplié son GSI par trois par rapport à celui d'un mâle normal.

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INTRODUCTION

Astacidae are generally gonochoric, with defined males and females, but some deviations occur (Noro et al., 2008). The most common is intersexuality, characterized by the presence of both male and female characteristics in a gonochoric or hermaphroditic individual. This can be limited to reproductive anatomy or extend to gonad morphological differentiation during stages of development (Sagi et al., 1996; Vogt, 2002; Noro et al., 2008).

In the Parastacidae, cases of intersexuality are found in species from Oceania and South America. In the genus *Parastacus*, from southern South America, the existence of male and female gonopores in an individual is common (Rudolph & Almeida, 2000). Although intersexuality is common in parastacids and is a strong indication of the occurrence of hermaphroditism, functional hermaphroditism is rare among crayfish and is of the protandric type (Rudolph, 1995). The first case of protandric hermaphroditism in *Parastacus* was described by Rudolph (1995) in the Chilean species *Parastacus nicoleti* (Philippi, 1882). Hermaphroditism has also been reported for *Parastacus brasiliensis* (Von Martens, 1869) and *Samastacus spinifrons* (Philippi, 1882) by Almeida & Buckup (2000) and Rudolph (1999, 2002), respectively. In the Parastacidae, there has been some investigation of the functional significance of the existence of supernumerary gonopores. These studies cover only a few species, including *P. nicoleti* (cf. Rudolph, 1995), *S. spinifrons* (cf. Rudolph, 1999, 2002), *Parastacus varicosus* (Faxon, 1898) (cf. Rudolph et al., 2001; Silva-Castiglioni et al., 2008), *P. brasiliensis* (cf. Almeida & Buckup, 2000) and *Viriliastacus rucapihuelensis* (Rudolph & Crandall, 2005) (cf. Rudolph et al., 2007).

Medley et al. (1994) observed a normal testis and a previtellogenic ovary in a *Cherax quadricarinatus* (Von Martens, 1868) intersex specimen and, on the basis of histological examination, described it as a case of true hermaphroditism. The specimen possessed a right-hand-side male genital opening and two female genital openings and functioned as a male by siring offspring.

In freshwater crayfish, the most common deviation from bisexuality is intersexuality, which has been reported in Astacidae, Cambaridae and Parastacidae, and documented in at least 12 of the 29 crayfish genera (Vogt, 2002). The frequency of intersex individuals with external male and female characteristics is generally lower than 1%, but, for species such as *C. quadricarinatus*, up to 14% has been reported (Sagi et al., 1996).

Some individuals of the Chilean species *P. nicoleti* were reported to have gonopores of both sexes (Rudolph, 1995). In the other two South American genera, *Samastacus* and *Virilastacus*, records indicate the presence of only one pair of gonopores. Intersexuality is also observed in Parastacidae including the Australian

genera *Engaewa*, *Engaeus*, *Euastacus* and *Cherax* (Horwitz, 1988; Sokol, 1988; Medley & Rouse, 1993; Sagi et al., 1996; Rudolph et al., 2007).

The signal crayfish, *Pacifastacus leniusculus* (Dana, 1852), the most common species of its genus, inhabits varying environments and has the typical life cycle of the Astacidae (Lewis, 2002; Johnsen & Taugbol, 2010). The biotope of the signal crayfish has been expanded by its introduction into the new habitats in North America, Europe, and Japan (Lewis, 2002). *Pacifastacus leniusculus* now occurs in thousands of lakes and rivers throughout Europe (Lewis, 2002).

Herein, we describe an intersex specimen of *Pacifastacus leniusculus* (Astacidae) confirmed by external macroscopic and internal histological observations.

MATERIAL AND METHODS

Animals

Adult crayfish (*Pacifastacus leniusculus*, $n = 5$) were obtained from the Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Experimental Fish Culture Facility, during the spawning season in November, 2013. The crayfish were collected from a fish pond by hand in the Vysočina region, Czech Republic during harvesting of fish when water was discharged from the pond. In this context, randomly selected *P. leniusculus* ($n = 5$) came from natural population by chance. The carapace length (mm) and wet weight (g) of the crayfish were measured in live animals, and gonads were weighed after dissection. Gonadosomatic index (GSI) was calculated as gonad weight/wet weight of crayfish.

Histology

Tissues were fixed in Bouin's solution for 3 h, stored in 80% ethanol at 4°C, dehydrated by acetone-xylol and embedded in paraffin. The blocks were cut into 8 μm sections and stained with hematoxylin-eosin according to standard procedures. The cells were identified on the basis of their structural characteristics.

RESULTS

One of the five specimens of *Pacifastacus leniusculus* appeared morphologically male, but with female genital openings on the third periopods and male openings on the fifth periopods (fig. 1). Dissection revealed a vas deferens and testis as well as an ovary containing immature ova. The testis tissue was atypically dispersed within the ovary (fig. 2). Gonad weight was two-fold and GSI three-fold that of the four normal males (table I). Histology showed the presence of both spermatophores and oocytes (fig. 3). The spermatophores were located inside the vas deferens. The

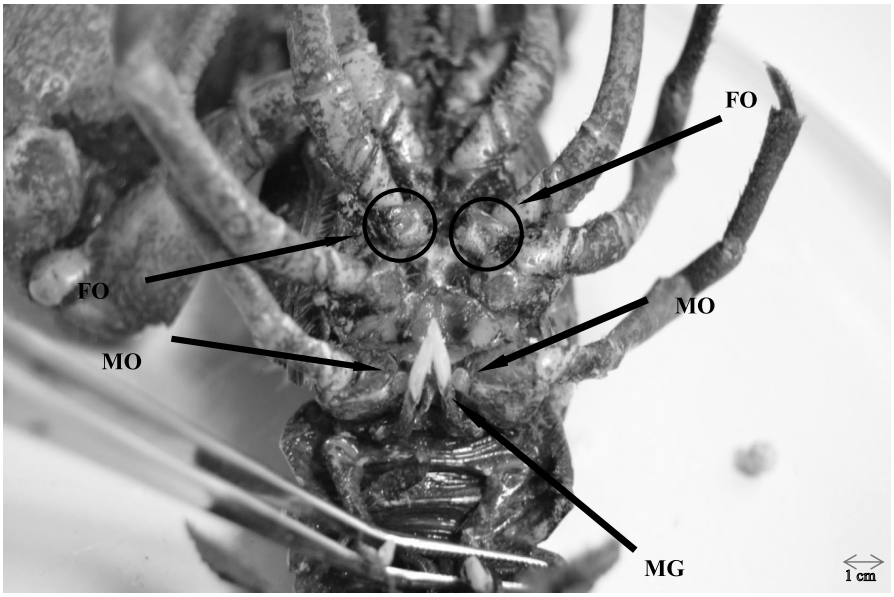

Fig. 1. Intersex *Pacifastacus leniusculus* (Dana, 1852). FO, female opening; MO, male opening; MG, male gonopods (Original). This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/15685403.

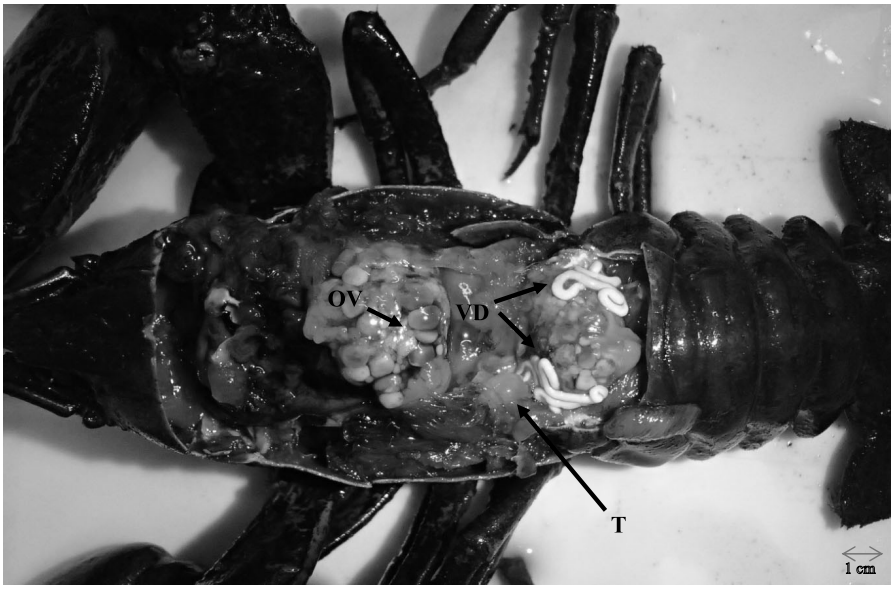

Fig. 2. Dissected intersex *Pacifastacus leniusculus* (Dana, 1852) showing vasa deferentia (VD) and ova (OV). T, testes (Original). This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/15685403.

TABLE I

Biometrics of *Pacifastacus leniusculus* (Dana, 1852) 1-4 male, 5 intersex

Crayfish	Carapace length (mm)	Weight (g)	Weight of gonad (mg)	Gonadosomatic index
1	51	41.1	0.43	1.05
2	58	56.3	0.69	1.23
3	58	59.6	0.65	1.09
4	56	53.4	0.57	1.06
5	56	64.8	2.46	3.79

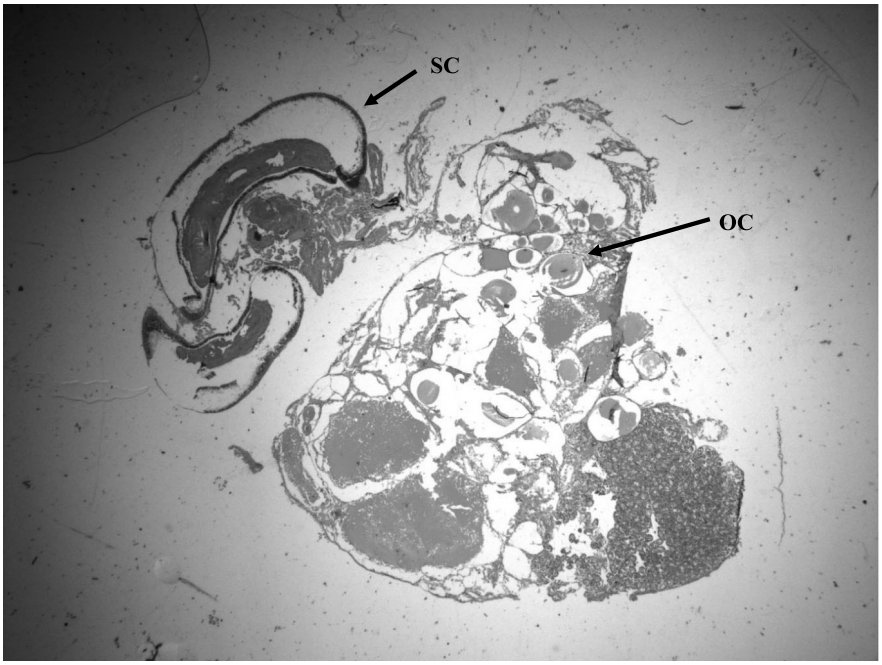

Fig. 3. Section of intersex *Pacifastacus leniusculus* (Dana, 1852) showing male and female gametes. SC, spermatocytes; OC, oocytes. Magnification 10× (Original). This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/15685403.

spermatophore consisted of a central sperm mass covered by a spermatophore wall (fig. 4).

DISCUSSION

Sagi et al. (1996) state that an ovary only exists in *Cherax quadricarinatus* when a female genital opening is present in the absence of a male genital opening on the same side. Our specimen of *Pacifastacus leniusculus* showed features in agreement with those studies. Further investigation is required to clarify the function of the female component of the reproductive system in intersex crayfish.

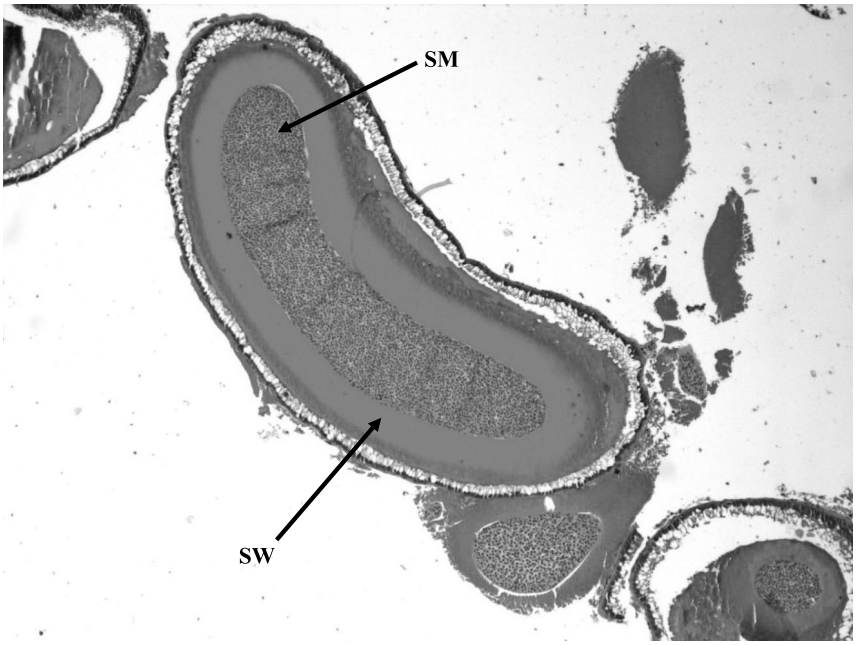

Fig. 4. Spermataphore section of male *Pacifastacus leniusculus* (Dana, 1852). SM, sperm mass; SW, spermatophore wall. Magnification 20× (Original). This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/15685403.

A well-developed male reproductive system including testis and vas deferens full of spermatophore was observed in the present study. The crayfish spermatophore consists of a sperm mass covered by a three-layered spermatophore wall, and each spermatozoon is enclosed by an extracellular capsule (Niksirat et al., 2013a, b, 2014).

Recent publications report hermaphroditism in Parastacidae. Future studies should address whether the intersexual signal crayfish is able to produce and fertilize eggs, and the possible mechanisms triggering intersexuality in signal crayfish, including whether environmental pollution may be responsible for crayfish hermaphroditism. Research has shown that some DDTs (dichloro diphenyl trichloroethanes) and brominated flame retardants can disrupt the endocrine system in aquatic animals (Hajslova et al., 2007; Havelkova et al., 2007). The effect of other pesticides toxicity on juvenile signal crayfish has been studied previously (Velisek et al., 2013). Establishment of external sex characteristics that identify intersexuality, along with the study of reproduction success, is necessary for a better understanding of intersexuality in Astacidea.

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CHAPTER 3

FINE STRUCTURE OF THE SPERMATOZOON IN THREE SPECIES OF CAMBARIDAE (ARTHROPODA: CRUSTACEA: DECAPODA) *CAMBARUS ROBUSTUS*, *ORCONECTES PROPINQUUS* AND *ORCONECTES RUSTICUS*: A COMPARATIVE BIOMETRICAL STUDY

Yazicioglu B., Hamr, P., Kozák, P., Kouba, A., Niksirat, H., 2016. Fine structure of the spermatozoon in three species of Cambaridae (Arthropoda: Crustacea: Decapoda) *Cambarus robustus, Orconectes propinquus* and *Orconectes rusticus*: A comparative biometrical study. PeerJ 4, e2363.

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Fine structure of the spermatozoon in three species of Cambaridae (Arthropoda: Crustacea: Decapoda) *Cambarus robustus*, *Orconectes propinquus* and *Orconectes rusticus*: a comparative biometrical study

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ABSTRACT

The ultrastructure of spermatozoa in three species of cambarid crayfish, *Cambarus robustus*, *Orconectes propinquus*, and *Orconectes rusticus*, were studied and compared with eight previously studied species from different crayfish families using morphological features and biometrical data. The ultrastructure of spermatozoa show a generally conserved pattern including an acrosome and nucleus in the anterior and posterior parts of the cell, respectively, radial arms that wrap around the nucleus, and the whole cell is enclosed by an extracellular capsule. The most outstanding morphological feature in spermatozoa of three studied cambarid crayfish is the crest-like protrusions in the anterior part of the acrosome that can be used as one of the features for distinguishing the members of this family. Results of biometrical data reveal that acrosome size in the representatives of Parastacidae are the smallest, while representatives of Astacidae show the biggest acrosome. The acrosome size in species belonging to Cambaridae occupy an intermediate position between the two other families of freshwater crayfish. In conclusion, a combination of morphological features and biometrical data of spermatozoa can help distinguishing different species of the freshwater crayfish.

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INTRODUCTION

Non-motile spermatozoa of decapods are very diverse in their morphology and that makes them suitable cases for phylogenetic studies (*Jamieson, 1991*; *Jamieson & Tudge, 2000*; *Tudge, 2009*; *Klaus & Brandis, 2011*; *Braga et al., 2013*). Currently, studies investigating decapod crustacean sperm morphology cover 100% of the decapod infraorders, 50% of the families, approximately 10% of the extant genera, but only 2% of the described, extant species (*Tudge, 2009*). Freshwater crayfish are highly diverse and commercially and ecologically important animals currently comprising 3 families, 33 genera, and over

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640 known species (*Crandall & Buhay, 2007*). In crayfish, aflagellate spermatozoa bear a relatively large acrosome in the anterior part and a nucleus in the posterior containing extensions of microtubular radial arms (*Moses, 1961a*; *Moses, 1961b*; *Dudenhausen & Talbot, 1982*). Spermatozoa of the freshwater crayfish have already been the subject of many ultrastructural studies (Table 1). The different dimensions of the most prominent organelle in crayfish spermatozoa, the acrosome, have been used for taxonomic studies, as well as the presence of some morphological features, especially in the anterior part of the spermatozoon, such as the spike, apical zone and crest can help distinguishing spermatozoa from different species (*Yasuzumi & Lee, 1966*; *Anderson & Ellis, 1967*; *Niksirat et al., 2013a*; *Niksirat et al., 2013b*; *Kouba, Niksirat & Bláha, 2015*).

The objective of the present study is to compare spermatozoal ultrastructure in three cambarid crayfish with other members of crayfish via morphological features and biometrical data.

MATERIALS AND METHODS

Samplings were carried out in Credit River, Norval, Ontario for *Cambarus robustus* and *Orconectes propinquus*, and Cavan Creek, Ontario for *Orconectes rusticus* during spawning season in May (Licence No. #1082971, MNR, Ontario). Five specimens of each species were anesthetized on ice for at least 10 min, and dissected to obtain the terminal portion of vasa deferentia near to gonopore containing the most developed spermatozoa. Samples for transmission electron microscopy (TEM) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 48 h at 4 °C, washed in buffer, and post-fixed in 4% osmium tetroxide for 2 h, washed in buffer, dehydrated through an acetone series (30, 50, 70, 90, 95, and 100% for 15 min each), and embedded in resin (EPON). A series of ultra-thin sections were cut using an UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany), mounted on the copper grids, double-stained with uranyl acetate and lead citrate (*Niksirat, Kouba & Kozák, 2015a*), and examined with a 1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operating at 80 kV. The length (L) and width (W) of the acrosome and the L: W ratio (*Jamieson, 1991*; *Klaus, Schubart & Brandis, 2009*; *Klaus & Brandis, 2011*) were determined in *C. robustus* ($n = 322$ spermatozoa), *O. propinquus* ($n = 120$ spermatozoa), *O. rusticus* ($n = 140$ spermatozoa) using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). For further comparison within different crayfish families, extra data were obtained from earlier published research describing spermatozoa of *Astacus astacus* ($n = 128$ spermatozoa), *A. leptodactylus* ($n = 98$ spermatozoa), *Austropotamobius torrentium* ($n = 86$ spermatozoa), *Pacifastacus leniusculus* ($n = 54$ spermatozoa), *Orconectes limosus* ($n = 139$ spermatozoa), *Procambarus clarkii* ($n = 115$ spermatozoa), *Cherax quadricarinatus* ($n = 91$ spermatozoa) and *C. destructor* ($n = 111$ spermatozoa) (*Niksirat et al., 2013a*; *Niksirat et al., 2013b*; *Kouba, Niksirat & Bláha, 2015*). The non-parametric Kruskal–Wallis test with subsequent pairwise comparison post-hoc statistical analysis were carried out using R statistical package version 3.2.5. For all statistical tests, $p < 0.05$ was considered significant. Data are expressed as the mean ± s.e.m.

Table 1 **Published literature about male gamete morphology in the freshwater crayfish species including four species of Astacidae, nine species of Cambaridae, and five species of Parastacidae.**

Family	Species	References
Astacidae	*Astacus astacus*	*Pochon-Masson, 1968*; *López-Camps et al., 1981*; *Niksirat et al., 2013b*; *Niksirat & Kouba, 2016*
	Astacus leptodactylus	*Eliakova & Goriachkina, 1966*; *Niksirat et al., 2013b*
	Austropotamobius torrentium	*Niksirat et al., 2013b*
	Pacifastacus leniusculus	*Dudenhausen & Talbot, 1979*; *Dudenhausen & Talbot, 1982*; *Dudenhausen & Talbot, 1983*; *Yazicioglu et al., 2014*; *Niksirat et al., 2013a*
Cambaridae	*Cambaroides japonicus*	*Kaye et al., 1961*; *Yasuzumi et al., 1961*; *Yasuzumi & Lee, 1966*
	Cambarus sp.	*Anderson & Ellis, 1967*
	Cambarus robustus	Present study*
	Orconectes limosus	*Niksirat et al., 2013a*
	Orconectes propinquus	Present study*
	Orconectes rusticus	*Berrill & Arsenault, 1982*; *Snedden, 1990*; Present study*
	Procambarus clarkii	*Moses, 1961a*; *Moses, 1961b*; *Niksirat et al., 2013a*; *Dong, Hou & Yang, 2014*
	Procambarus leonensis	*Felgenhauer & Abele, 1991*
	Procambarus paeninsulanus	*Hinsch, 1992*; *Hinsch, 1993a*; *Hinsch, 1993b*
Parastacidae	*Cherax albidus*	*Beach & Talbot, 1987*
	Cherax destructor	*Jerry, 2001*; *Kouba, Niksirat & Bláha, 2015*
	Cherax quadricarinatus	*López-Greco, Vazquez & Rodríguez, 2007*, *López-Greco and Lo Nostro, 2008*; *Kouba, Niksirat & Bláha, 2015*
	Cherax cainii[a]	*Beach & Talbot, 1987*; *Jamieson, 1991*
	Parastacus defossus	*Noro, López-Greco & Buckup, 2008*

Notes.

[a]We assume that the smooth marron, a common and widespread species largely involved in aquaculture formerly called *C. tenuimanus* was examined. See *Austin & Ryan (2002)* for details.

RESULTS

Morphological features

The acrosome complex, as the most prominent organelle, is located in the anterior part of the spermatozoon. It consists of two distinct components including the main body of the acrosome vesicle and the subacrosomal zone (Figs. 1A, 2A and 3A). The anterior-most central portion of the acrosome vesicle is folded into a series (usually 2–5) of protrusions resembling a crenulated crest (Figs. 1B, 2B and 3B). The main body of the acrosome vesicle appears to be divided into two, sometimes indistinct, zones. In the innermost zone some filaments are visible while those filaments are not present in the outer layer (Figs. 1C, 2C and 3C). The space posterior to the main body of the acrosome vesicle is filled by a flocculent electron lucent subacrosomal zone. The density of the subacrosomal zone is less in the vicinity of the posterior-most part of the main body of the acrosome vesicle (Figs. 1A, 2A and 3A). Radial arms consisting of microtubules wrap around the main body of acrosome vesicle, but remain contained within the extracellular capsule (Figs. 1D, 2D and 3D). Membranous lamellae, as a concentric bundle of convoluted membranes, are clearly visible inside the cell (Figs. 1E, 2E and 3E). The nucleus is located in the posterior part of the

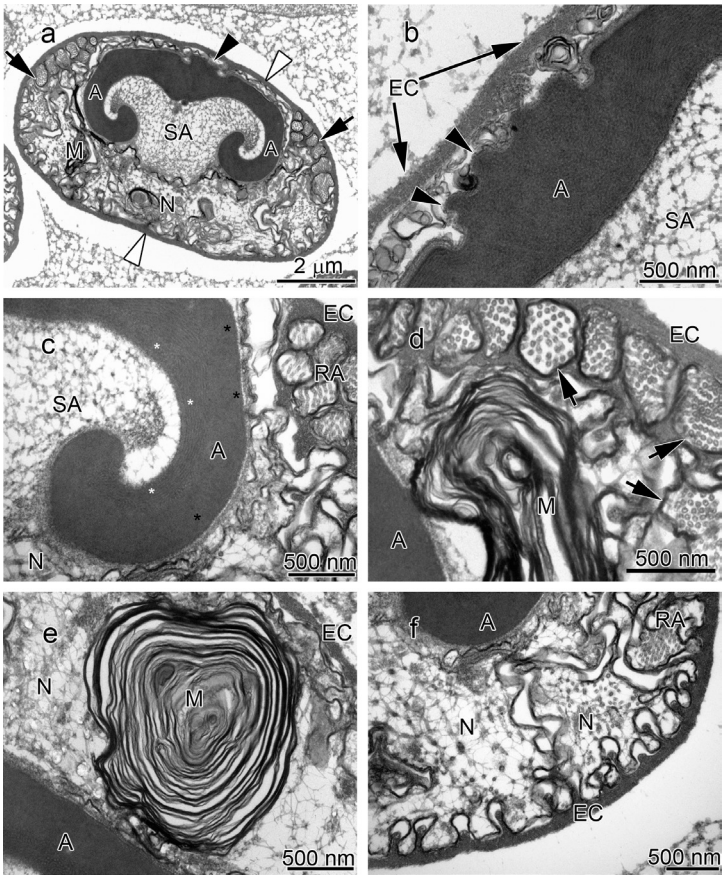

Figure 1 **Transmission electron micrographs of *Cambarus robustus* spermatozoon.** (A) longitudinal sagittal view of the entire spermatozoon, black arrows show sections of radial arms. The crest and extracellular capsule are shown by black and white arrowheads, respectively, (B) protrusions of the acrosome crest (black arrowheads), (C) filamentous (white stars) and non-filamentous (black stars) of the main body of acrosome, (D) higher magnification of microtubules in the radial arms of spermatozoon (black arrows), (E) membranous lamellae, (F) nucleus. A, acrosome main body; EC, extracellular capsule; M, membranous lamella; N, nucleus; RA, radial arms; SA, subacrosome zone.

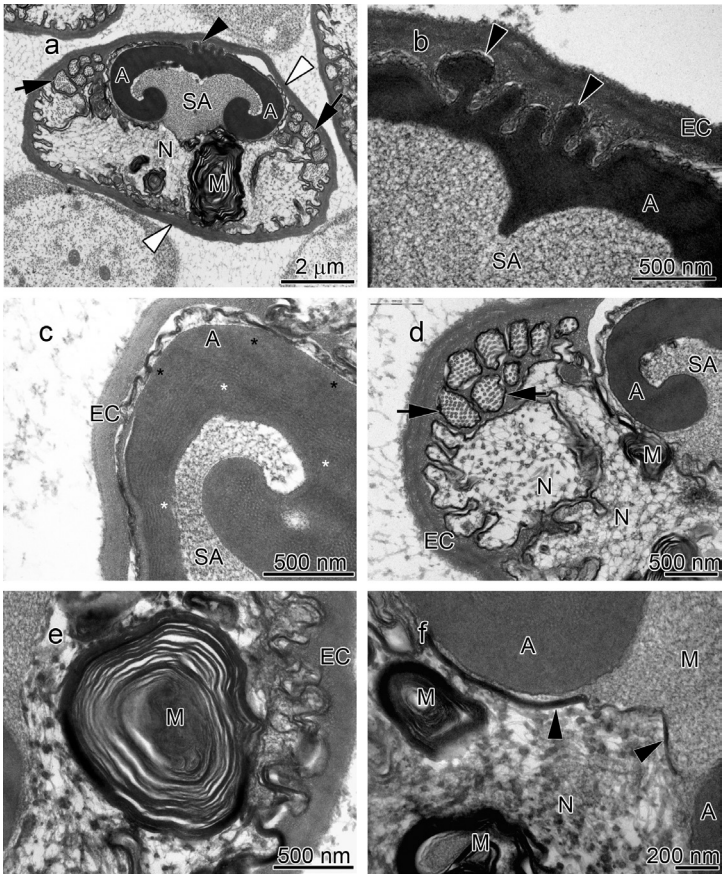

Figure 2 **Transmission electron micrographs of *Orconectes propinquus* spermatozoon.** (A) longitudinal sagittal view of the entire spermatozoon, black arrows show sections of radial arms. The crest and extracellular capsule are shown by black and white arrowheads, respectively, (B) protrusions of the acrosome crest (black arrowheads), (C) filamentous (white stars) and non-filamentous (black stars) of the main body of acrosome, (D) higher magnification of microtubules in the radial arms of spermatozoon (black arrows), (E) membranous lamellae, (F) nucleus. A, acrosome main body; EC, extracellular capsule; M, membranous lamella; N, nucleus; RA, radial arms; SA, subacrosome zone.

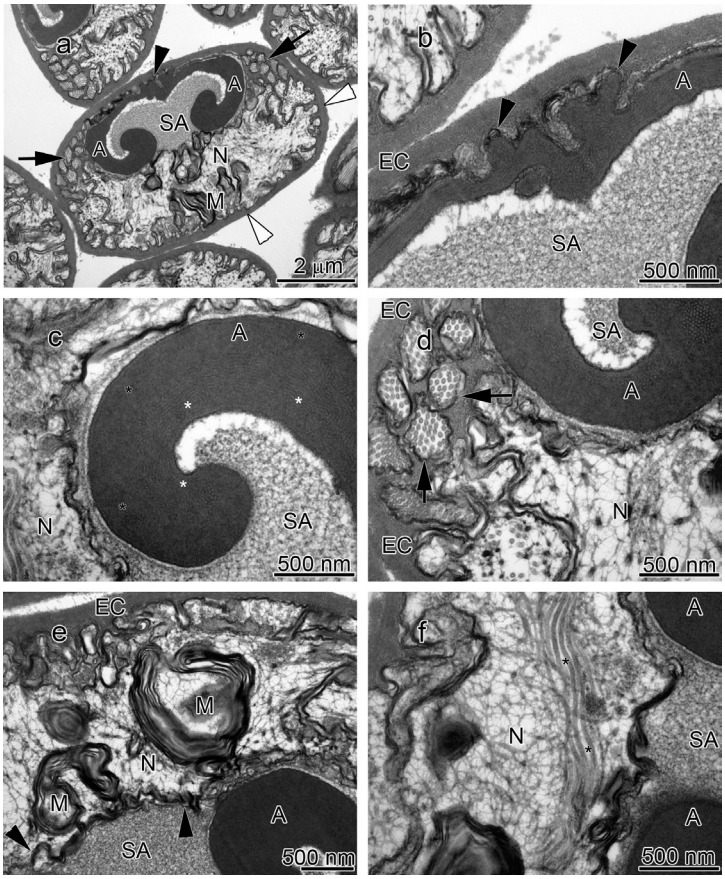

Figure 3 **Transmission electron micrographs of *Orconectes rusticus* spermatozoon.** (A) longitudinal sagittal view of the entire spermatozoon, black arrows show sections of radial arms. The crest and extracellular capsule are shown by black and white arrowheads, respectively, (B) protrusions of the acrosome crest (black arrowheads), (C) filamentous (white stars) and non-filamentous (black stars) of the main body of acrosome, (D) higher magnification of microtubules in the radial arms of spermatozoon (black arrows), (E) membranous lamellae, (F) nucleus, black stars show microtubules inside nucleus. A, acrosome main body; EC, extracellular capsule; M, membranous lamella; N, nucleus; RA, radial arms; SA, subacrosome zone.

Table 2 **Summarizes comparative morphological features among eleven species of the freshwater crayfish.**

Family	Species	Acrosome spike	Apical zone	Crest	Extracellular capsule	Radial arms
Astacidae	*Astacus astacus*	−	+	−	+	+
	Astacus leptodactylus	−	+	−	+	+
	Austropotamobius torrentium	−	+	−	+	+
	Pasifastacus leniusculus	−	+	−	+	+
Cambaridae	*Cambarus robustus*	−	−	+	+	+
	Orconectes limosus	−	−	+	+	+
	Orconectes propinquus	−	−	+	+	+
	Orconectes rusticus	−	−	+	+	+
	Procambarus clarkii	+	−	−	+	+
Parastacidae	*Cherax destructor*	−	−	−	−	−
	Cherax quadricarinatus	−	−	−	−	−

spermatozoon containing nuclear materials (Figs. 1F, 2D, 2F, and 3F). The spermatozoon is tightly enclosed by an extracellular capsule (Figs. 1A, 2A and 3A). Table 2 summarizes comparative morphological features among eleven species of freshwater crayfish, eight from literature and three from this study.

Biometrical data

A significant correlation ($p < 0.0001$, $r = 0.97$) was observed between the length and width of the acrosome vesicle in all studied species (Fig. 4). The Kruskal–Wallis test showed significant differences among length, width and L: W of studied groups ($p < 0.05$). The smallest and largest acrosome length were recorded in *C. destructor* and *A. astacus*, respectively. Significant differences were observed in the acrosome length among studied species ($p < 0.05$) except between these species (1) *C. destructor* and *C. quadricarinatus*, (2) *C. quadricarinatus* and *P. clarkii*, (3) *O. limosus* and *O. rusticus*, (4) *O. rusticus*, and *O. propinquus*, (5) *O. propinquus* and *C. robustus*, and (6) *Au. torrentium*, *P. leniusculus*, *A. leptodactylus* and *A. astacus* (Fig. 5A).

The smallest and largest acrosome width were recorded in *C. destructor* and *A. astacus*, respectively. Significant differences were observed in the acrosome width among studied species ($p < 0.05$) except between these species (1) *C. destructor*, *C. quadricarinatus*, and *P. clarkii*, (2) *O. rusticus*, and *C. robustus*, (3) *C. robustus*, *O. limosus*, and *O. propinquus*, (4) *Au. torrentium*, *A. leptodactylus*, and *P. leniusculus*, (5) *P. leniusculus* and *A. astacus* (Fig. 5B).

The smallest and largest acrosome length to width ratio were recorded in *O. limosus* and *P. clarkii*, respectively. Significant differences ($p < 0.05$) were observed in the acrosome length to width ratio among studied species ($p < 0.05$) except between these following groups: (1) *O. propinquus* and *O. rusticus*, (2) *C. robustus* and *A. astacus*, (3) *A. astacus* and *P. leniusculus*, (4) *P. leniusculus* and *Au. torrentium*, (5) *Au. torrentium*, *A. leptodactylus*, *C. quadricarinatus*, and *C. destructor*, (6) *A. leptodactylus*, *C. quadricarinatus*, *C. destructor* and *P. clarkii* (Fig. 5C).

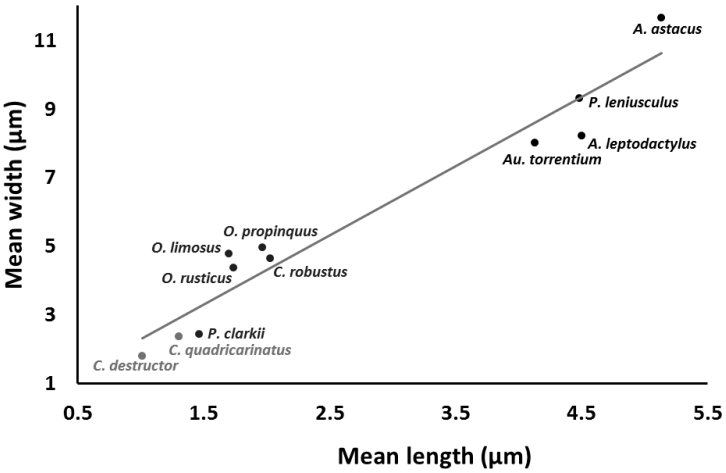

Figure 4 Correlation between length and width of the spermatozoon acrosome in eleven species of freshwater crayfish. ($p < 0.0001$, $r = 0.97$).

DISCUSSION

Morphological features

The results of the present study show that the general morphology of spermatozoa in studied members of Cambaridae is similar to other crayfish including a relatively large acrosome, nucleus and radial arms that are enclosed by an extracellular capsule (*Jamieson & Tudge, 2000*; *Vogt, 2002*). Radial arms are usually the extensions of the nucleus that wrap around the acrosome vesicle. These arms are present in Astacidae and Cambaridae, but not in studied *Cherax* species (*Beach & Talbot, 1987*; *Kouba, Niksirat & Bláha, 2015*). The radial arms in decapod spermatozoa may be composed of microtubules, nuclear material, or both (*Tudge, 2009*). Molecular studies identified tubulin proteins, as major units of microtubules in the proteomic profile of the crayfish male gamete that confirms the microtubular nature of radial arms (*Niksirat et al., 2014a*; *Niksirat et al., 2015b*) and as seen in the TEM images in this study. Although, microtubular radial arms undergo protein tyrosine phosphorylation during spermatophore post-mating storage on the body surface of female crayfish (*Niksirat et al., in press*), the exact role(s) of radial arms in fertilization is yet to be determined. The extracellular capsule seems to be an envelope for tight compaction of long organelles such as radial arms. This hypothesis is further supported by the absence of a capsule in the studied *Cherax* spermatozoa, where radial arms are not present (*Beach & Talbot, 1987*; *Vogt, 2002*; *Kouba, Niksirat & Bláha, 2015*).

The membranous lamella is an organelle that has been reported in spermatozoa of several crayfish (*Jamieson & Tudge, 2000*). It has been observed that some mitochondria

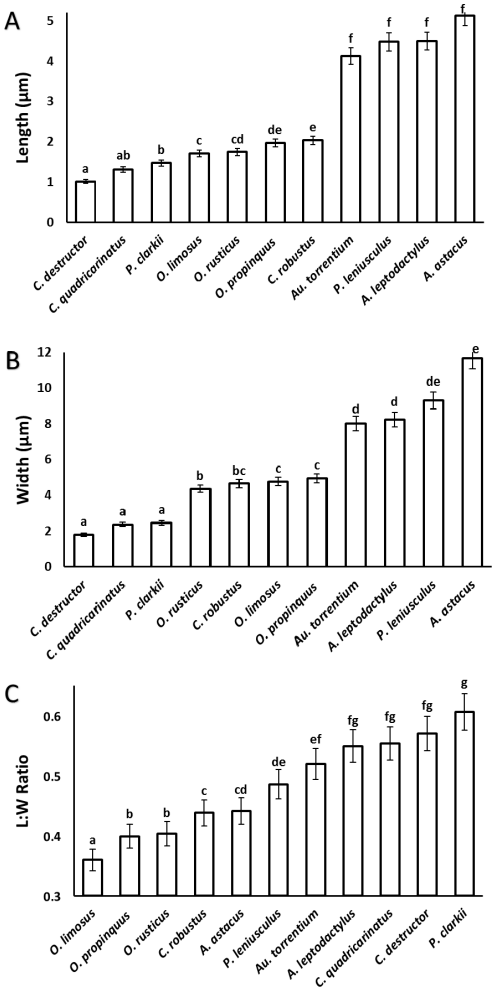

Figure 5 Comparison of different dimensions of the spermatozoon acrosome among eleven species of freshwater crayfish. (A) mean length of the acrosome, (B) mean width of the acrosome, and (C) length: width ratio of the acrosome. Within each chart, bars marked with a similar superscripts did not differ significantly from each other at $p < 0.05$.

lose their internal matrix and are transformed into membranous lamellae during the early spermatid stage of the crayfish *Cambarus* sp. which is still able to provide energy to the cell (*André, 1962*; *Anderson & Ellis, 1967*). A positive staining of Janus green B, an indicator of active mitochondria, in the same area of the crayfish spermatozoon has been reported (*André, 1962*). Several proteins related to metabolism and energy production were identified in the protein profile of the crayfish male gamete that may confirm the presence of an energy supply center in the sperm cell (*Niksirat et al., 2014a*; *Niksirat et al., 2015b*).

A set of electron lucent pores, a unique morphological feature, have only been reported at the margins of the main body of the acrosome in *P. leniusculus* (*Niksirat et al., 2013b*). The anterior-most margin of the main body of the acrosome vesicle in different crayfish species showed a diversity in shape that can be used as an important morphological feature for distinguishing different species of freshwater crayfish (*Niksirat et al., 2013a*; *Niksirat et al., 2013b*; *Kouba, Niksirat & Bláha, 2015*; present study). For example, in Cambaridae, a horn-like spike was observed in the anterior part of the fully developed spermatozoon of *Cambaroides japonicus* (*Yasuzumi & Lee, 1966*). A similar spike-shaped structure has been reported in spermatozoa of *Cambarus* sp. (*Anderson & Ellis, 1967*) and *Procambarus leonensis* (*Felgenhauer & Abele, 1991*). While several other studies on spermatozoal ultrastructure and spermatogenesis in *Procambarus* (*Moses, 1961a*; *Moses, 1961b*; *Hinsch, 1992*; *Hinsch, 1993a*; *Hinsch, 1993b*) did not report an acrosomal spike, development of a spike in the anterior part of the acrosome vesicle has been observed in *Procambarus clarkii* when the spermatozoa are inside the vas deferens (*Niksirat et al., 2013a*). An apical zone, an area filled with bundles of curled filaments has been reported in *A. astacus*, *A. leptodactylus*, *A. torrentium*, *P. leniusculus* (*Pochon-Masson, 1968*; *López-Camps et al., 1981*; *Niksirat et al., 2013a*; *Niksirat et al., 2013b*). Those filaments and some material originating from the acrosome are released outside the spermatozoon and form a new formation called filament-droplet structure that could facilitate egg-spermatozoon binding during fertilization in crayfish (*Niksirat, Kouba & Kozák, 2014b*). In the present study, crest-like protrusions observed in the anterior part of the acrosome vesicle of spermatozoa can be used as one of the morphological features for distinguishing cambarids from other species of freshwater crayfish.

Biometrical data

Results of acrosome measurement in the spermatozoa of eleven species of freshwater crayfish show that despite some similarities, a combination of different acrosome dimensions (length, width, and length:width ratio) can be useful for distinguishing different species of crayfish. The length:width ratio of the acrosome vesicle has been applied to divide crustaceans into three different categories: depressed (<1), spherical (1) and elongated (>1). The eleven species of crayfish fall into the depressed acrosome category sharing this position with a few thoracotreme and heterotreme brachyurans, all investigated podotreme brachyurans, some astacid, palinurid and enoplometopid lobsters (*Jamieson, 1991*), and Pylocheles (Bathycheles) from the Anomura (*Tudge, Scheltinga & Jamieson, 2001*).

The size of the acrosome vesicle in the representatives of Parastacidae (*Cherax*) are the smallest within studied crayfish species. The representatives of Astacidae including *Astacus*, *Pacifastacus*, and *Austropotamobius* showed the largest acrosome vesicles. The acrosome size in species belonging to *Orconectes* and *Procambarus* as representatives of Cambaridae occupy an intermediate position among the above mentioned families of freshwater crayfish.

In conclusion, despite conserved general pattern of the crayfish spermatozoon, combination of morphological features such as apical zone, crest and spike in the anterior part of the acrosome, and biometrical data of the acrosome dimensions can provide a tool to distinguish different species of freshwater crayfish families.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Buket Yazicioglu performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Přemek Hamr wrote the paper, reviewed drafts of the paper.
- Pavel Kozák conceived and designed the experiments, reviewed drafts of the paper.
- Antonín Kouba performed the experiments, reviewed drafts of the paper.
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The raw data has been supplied as a Supplemental Dataset.

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CHAPTER 4

POST-MATING SPERMATOPHORE STORAGE STRATEGIES IN TWO SPECIES OF CRAYFISH: IMPLICATIONS FOR BROODSTOCK MANAGEMENT

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Post-mating spermatophore storage strategies in two species of crayfish: implications for broodstock management

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Female crayfish stores male gametes after mating until the beginning of egg laying and fertilization. The aim of the present study was to investigate the duration of post-mating spermatophore storage as well as the timing and temperature of spawning in two crayfish species of economic importance, namely the signal crayfish Pacifastacus leniusculus *and the noble crayfish* Astacus astacus*. Results showed that the average duration of the post-mating spermatophore storage is significantly ($P < 0.05$) longer in the noble crayfish (34.6 ± 1.7 days, range: 19 to 60 days) than the signal crayfish (3.9 ± 0.5 days, range: 1 to 18 days). The highest percentages of the post-mating spermatophore storage duration in the signal crayfish (46.5%) and the noble crayfish (44.5%) were 1 and 31 to 40 days, respectively. While there is an overlap in the timings of mating and egg laying in the signal crayfish, these two reproductive processes were not observed at the same days in the noble crayfish and there was at least 2 weeks interval between last mating and first egg laying individuals. Average mating and egg laying temperatures were significantly ($P < 0.05$) higher in the signal crayfish than the noble crayfish. The average temperatures for mating in both species were significantly ($P < 0.05$) higher than the temperatures that they utilized for egg laying. In conclusion, female noble crayfish stores post-mating spermatophores a longer duration compared with the signal crayfish. Also, the signal crayfish mates and lays egg in temperatures that are higher than the noble crayfish. Spawning season is shorter in the signal crayfish compared with the noble crayfish. The results of present study provide information contributing to the crayfish broodstock management in aquaculture.*

Keywords: crustacean, decapoda, noble crayfish, signal crayfish, sperm storage

Implications

Crayfish is a delicious and healthy food item for human. An increasing market demand for this aquatic organism encourages development of techniques for artificial reproduction and farming of crayfish during recent decade. Here we studied the time interval between mating and egg laying in broodstock of two widespread freshwater crayfish species including signal and noble crayfish. Results of present study can directly be used by crayfish farmers for management of broodstocks across Europe and America. Also, this study provides basic information for biologists to further study of biology of reproduction in crayfish.

Introduction

The post-mating sperm storage is well known in a wide range of animals including vertebrates (Holt and Lloyd, 2010; Holt, 2011) and invertebrates (Bauer, 1986; Wolcott *et al*., 2005; Niksirat *et al*., 2014). Crayfish as members of decapod crustaceans make up a large group of invertebrates including three families, 33 genera, and over 640 known species (Crandall and Buhay, 2008). Crayfish male produces immotile spermatozoa (Tudge, 2009; Niksirat *et al*., 2013a and 2013b; Kouba *et al*., 2015; Yazicioglu *et al*., 2016) that are packed into spermatophore and transferred to the female body surface in Astacidae and Parastacidae, or into the *annulus ventralis* which is known as a spermatophore storage segment in Cambaridae (Hamr, 2002), during mating. Crayfish male gametes undergo post-mating morphological and molecular changes that are necessary for them to acquire fertilization ability (Niksirat *et al*., 2015a and 2016; Niksirat and Kouba, 2016). Spermatozoa are released from spermatophore during fertilization and after fertilization eggs are attached to the body of female crayfish until hatching (Niksirat *et al*., 2015b).

Noble crayfish *Astacus astacus* occurs in the open waters and it is widely distributed mainly in the northern and central Europe (Holdich *et al*., 2009). The signal crayfish *Pacifastacus leniusculus* is a North American species with a wide native

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range between the Pacific Ocean and Rocky Mountains (Taylor *et al.*, 2007). Although, there are some reports regarding the timing of spawning in crayfish (Lewis, 2002), the duration of post-mating spermatophore storage is not fully investigated in crayfish, yet. Because of high market demand of crayfish, there is an increasing interest in crayfish culture (Skurdal and Taugbøl, 2002). Production of juveniles is an important step for development of sustainable crayfish farms. Knowledge such as the duration of post-mating spermatophore storage in relation with environmental factors (e.g. temperature and season) can facilitate management of crayfish broodstock with aim of juvenile production.

The goal of present study was to investigate the duration of post-mating spermatophore storage as well as the timing and temperature of spawning in the signal crayfish and the noble crayfish. The results of present study can provide basic data regarding the time interval between mating and the onset of egg laying and subsequent fertilization in two commercially and ecologically important crayfish species that can be used for the management of broodstock in farms within their native ranges and/or natural habitats.

Material and methods

Adult signal crayfish (*Pacifastacus leniusculus* Dana, 1852; $n = 142$) and noble crayfish (*Astacus astacus* Linnaeus, 1758, $n = 72$) were collected from the Babačka Brook (Sklené nad Oslavou, Czech Republic) and Kramata Reservoir (Hrabice, Czech Republic), respectively. The experiment was started on September 26, 2012. A total of 36 and 71 pairs of noble and signal crayfish were used for experiment, respectively. Each pair consisted of one male and one female. The pairs of crayfish were placed in plastic mesh boxes which were divided into four chambers to keep each pair separately. Plastic mesh boxes were placed in two 850 l outdoor tanks. Animals were fed during experiment. All animals were kept under the same natural temperature and photoperiod. Water temperature was recorded using data loggers (Minikin; Environmental Measuring Systems, Brno, Czech Republic). All pairs were checked for mating and egg laying twice a day by observing presence of spermatophore and eggs in the abdominal part of female, respectively.

Statistical analysis

The non-parametric Mann–Whitney test was carried out to compare the post-mating spermatophore storage duration as well as suitable temprature for the mating and egg laying between two studied species using SPSS statistical package version 16.0. For all statistical tests, $P < 0.05$ was considered significant. Data are presented as the mean ± SEM.

Results

Results showed that the average duration of the post-mating spermatophore storage is significantly ($P < 0.05$) longer in the noble crayfish (34.6 ± 1.7 days, range: 19 to 60 days) than the

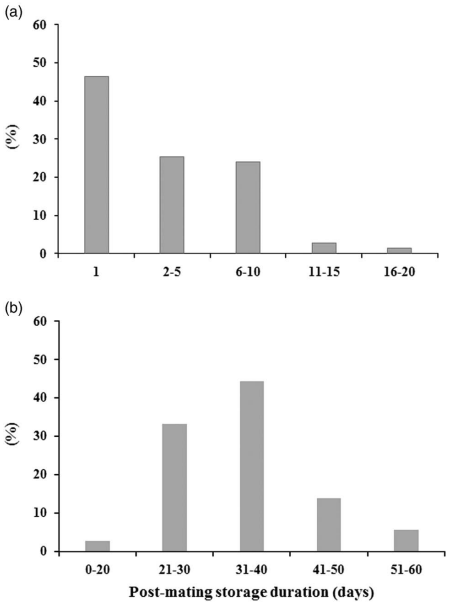

Figure 1 Bars show classification of the post-mating spermatophore storage duration in the signal (a) and the noble crayfish (b).

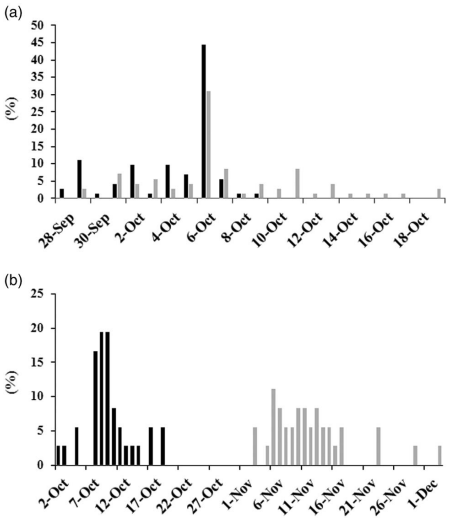

Figure 2 Mating and egg laying dates in the signal (a) and the noble crayfish (b) during spawning season. Black and gray bars show mating and egg laying percentages, respectively.

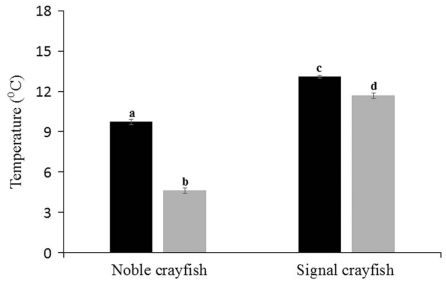

Figure 3 Average water temperatures during mating and egg laying in the noble crayfish (36 pairs) and the signal crayfish (71 pairs). Values marked with different superscripts differ significantly from each other at $P < 0.05$. Data of temperature are shown as mean ± SEM. Black and gray bars show the mating and egg laying, respectively.

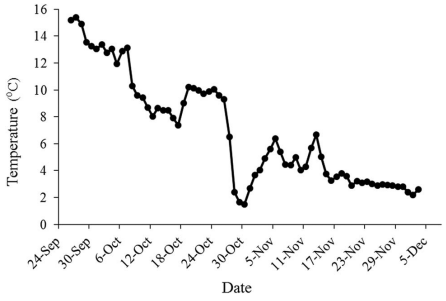

Figure 4 Water temperature during the experiment.

signal crayfish (3.9 ± 0.5 days, range: 1 to 18 days). The highest percentages of the post-mating spermatophore storage duration in the signal crayfish (46.5%) and the noble crayfish (44.5%) were 1 and 31 to 40 days, respectively (Figure 1a and b). The first and last matings and egg layings in the signal crayfish were observed on September 28, October 9, September 29 and October 19, respectively. The first mating in the noble crayfish occured on October 2. Mating in this species ended on 19th of the same month. However, first egg laying female was not observed before November 3. Egg laying terminated on December 3 in the noble crayfish. While there was an overlap in the mating and egg laying timings in the signal crayfish, these two reproductive processes were not observed at the same days in the noble crayfish and there was at least 2 weeks interval between the last mating and first egg laying individuals (Figure 2a and b).

Average mating and egg laying temperatures were significantly ($P < 0.05$) higher in the signal crayfish than the noble crayfish. The average temperatures for mating in the both species were significantly ($P < 0.05$) higher than the temperatures that they utilized for egg laying (Figure 3). Temperature trend during experiment is shown in Figure 4.

Discussion

Results of the present study showed that the female noble crayfish stores the post-mating spermatophores for longer periods compared with the signal crayfish. In addition, we managed to show that the signal crayfish preferes warmer waters for mating and laying of eggs in comparison with the noble crayfish. Buřič *et al.* (2013) observed that the female spiny-cheek crayfish, *Orconectes limosus*, can successfully store spermatophore from their autumn mating for more than half a year. They stated that this trait allows the female to increase its chance for multiple successful matings and also finding the best mate.

Post-mating spermatophore storage has been reported in many other species of decapod crustaceans including crabs, shrimps and lobsters (Bauer, 1986; Moyano *et al.*, 2009). In some decapods such as clawed lobsters and some crabs with a long life-span, post-mating male gametes can be saved across consecutive molts by storage in some parts of the seminal receptacles of females that are not cast out during molting. This strategy allows post-mating spermatophores to be utilized successfully for next years for fertilization of eggs (Factor, 1995; Becker *et al.*, 2011). This strategy is well observed in the mated females Tanner crab, *Chionoecetes bairdi*, that produced 100% of fertilized eggs in the same year after mating but continued to produce 97% and 71% fertilized eggs in the next 2 years even without any contact by males (Paul, 1984). Also, Jensen and Bentzen (2012) reported that female Dungeness crab, *Metacarcinus magister* can fertilize eggs using stored sperm as old as 2.5 years. Female blue crab *Callinectes sapidus* that mates in summer and fall must store spermatophore for 7 to 11 months before using them in following summer for fertilization. This post-mating spermatophore storage strategy is very important for the blue crab because female mates only once during whole lifetime (Millikin and Williams, 1984; Hines *et al.*, 2003). Long-term post-mating spermatophore storage strategy by female decapods has an important role in the population biology and sustainable fishery, because it may compensate negative effects of fishing to some extent by allowing posthumous paternity to the largest males that are caught by fishing activities (Gosselin *et al.*, 2005; Sainte-Marie *et al.*, 2008; Taylor *et al.*, 2014; Ellis *et al.*, 2015; Vogt, 2016).

In addition, it has been proved that decapod spermatozoa undergo some morphological and molecular modifications providing them fertilizing ability, also known as spermatozoon capacitation (Alfaro *et al.*, 2007). Vanichviriyakit *et al.* (2004) proved that an extensive molecular modification of the spermozoon including protein tyrosine phosphorylation takes place during post-mating storage in the female thelycum of giant tiger prawn *Penaeus monodon*. It takes 6 to 7 h for spermatophore of Pacific white shrimp *Litopenaeus vannamei* to be capacitated in the thelycum of female at 28°C. During post-mating interval, spermatozoa undergo a morphological change including further development of a region in the spermatozoon so called filamentous meshwork,

located between the nucleus and the hemispherical cap (Alfaro *et al*., 2007). In addition, observation of some biochemical changes confirm that capacitation of *L. vannamei* spermatozoa happens following 4 to 6 h of post-mating storage in thelycum of female at 28°C (Aungsuchawan *et al*., 2011). It is well-known that higher temperatures accelerate biological processes (Cossins, 2012). Therefore, shorter post-mating spermatophore storage duration in the female signal crayfish could be attributed to higher water temperature compared with the noble crayfish that preferes colder temperatures and stores post-mating spermatophore for longer periods.

Post-mating spermatophore storage duration was poorly documented in decapods in general and crayfish in particular. Here we managed to demonstrate the patterns of spermatophore storage in relation with environmental factors such as temperature in broodstocks of two ecologically and economically important crayfish species that were kept under equal outdoor conditions. Our results can provide information for farmers to predict reproductive behaviors of studied crayfish species and therefore can facilitate broodstock management. For example, we have frequently observed that males eat eggs from female crayfish. If farmers are aware of the egg laying timings for those two species, they can separate mated females from males and transfer them from the mating to spawning facilities where females are allowed to incubate their fertilized eggs in safer conditions. To sum up, female noble crayfish stores post-mating spermatophores for longer periods compared with the signal crayfish. Also, signal crayfish mates and lays egg in temperatures that are significantly higher than noble crayfish with spawning season shorter in signal crayfish (September 28 to October 19) compared with noble crayfish (October 2 to December 3). The results of present study provide information contributing to the crayfish broodstock management in farms.

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CHAPTER 5

GENERAL DISCUSSION

The effects of aquatic invasive species on native species and ecosystems have become one of the most important issue around the world (Hazlet et al., 2003; Gollasch, 2006; Lovel et al., 2006; Gherardi and Acquistapace, 2007; Pintor et al., 2008; Rahel and Olden, 2008; Gherardi et al., 2011). The invasive species have been introduced outside of their native ranges and have the potential to cause ecological and economic damage. They are also called alien or non-indigenous species (Gherardi et al., 2011). The introduced invasive species are a bigger threat to native biodiversity than pollution, over-fishing, and disease combined. When invasive species are introduced into new habitats, they can cause destruction and damage to global diversity, easily (Lodge et al. 1998; Nyström 1999). Invasive species impact the habitats they invade by reducing the abundance of native species and altering ecosystem processes. They impact native species because of competition for space and food, predation and to be resistant to harmful pathogens and parasites. Invasive species may also change normal functioning of the ecosystem by changing hydrology, nutrient cycling and efficiency. Generally, invasive species have several common characteristic features such as fast growth, ability to survive in a wide range of environmental conditions, easily adaptable to different conditions, high dispersal ability and rapid reproduction. General characteristic structure of invasive species provides information about reproduction biology which is considered as one of the important factors for spreading of invasive species around the world. Unfortunately, almost all principles for spreading of invasive species are also applicable for crayfish invasive species. For example, the signal crayfish *Pacifastasus leniusculus* has been shown to be competitively outstanding by way of aggressive interactions over several native crayfishes and has negative impacts on native prey (Usio et al., 2001). However, it is known that there are several negative impacts which are caused by invasive crayfish species. One of them is environmental effects that include hybridization. For this reason, in the first step of our study we tried to understand reproduction strategy of crayfish. We summarized in our review not only normal crayfish reproductive mode but also focused on different unusual reproductive strategies in crayfish and tried to explain why they occur so often over the last decades. First of all, a general knowledge has given about unusual reproduction strategies such as parthenogenesis, hermaphrodite and intersexuality in freshwater crayfish. In most of experimental studies it has been showed that when we manipulate hormones like damaging androgenic gland in the crayfish, it can affect their reproduction strategies such as intersexuality which calls non-functional hermaphroditism. The marble crayfish is the only known parthenogenetic species (Scholtz et al., 2003). Additionally, Buřič et al. (2011) proved that spiny-cheek crayfish can reproduce parthenogenetically when the specific condition occurs. It's well known that invasive American crayfish species are occurring in Europe and may cause a direct or indirect effect on native species (Parker et al., 1999; Gherardi, 2007). One of the possibilities in case of direct effect is ''hybridization'' in the environmental condition between native and invasive species. The result of this hybridization could create a new invader, and this new species can become dominant in the natural habitat. In addition, hybridization between different species is one of the reasons of the various reproductive behaviours. Besides, chemical factors may impact reproductive strategies in crayfish.

We reported first intersex case in signal crayfish *Pacifastacus leniusculus* (Dana, 1852). The specimen was male at first glance, but after dissection, both testis and ovary were detected. The reproductive system of the specimen was well-developed and full of spermatophores in the vas deferens. Sagi et al. (1996) showed the same feature in *Cherax quadricarinatus*. According to recent publications, hermaphroditism was observed in Parastacidae. Some researchers have shown that some chemicals can disrupt the endocrine system of aquatic

animals such as chub, bream and perch (Hajslova et al., 2007; Havelkova et al., 2007). Additionally, pesticides toxicity can affect signal crayfish as observed by Velisek et al. (2013). Their study demonstrated that triazines are toxic to signal crayfish and it is more sensitive than fish. They also supposed that signal crayfish could be used as a bio-indicator of environmental contamination. However, still, there is a gap of knowledge about the main reason of this abnormality. Further studies are needed to find answers to the following questions: (1) The intersex or hermaphrodite in freshwater crayfish is functional or non-functional? (2) Can intersexual signal crayfish be able to produce and fertilise eggs? (3) If not, what is the triggering factor of this abnormality? Could environmental pollution be responsible for it? The further studies are needed to perform related to these points of views.

We also investigated ultrastructure of spermatozoa in three species of cambarid crayfish, *Cambarus robustus, Orconectes propinquus* and *Orconectes rusticus*. Additionally, compared with eight previously studied species (*Astacus astacus, Astacus leptodactylus, Austropotamobius torrentium, Cherax destructor, C. quadricarinatus, Orconectes limosus, Procambarus clarkii, P. leniusculus*) from different crayfish families using morphological features and biometrical data (Niksirat et al., 2013a,b; Kouba et al., 2015). The ultrastructure of spermatozoa shows a generally conserved pattern including an acrosome and nucleus in the anterior and posterior parts of the cell, respectively, radial arms that wrap around the nucleus, and the whole cell is enclosed by an extracellular capsule. In crayfish, aflagellate spermatozoa bear a relatively large acrosome in the anterior part and a nucleus in the posterior containing extensions of microtubular radial arms (Moses, 1961a,b; Dudenhausen and Talbot, 1982) and as we observed by the transmission electron microscopy (TEM). These arms are present in Astacidae and Cambaridae, but not in studied *Cherax* species (Beach and Talbot, 1987; Kouba et al., 2015). The radial arms in decapod spermatozoa may be composed of microtubules, nuclear material, or both (Tudge, 2009). Actually, the exact role(s) of radial arms in fertilization is yet to be determined. The membranous lamella is an organelle that has been reported in spermatozoa of several crayfish (Jamieson and Tudge, 2000). The extracellular capsule seems to be an envelope for tight compaction of long organelles such as radial arms. This hypothesis is further supported by the absence of a capsule in the studied *Cherax* spermatozoa, where radial arms are not present (Beach and Talbot, 1987; Vogt, 2002; Kouba et al., 2015). For example, Yasuzumi and Lee (1966) proved that there is a horn-like spike in the anterior part of the well-developed spermatozoon of *Cambaroides japonicus*. A similar spike-shaped structure has been reported in spermatozoa of *Cambarus* sp. (Anderson and Ellis, 1967) and *Procambarus leonensis* (Felgenhauer and Abele, 1991), as well. Although, there are several studies about spermatozoal ultrastructure and spermatogenesis in *Procambarus* (Moses, 1961a,b; Hinsch, 1992; Hinsch, 1993a,b) in the literature which did not report any acrosomal spike, development of a spike in the anterior part of the acrosome vesicle has been observed in *Procambarus clarkii* when the spermatozoa are inside the vas deferens (Niksirat et al., 2013a). On the contrary, an apical zone filled with bundles of curled filaments was reported in *Astacus astacus, A. leptodactylus, Austropotamobius torrentium, Pacifastacus leniusculus* (Pochon-Masson, 1968; López-Camps et al., 1981; Niksirat et al., 2013a,b). However, in our study, we found that crest-like protrusions observed in the anterior part of the acrosome vesicle of spermatozoa can be used as one of the morphological features for distinguishing cambarids from other species of freshwater crayfish. Jamesion (1991) and Tudge et al. (2001) used length: width ratio of the acrosome to divide crustaceans into three different categories: depressed (<1), spherical (1) and elongated (>1). We also used a combination of different acrosome measurements such as length, width, and length: width ratio. According to our measurements for biometrical data, the size of the acrosome vesicle in the representatives of Parastacidae (*Cherax*) are the smallest of studied crayfish species. The representatives

of Astacidae including *Astacus, Pacifastacus*, and *Austropotamobius* showed the largest acrosome vesicles. The acrosome size in species belonging to *Orconectes* and *Procambarus* as representatives of Cambaridae occupy an intermediate position among the above mentioned families of freshwater crayfish.

In the last study, we observed differences in post-mating spermatophore storage duration between a native (*Astacus astacus* – noble crayfish) and a non-native (*Pacifastacus leniusculus* – signal crayfish) crayfish. In crayfish, spermatozoa are packaged into spermatophores that function in the transfer of spermatozoa from male to the female during mating. As it is well known, crayfish spermatophores are deposited on the ventral surface in Astacidae and Parastacidae of the female or into the *annulus ventralis* in Cambaridae (Vogt, 2002). In the literature, post-mating spermatophore storage has been reported in many other species of decapod crustaceans including crabs, shrimps and lobsters (Bauer, 1986; Moyano et al., 2009). For example, female Dungeness crab, *Metacarcinus magister* can fertilize eggs using the sperm which was stored for 2.5 years (Jensen and Bentzen, 2012). Female blue crab *Callinectes sapidus* can store spermatophore for 7–11 months. They mate in summer but use spermatophore in following summer for fertilization. This post-mating spermatophore storage strategy is very important for the blue crab because it is female's single, lifetime mating opportunity (Millikin and Williams, 1984; Hines et al., 2003). Crayfish, e.g., spiny-cheek crayfish *Orconectes limosus*, can successfully store spermatophore from their autumn mating for more than half a year. It supposed to be this storage allows to high chance for multiple matings and to find the best mate in female *O. limosus* (Buřič et al., 2013). In our study, we observed duration of spermatozoa storage and further found which water temperature is preferred for mating and laying of eggs. Firstly, we observed that female noble crayfish stores post-mating spermatophores for longer periods compared to the signal crayfish. That is why we would like to compare native and non-native species to show differences in the case of the reproductive behaviour. Secondly, it was clear that signal crayfish prefer warmer waters for mating and laying of eggs in comparison with noble crayfish. It is well-known that higher temperatures accelerate biological processes (Cossins, 2012); therefore, shorter post-mating spermatophore storage duration in the female signal crayfish could be attributed to higher water temperature compared to the noble crayfish that prefer colder temperatures. Our results can be helpful for farmers to use as a guideline for the management of broodstock. Additionally, information about the reproductive behavior of males after mating was reported from our observation that male crayfish could be potential hunters for eggs of female crayfish. When farmers are aware of timeing of egg laying, they will know when to separate mated females from males so that time-mated females are allowed to incubate their fertilized eggs without any damage from males.

CONCLUSIONS

This thesis includes four publications and one manuscript describing some basic knowledge about reproduction biology of invasive crayfish. These can be used for understanding reproductive and physiological aspects of invasive crayfish. The following are our conclusions: In the literature, we realised that it is possible to find abnormalities in reproductive patterns of crayfish, either in the natural habitat or under the laboratory conditions. Also, there are many factors such as manipulation, hybridization or pollution that can cause changes in reproductive mode. For this reason, more studies are needed to understand exact triggering factor(s) of unusual reproduction strategies.

Future studies should address whether the intersexual signal crayfish is able to produce and fertilize eggs, and the possible mechanisms that trigger intersexuality in signal crayfish,

including whether environmental pollution may be responsible for crayfish hermaphroditism. Identifying external sex characteristics that identify intersexuality, along with the study of reproduction success, is necessary for a better understanding of intersexuality in Astacidea.

Despite conserved general pattern of the crayfish spermatozoa, combining morphological features such as apical zone, crest and spike in the anterior part of the acrosome, and biometrical data of the acrosome dimensions can provide a tool to distinguish different species of freshwater crayfish families.

Unfortunately, post-mating spermatophore storage duration has been poorly documented in decapods in general and crayfish in particular. Here we managed to demonstrate the patterns of spermatophore storage in relation with environmental factors such as temperature in broodstocks of two ecologically and economically important crayfish species. Also, this study provides basic information for biologists to further study of biology of reproduction in crayfish.

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ENGLISH SUMMARY

Some reproductive and physiological aspects of invasive crayfish

Invasive crayfish can cause negative effects outside of their native ranges. Reproduction is a key factor for spreading and establishing invasive species in new habitats. Several studies have been carried out to understand reproductive biology of invasive crayfish. Many researchers have described how invasive species establish their population in new habitats, interactions between native and invasive species, their rapid spreading, changing of behavior and biology, especially reproductive behavior and even genetics. However, there are still many questions which need to be addressed in case of invasive crayfish species. This thesis focused on different aspects of reproduction biology of invasive crayfish.

The main aim of the first study was to understand reproduction strategies of crayfish of different species. Generally, the normal crayfish reproduction mode is gonochorism, but, it has been shown in the literature that a few species such as *Cherax quadricarinatus, Samastacus spinifrons, Parastacus virilastacus* and *Pacifastacus leniusculus* may have different reproduction modes such as hermaphroditism or intersex. Parthenogenesis has been found in *Procambarus fallax* f. *virginalis*. Also, apomictic parthenogenesis has been reported in *Orconectes limosus*. Moreover, there have been several manipulations (e.g. eyestalk ablation or androgenic gland ablation) which directly affect reproduction biology of crayfish under lab conditions. Additionally, hybridization can be expected between Astacus astacus and *Astacus leptodactylus, Orconectes rusticus* and *Orconectes propinquus* under natural conditions. Some studies speculated that chemical factors also one of the reasons which could lead to some changes in reproductive system of crayfish.

The objective of the Chapter 2 was to report first evidence of intersex in the signal crayfish, *Pacifastacus leniusculus* (Dana, 1852). An intersexual specimen was randomly found within five dissected specimens from an established wild population. That specimen appeared morphologically male but with female genital openings, as well. Spermophores were obtained from vas defertia of this speciemen. The ovary was undeveloped. Histological study demonstrated that both spermatophores and oocytes were present. The gonadosomatic index (GSI = 3.79) was measured as a reproductive parameter. The results showed that intersex male had a GSI three times greater than normal males.

The aim of the third study was to prove possibility of distinguishing different species of the freshwater crayfish using combination of morphological features and biometrical data of their spermatozoa. The ultrastructure of spermatozoa in three cambarid species *Cambarus robustus, Orconectes propinquus, Orconectes rusticus* were described and compered with eight previously studied species from family of Astacidae, Cambaridae and Parastacidae. In the studied cambarids, the crest-like protrusions in the anterior part of the acrosome is one of the most remarkable differences in case of morphological feature in spermatozoa and can be used for distinguishing the members of Cambaridae. The results of biometrical data showed that the smallest and biggest acrosome sizes in the studied species were in Parastacidae and Astacidae, respectively. In Cambaridae, the acrosome size was in the middle position between the Astacidae and Parastacidae.

The duration of post-mating spermatophore storage as well as the timing and temperature of spawning in two crayfish species (*Pacifastacus leniusculus* and *Astacus astacus*) were reported in the Chapter 4. Seventy-one pairs of adult signal crayfish (*Pacifastacus leniusculus* Dana, 1852) and thirty-six pairs of noble crayfish (*Astacus astacus* Linnaeus, 1758) were used in the experiment. The pairs of crayfish were placed in plastic boxes which divided into four parts to separate each pair. The results indicated that there were significant differences

(P < 0.05) between noble crayfish and signal crayfish in average duration of the post-mating spermatophore storage. The duration of the post-mating spermatophore storage is longer in the noble crayfish with 34.6 ± 1.7 days (range 19–60) than the signal crayfish with 3.9 ± 0.5 days (1–18). Additionally, the longest proportions of the post-mating spermatophore storage duration in the signal crayfish (46.5%) and the noble crayfish (44.5%) were 1 and 31–40 days, respectively. There were also differences in the time of mating and egg laying between the signal crayfish and noble crayfish. In the population of signal crayfish both mating and egg laying overlapped, but in the population of noble crayfish there was at least a two-week gap between last mating and first egg laying individuals. Water temperature was significantly (P < 0.05) higher during mating and egg laying in the signal crayfish than the noble crayfish. The average temperatures for mating in both species were significantly (P < 0.05) higher than the temperatures that they utilized for egg laying. In conclusion, more research is needed to better understand the reproduction strategies of invasive crayfish.

CZECH SUMMARY

Některé reprodukční a fyziologické aspekty invasivních raků

Invazivní druhy raků mohou mít negativní vliv mimo jejich přirozený výskyt. Reprodukce je klíčovým faktorem k šíření a osídlování nových stanovišť invazivními druhy. Bylo provedeno několik studií s cílem porozumět reprodukční biologii invazivních druhů raků. Mnoho studií popisuje, jak invazivní druhy vytvářejí populace na nových stanovištích, interakce (vzájemné působení) mezi původními a nepůvodními druhy, rychlost jejich šíření, změny v chování a biologii, zejména reprodukční chování a dokonce genetiku. Zdá se však, že zde stále existuje mnoho otázek, které je v souvislosti s invazivními druhy třeba řešit. Tato práce je zaměřena na různé aspekty reprodukční biologie raků.

Hlavním cílem první studie bylo porozumění reprodukčním strategiím různých druhů raků. Obecně jsou raci gonochoristé, avšak literatura poukazuje na některé druhy, jako jsou *Cherax quadricarinatus, Samastacus spinifrons, Parastacus virilastacus* a *Pacifastacus leniusculus*, které mohou mít různé reprodukční módy, jako je hermafroditismus nebo intersex. U druhu *Procambarus fallax* f. *virginalis* byla dokázána partenogeneze. Apomiktická partenogeneze byla také prokázána u raka pruhovaného *Orconectes limosus*. Navíc došlo k několika chirurgickým manipulacím (např. ablace oční stopky nebo androgenní žlázy), které přímo ovlivňují reprodukční biologii raků v laboratorních podmínkách. Mimo to lze očekávat přirozenou hybridizaci mezi druhy *Astacus astacus* a A*stacus leptodactylus, Orconectes rusticus* a *Orconectes propinquus*. Některé studie předpokládají, že chemické faktory jsou jedním z důvodů, které by mohly vést k určitým změnám v reprodukčním procesu raků.

Cílem druhé kapitoly bylo popsat první evidenci o intersexu u raků signálních *Pacifastacus leniusculus* (Dana, 1852). Intersexní jedinec byl náhodně nalezen mezi pěti pitvanými jedinci ze stabilní populace z přírody. Tento jedinec vykazoval samčí morfologické znaky, ale se samičími vývody pohlavních cest. Z vas defertia tohoto jedince byly získány spermatofory. Struktura vaječníků nebyla vyvinutá. Histologické vyšetření odhalilo přítomnost jak spermatoforů, tak oocytů. Gonadosomatický index (GSI = 3,79) byl měřen jako reprodukční parametr. Výsledek ukázal, že intersexní samci měli GSI třikrát vyšší, oproti normálním samcům.

Cílem třetí kapitoly bylo dokázat možnosti rozlišení různých druhů sladkovodních raků použitím kombinace morfologických znaků a biometrických dat jejich spermatozoí. Ultrastruktura spermatozoí u třech druhů z čeledi Cambaridae, *Cambarus robustus, Orconectes propinquus, Orconectes rusticus* byla popsána a porovnána s osmi předešle studovanými druhy z čeledí Astacidae, Cambaridae a Parastacidae. Nejvýraznějším morfologickým rysem spermatozoidu u studovaných raků z čeledi Cambaridae jsou hřbetovité výčnělky v přední části akrozomu, které mohou být použity jako rozlišovací znak této čeledi. Výsledky biometrických dat ukázaly, že nejmenší velikosti akrozomů u studovaných druhů byly u čeledi Parastacidae, naopak největší v případě čeledi Astacidae. Velikost akrozomu u čeledi Cambaridae byla mezi Astacidae a Parastacidae.

V kapitole 4 byla zkoumána doba uchování spermatoforu po páření, dále načasování a teplota během páření u dvou druhů raků. V experimentu bylo použito 71 párů dospělých raků signálních (*Pacifastacus leniusculus* Dana, 1852) a 36 párů raků říčních (*Astacus astacus* Linnaeus, 1758). Páry raků byly umístěny v plastových krabicích rozdělených na 4 části k držení jednotlivých párů odděleně. Výsledky ukázaly signifikantní rozdíl ($P < 0,05$) mezi samicemi raka říčního a raka signálního v průměrném trvání uchování spermatoforu po spáření. Doba uchování spermatoforu po spáření je delší u raka říčního 34,6 ± 1,7 dne (v rozmezí 19–60 dní) než u raka signálního 3,9 ± 0,5 dne (1–18 dní). Také zde byly rozdíly v načasování páření a kladení vajíček mezi rakem říčním a rakem signálním. V populaci raka signálního se jak páření,

tak kladení vajíček časově překrývalo, ale v populaci raka říčního zde byla časová mezera nejméně dva týdny mezi posledním pářením a prvním kladením vajíček. Teplota vody byla signifikantně vyšší ($P < 0,05$) během páření a kladení vajíček u raka signálního než u raka říčního. Průměrné teploty k páření byly u obou druhů výrazně vyšší ($P < 0,05$) než teploty během kladení vajíček. Závěrem nutno zmínit, že je zapotřebí další výzkum pro lepší pochopení reprodukčních strategií invazivních druhů raků.

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LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Yazicioglu, B., Kouba, A., Kozák, P., Niksirat, H., 2017. Post-mating spermatophore storage strategies in two species of crayfish: Implications for broodstock management. Animal, 1-5. doi:10.1017/S1751731117001744. (IF 2016 = 1.921)

Yazicioglu, B., Hamr, P., Kozák, P., Kouba, A., Niksirat, H., 2016. Fine structure of the spermatozoon in three species of Cambaridae (Arthropoda: Crustacea: Decapoda) *Cambarus robustus, Orconectes propinquus* and *Orconectes rusticus*: A comparative biometrical study. PeerJ 4, e2363. (IF 2015 = 2.183)

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Yazicioglu B., Hamr, P., Kozák, P., Kouba, A., Niksirat, H., 2016. Fine structure of the spermatozoon in three species of Cambaridae (Arthropoda: Crustacea: Decapoda) *Cambarus robustus, Orconectes propinquus* and *Orconectes rusticus*: A comparative biometrical study. In: 21st Symposium of the International Association of Astacology, September 5-8, 2016, Madrid, Spain.

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Yazicioglu, B., Kozak, P. 2014. What do we know about reproduction of crayfish? In: FABA 2014: International Symposium on Fisheries and Aquatic Science. Book of Abstracts. September 25–27, 2014, Trabzon, Turkey. (poster presentation)

Kozák, P., Bláha, M., Kubec, J., **Yazicioglu, B.**, Kouba, A., 2013. Hybridisation experiment between *Astacus astacus* and *Astacus leptodactylus*. In: Regional European Crayfish Meeting. Book of Abstracts. September 26–29, 2013, Rovinj, Croatia, p. 21. (oral presentation)

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