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Chování raků jako nástroj pro výzkum invazních druhů a měnících se podmínek prostředí

Habilitační práce

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Prohlášení

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Ve Vodňanech, dne 31.3.2020 Ing. Miloš Buřič, Ph.D.

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1. Úvod

Raci (Decapoda: Astacidea) jsou zajímavou a rozmanitou skupinou organismů sladkovodních ekosystémů, ve které můžeme naleznout jak kriticky ohrožené druhy, tak celosvětově nebezpečné invazní druhy (Lodge et al. 2012). To je obzvláště patrné ze situace v Evropě, zejména díky informovanosti pramenící z čilé vědecké aktivity (Holdich et al. 2009). Tak nebo jinak, raci mají významné ekologické role, které jsou patrné jak u raků původních tak u nepůvodních. Raci, pokud se na lokalitě vyskytují, obvykle tvoři naprostou většinu biomasy bentických organismů a jsou potravou širokého spektra predátorů. Sami pak působí jako konzumenti všech dostupných zdrojů potravy (Neveu 2009; Reynolds 2011). Proto zprostředkovávají tok živin a energie ve vodním ekosystému se začleněním terestrických zdrojů do potravních řetězců, a to díky jejich schopnosti zpracovávat rostlinné zbytky terestrického původu, stejně jako schopnosti aktivně vyhledávat potravu mimo vodní prostředí (Correia & Anastacio 2008; Grey & Jackson 2012). Není proto překvapením, že jsou raci velmi často považováni za klíčové druhy vodních ekosystémů a zároveň ekosystémové „inženýry“ (Dorn & Wojdak 2004).

V posledních přibližně sto letech bylo ztraceno nevyčíslitelné množství populací původních evropských druhů raků a mnoho dalších bylo významným způsobem negativně poznamenáno (Holdich et al. 2009). Hlavním důvodem bylo a stále je šíření račího moru zavlečeného do Evropy s transportem prvních nepůvodních druhů a jeho následným šířením. Následovaly další nové introdukované druhy a na ně navázané další kmeny oomycety způsobující račí mor (*Aphanomyces astaci*, Schikora 1903) (Svoboda et al. 2017). Přeživší populace původních druhů raků byly pod tlakem zhoršujících se podmínek prostředí, jako regulace toků a znečištění, ale hlavně byly vystaveny přímým a nepřímým efektům přítomnosti nepůvodních druhů raků (Kozák et al. 2015). Kromě toho jsou působením nepůvodních druhů často ovlivněny celé ekosystémy (Lodge et al. 2000). Vzhledem k tomu, že nepůvodní raci jsou stále přenašeči račího moru a zároveň je takřka nemožné se s jejich populacemi vypořádat, jsou tyto změny nevratné. Vývoj posledních let ovlivněný klimatickými změnami a lidskou činností nadále nahrává adaptabilnějším a odolnějším nepůvodním druhům, které se lépe vyrovnávají s extrémy prostředí, narušením habitatů i znečištěním (Seebens et al. 2017). Tato situace vedla mj. i k vydání Nařízení evropského parlamentu a rady (EU) č. 1143/2014 o prevenci a regulaci zavlékání či vysazování a šíření invazních nepůvodních druhů spolu s periodicky aktualizovaným seznamem invazních druhů evropského významu. V tomto seznamu je uvedeno celkem pět invazních nepůvodních druhů raků, což ilustruje jejich význam i negativní dopady na společenstva.

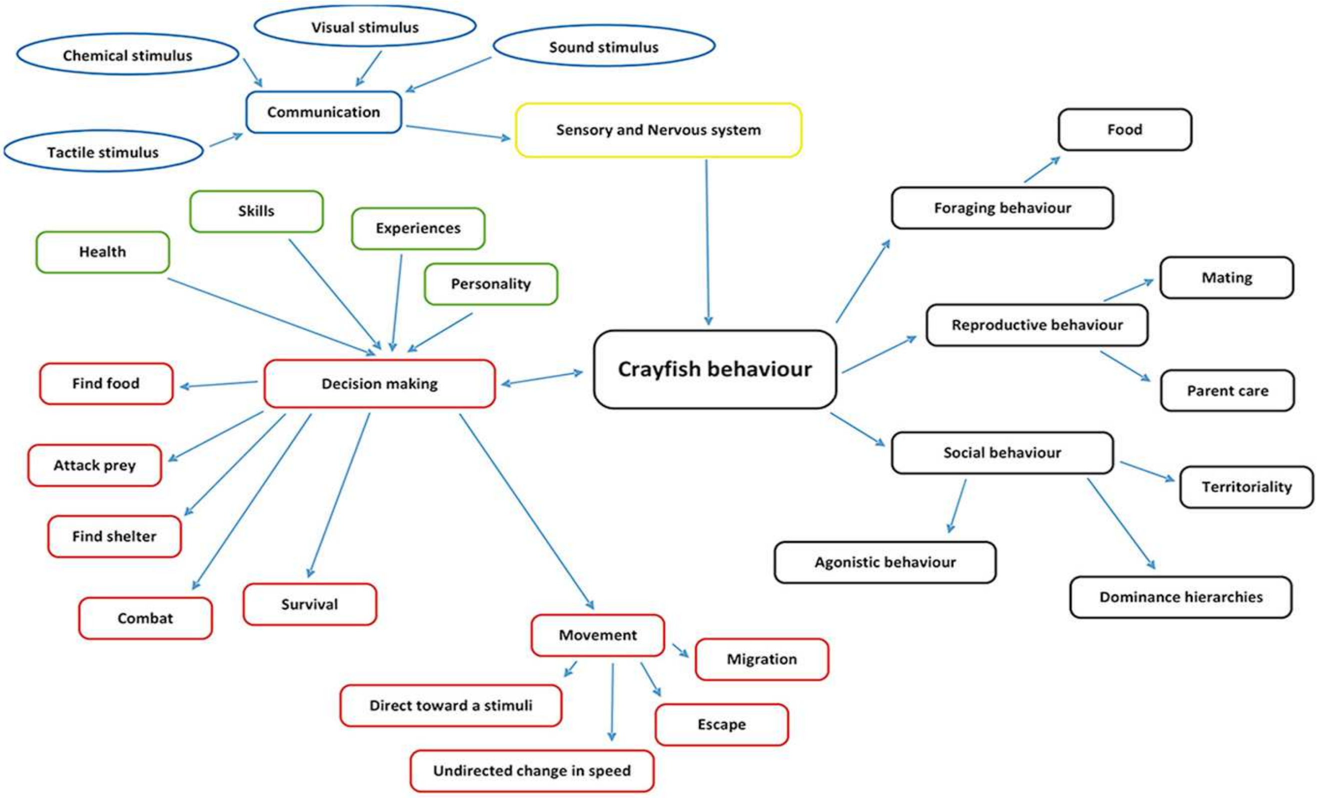
Vliv nepůvodních druhů raků byl v posledních letech intenzivně studován z mnoha směrů, z celkového kontextu však začal vyčnívat rak mramorovaný (*Procambarus virginalis* Lyko 2017) díky rychlosti jakou se objevil a rozšířil, stejně jako jeho schopnosti reprodukce pomocí obligátní apomiktické partenogeneze. Ta je unikátní mezi všemi destinožci a znamená, že v podstatě celá světová populace raka mramorovaného pravděpodobně představuje jeden klon (Gutekunst et al. 2018). Jak se zdá, kromě povahy reprodukce (a tím přítomností pouze samičího pohlaví) se tento druh svými základními životními projevy zásadně neliší od ostatních druhů. Rozdíly jsou spíše v kvantitativní rovině, tzn. do jaké míry či jak frekventovaně se vyskytují, což je obdobné při porovnání jakýchkoli jedinců nebo druhů. Díky snadnému odchovu rychlému dospívaní a genetické uniformitě se z raka mramorovaného stal velmi rychle žádaný modelový druh (Vogt 2008; Hossain et al. 2018 – **Příloha 2**).

Přesto je vhodné pro studie používat rovněž druhy raků rozmnožující se sexuálně a zároveň odlovené z přirozených podmínek, popř. jejich sledování v přirozeném prostředí pro širší vhled do problematiky. Přestože raci jsou jako skupina velmi různorodí, s různými nároky na prostředí, různým stupněm agrese či aktivity, zdá se, že základní principy jejich chování (zejména reprodukčního a sociálního společně s komunikací) jsou podobné napříč rody a čeleděmi (Breithaupt & Thiel 2010; Kubec et al. 2019 – **Příloha 1**). Studium vzájemných interakcí raků, či neprobádaných specifik jejich sociálního chování a komunikace stále představuje velkou výzvu pro výzkum. Ukazuje se také, že raci jsou velmi slibnými modelovými organismy použitelnými v ekotoxikologii, ale třeba i medicíně (Vogt et al. 2015).

1.1. Základní specifika chování raků

Povědomí o behaviorálních procesech a rozhodovacím procesu a o zákonitostech, které je ovlivňují, je nutné pro porozumnění základním biologickým vztahům sledovaných organismů – v tomto případě raků. Pro studium chování raků je nutné brát v potaz schopnosti komunikace raků, jejich smyslovou vybavenost, zákonitosti (známé) sociálního a reprodukčního chování, abychom byli schopni determinovat finální odezvu organismu s ohledem na jejich adaptabilitu v měnícím se prostředí (Breithaupt & Thiel 2010). Kromě těchto premis je nutné mít neustále otevřené oči, neboť některá sledování předchozích výzkumů jsou přehlížena, nebo dokonce nesprávně interpretována.

Z hlediska etologie je studium komunikace a percepce signálů z okolního prostředí nezbytné pro pochopení behaviorálních reakcí organismů, které jsou na těchto signálech (biologické, fyzikální a chemické faktory) z prostředí závislé (Slater et al. 1998). Komunikační signály pak hrají důležitou roli ve společenstvech organismů při tak zásadních procesech jako rozmnožování (rozpoznání pohlaví a dospělosti, výběr partnera) a vnitro- a mezi- druhová kompetice. Z tohoto důvodu se v živočišné říši vyvinulo mnoho způsobů jak sdílet a sdělovat informace o své identitě, statutu, náladě, pozici, fyziologickém stavu i zkušenostech (Bradbury & Vehrencamp, 1998). Organismy jsou i díky komunikačním a percepčním schopnostem vybaveny k rozhodování a cílovému chování ať už se jedná o preference partnerů při reprodukci, příjmu potravy, využití prostředí nebo třeba obrany proti nebezpečí (Obr. 1) - navíc jsou schopny využívat své předchozí zkušenosti při nových rozhodnutích tj. učit se (Atema 1988; Veselovský 2005), což se v omezené míře týká i raků (Bierbower et al., 2013; Kubec et al. 2019 – **Příloha 1**).



Obr. 1. Ilustrační schéma chování raků (Kubec et al. 2019 – **Příloha 1**).

Různé druhy raků vykazují specifické životní strategie a adaptace jak v dlouhodobém měřítku (adaptace na život v brakických vodách, Holdich et al. 1997) nebo v tom krátkodobém (např. tolerance nízkých teplot teplovodními druhy, Veselý et al. 2015). Některé druhy jsou invazní a způsobují citelné škody sladkovodním habitatům (Crandall & Buhay, 2008; Holdich et al., 2009), některé jsou schopny hloubit nory (Holdich 2002; Kouba et al. 2016 – **Příloha 4**) a některé obývají podzemní nebo semi-terestrické ekosystémy (Holdich 2002).

Raci vykazují největší aktivitu ve tmě (McMahon et al. 2005) a proto mají velmi dobře vyvinuté sensorické orgány umožňující orientaci v prostoru, hledání potravy a komunikaci v podmínkách nízké nebo nulové viditelnosti (Basil & Sandeman 2000;). Komunikace, na rozdíl od pouhého příjmu informací z prostředí, vyžaduje oboustrané porozumnění ze strany účastníků, ať už při reprodukci, inter-specifických nebo intra-specifických interakcích, nebo při varování před nebezpečím (Breithaupt & Thiel 2010; Kawai et al. 2015). Proces komunikace zahrnuje účastníky nebo skupiny účastníků, kteří vystupují jako odesilatelé a příjemci informace. Volba informačního kanálu (způsobu komunikace) závisí na podmínkách prostředí (vzduch, voda, kalná voda, překážky) a také na schopnostech odesilatele a senzorickém vybavení příjemce (Bradbury & Vehrencamp 1998; Alcock 2009). Mezi základní způsoby komunikace patří komunikace vizuální, akustická, taktilní a chemická, které mají své výhody i omezení (Tab. 1). Nejdůležitějším a nejefektivnějším informačním kanálem pro raky je jednoznačně chemická komunikace (Breithaupt & Thiel 2010; Kubec et al. 2019 – **Příloha 1**).

Tab. 1. Základní způsoby komunikace a jejich charakteristika. Upraveno dle Alcock (2009).

	Komunikace			
Vlastnosti	Vizuální	Akustická	Taktilní	Chemická
Vzdálenost	limitovaná	vysoká	krátká	vysoká
Přenos	rychlý	rychlý	rychlý	pomalý
Limitace překážkou	ano	ne	ano	ne
Lokalizace	přesná	různá	přesná	různá
Energetická náročnost	nízká	vysoká	nízká	nízká

Sociální chování

Sociální chování by se dalo definovat jako interakce mezi dvěma a více jedinci, typicky ve skupině jedinců téhož druhu (Krebs & Davies 2009; Manning & Dawkins 2012). Příkladem může být koordinace reprodukčního chování nebo mateřská péče, kdy samice raků aktivně pečují o snůšku a ráčata přichycená na pleopodech (Holdich 2002; Vogt, 2013). Dalším stupněm mateřské péče je ochrana již samostatně se pohybujících ráčat založená na chemické komunikaci mezi matkou a potomstvem, kdy ráčata aktivně matku vyhledávají v případě nebezpečí a matka jim umožňuje ukrýt se na jejích pleopodech (Mathews 2011, Kubec et al. 2019 – **Příloha 1**).

Sociální chování je ale u raků mnohem častěji projevováno jako agresivní konflikt na základě boje o zdroje (potrava, prostor, samice před pářením) či o pozici v hierarchii dané populace (Holdich 2002). Obvykle se jedná o zvýšenou agresitvitu mezi dvěma oponenty, která je nejprve vyjádřena chemickými a vizuálními signály k zastrašení soupeře neboť pro obě strany konfliktu je méně náročné a riskantní protivníka zastrašit než rovnou podstoupit souboj (Breithaupt & Thiel 2010). Kromě ztráty energie může souboj s podobně vybaveným protivníkem znamenat zranění až smrt a v neposlední řadě jsou v průběhu souboje jedinci více viditelní a ulovitelní predátory (Jakobsson et al. 1995). Při zastrašení jedince hraje hlavní roli velikost těla, velikost klepet, ale i kondice a zkušenosti protivníků nebo jejich stupeň vývoje (Holdich et al. 2002). Svou významnou roli ale má i chemická komunikace, kterou rak prezentuje svou předchozí pozici (vítěze či poraženého tj. dominantního či podřízeného jedince z předchozích soubojů). Sociální hierarchie, která se pomocí série podobných soubojů a zastrašení ustaluje, následně zabraňuje další zbytečným soubojům – což je jednoznačně prvek přispívající k životaschopnosti dané komunity (Manning & Dawkins 2012; Neumann et al. 2011). Obecně je hierarchie založena na dominanci od nejsilnějších po nejslabší jedince, ale zároveň je jasně založena na schopnosti raků rozeznat jedince (individua) a/nebo jejich postavení v hierarchii (Aquiloni et al. 2008 – **Příloha 3**; Fero & Moore 2014). S hierarchií je samozřejmě spojená teritorialita, kdy dominantní jedinci mají lepší přístup k potravě, úkrytům i reprodukčním partnerům (Fero & Moore 2014; Manning & Dawkins 2012). Ustanovená hierarchie znamená stabilitu dané sociální skupiny, naproti tomu změny v hierarchii (např. v období po svlékání raků) mohou vést ke zvýšedné agresivitě v populaci vedoucí k množství zranění a mortalitě včetně kanibalismu (Huber & Hock 2009).

Kromě vnitrodruhových interakcí je z hlediska dnešních „multikulturních“ populací původních a nepůvodních druhů nutné zmínit i interakce mezidruhové. Ty mohou vést v rámci tlaku na potravní zdroje, úkrytové možnosti a v měnících se podmínkách až k rychlému vytlačení jednoho druhu jiným, aktivnějším, agresivnějším popř. adaptabilnějším druhem. Častěji ale druhy (pokud neproběhne např. přenos letálních patogenů z jednoho druhu na druhý) koexistují, a to buď dlohodobě bez znaků dominance jednoho druhu, nebo je jeden druh postupně vytlačován (Holdich 2002, Gherardi et al. 2011).

Reprodukční chování

Reprodukční chování je významnou manifestací života každého zvířete (Krebs & Davies 1997; Alcock 2009) s výjimkou druhů s asexuálním způsobem rozmnožování. Těch obligátně asexuálních není mnoho, ale patří mezi ně i jeden druh raka, rak mramorovaný (Scholtz et al. 2003). Většina zvířat má rozlišitelná pohlaví a často výrazný pohlavní dimorfismus, který v podstatě zohledňuje požadavky a preference potenciálních partnerů. Výrazné důležité samčí znaky (ať už je to velikost, nebo u raků velikost klepet) tak zvyšují atraktivitu u samic a naopak (Manning & Dawkins, 2012). Finálním fázím reprodukčního chování obvykle předchází námluvy (předvedení síly, tělesných parametrů či oboustrané rituály), následně tvoření párů a mechanismy synchronizace a konsolidace vztahů mezi samcem a samicí (Bradbury & Vehrencamp 1998; Alcock 2001). U raků (a samozřejmě i mnoha jiných organismů) je zároveň reprodukce úzce spjata s teplotou, fotoperiodou a roční dobou (Holdich 2002).

Ve fázi námluv a formování párů hraje důležitou roli výběr partnera na základě preferencí jednotlivých organismů. Smysl výběru partnera tkví ve snaze jedinců zvýšit své šance úspěšné reprodukce a zároveň fitness svého potomstva výběrem toho nejlepšího dostupného partnera (Partridge 1980; Chenoweth & Blows, 2006). Mezi obvyklé znaky, které určují preference při pohlavní výběru, můžeme zařadit parametry vizuální (velikost, barva), chemické (feromony, hlavní histokompatibilní komplex) a behaviorální (rituály námluv, agonistické interakce, hierarchický status) (Bakker et al. 1999; Roberts & Gosling 2003). Logicky je správný partner obvykle stejného druhu (i když ne nutně) a ze stejné populace (Lande, 1981; Veselovský, 2005; Roberts and Gosling, 2003). U raků byla potvrzena preference pohlavního partnera ze stejné populace, přestože není jasné, jak byly samice schopny rozlišit mezi stejně velkými samci z různých populací (Kubec et al. 2019 – **Příloha 7**). Pokud věc shrneme, tak faktory jako předchozí sexuální historie (například preference ještě nespářených samic u raků, Aquiloni & Gherardi 2008a), dominance, velikost důležitých částí těla a fitness představují výhodu při hledání partnera (Jennions & Petrie, 1997).

V průběhu vyhledávání partnera a tvoření párů, ale často dochází k agonistickým interakcím (soubojům) samců o dostupnou samici(e) (Alcock 2009; Hunt et al. 2009). Přímé souboje samců o samice jsou obvyklé i u raků, kdy samice je schopná na základě předchozího souboje zvolit dominantního jedince, a to jak na základě vizuální, tak chemické informace (Aquiloni & Gherardi 2010; Aquiloni et al. 2008 – **Příloha 3**). Volba na základě soubojů ale u raků není vždy jednoznačná a stane se, že samice si vybere nebo zapudí samce nehledě na výsledek souboje. Na druhou stranu, pokud samice nemá možnost se ukrýt před samcem, se kterým se pářit nehodlá, samec je schopen spářit se se samicí pomocí síly (Mellan et al. 2014; Villanelli & Gherardi, 1998). Strategie samic a samců u raků je z hlediska páření obvykle odlišná. Samice se obvykle snaží vybrat partnera dle výše zmíněných preferencí, kdežto samci nejsou vybíraví a snaží se spářit s co největším množstvím samic (Kubec et al. 2019 – **Příloha 7**). U některých druhů byl sledován zajímavý mechanismus, kdy samec nejprve odstranil ze samice spermatofory předchozího samce a by je následně při páření nahradil těmi svými (Snedden 1990). Mechanismů jak zvýšit svůj podíl na potomstvu v populaci ale bude jistě více.

Mateřská péče

Mateřské chování raků bylo nastíněno již výše v podkapitole sociální chování, ale zároveň patří i do chování reprodukčního, neboť významně přispívá k úspěšnému rozmnožování této skupiny živočichů. U raků obecně je mateřská péče důležitá jak při inkubaci vajíček, tak i v průběhu raného postembrynálního vývoje (Holdich 2002; Vogt 2013), kdy samice ochraňuje snůšku před nežádoucími vlivy (prokysličování snůšky pohybem pleopodů, čištění snůšky od odumřelých vajíček), predací či kanibalismem (Mathews 2011). Péče o již vylíhlá račata se druhově specificky liší a pohybuje se od přibližně 2 týdnů u raka říčního (*Astacus astacus* Linnaeus 1758) (Holdich 2002) po až 5 měsíců u raka zélandského (*Paranephrops zealandicus* White 1847) (Whitmore & Huryn, 1999). V průběhu mateřské péče používají samice chemickou komunikaci pro ochranu račat před stresem (Vogt & Tolley 2004; Kawai et al. 2015). Uvádění je zejména tzv. „brood pheromone“, který račata láká zpět k matce (Little 1975). Tento feromon, pravděpodobně druhově specifický, stále nebyl chemicky izolován, ale jeho existence byla popsána na základě behaviorálních vztahů mezi matkou a potomstvem (Little 1976; Mathews 2011). Nicméně Vogt (2013) popisuje návrat račat k matce pouze na základě odezvy na vizuální signály matky. U samic raka červeného (*Procambarus clarkii* Girard 1852) a u norujícího raka brvoklepetého (*Parastacus* *pilimanus* von Martens 1869) bylo dokonce sledováno ochranné mateřské chování i k cizímu potomstvu bez znaků agresivního chování (Aquiloni & Gherardi 2008b; Santos et al. 2012). Mathews (2011) rovněž popsala samice raka pruhovaného (*Faxonius limosus* Rafinesque 1817) nosící ráčata jiných samic. Otázkou zůstává, jestli je toto chování zapříčiněné jistým způsobem altruismu, nebo samice ve skutečnosti nejsou schopny (oproti publikovaným výsledkům) rozlišit své potomstvo od od potomků jiné matky. Jedna z mála studií paternity u raků z volné přírody, ale uvádí, že samice nosí pouze své potomstvo (Walker et al. 2002). Ochrana vlastního potomstva rozhodně zůstává klíčovou, neboť jakmile ráčata dosáhnou samostatnosti, samice spíše požírají cizí než své vlastní potomstvo (Mathews 2011; Kawai et al. 2015).

Aktivita a migrace

Na závěr nelze opominout základní projev živých organismů tj. pohyb. Pohyb je výrazem aktivity za určitým účelem – vyhledávání potravy, partnera, úkrytu, opuštění místa s nevhodnými či stresujícími životními podmínkami apod. Z hlediska aktivity můžeme jednoduše shrnout, že raci jsou aktivní ve všech zmíněných činnostech zejména v noci s vrcholem při stmívání a rozednění (Holdich 2002). Některé druhy jsou více aktivní přes den (druhy v Evropě nepůvodní), což jim umožňuje lépe využít potravní zdroje, ale zároveň je více vystavuje riziku predace (Kubec et al. 2019 – **Příloha 1**). Pohyb, ať už na kratší nebo delší vzdálenost, umožňuje změnit habitat, ale vždy závisí na druhově i individuálně specifických vazbách mezi benefity takového přesunu a riziky a náklady vynaloženými na přesun (Cote et al. 2010). Proto jsou popisovány velké rozdíly v pohyblivosti jedinců v populaci, což může reflektovat i pozici jedince v populaci (Jonsson & Jonsson 1993; Kubec et al. 2019 – **Příloha 1**). Pohyblivost se často liší i v průběhu roku, kdy, konkrétně u raků, dochází v období páření ke značnému zvýšení migrační aktivity u samců. Denní uražená vzdálenost pak může převyšovat stovky metrů (Buřič et al. 2009a; Gherardi et al. 2000). Po skončení páření raci svou aktivitu snižují a využívají spíše krátké přesuny s využitím vlastního domovského okrsku – systému úkrytů na daném místě, které periodicky obývají a vrací se k nim i po předchozím přesunu na jiné místo (Buřič et al. 2009b).

Obecně jsou v prostředí s vysokou hustotou populace jedinci i celá populace vystaveni volbě, zda v daných podmínkách zůstat a omezit svůj růst a reprodukci, nebo se přesunovat za lepšími podmínkami a šířit se tak do nových oblastí (Einum et al. 2006). To sebou samozřejmě nese risk větších energetických nákladů a predace, nicméně je tato strategie v rámci raků (ale i ostatních organismů) častá, a v případě nepůvodních druhů dokonce velmi dobře viditelná (Gherardi 2007).

1.2. Chování raků pro studium interakcí a vlivu kontaminace prostředí

Studium interakcí raků

Výše popsaná základní specifika chování raků jsou aplikovatelná v širší míře z hlediska studia vlivu prostředí (např. různá komplexita prostředí, fyzikální a chemické parametry vody, světelné podmínky, různé typy znečistění, různé proudění vody apod.) na změny charakteristického chování nebo k porovnání chování jednotlivých druhů raků a jejich vzájemných interakcí. Obvykle se v minulosti jednalo o interakce nepůvodních druhů raků s těmi původními (např. Pöckl & Pekny 2002; Pintor & Sih 2009), ale s jejich rostoucím významem a rozšířením, jde čím dál tím více o interakce s ostatními nepůvodními raky (např. Chucholl et al. 2008; Hudina et al. 2011) nebo s jinými vodními organismy (např. Hirsch & Fischer 2008; Rosewarne et al. 2016).

Velmi často se u sledovaných jedinců zaznamenává jejich aktivita (obvykla uváděná jako doba jejich aktivního pohybu mimo úkryt, nebo v % na daný časový úsek), doba pohytu mimi úkryt/v úkrytu, preference prostředí, potravní aktivita a preference, celková uražená vzdálenost, trajektorie pohybu, rychlost nebo čas nutný k dosažení cíle (např. potrava) (Holdich 2002; Kubec et al. 2019 – **Příloha 1**). Je velký rozdíl zdali při podobných sledování raky umístíme do známého, či neznámého prostředí, do prostředí optimálního (kvalita vody, dostatek potravy, úkrytů) nebo stresujícího (např. chybějcí úkryt, intenzivní osvětlení, přítomnost predátora apod.). Kombinace faktorů pak situaci mohou přímo komplikovat vzhledem k vysoké míře individuality u raků a to i u klonálního raka mramorovaného (Gherardi et al. 2012; Hossain et al. 2018 – **Příloha 2**). Změna nastavení obvykle výrazně mění chování raků, kteří se v daných podmínkách snaží najít optimální rozhodnutí – primárně ukrýt se, pokusit se o útěk, prozkoumat nové prostředí a až následně, pokud to podmínky dovolují, aklimatizovat a zklidnit se (Gherardi et al. 2012; Kubec et al. 2019 – **Příloha 1**).

Pokud nesledujeme individua, ale skupiny o dvou a více jedincích, je nutné se věnovat jejich interakcím a následkům, které z nich vyplývají. Obecně můžeme posuzovat jednotlivé stupně vzájemného chování, jak je uvedeno na příkladech v Tab. 2 a Tab. 3. Význam a průběh agresivních interakcí je uveden výše v podkapitole o sociálním chování. Kromě těchto parametrů je možné sledovat jedince až do ustanovení hierarchie ve skupině a jejich reakce na další podněty, které mohou kompletně převrátit předtím ustanovená pravidla (Graham & Herberholz 2009; Gherardi et al. 2012).

V rámci intra-specifických skupin je stále zajímavou oblastí výzkumu reprodukční chování (výběr partnera) a význam a hlavně podstata chemické komunikace tj. pochopení jakou informaci vlastně raci poskytují, jak je srozumitelná, pro koho, jak je odpovědná za finální chování testovaných zvířat a hlavně následně chemická izolace feromonů odpovědných za přenos těchto informací (např. Breithaupt & Thiel 2010). U inter-specifických skupin se většinou jedná o porovnání druhů z hlediska jejich možné koexistence v přírodních podmínkách a predikce jejich působení na již etablované druhy (např. Fořt et al. 2019).

Tab. 2. Definice parametrů agonistického chování raků použité v publikaci Hossain et al. (2019a – **Příloha 6**) - modifikováno dle Bruski & Dunham (1987), Pavey & Fielder (1996), Sato & Nagayama (2012), Cioni & Gherardi (2004) a Aquiloni et al. (2008 – **Příloha 3**).

Termín	Definice
Kontakt	Dotek klepety, tělem (hlavohruď, abdomen) spíše než anténami a antenulami.
Stranění se (pomalý únik)	Únik jednoho jedince od druhého (stranění se z cesty) bez zjevných projevů hrozby, nebo po hrozbě.
Hrozba	Postoj hrozby, varování – hlavně postoj se zdviženými klepety, pohyb klepety do stran i nahoru a dolů.
Vítěz	Jedinec, který neutekl ze souboje, nebo zanechal oponenta se znatelně podřízeným postojem (nebo bez pohybu). Pokud ze souboje odstoupí oba oponenti zároveň, není určen vítěz.
Souboj	Raci se dotýkaji klepety, chytají se klepety za protivníka či jej drží, nebo se jej snaží klepety udeřit.
Iniciátor souboje	Rak, který první aktivně vstoupí do souboje.
Tail flip	Rychlé smrštění abdominálního svalstva, které vede k rychlému pohybu (plavání) směrem dozadu.

Tab. 3. Etogram agonistického chování raků modifikovaný z Wofford & Moore (2017) a Steele et al. (2018) a použitý v práci Hossain et al. (2020 – **Příloha 10**). Numerická intenzita reprezentuje intenzitu sledovaného chování.

Sledovaná událost	Stupeň intenzity	Popis chování
Tailflip	-2	Rychlé smrštění abdominálního svalstva, které vede k rychlému pohybu (ústupu) směrem dozadu.
Pomalý ústup	-1	Nízká intenzita, pomalý ústup směrem od oponenta.
Pomalý přístoupení	1	Nízká intenzita, pomalé přibližování k oponentovi bez znaků hrozby.
Rychlé přistoupení	2	Rychlé a intenzivní přistoupení k oponentovi s pozdviženými klepety na znamení hrozby.
Dotek zavřenými klepety	3	Nepříliš silný kontakt pomocí zavřených klepet. Klepeta stále nejsou užita k tlačení, úderům či uchopení oponenta.
Švihání anténami	4	Rychlé šlehání oponenta anténami.
Pevná klepeta	5	Udržování oponenta v odstupu držením zavřených klepet natažených přímo před sebe.
Úder zavřeným klepetem	6	Silné tlačení až úder zavřeným klepetem bez zachycení či držení oponenta.
Dotek otevřeným klepetem	7	Nepříliš silný kontakt otevřeným klepetem. Bez uchycení či držení oponenta.
Úder otevřeným klepetem	8	Silné údery otevřeným klepetem, bez držení oponenta.
Uchopení za končetiny	9	Silné chycení oponenta klepetem za končetiny nebo klepeta. Není užita síla k tahání či trhání končetin.
Trhání končetin	10	Klepeta použita k uchycení a držení končetin oponenta s pohybem a silou vyvinutou k tahání a trhání končetin.
Přetočení	11	Přetočení oponenta a použití klepet k tahání a trhání přetočeného těla oponenta.

Studium vlivu kontaminantů na chování raků

Kontaminace vodního prostředí farmakologicky aktivními látkami je stále více vnímána jako hlavní hrozba pro vodní ekosystémy, a to celosvětově. Tyto látky, používané v podobných kvantitách jako pesticidy a jiné organické mikropolutanty, přímo i nepřímo ovlivňují chování sladkovodních organismů (Boxall et al. 2012; Buřič et al., 2018 – **Příloha 8**).

Psychoaktivní farmaka jsou vyvinuta tak, aby působila na signální dráhy v mozku, a proto je možné očekávat jejich vliv na chování exponovaných necílových organismů ve vodním prostředí (Fong & Ford 2014). Některá antidepresiva se dokonce akumulují v nervových tkáních ryb a komunitě bentosu, což zvyšuje pravděpodobnost viditelných efektů těchto látek na zmíněné organismy (Grabicova et al. 2015; Grabicova et al. 2017). Stejně jako ryby a larvy vodního hmyzu i raci vykazují behaviorální změny indukované cizími látkami ve vodním prostředí, včetně mikropolutantů (Lahman et al. 2015; Hossain et al. 2019b – **Příloha 9**). Exponované organismy často vykazují změny v sociálních interakcích, reprodukci, reakcích na predátory, pohyblivosti apod. (Brodin et al. 2013; Valenti et al. 2012). Efekt těchto mikropolutantů tedy není letální, ale může nepřímo narušit celé populace a fungování ekosystému narušením vztahů predátor/kořist či narušením reprodukce organismů. Kupříkladu zvýšená aktivita, agresivita a potravní chování mohou významně ovlivovat skladbu a dostupnost potravních zdrojů a ovlivňovat celý potravní řetězec. Na druhou stranu snížená aktivita, pomalejší reakce a snížený příjem potravy může vést ke snížené schopnosti reprodukce a k větší náchylnosti k predaci (Bláha et al. 2019; Hossain et al. 2019b – **Příloha 9**).

Evaluace vlivů mikropolutantů ve vodním prostředí vyžaduje jednoduchou ověřenou metodu (umožňující její opakování v precizně definovaných podmínkách) a zároveň vnímavý vhodný modelový druh. Z tohoto pohledu jsou raci a sledování jejich chování v kontrolovaných podmínkách pravděpodobně vhodnou volbou (Buřič et al. 2018 – **Příloha 8**; Hossain et al. 2019 – **Příloha 9**). Raci jsou vhodným adeptem i z hlediska jejich klíčové role v ekosystémech, kdy vliv na populace původních druhů může vést k nestabilitě ekosystému (Creed & Reed 2004; Usio & Townsend 2004).

1.3. Cíle práce

K cílům práce patřilo:

1/ zhodnotit základní aspekty komunikace, chování a rozhodovacího procesu raků a zároveň sumarizovat odborné informace o partenogenetickém raku mramorovaném, přičemž obě tyto části měly být základnou pro využí chování raků a zejména raka mramorovaného pro výzkum základních biologických vlastností raků, jejich interakcí a změn chování vyvolaných vnějšími faktory,

2/ výzkum základních biologických vlastností raků spojených s jejich chováním včetně jejich vnitrodruhových a mezidruhových interakcí a jejich behaviorálních adaptací na měnící se podmínky prostředí (mezi sebou i s prostředím).

3/ determinovat potenciální změny chování vyvolané environmentálně relevantními koncentracemi mikropolutantů – konkrétně vybraných psychoaktivních látek.

Výsledky práce a jejich diskuze jsou stručně shrnuty v následujících kapitolách. Podrobnosti o jednotlivých studiích jsou patrné z přiložených publikovaných článků, které tvoří přílohy této habilitační práce.

2. Výsledky a diskuze

2.1. Použití raků a jejich chování pro vědecké účely

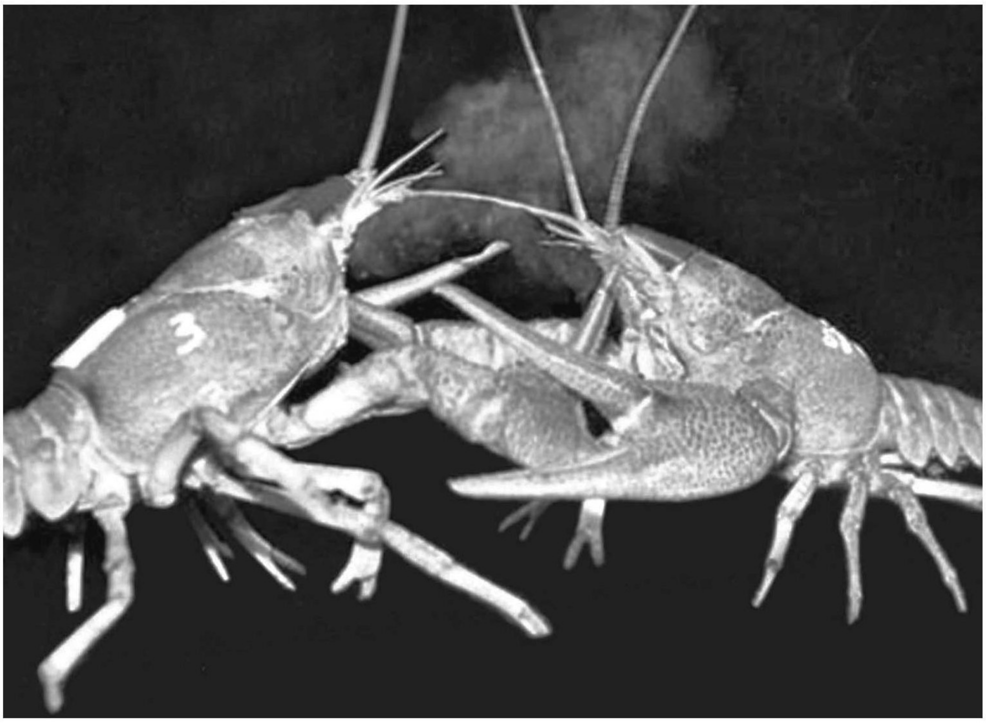
2.1.1. Základy chování a komunikace raků

Pro pochopení chování a komunikaci raků je nutné nejprve sumarizovat známé literární zdroje. Pro jejich vhodnou interpretaci a použití pro budoucí rozvoj této vědecké oblasti je pak třeba i širší znalost problematiky, tzn. parametry chování obecně známé a platné známé i pro ostatní živočichy, spolu s vlastními zkušenostmi a pozorováními. Tento přehledový článek (Kubec et al. 2019 – **Příloha 1**) si kladl za cíl shrnout nejdůležitější známá fakta o chování, komunikaci a rozhodovacím procesu raků, tak aby jej bylo možné použít i jako podklad pro budoucí studie a zároveň edukační materiál pro nové studenty této problematiky.

Konkrétně přehledový článek poskytuje souhrn sociálních interakcí a rozhodovacích procesů, se zahrnutím možných způsobů rozlišení a zpracování environmentálních stimulů (fyzikální, chemické i ty biologické) s primárním zaměřením na sladkovodní raky. Byly popsány vztahy prostředí a organismu samotného a základní biologie zpracování signálů. Tyto aspekty jsou důležité, jelikož každé rozhodnutí a interakce v důsledku vedou k přežití a reprodukci tj. k určité míře úspěšnosti jedince a druhu.

Pro etologické studie reprezentují raci, se svou rolí klíčového druhu, vhodný modelový organismus mezi sladkovodními organismy. Raci mají výhody oproti ostatním bezobratlým, nejen díky své velikosti, a snadnosti udržování v laboratorních chovech, ale zároveň díky vysokému stupni sociálních interakcí, a to nejen v přírodních podmínkách, ale i v laboratoři (Kubec et al. – **Příloha 1**). Jejich schopnost chemické komunikace a poměrně široké spektrum informací, které jsou takto schopni předávat a přijímat (Breithaupt & Thiel 2011), z nich činí cenný model. Raci zároveň vykazují unikátní eko-etologické charakteristiky, jako široká variabilita potravního chování, cykly aktivity, výběr a použití habitatu, nebo využití zdrojů s ohledem na specifika druhová, pohlavní a vývojová (Gherardi et al. 2012). Jejich schopnost rozpoznat nejen jedince stejného druhu, ale i jednotlivce (na základě jejich zkušeností s nimi) nebo jejich vlastní potomstvo je rovněž unikátní (Aquiloni et al. 2008 – **Příloha 3**; Breithaupt & Thiel 2011; Kubec et al. 2019 – **Příloha 7**). Chemické stimuli a zejména chemická komunikace výrazně ovlivňuje chování před a v průběhu páření, rozpoznání statutu daného jedinec (dominance, zralost, pohlaví, příslušnost k populaci), rozpoznání potomstva apod., a tak stále představuje příležitost a zároveň výzvu pro další výzkum. Velikost raků a množství vypouštěné močoviny s feromony (Obr. 2) rovněž nabízí možnost v budoucnu tyto látky identifikovat chemicky. To je výzva, která by mohla skutečně osvětlit podstatu fungování přenosu informace.

V neposlední řadě je chování raků vědecky široce využitelné pro evaluacei efektu různých environmentálních podmínek od vlivu klimatických změn, přes reakce na biologické invaze až po znečištění prostředí nejrůznějšími kontaminanty (viz. následující kapitoly). V teoretické i praktické rovině studium chování vodních organismů a konkrétně raků představuje otevřenou širokou vědeckou oblast, jak v přirozených, tak i v laboratorních podmínkách. Detaily naleznete v připojené **Příloze č. 1**.



Obr. 2. Chemická komunikace dvou samců vizualizovaná s pomocí fluorescenčního barviva (fluorescein) aplikovaného injekčně do hemolymfy raků a v průběhu interakce vypouštěná společně s močovinou a feromony (s laskavým svolením autora fotografie použito z Breithaupt & Thiel 2011).

2.1.2. Použití raka mramorovaného jako biologického modelu

Termín modelový organismus má ve výzkumu význam jednoduchého, dostupného a jednoduše manipulovatelného druhu použitelného jako zástupný vzorek pro studium rozličných biologických procesů. Modelové organismy obecně rychle rostou a dospívají, je relativně jednoduché a levné s nimi pracovat a jsou široce použitelné v různých typech experimentální práce. Modelové organismy jako octomilka, háďátko obecné, danio pruhované nebo hlodavci se staly nenahraditelnými součástmi biologického a klinického výzkumu a pomohly vědcům k dosažení obrovského množství vědomostí (Hunter 2008).

Obecně obratlovci mají v rámci laboratorního využití výhodu v bližší biologické příbuznosti k člověku, ale bezobratlí mají výhodu ve snažším odchovu, nižších nákladech a nižších omezeních daných ochranou druhů a nižších požadavků na jejich welfare (Vogt 2010). Mezi bezobratlými jsou destinozí korýši používáni v mnoha oblastech laboratorního výzkumu a mnoha biologických oblastech a jejich využití naznačuje, že by se mohli brzy stát důležitými modelovými organismy (Kawai et al. 2015). Tento přehledový článek měl hlavní účel v sumarizaci známých poznatků o výskytu, biologii, odchovu, výhodách i nevýhodách raka mramorovaného jako modelového organismu – článek je připojen jako **Příloha č. 2**.

Rak mramorovaný jednoznačně splňuje základní požadavky pro laboratorní zvířata obecně. Jednoduchý odchov, vhodná velikost, individualita jedinců, vysoká tolerance ke stresu z manipulace, vysoká plodnost, krátká generační doba a adaptabilita širokému spektru environmentálních i nutričních podmínek – což jsou mimochodem i důvody proč je rak mramorovaný nebezpečným invazním druhem (Kawai 2016). Rak ramorovaný si rozvněž zachoval široký behaviorální repertoár a vysokou schopnost regenerace (Vogt et al. 2008). Navíc má rak mramorovaný výhodu, že matky i jejich potomstvo jsou geneticky uniformní klony. To je predispozice pro výzkum epigenetických aspektů fenotypů (Martin et al. 2007).

Rak mramorovaný se zdá být i dobrým kandidátem na půl cesty mezi menšími krátkověkými a většími dlouhověkými modelovými organismy, neboť je dostatečně malý na to, aby ho bylo možné odchovávat v ohromných množstvích a dostatečně velký na umožnění fyziologických, biochemických či behaviorálních analýz. Je možné jej používat i pro dlouhodobé studie s použitím odběrů hemolymfy a biopsií, nebo s použitím analýzy exuvií po sobě jdoucích vývojových fází jakožto excelentního archivu morfologického vývoje nebo vývoje externích senzorických orgánů (Vogt 2010). Délka života raka mramorovaného rovněž umožňuje detailní sledování procesů regenerace chybějících končetin, efekt chronických dávek toxikantů na fitness a zdraví a vývoj jedinců nebo na změny epigenetického kódu s věkem (Vogt 2010; Hossain et al. 2018 – **Příloha 2**).

V posledním destiletí došlo k nárůstu použití raka mramorovaného pro nejrůznější typy studií v různých oborech jako např. vývojová biologie (např. Jirikowski et al. 2010), neurobiologie (např.Vilpoux et al. 2006), epigenetika (např. Schiewek et al. 2007), výzkum kmenových buněk (např. Vogt 2010), chování (např. Hossain et al. 2019a – **Příloha 6**), biogerontologie (Vogt 2010), biochronologie (Farca Luna et al. 2010), toxicologie (např. Buřič et al. 2018 – **Příloha 8**), ekologie (např. Jones et al. 2009) nebo evoluční biologie (např. Sintoni et al. 2007). Vzhledem k tomu, že v nedávné době došlo k přečtení celého genomu raka mramorovaného (Gutekunst et al. 2019), dá se předpokládat, že se jeho využití jakožto modelového organismu ještě více rozvine. Otázkou je potom ochrana výzkumných pracovišť proti úniku raka mramorovaného do volného prostředí, které vzhledem k jeho invaznímu poteciálu musí být důkladně řešeno.

Obr. 3. Rak mramorovaný (*Procambarus virginalis*) s ráčaty připevněnými na ventrální straně abdomenu (foto: Martin Bláha).

2.2. Behaviorální adaptace a interakce raků

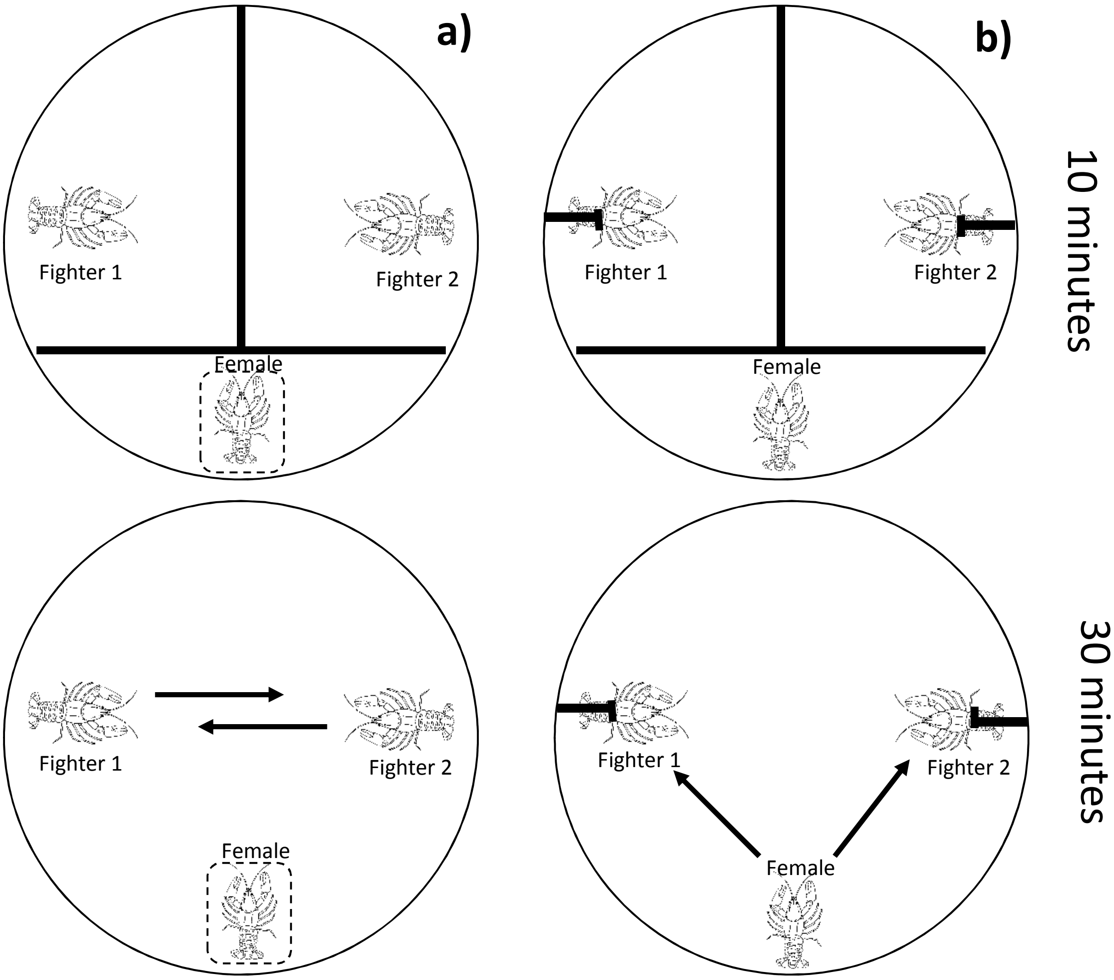
2.2.1. Výběr partnera na základě sledování předchozího souboje

V této studii (celá publikace připojena jako **Příloha č. 3**) je věnována pozornost skutečnosti, že samice v období páření často přivábí více samců najednou. Následně zpravidla docházi k souboji, kdy dominantní samec obvykle zvítězí a posléze se spáří se samicí. Pakliže samci podstupují několik soubojů v průběhu období páření, bylo dle naší hypotézy očekávatelné, že samice jsou schopny zpracovat informaci ze souboje samců pro vyhodnocení jejich vhodnosti pro budoucí páření.

Cílem studie tedy bylo zjistit roli sociálního naslouchání (pozorování) u desetinožců. Jako modelo vý druh byl použit rak červený. Studie byla rozdělena na dva experimenty. První byl zaměřen na to, zda se schopnost samice rozpoznat dominantního samce zvýší, pokud pozoruje samce při souboji. Ve druhém experimentu byla samice konfrontována s jinými samci, než které pozorovala při souboji. To proto, abychom pochopily, zda přihlížení umožní samicím rozpoznat dominantní status jako takový nebo zda se jedná pouze o rozpoznání daného jedince.

Prvním výsledkem studie bylo, že samice raka červeného si při výběru volily dominantního samce, čímž potvrdily naši předchozí hypotézu. Předchozí studie (Aquiloni & Gherardi 2008a) zjistila, že samice nebyly schopny jednoznačně vybrat mezi stejně velkými samci toho dominantního, což vedlo k domněnce, že velikost je jediným důležitým faktorem pro výběr samce. Oproti tomu naše výsledky, že pokud samice je schopna rozlišit dominantního od podřízeného samce (na základě pozorování souboje), pak si ho i následně vybírá.

K tomu jí jednoznačně nejvíce pomáhá přímé sledování soubojů. Experiment 1 konkrétně ukazuje, že účastné samice, na rozdíl od těch naivních, prvně navštěvují dominantního samce (i v případě, že je ten podřízený stejné velikosti), zůstávají v jejich blízkosti delší dobu a jsou s nimi častěji v přímém kontaktu. Samice může extrahovat informaci z komunikační sítě mezi dvěma bojujícími samci v průběhu jejich souboje bez toho, aby se souboje sama účastnila. Takto samice přímo porovnávají mezi samci, a to s minimálními náklady a se získáním přesné informace o relativní a absolutní kvalitě samců (např. hierarchický status). Tyto informace posléze používají pro následný výběr vhodného partnera (McGregor 2005; Otter et al. 1999). U korýšů byla role sledování soubojů v následném rozpoznání dominantního statutu samce při sexuální selekci potvrzena poprvé v této práci.



Obr. 4. Schematický nákres experimentálních arén pro a) pozorování bojujících samců samicí a pro b) selekci samců samicí. Naznačen je časový úsek pro aklimatizaci v experimentální aréně (10 min.) a dobu sledování souboje/selekce (30 min.).

Samice, které neměly možnost vidět předchozí interakce samců, nevykazují preference pro vítěze či poraženého, z toho vyplývá, že dominantní samec nenažnačoval nic, co by ukazovalo na jeho dominanci (behaviorálně či chemicky) a vedlo tak k detekci a preferenci samicí. Nicméně literatura naznačuje, že existují jiné charakteristiky než velikost umožňující samicím desetinožců rozpoznání hierarchického postavení samce. Například u humra amerického (*Homarus americanus*

Milne-Edwards 1837) byly výsledky soubojů stejně velkých samců odhadnutelné na základě úrovně proteinů v plasmě a koncentrace vápníku v exoskeletonu (Vye et al. 1997). Tyto charakteristiky samozřejmě nejsou rivalům viditelné, ale mohou být nepřímo odhadnuty sílou stisku klepet při interakcích, odolností exoskeletonu tlaku protivníka nebo obecně celkovou silou v souboji (Vye et al. 1997). Je možné, že v přírodních podmínkách jsou samice schopny posoudit tyto charakteristiky při vzájemných interakcích se samcem. Je známo, že interakce samců a samic raků před pářením jsou z počátku podobné jako agresivní interakce a probíhají před tím, než samice akceptuje páření s daným samcem (Ameyaw-Akumfi 1976; Kubec et al. 2019 – **Příloha 1**). Proto nemůžeme vyloužit zároveň roli těchto prekopulačních interakcí v posouzení kvality samce a finální volbě.

Ačkoli je potvrzeno, že raci rozpoznají sociální status pomocí chemických signálů (Zulandt-Schneider & Moore 2000), naše výsledky napovídají, že chemické informace vylučované v průběhu souboje nejsou pro samici jednoznačně dostatečné pro selekci dominantního samce a musí být podpořeny výsledkem v souboji.

Je známo, že chemická komunikace může přenést informaci o dominanci či reprodukčním stavu jedince a dokonce i o jeho velikosti (Breithaupt & Atema 2000; Simon & Moore 2007; Aquiloni & Gherardi 2008a). Novější výzkum pak dokládá i schopnost raků rozpoznat jedince ze stejné populace přes jejich dlouhodobou separaci (Kubec et al. 2019 – **Příloha 7**). Přesto tato práce ukazuje, že pachový stimul sám o sobě pravděpodobně není schopen zprostředkovat informaci of hierarchické postavení samce naivní samici. Možným vysvětlením může být i malé množství vypouštěné močoviny, která byla použita už v předchozích interakcích. Je možné, že koncentrace metabolitů jako serotoninu a/nebo octopaminu se v hemolymfě mění v závislosti na typu sociální interakce (Moore & Bergman 2005).

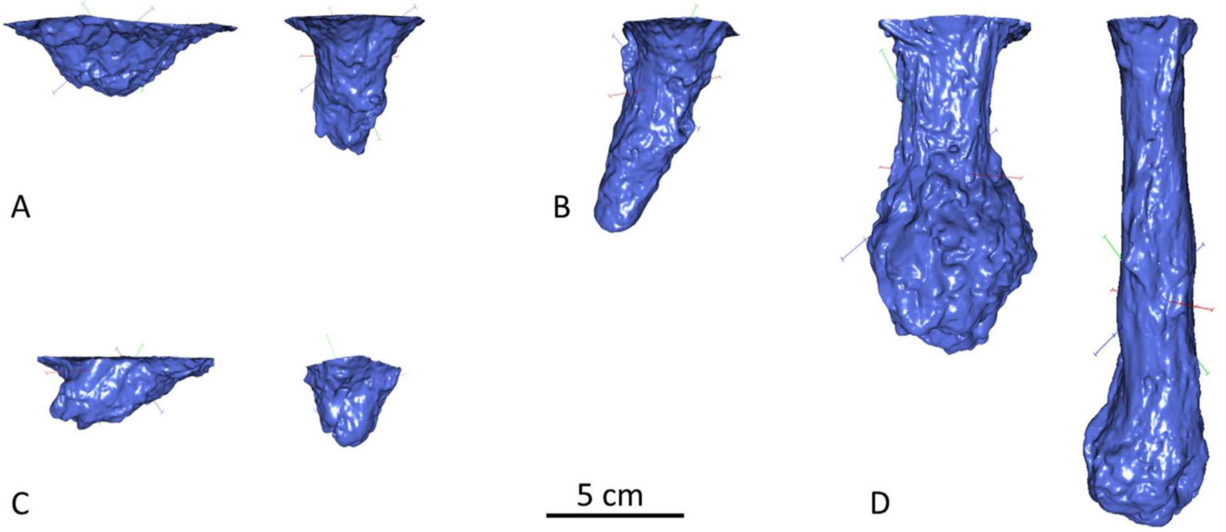
Samice jsou zdá se schopné rozpoznat dominanci pouze u vítěze, kterého viděly předtím bojovat. Můžeme se domnívat, že je to důležité z toho důvodu, že dominance je přechodná (často krátkodobá) charakteristika, která závisí na věku, velikosti, zdravotním stavu, výživě, motivaci jedince k boji apod. Výzkum v této oblasti je stále otevřený, ale tato studie jako první demonstrovala schopnost korýšů rozpoznat konkrétního jedince.

2.2.2. Norovací chování raků v podmínkách simulovaného sucha

Biologické invaze patří k hlavním faktorům ovlivňujícím globální biodiverzitu a funkce ekosystému, a to zejména ve sladkovodních ekosystémech (Strayer & Dudgeon 2010). Nepůvodní druhy, jsou schopny úspěšně interagovat s původními druhy a svou aktivitou a šířením tyto vytlačovat (Taylor et al. 2007). Vysvětlením bývá obvykle evaluace jednoho až čtyř biotických faktorů a jejich kombinací, a to: kompetice, predace, reprodukční odlišnosti a přenos nemocí spolu s jejich nižšími environmentálními nároky a vyšší adaptabilitou (Souty-Grosset et al. 2006). Role abiotických faktorů jako např. dlouhotrvajícího sucha byla do nedávna přehlížena a nevalně pochopena a dodnes je ve velké míře neúplná (Larson et al. 2009). Schopnost budování vertikálních nor a odolnost k vysychání je nutná v kontextu přetrvání v kontaktu se spodní vodou (nebo alespoň s vlhkým prostředím) v případě vysychání lokalit (dlouhodobého i periodického). Tato studie je první porovnávací studií schopnosti budování vertikálních nor v podmínkách simulovaného sucha u nepůvodní a původních druhů raků. Celkem jsme porovnávali tři evropské původní druhy: raka říčního (*Astacus astacus* Linnaeus 1758), bahenního (*Pontastacus leptodactylus* Eschscholtz 1823), a kamenáče (*Austropotamobius torrentium* Schrank 1803); a pět nepůvodních druhů: raka ničivého (*Cherax destructor* Clark 1936), červeného (*Procambarus clarkii* Girard 1852), signálního (*Pacifastacus leniusculus* Dana 1852), mramorovaného (*Procambarus virginalis* Lyko 2017) a pruhovaného (*Faxonius limosus* Rafinesque 1817).

Původní druhy v podmínkách simulovaného sucha intenzivně hynuly bez náznaků schopností vybudovat si vertikální noru a zůstat tak v kontaktu se spodní vodou. Přežití nepůvodních druhů bylo výrazně vyšší. Rak signální rovněž nebyl schopen norování, ale byl krátkodobě odolný vysušení. Simulované týdenní sucho přežili pouze všichni jedinci raka červeného, ničivého a mramorovaného. U raka mramorovaného byla zaznamenána mortalita (cca. 17 %) během týdne stráveného po pokusu ve vodním prostředí. U raka pruhovaného a raka signálního byla sledovaná mortalita 60 % resp. 70 %. Pouze jeden jedinec raka bahenního a překvapivě raka kamenáče, přežil do konce experimentu. Všichni jedinci raka říčního uhynuli do 5 dnů od začátku experimentu.

Raci, kteří byli schopni norování, obvykle vybudovali jedinou noru, pouze jeden rak červený a jeden rak ničivý si vybudovali nory dvě. Dva jiní raci červení pak vybudovali nory dokonce tři. Pouze rak červený (více jak v 33 % případů) byl schopen noru uzavřít pomocí bahenní zátky. Navíc jeho samice budovali hlubší nory než samci – u ostatních druhů rozdíly nebyly. Rak červený a mramorovaný konstruovali větší a hlubší nory než rak ničivý a rak pruhovaný (Obr. 5). Podrobnější výsledky jsou dosažitelné v **Příloze č. 4**.



Obr. 5. Příklady 3D modelů nor vybudovaných rakem ničivým (A), mramorovaným (B), pruhovaným (C), a červeným (D – vpravo nora samice).

Schopnosti k norování se v rámci raků velmi liší. Rozeznáváme nenorující raky, primárně norující raky (život z větší části tráví v noře), sekundárně norující raky (okupující nory, ale často vycházející mimo nory pokud jsou zaplaveny) a terciálně norující raky, kteří norují jen v období sucha nebo v některých případech v reprodukční sezóně nebo nedostatku úkrytů (Hobbs 1942). Vzhledem ke zmíněnému členění můžeme všechny studované druhy zahrnout do poslední skupiny.

Výsledky jasně ukazují na výrazně nižší schopnost přežití u původních evropských druhů raků v podmínkách simulovaného sucha. Naopak rak červený a rak ničivy se zdají být velmi dobře adpatování i vzhledem k jejich původním oblastem výskytu, které často periodicky vysychají (Holdich 2002). Vysoké přežití u raka mramorovaného (pouze post-treatment), signálního a pruhovaného dokumentuje zvýšené schopnosti odolávat vysušení popsané již v minulosti (Holdich & Black 2007; Banha & Anastácio 2014).

Pokud posuzujeme dohromady odolnost vysoušení a norovací schopnosti, pak rak červený je nejlépe adpatovaným druhem a zároveň jediným testovaným druhem, které své nory zavírá bahenní zátkou (Correia & Ferreira 1995). Je to jev častěji pozorovaný u samic, které navíc budují hlubší úkryty, pravděpodobně proto, aby dosáhly kontaktu se spodní vodou. Důvod je jasný. Samice raka červeného nory často používají pro inkubaci vajíček, ale i raný postemryonální vývoj. V tomto případě nejsou vajíčka/ráčata trvale potopena ve vodě, ale přijímají přímo atmosférický kyslík (i za pomoci pohybů pleopodů samice) a voda v úkrytu je používána jen pro jejich udržování ve vlhkosti (Huner et al. 1984).

Kromě raka červeného, ostatní nepůvodní druhy zahrnuté ve studii vykazovali kombinované strategie zaměřené na zvýšenou toleranci k vysoušení (rak ničivý, mramorovaný, pruhovaný a signální) a na schopnost hloubit nory (rak mramorovaný a v menší míře také rak ničivý a pruhovaný). Celkově nejnižší schopnosti vyrovnat se s podmínkami delšího sucha připadají na testované původní evropské druhy a vzhledem k norování rovněž raku signálnímu. Předpokládáme, že schopnosti vyrovnávat se s nízkými stavy vodní hladiny nebo vysoušením lokalit bude v Evropě i jinde na světě velmi důležitý faktor pro přežití v podmínkách předpokládaných fluktuací klimatu a tedy i extrémů počasí (Underwood 2015). Norování pak nemá nutně svou roli jen v průběhu extrémního sucha, ale hraje roli i v přezimování, kdy i v případě původně teplovodních druhů může tato vlastnost napomoci jejich etablování i v chladnějších oblastech (Stoeckel et al. 2011; Veselý et al. 2015).

2.2.3. Raci schovávají svoje exuvie

Jak již bylo popsáno výše, raci jsou velmi různorodou skupinou živočichů s mnoha životními strategiemi a adaptacemi včetně zajímavých behaviorálních charakteristik. V této práci (Buřič et al. 2016 – **Příloha 5**) jsme sledovali nový behaviorální fenomén, kdy raci po prodělaném svlékání zahrabávali své svlečky do jemného písku, který byl použitý jako substrát (Obr. 6). Tento druh chování může být určitým typem chování popsaného anglickými termíny *hoarding* nebo *caching* (hromaděnía a uschovávání). Takto uschovaná exuvie byla opět vyhrabána po dvou až třech dnech (kdy už tělo raka po svleku nebylo tak měkké) a zkonzumována. *Caching* nebo *hoarding* je dobře známý typ chování zejména u savců (VanderWall & Jenkins 2003) a ptáků (Bugnyar & Kotrschal 2002), jako druh potravního chování charakterizovaný sběrem potravy v době dostatku a jejího ukrytí na jedno či více míst pro pozdější konzumaci, např. jako zásoba na zimní období (Schneider et al. 2013). Hromadění potravy ale může být i výsledkem předchozí potravní deprivace udruhů, který tento způsob chování obvykle nepraktikují (Yang et al. 2011). Stejně tak může být toto chování indukováno u destinohých korýšů (Kim 2010) nebo může být druhově specifickým znakem jako u některých mravenců (Mueller et al. 2011) či pavouků (de Crespigny et al. 2001). Tak či onak je to chování u bezobratlých velmi vzácné a uschovávání svleček u bezobratlých pozorován ještě předtím nikdy nebylo.

Popsané chování zahrabávání svleček bylo sledováno náhodně při aklimatizaci raka signálního a raka ničivého pro jiný pokus, tzn. u zástupců raků jak severní, tak jižní polokoule. Zahrabání svlečky bylo umožněno díky použití jemného písku jako substrátu a nutno dodat, že všichni raci, kteří se v průběhu pokusu svlékli, se zachovali stejně, tj. všichni svlečku zahrabali podobným způsobem. Toto neočekávané chování unikalo pozornosti navzdory aktivnímu výzkumu i chovu raků na celém světě (Souty-Grosset et al. 2006).

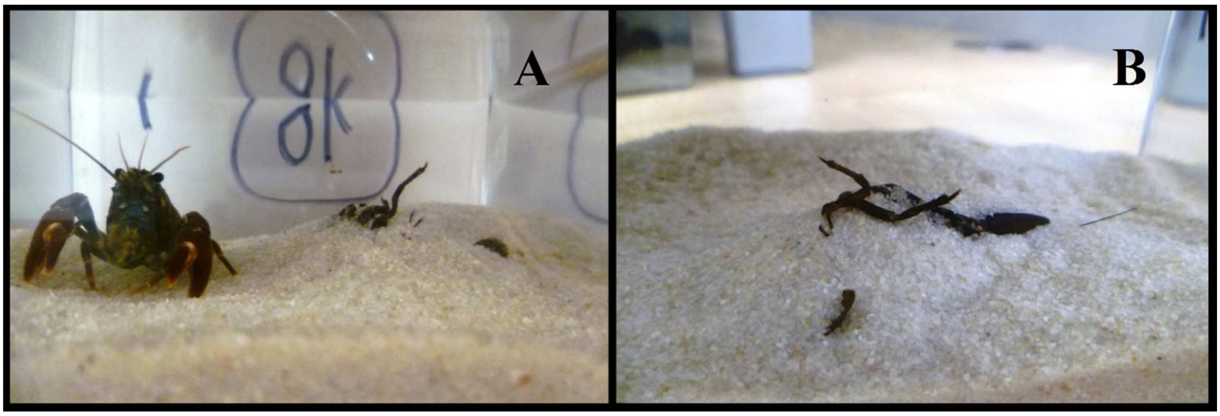
Odložená konzumace exuvie, jakožto zdroj minerálů pro rekalcifikaci krunýře, bylo vynuceno pravděpodobně nutností recalcifikovat nejdříve části ústního ústrojí z interních zdrojů raka (gastrolitů) (Holdich 2002). V průběhu této periody trvající 2 – 3 dny, kdy je jedinec měkký a neschopný obrany, byla svlečka pohřbena ve vhodném substrátu. *Hoarding* a *caching* mohou být vyvolané nutností zásobit se nebo periodou potravní deprivace (Yang et al. 2011), ale v tomto případě je sledované chování pravděpodobně spojené spíše s ochranou před predátory a kanibalismem. Volně viditelná svlečka totiž může být označením místa, kde se skrývá bezmocná oběť.

Důvod přehlížení tohoto chování může být například nevhodností podmínek, vyrušení jedinců nebezpečím, nebo marginalizací sledovaného chování, jak tomu ostatně bylo i v případě této studie. První sledování tohoto chování totiž bylo posouzeno jako náhodné zakrytí svlečky při hrabání raka ničivého v substrátu. Vzhledem k tomu že všichni jedinci, kteří se v daných podmínkách svlékli (jak rak ničivý, tak rak signální), pohřbili své exuvie, určitě se o náhodný efekt nejednalo.

Konkrétní důvody sledovaného chování jsou neznámé, ale obecně mohlo být motivováno dvěma hlavními důvody. Prvně uschování a uchování snadno dostupného zdroje minerálů pro rekalcifikaci po dobu, kdy rak vytvrzuje strategické části těla (ústní ústrojí a kráčivé končetiny) prvně z interních zdrojů (zejména gastrolitů) (Greenaway 1985). Po dvou až třídenní periodě už je rak schopen svlečku zpracovat. Druhým důvodem je uschování exuvie, jako uschování znaku neschopnosti bránit se případnému predátorovi nebo útoku ze strany jiných raků v populaci.

V průběhu svlékání obvykle dochází k největším mortalitám vyvolaným také predací a kanibalismem (Holdich 2002) takže uschovaná exuvie může snížit pravděpodobnost lokalizace, měkkého, čerstvě svlečeného jedince. Pokud je svlečka nalezena a kupříkladu zkonzumována, může tak pro raka dojít pouze ke ztrátě daného zdroje minerálů, zatímco sám přežije nepozorován v úkrytu.

Mechanismy indukující či inhibující popsané chování jsou neznámé a znamenají výzvu pro další výzkum. Prezentovaná práce poskytuje základnu pro řadu nových hypotéz a budoucí výzkum.



Obr. 6. Rak signální a jeho mohyla z písku pro uschování exuvie (A) a detailní pohled na zahrabanou svlečku (B).

2.2.4. Interakce raka mramorovaného a raka červeného

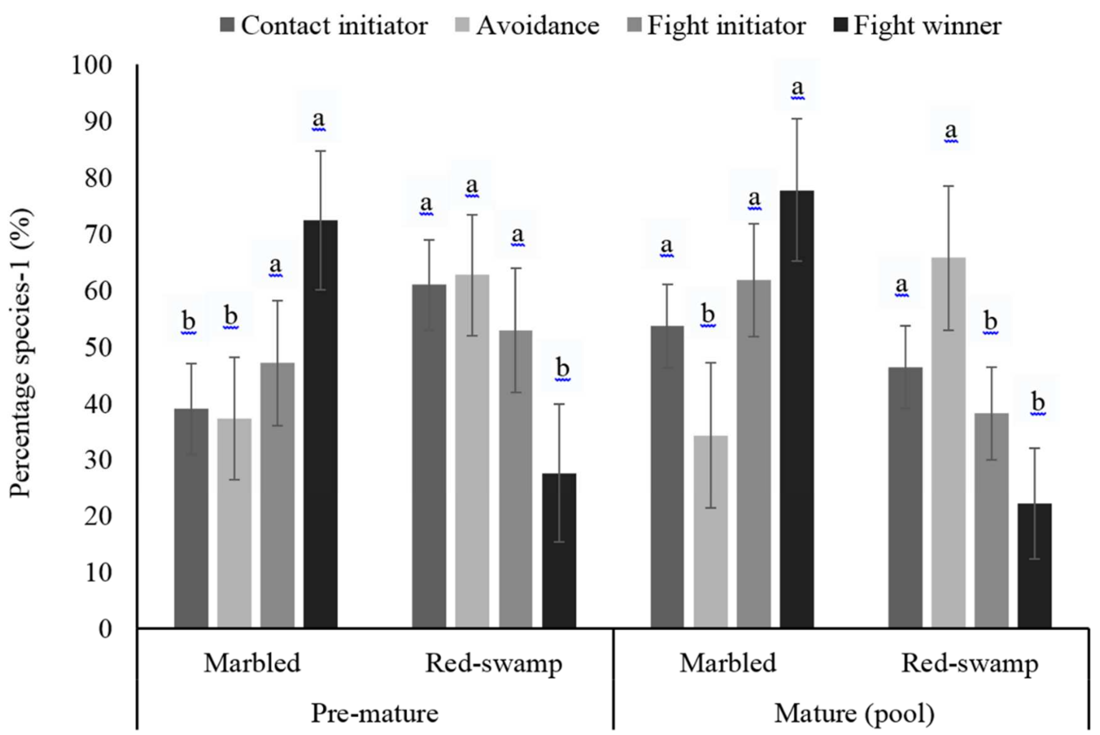
Intra- a inter-specifické interakce příspívají k úspěšnému etablování a následnému šíření druhu v prostředí, což nabývá na významu zejména v kontextu recentních biologických invazí. Tato studie (detailně v **Příloze č. 6**) zkoumala interakce velikostně vyrovnaných párů raka mramorovaného a raka červeného – vysoce agresivního invazního druhu.

Uspěšný invazní druh raka je obvykle determinován kromě ekologických strategií a adaptability také agresivními interakcemi s ostatními druhy (Holdich et al. 2009; Hudina et al. 2011). V závislosti na jeho celosvětovém šíření nabývá rak mramorovaný na významu a zájmu ze strany biologů (Hossain et al. 2018 – **Příloha 2**). Informace o jeho konkurenceschopnosti s ostatními druhy jsou stále nedostatečné (Kawai et al. 2015). Obecně je rak mramorovaný považován za klidný druh, odolný vůči stresu a často stimulovaný k aktivitě pouze mechanickými stimuly (Kasuya & Nagayama 2016). Proti klidné povaze raka mramorovaného vystupují informace o jeho neočekávané schopnosti úspěšně v soubojích čelit agresivnímu raku červenému, pravděpodobně celosvětově nejúspěšnějšímu invaznímu raku (Jimenez & Faulkes 2011). Nicméně tento důkaz byl proveden na velikostně nevyvážených párech oponentů a navíc se jednalo o dospělé raky mramorované (8 – 9 měsíců staré) a juvenilní raky červené, což mohlo výsledky pokusu ovlivnit. Nicméně autoři tvrdí, že i rak mramorovaný nebyl dospělý, neboť u něj nebyly pozorovány vajíčka ani bílé žlázy na abdomenu (Faulkes, osobní sdělení). Tato studie rozšiřuje předchozí studie použitím dvou velikostních skupin raků (juvenilní a dospělci) a použití velikostně vyrovnaných párů oponentů.

Naše výsledky nasvědčují tomu, že rak mramorovaný skutečně může být minimállně rovnocenným partnerem stejně velkému raku červenému. A to ve skupině juvenilních, tak i dospělých jedinců. Rak mramorovaný byl ve vetšině případů dominantním nebo ekvivalentním protivníkem raku červenému. Viditelné to bylo zejména u skupiny juvenilů a u soubojů raka mramorovaného se samicemi raka červeného. U soubojů se samci bylo skóre přibližně vyrovnané, pravděpodobně díky jejich větší agresivitě (Holdich 2002). Dospělci byli méně aktivní v soubojích i ustanovení hierarchie než juvenilové, jak je ostatně známé i z jiných studií (Sato & Nagayama 2012). Při porovnání morfometrických údajů u použitých raků, nebyly zjištěny významné rozdíly, a proto pravděpodobně neměly vliv na výsledky studie. Počty iniciovaných a vítězných soubojů a další behaviorální charakteristiky jsou patrné z Obr. 7.

Juvenilní raci červení byli více aktivní a současně i více aktivní v iniciování kontaktů a soubojů než rak mramorovaný, ale jejich útoky byly ve většině případů neúspěšné a rak červený tak zbytečně plýtval energií. Jedním z důvodů neúspěchu raka červeného tak mohl být přílišné energetické náklady v porovnání s rakem mramorovaným, který byl aktivní jen v nutných případech (kontakt s oponentem).

Během přímých interakcí jsou raci schopni odhadnout schopnosti protivníka a zapamatovat si jej po předchozích interakcích. To rakům umožňuje uspořit energii v již ustanovené hierarchii nebo rozlišit a vybrat správného partnera (Aquiloni et al. 2008 – **Příloha 3**; Kubec et al. 2019 – **Příloha 1**). V ustálené sociální hierarchii se podřízený jedinec vyhýbá dominantnímu a aktivita směrem k vyvolání kontaktu či konfliktu klesá (Issa et al. 1999), což bylo patrné i z našich sledování. To má smysl ve snižování nákladů na agresivní interakce. Obvykle tak stačí, že podřízený jedinec ustoupí, jakmile dominantního spatří (cítí), nebo ustoupí poté, co dominantní jedinec pozvedne klepeta k hrozbě.



Obr. 7. Porovnání podílu raka mramorovaného a raka červeného v průběhu interakcí na počtu iniciovaných kontaků a soubojů, vítězných soubojů a stranění se svého oponenta. Hodnoty jsou uvedeny jako průměr ± směrodatná odchylka. Různá písmena nad sloupci udávají statisticky významný rozdíl mezi typy chování v rámci jednotlivých skupin (pre-mature, mature).

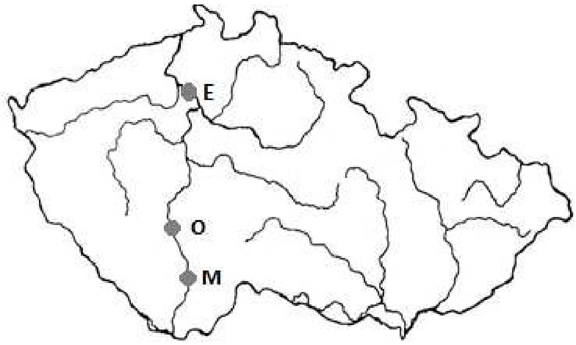
Závěrem můžeme říci, že rak mramorovaný může být rovnocenným nebo dokonce dominantním protivníkem stejně velkému agresivnímu invaznímu raku červenému. Rak mramorovaný se zdá být šetrnějším ve smyslu vynaložené energie, ale efektivně reaguje na výzvy ze strany oponenta. Jakmile je ustanovena dominace, oba druhy raků ztrácí zájem o další souboje. Samozřejmě se situace může lišit v přírodních podmínkách, kde populace není tak dobře strukturovaná jako v experimentálních podmínkách a raci jsou vystaveni mnohem komplexnějším podmínkám včetně predace. Rak červený bude jednoznačně zvýhodněn v přímých interakcích v přirozených podmínkách větší maximální velikostí a velikostí klepet u velkých samců (Holdich 2002), ale na druhou stranu částečně znevýhodněn přehnaně vysokou aktivitou a proto i nadměrnými energetickými vydáními a expozicí predaci. Tak či onak úspěch raka mramorovaného v kompetici s rakem červeným v přírodních podmínkách tak může být limitován.

Úspěšný invazní druh ale není determinován jen agresivitou, ale i úspěšnou reprodukční strategií a kapacitou a zároveň schopností adaptovat se na různé podmínky (Holdich 2009). V tomto světle je rak mramorovaný znám svou vysokou adaptabilitou a reprodukcí až několikrát ročně pomocí apomiktické partenogeneze tj. bez účasti samců (Hossain et al. 2018 – **Příloha 2**). Dohromady se schopností úspěšně se bránit ba i dominovat v agresivních interakcích s jinými druhy znamená značné nebezpečí pro původní biodiverzitu.

2.2.5. Samice raků rozpoznají a preferují samce z mateřské populace

Reprodukční chování je důležitým bodem v životě živočichů a pochopení sexuálního výběru patří mezi klíčové výzvy behaviorálních i ekologických studií. Jak již bylo popsáno výše, selekce partnera je založena na rozpoznání druhu, pohlaví, fyziologického stavu a sociální pozice jedince. U raků jsou samice obvykle vybíravější než samci pravděpodobně z důvodu větší nákladů spojených s reprodukcí (oogeneze, inkubace vajíček, mateřská péče). Tato studie (podrovněji v **Příloze č. 7**) analyzuje preference u sexuální selekce raka pruhovaného z hlediska výběru mezi velikostně podobnými jedinci z různých, geograficky oddělených populací (Obr. 8). Oběma pohlavím, byly k výběru poskytnuty vždy dva jedinci – buď jeden z cizí populace a druhý z jejich vlastní populace, nebo dva jedinci z cizí populace. Vše bylo vybírajícím jedincům stíženo tím, že ačkoli byli drženi individuálně (bez možnosti vizuálního a taktilního kontaktu), byli ve stejných podmínkách ve společné nádrži s jedinci z ostatních populací.

Obecně naše výsledky poukazují na to, že samice jsou schopny rozpoznat samce z vlastní populace a preferují je před ostatními, kdežto samci jsou v tomto směru nevybíraví. Pokud samice volily mezi samci ze dvou jim neznámých (ne mateřských) populací neprefererovali ani jednu z nich. Oproti tomu volící samice ze všech tří populací vždy preferovaly samce z vlastní populace na základě času stráveného u samce, množství kontaktů a času stráveného v kontaktu s daným samcem. Samice se pářily v průběhu pokusu v ca. 35 % případů. Také pro páření samice preferovaly samce ze známé populace. V několika případech samec z cizí populace zachytil samici a nasilím ji držel ve své blízkosti. Ve variantě pokusu, kdy volil samec ze dvou samic, došlo k páření v 63 % trialů. Samci v žádném ze sledovaných parametrů, včetně páření nevykazovali preferenci pro žádnou z populací, včetně té vlastní.



Obr. 8. Mapa hlavní říční sítě České Republiky s lokalizací zdrojových populací rak pruhovaného pro prezentovanou studii. E – řeka Labe; O – údolní nádrž Orlík; M – řeka Malše.

Přestože výzkum individuálního rozpoznání a chemické komunikace odhalil různé schopnosti raků (např. Aquiloni & Gherardi 2008a; Aquiloni et al. 2008 – **Příloha 3**; Seebacher & Wilson 2007), doposud nebylo známé, zda raci dokáží rozpoznat jedince z geogaficky oddělených lokalit. Pohlavní výběr v průběhu období páření raka pruhovaného se zdál být dobrým způsobem, jak to zjistit. Vzhledem k výše popsaným výsledkům, byla tato hypotéza správná, neboť minimálně samice tuto schopnost mají. Teoreticky tak nově introdukovaní jedinci mohou být reprodukčně diskriminování původní populací a naopak. To může mít svou roli i v ochraně původních druhů, při reintrodukcích dospělých jedinců.

Nevybíravost samců ale potvrdila výsledky předchozích studií vedoucích k závěrům, že samci cílí zejména na kvantitu (počet spářených samic), popř. na velikost samice nebo na preferenci dosud nespářených samic (Acquistapace et al. 2002; Ameyaw-Akumfi 1976; Aquiloni & Gherardi 2008a). Proto jsou samci v období páření velmi aktivní (Buřič et al. 2009a; Gherardi & Barbaresi 2000), případně se snaží i odstranit spermatofory předchozích samců (Snedden 1990; Villanelli & Gherardi 1998) na rozdíl od samic, které své volby více váží pravděpodobně vzhledem k budoucím výdajům pro inkubaci vajíček a péči o potomstvo (Kubec et al. 2019 – **Příloha 1**).

Objevily se ovšem nové otázky k zodpovězení. Zejména proč samice preferují, ale hlavně jakým způsobem rozpoznají samce z mateřské populace. Na začátku studie jsme spíše předpokládali preferenci cizích populací pro zvýžení heterozygotnosti populace. Zejména proto, že rak pruhovaný prošel v průběhu své invaze od konce 19. století (kdy bylo vysazeno pouze ca. 90 jedinců, ze kterých pochází zdá se všechny jeho nynější populace v Evropě) několika populačními oslabeními (*bottleneck effect*) z důvodu přenosu několika jedinců na jiné lokality (Filipová et al. 2011). Ale raci pravděpodobně nejsou schopni takto posoudit jejich populační diversitu a spíše tíhnou k familiaritě podobné popsané např. při tvoření rybích hejn (např. Griffiths & Magurran, 1997; Ward & Hart, 2003).

Pohlavní preference známých jedinců (ale v tomto případě známých z předchozí zkušenosti s nimi) byla u korýšů zaznamenána (Caldwell 1992; Karavanich & Atema 1998), kdy familiarita může potenciálně snížit úroveň agresivity a tím energetické náklady na konflikty, stejně jako zkrátit dobu námluv (Hojesjo et al. 1998; Daws et al. 2011). To tedy můžou být pravděpodobné důvody volby samců z mateřské populace.

Nicméně mechanismus, jak samice jedince ze známé populace poznají je zastřen tajemstvím. Jsou známé případy individuální rozpoznání jedinců, ale tento efekt není dlouhodobý, je limitován počtem „zapamatovaných" jedinců a obvykle trvá jen několik dní (např. Van der Velden et al. 2008; Kubec et al. 2019 – **Příloha 1**). Rozpoznání na základě předchozí znalosti daných jedinců z interakcí v dané populaci, tak můžeme vyloučit vzledem k jejich individuálnímu přechovávání po dobu ca. 5 týdnů.

Proto předpokládáme, že rozpoznání je spíše založeno na bázi chemických stimulů. Je sice velmi nepravděpodobné, že samci aktivně chemicky informují samice o místě svého původu, ale jejich rozpoznání může být dáno funkcí předchozího života na stejné lokalitě a proto spíše detekce specifického „pachu lokality". Tento pach může být spojen i s podobným mikrobiomem zvířat žijících na stejné lokalitě (Arbuthnott et al. 2016; Ezenwa et al. 2012), s minimálním vlivem na metabolické a funkční změny i při změně environmentálních podmínek (Van Bonn et al., 2015). Specifické chemické, fyzikální a biologické podmínky totiž vedou k výrazné diferenciaci vnitřní mikroflóry a proto k různému pachu jedinců z dané lokality (Lize et al. 2013). Konkrétní mechanismy rozpoznání a jeho funkce jsou výzvami pro budoucí výzkum.

2.3. Chování raků ovlivněné znečištěním prostředí

2.3.1. Nízké koncentrace psychoaktivních látek mění chování raků

Kvalita povrchových vod je nezbytná pro správné fungování ekosystémů, biodiverzitu, stejně jako pro lidskou potřebu pitné vody, nebo vody pro průmysl a zemědělství (National Intelligence Council 2012). Výzkum efektů farmaceuticky aktivních látek na necílové vodní organismy v současnosti expandoval z výzkumu nejvíce se vyskytujících látek (antibiotika, antihypertenziva) spíše k mikropolutantům vyskytujícím se v povrchových vodách v nízkých koncentracích tj. antidepresiva, opiody a jiné psychoaktivní látky (Brodin et al. 2013; Fong & Ford 2014). Minimálně některé jsou známé tím, že u vodních organismů mohou vyvolat behaviorální změny v nízkých, environmentálně relevantních koncentracích (Barry 2013; Brodin et al. 2017). Následující dvě studie vyhodnocují vliv čtyř psychoaktivních látek (tramadol, citalopram, metamfetamin, sertralin) v environmentálně relevantních koncentracích na základní behaviorální patrametry modelového organismu, raka mramorovaného (**Příloha č. 8** a **Příloha č. 9**).

Změny základních parametrů chování organismů jako je aktivita, odvaha či sociabilita mohou změnit následně i fungování celých ekosystémů (Manning & Dawkins 2012; Schmitz 2007). Obvzláště u druhů, či skupin organismů, označených za klíčové sladkovodní organismy (Holdich et al. 2009). Studie chronické expozice polutantům a sledování jejich vlivu, pracuje obvykle s velmi nízkými koncentracemi látek a potřebuje dostatečně robustní metodiku, která je snadno proveditelná a opakovatelná. Několikadenní až několikatýdenní expozice (podle mechanismu účinkování testované látky) a následné sledování chování exponovaných raků v jasně definovaných podmínkách pomocí automatického sofwaru a porovnání s kontrolou manipulovanou stejným způsobem, jako exponovaní raci, se zdá být vhodnou metodou (Buřič et al. 2018 – **Příloha 8**; Hossain et al. 2019b - **Příloha č. 9**).

V případě obou studií a všech testovaných látek byla sledována velká individualita raků v kontrolní i exponované skupině ve všech sledovaných parametrech. To naznačuje určitou personalitu, individuální různorodost chování konsistentní na úrovni jedince v rámci času nebo kontextu (Réale et al. 2007). V našich studiích je patrné, že tato variabilita je obvyklá i u geneticky uniformních jedinců. Dá se ovšem očekávat, že bude nižší než u ostatních geneticky variabilních druhů. Přes značnou individuální variabilitu byly nalezeny signifikantní změny chování vyvolané všemi čtyřmi testovanými látkami.

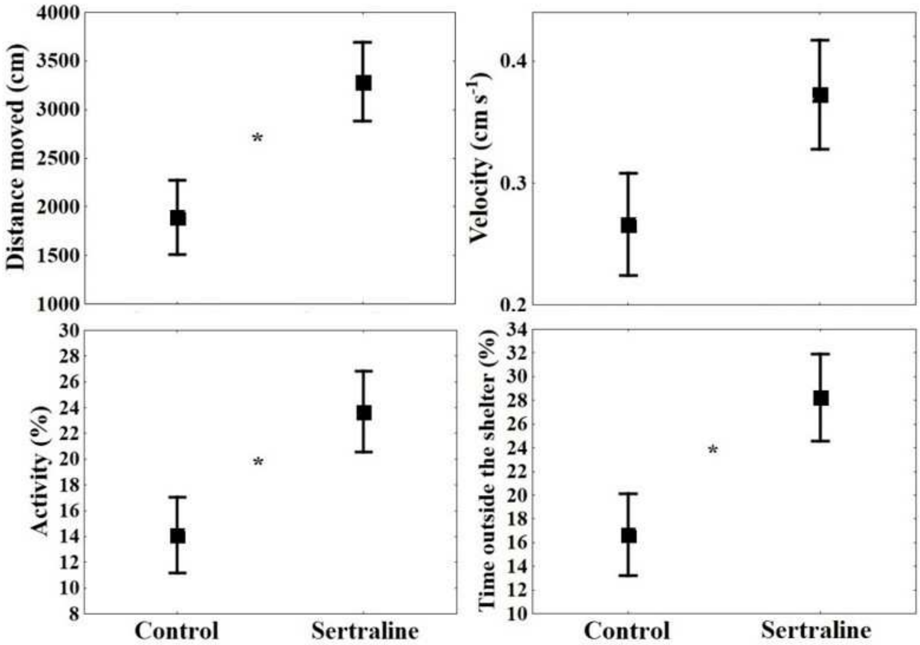
Tramadol (**Příloha č. 8**) chování ovlivnil směrem k nižší rychlosti, tedy v kratší rakem uražené vzdálenosti v aréně bez úkrytu, a to přesto, že tramadolu exponovaní raci byli celkově aktivnější – jejich pohyb byl častý, ale pomalejší jakoby ospalejší. Pokud byl úkryt přítomen, pak tyto změny již nebyly detekovatelné. Přesto raci exponovaní tramadolu byli kratší čas mimo úkryt, i když ne statisticky významně (P = 0.053), což může být rovněž znakem snížené pohyblivosti exponovaných raků. Podobné, téměř shodné, výsledky byly sledovány i v případě citalopramu (**Příloha č. 8**).

Sledované změny chování mohou být výsledkem povahy testovaných látek, kdy tramadol je znám pro svůj efekt na snížení pozornosti, snížení chuti k jídlu, ospalost a sníženou citlivost okolním vjemům (Langley et al. 2010). Citalopram je popisován naopak, jako redukující agresivní a impulsivní chování (Armenteros & Lewis, 2002).

Raci exponovaní environmentální koncentraci metamfetaminu (**Příloha č. 9**) se v naprosté většině sledovaných parametrů nelišili od kontrolních jedinců. To je v rozporu s jinými studiemi, které naopak popisovaly vyšší aktivitu u raků (Imeh-Nathaniel et al. 2017) či savců (Pang et al. 2016). Rozdíl byl ale ve vyšší dávce metamfetaminu a způsobu podání (injekčně). Jediným efektem metamfetaminu v naší studii, byl delší čas strávený mimo úkryt u exponovaných raků. Nejednalo se ale o aktivní průzkum prostředí nebo jiný druh pohybu – jejich přítomnost mimo úkryt byl pasivní. To může mít nebezpečný dopad na raky, jejichž úspěch je do značné míry ovlivněný využitím úkrytů zejména v denních hodinách (Kubec et al. 2019 – **Příloha 1**; Lozán, 2000). Je otázkou, zda by delší expozice znamenala další efekty na sledované raky.

V případě sertralinu (**Příloha č. 9**) bylo sledováno naopak velké množství změn chování u exponovaných raků - signifikantně vyšší aktivita jak v s přítomností, tak v nepřítomnosti úktrytu, delší uražená vzdálenost v přítomnosti úkrytu, a zároveň marginálně signifikantní vyšší rychlost a uražená vzdálenost v podmínkách bez úkrytu (Obr. 9). Předchozí studie u ryb poukazovaly spíše na sníženou pohybovou aktivitu z důvody sníženého pocitu úszkosti – strachu jako u lidí (Giacomini et al. 2016; Simmons et al. 2017). Raci ale ze stejného důvodu pravděpodobně byli pohybově aktivní více. Velmi nízké koncentrace sertralinu totiž podobně působily na rychlost plavání různonožce *Echinogammarus marinus* Leach 1815 (Bossus et al. 2014).

Celkově se dá říci, že výsledky studií na různých druzích a různých koncentracích stejné látky obvykle působí někdy podobné, ovšem často i úplně opačné změny chování. Z toho vyplývá, že změny chování vyvolané jednotlivými kontaminanty jsou druhově specifické, ovlivněné různými koncentracemi látek, ale i různé v rámci jedné skupiny látek, přičemž se efekt může lišit i v případě použití stejných koncentrací, ale jiných způsobů expozice – její délka, kontinuita, působení v tekoucí či stojaté vodě apod. (Brodin et al. 2013; Hossain et al. 2020 – **Příloha 10**).



Obr. 9. Celková uražená vzdálenost (cm), rychlost (cm s^{-1}), aktivita (%), a podíl času stráveného mimo úkryt (%) u raka mramorovaného ve skupině exponované environmentálně relevantní koncentraci sertralinu a v kontrolní skupině. Signifikantní rozdíl hodnot je označený hvězdičkou.

U raků exponovaných 21 dní environmentálně relevantní koncentraci sertralinu bylo kromě změn chování pozorováno větší množství svleků (nesignifikantní) a signifikantně větší množství samic kladoucích vajíčka a vyšší mortalita než u kontrolní skupiny (Tab. 4). Zrychlení reprodukce bylo rovněž pozorováno ve spojitosti s expozicí antidepresivům (fluoxetinu) u slávičky mnohotvárné (*Dreissena polymorpha* Pallas 1771) (Fong 1998). Escitalopram naopak například narušoval produkci jiker u dania pruhovaného (*Danio rerio* Hamilton 1822). Vzhledem k tomu, že antidepresiva působí rovněž jako imunosupresanty a působí na hormony ovlivňující svlékání u raků je logický i rozdíl v množství svleků a vyšší mortalitě, která mohla být nejspíše ovlivněna abnormalitami při svlékání (Fanjul-Moles 2006; Gobin et al. 2014).

Tab. 4. Počet svlékání, kladení vajíček a uhynulých jedinců raka mramorovaného exponovaného metamfetaminu a sertralinu v porovnání s kontrolními jedinci. Různá písmena u hodnoty indikují signifikantní rozdíl mezi exponovanou a kontrolní skupinou.

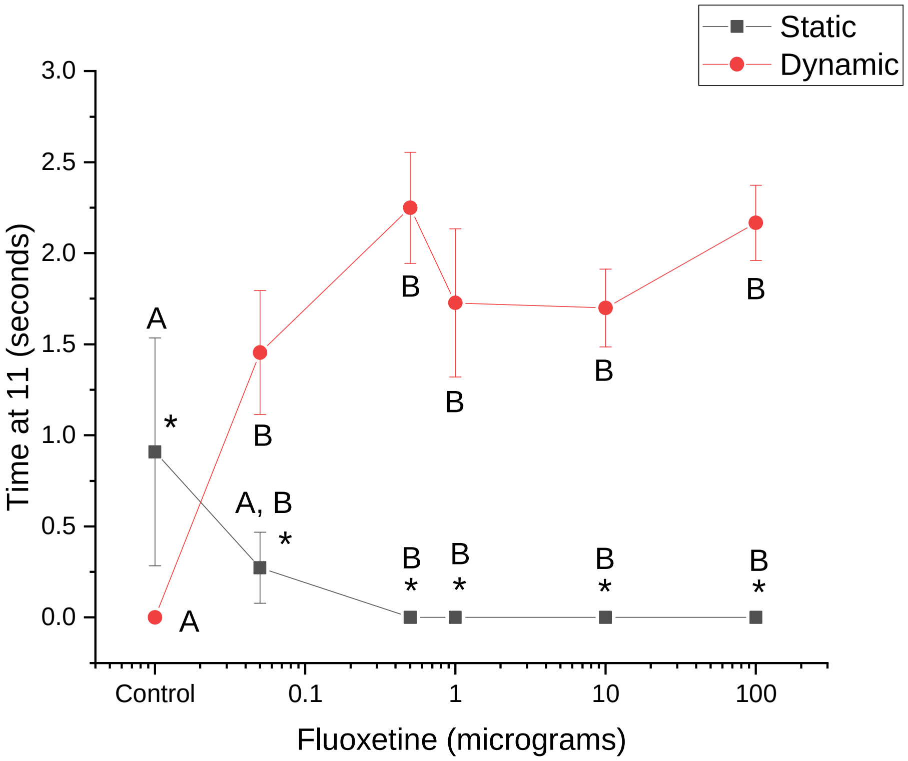
Pharmaceutical	Group (n)	Molted (n)	Spawned (n)	Mortality (n)
Methamphetamine	Control (73)	7	0	0
	Exposed (73)	9	0	0
Sertraline	Control (75)	15	2^b	0^b
	Exposed (75)	22	9^a	8^a

Předchozí výsledky spolu s našimi studiemi ilustrují, jak variabilní a intenzivní může být vliv psychoaktivních látek ve velmi nízkých koncentracích v povrchových vodách. Efekt testovaných látek v daných koncentracích je jasně viditelný a nabádá k ostražitosti z hlediska bezpečnosti povrchových vod a fungování vodních ekosystémů. Z dlouhodobého hlediska by se mělo řešit jednak nadužívání těchto látek, tak jejich účinné odstraňování z odpadních vod.

2.3.2. Způsob expozice fluoxetinu ovlivňuje různým způsobem chování raků

Efekt psychoaktivních látek nebo jiných polutantů byl prokázán u řady látek a řady organismů. Obvykle tyto eko-toxikologické studie bývají provedeny ve statických expozičních systémech. Ty ignorují prostorově-časovou dynamiku toxikantů v tekoucích vodách. V dynamických systémech se mohou vlivy toxikantů lišit z důvodu fluktuace frekvence, rozsahu a trvání oblaku (vlně) toxikantu, který je výsledkem proudění vody interagujícího s geomorfologickými charakteristikami prostředí. Cílem studie bylo porozumění tomu, jak dynamika expozice antidepresivu (fluoxetin) ovlivňuje agresivní chování raků. Studie proběhla na terénní stanici v Ohiu (USA) s použitím raka statného (*Faxonius virilis* Hagen 1870) exponovaného různým koncentracím fluoxetinu (kontrola; 0,05; 0,5; 1; 10 a 100 µg/l) po dobu 23 hodin, a to ve statickém a dynamickém systému. Následně byly páry velikostně podobných raků ze stejných skupin sledovány při agonistických interakcích a analyzovány dle etogramu popsaného v Tab. 3. Podrobný popis studie a detailnější informace jsou patrné z **Přílohy 10**.

Raci signifikantně častěji volili únikové reakce (*tailflip*, útěk) ve skupině se statickou expozicí v porovnání s identickými koncentracemi v dynamické expozici. Početnost a trvání největší intenzity soubojů se rovněž lišila mezi statickou a dynamickou expozicí. Čas strávený v nejvyšší intenzitě soubojů a čas do jejich eskalace byl nejnižší v dynamické kontrole, nižší než ve statické kontrole. Výsledky indikují, že raci vystavení i nízkým koncentracím fluoxetinu jsou výrazně ovlivněni ve svém agresivním chování, ale dynamická expozice výrazně mění míru tohoto chování.



Obr. 10. Průměrný čas strávený v nejvyšší intenzitě agonistických interakcí u raků exponovaným různým koncentracím fluoxetinu v podmínkách statické a dynamické expozice. Hvězdičky poukazují na signifikantní rozdíly mezi statickou a dynamickou expozicí. Velká písmena označují signifikantní rozdíly v rámci expozičního paradigmatu.

V předchozích studiích bylo prokázáno mnoho různých změn chování vodních organismů vyvolaných fluoxetinem a zároveň o různé intenzitě v závislosti na koncentraci, druhu, experimentálním designu a délce expozice (Backhaus 2014; Bossus et al. 2014; Fong & Ford

2014). Nicméně všechny nám známé studie vlivu fluoxetinu byly provedeny ve statických režimech expozice. Ty nepřesně reprezentují fluktuace toxikantu při jeho šíření a ředění v přírodních podmínkách (Harrigan & Moore, 2017). Prostorová a časová variabilita koncentrace toxikantu může vyvolat různé odpovědi exponovaných organismů, což je dobře reprezentováno naší studií i předchozími studiemi používajícími dynamická expoziční paradigmata (Neal & Moore 2017; Steele et al. 2018). V naší studii, napodobující přírodní dynamické sytémy, bylo chování sledovaných raků změněno opačným způsobem než u raků ve statickém systému. Efekt prostředí byl detekován jak u exponovaných zvířat, tak u kontrolních.

Ekotoxikologické studie recentně zjišťují, že dynamická expoziční paradigmata ovlivňují nejen agresivní chování raků, ale i třeba růst ryb, nebo mohou působit i větší negativní efekty než ve statických systémech (Angel et al. 2015; Diamond et al. 2006; Welsh et al. 2008). Geomorfologické charakteristiky habitatu tvoří různé prostorové mapy a ovlivňují dynamiku chemické disperze látek. Zde potom lze očekávat často nelineární a na dávce nezávislé výsledky dynamické expozice. Dokonce organismy žijící ve stejném regionu, ale čelící různým hydrodynamickým režimům, jsou vystaveny různým koncentracím polutantů (Harrigan & Moore 2017; Steele et al. 2018). Budoucí výzkum by se měl obrátit více na pochopení reálných důsledků znečištění vyvolaných strukturou habitatu a expozičním paradigmatem.

3. Závěry

3.1. Význam výsledků pro vědní obor a možnosti směřování dalšího výzkumu

V **Přílohách 1** a **2** je prezentován přehled dostupných informací o chování raků, jejich komunikaci a rozhodovacím procesu, stejně jako dostupné informace o raku mramorovaném, jakožto recentně hojně využívaném modelovém organismu pro širokou škálu vědních oborů. Obě tyto přehledové publikace tvořily a stále tvoří základy pro ekologický, etologický i eko-toxikologický výzkum nejen v rámci našeho pracoviště, ale i pro ostatní podobně zaměřené vědecké týmy.

Příloha 3 prezentuje u korýšů poprvé popsanou schopnost rozpoznávat dominantního jedince na základě předchozího sledování souboje a otevřela tak další otázky pro výzkum chování a komunikace bezobratlých živočichů.

V **Příloze 4** bylo provedeno unikátní srovnání sedmi druhů původních a nepůvodních raků s cílem sjistit jejich schopnost snášet vysoušení lokality a schopnost přečkávat nepříznivé podmínky ve vybudovaných norách. Studie akcentuje problematiku lokálních extrémů počasí – krátkodobého i dlouhodobého vysychání toků. Původní druhy jsou v tomto směru viditelně neadaptibilní, naopak šíření invazních druhů se dá očekávat i v podmínkách s nízkými srážkovými úhrny.

V **Příloze 5** je prezentován nově popsaný druh chování u bezobratlých – zahrabávání vlastní svlečky, pravděpodobně jako maskování jejího bezbranného majitele. Toto chování bylo popsáno u dvou druhů raků ze dvou různých čeledí, žijících na severní a jižní polokouli, což ukazuje dlouho přetrvávající druh chování, pravděpodobně široce rozšířený, přestože dlouho přehlížený. Výsledky otevírají nové možnosti výzkumu chování korýšů.

Příloha 6 obsahuje informace o interakcích dvou invazních druhů raků. Na jedné straně téměř kosmopolitního agresivního druhu a na strně druhé recentně nejrychleji se šířícího druhu, profitujícího zejména z obligatorní asexuální reprodukce. Výsledky napovídají, že rak mramorovaný je schopen koexistovat a úspěšně konkurovat i etablovaným agresivním druhům.

V **Příloze 7** byla popsána nová schopnost raků rozpoznat a preferovat při sexuálním výběru jedince z mateřské populace i přes 5 týdenní periodu aklimatizace, kdy byli jedinci drženi individuálně ve stejných podmínkách. Předpokládáme, že daná schopnost není dána pamětí daných jedinců na jedince z mateřské populace, ale spíše chemickou cestou detekovaný „pach lokality“ díky podobnému mikrobiomu raků.

V **Přílohách 8**, **9** a **10** jsou raci použiti jako modelové organismy v eko-toxikologických studiích zaměřených na vliv environmentálně relevantních koncentrací psychoaktivních látek na základní parametry chování raků. I v nízkých koncentrací tramadolu, citalopramu, metamfetaminu a sertralinu byly prokázány nezanedbatelné účinky na necílové organismy s potenciálem větších důsledků pro populace a ekosystémy (**Přílohy 8** a **9**). **Příloha 10** pracovala sice s několika koncentracemi psychoaktivní látky (antidepresivum fluoxetin) nicméně jeho vliv na vodní organismy již byl znám a nebyl předmětem studie. Studie prokázala, že různé expoziční přístupy (zde satická a dynamická expozice) mohou vést až k opačným výsledkům, Proto by se v eko-toxikologických studiích mělo dbát na co největší napodobení přírodních podmínek.

Výše zmíněné studie v naprosté většině případů nepřináší pouze informace o daném studovaném tématu, ale zároveň mnoho otázek pro další výzkum, často zásadních. Na základě získaných výsledků a nových hypotéz se momentálně rozvíjí další aktivity našeho týmu zejména na poli výzkumu komunikace a chování raků.

3.2. Využití dosažených výsledků při výuce

Teoretické i praktické vědomosti, které byly získány v průběhu řešení zde prezentovaných i dalších vědeckých prací (viz. Kapitola 10) získané při řešení prezentované problematiky byly kromě jiného využity při tvorbě nového studijního předmětu s názvem „Základy etologie vodních organismů“ a předmětu „ Biologické invaze ve sladkovodních systémech“ pro nově akreditovaný doktorský studijní program „Ochrana vodních ekosystémů“.

K dosažení prezentovaných výsledků významně přispěli dva studenti (momentálně již po obhajobách svých doktorských prací) doktorského studia Ing. Jan Kubec, Ph.D. a M. Sc. Md Shakhawate Hossain, Ph.D. a zároveň několik studentů bakalářského a magisterského stupně studia Ing. Kateřina Švagrová, Ing. Lucie Müllerová, Ing. Martin Fojt, Ing. Jiří Jakš, Ing. Martin Musil, Bc. Josef Mareš. Všichni výše jmenovaní studenti pracovali pod vedením autora habilitační práce.

3.3. Využití dosažených výsledků pro praxi

Výsledky této habilitační práce v praxi, pokud vím, dosud využity nebyly, přesto mají potenciál z hlediska ochranářského (studium invazních druhů a adaptability druhů na změny životního prostředí), z hlediska ochrany vod (studium vlivu polutantů na vodní organismy a jejich odezvy), či z hlediska rozvoje využití raků jako modelových organismů pro celou řadu vědeckých odvětví.

Chování organismů často neovlivňuje jen jejich populace, ale i populace ostatních organismů, a tak může mít dopad na strukturu celých ekosystémů. U raků, často označovaných jako klíčové organismy sladkovodních ekosystémů to pak platí určitě. Informace o chování raků mohou být teoreticky přínosem i pro akvakulturní chovy.

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5. Seznam prací autora, které jsou součástí habilitační práce

Součástí habilitační práce je celkem 10 publikovaných prací. Všechny práce byly publikovány v oponovaných mezinárodních vědeckých časopisech s přiřazeným impact faktorem (IF) v databázi Web of Science společnosti Thomson Reuters. Práce jsou číslovány dle jejich příslušnosti k jednotlivým cílům práce uvedeným výše. Uvedené hodnoty IF u publikací se vztahují k datu publikování článků v jednotlivých časopisech nebo poslednímu známému IF žurnálu v dobu sepisování habilitační práce, pokud pro rok publikace IF ještě nebyl znám. U každé publikace je rovněž uveden aktuální citační ohlas (SCI) v době sepisování habilitační práce.

Příloha č. 1: Kubec, J., Kouba, A., **Buřič, M.**, 2019. Communication, behavior and decision making in crayfish: A review. Zoologischer Anzeiger 278: 28-37. (IF2018=1.601; SCI 2020 = 7)

Příloha č. 2: Hossain, M.S., Patoka, J., Kouba, A., Kubec, J., **Buřič, M.**, 2018. Clonal crayfish as biological model – a review on marbled crayfish. Biologia 73: 841-855. (IF2018 = 0.728; SCI = 16)

Příloha č. 3: Aquiloni, L., **Buřič, M.**, Gherardi, F., 2008. Crayfish females eavesdrop on fighting males before choosing the dominant. Current Biology 18 (11): 462-463. (IF 2008 = 10,777; SCI = 41)

Příloha č. 4: Kouba, A., Tíkal, J., Císař, P., Veselý, L., Fořt, M., Příborský, J., Patoka, J., **Buřič, M.**, 2016. The significance of droughts for hyporheic dwellers: evidence from freshwater crayfish. Scientific Reports 6: 26569. (IF2016 = 4.259; SCI = 28)

Příloha č. 5: Buřič, M., Fořt, M., Bláha, M., Veselý, L., Kouba, A., 2016. Crayfish bury their own exuviae - a newly discovered behavioral pattern in decapods. SpringerPlus 5:1674. (IF2016 = 1.130; SCI = 1)

Příloha č. 6: Hossain, M.S., Kubec, J., Kouba, A., Kozák, P., **Buřič, M.**, 2019. Still waters run deep: marbled crayfish dominates over red swamp crayfish in agonistic interactions. Aquatic Ecology 53: 97-107. (IF2018 = 2.505; SCI = 6)

Příloha č. 7: Kubec, J., Kouba, A., Kozák, P., **Buřič, M.**, 2019. Females bet on the known: crayfish females recognize and prefer males from familiar population, males are not picky. Hydrobiologia 842: 31-38. (IF2018 = 2.325; SCI = 0)

Příloha č. 8: Buřič, M., Grabicová, K., Kubec, J., Kouba, A., Kuklina, I., Kozák, P., Grabic, R., Randák, T., 2018. Environmentally relevant concentrations of tramadol and citalopram alter behaviour of an aquatic invertebrate. Aquatic Toxicology 200: 226-232. (IF2018 = 3.794; SCI = 22)

Příloha č. 9: Hossain, M.S., Kubec, J., Grabicová, K., Grabic, R., Randák, T., Guo, W., Kouba, A., **Buřič, M.**, 2019. Environmentally relevant concentrations of methamphetamine and sertraline modify the behavior and life history traits of an aquatic invertebrate. Aquatic Toxicology, 213: 105222. (IF2018 = 3.794; SCI = 6)

Příloha č. 10: Hossain, M.S., **Buřič, M.**, Moore, P.A., 2020. Exposure paradigm of fluoxetine impacted the Faxonius virilis agonistic behavior differently. Science of the Total Environment 699: 134300. (IF2018 = 5.589; SCI = 0)

6. Český abstrakt

Buřič, M., 2020. Chování raků jako nástroj pro výzkum invazních druhů a měnících se podmínek prostředí. Habilitační práce, Jihočeská univerzita v Českých Budějovicích, Fakulta rybářství a ochrany vod, Vodňany, 166s.

Habilitační práce je tvořena komentovanou kompilací 10 vědeckých publikací vybraných z prací autora tak, aby naplňovala tři tématické celky. První celek je tvořen přehledovými pracemi uvádějícími problematiku dalších dvou tematických celků, a to „Behaviorální adaptace a interakce raků“ a „Chování raků vyvolané znečištěním prostředí“.

Raci jsou zajímavou skupinou sladkovodních organismů, ve které můžeme nalézt jak kriticky ohrožené druhy, tak celosvětově nebezpečné invazní druhy. Raci, pokud se na lokalitě vyskytují, obvykle tvoři naprostou většinu biomasy bentických organismů a jsou potravou širokého spektra predátorů. Sami pak působí jako konzumenti všech dostupných zdrojů potravy a zprostředkovávají tak tok živin a energie ve vodním ekosystému. Raci jsou proto velmi často považováni za klíčové druhy vodních ekosystémů a zároveň ekosystémové „inženýry“. Studium vzájemných interakcí raků, či neprobádaných specifik jejich sociálního chování a komunikace stále představuje velkou výzvu pro výzkum. Ukazuje se také, že raci jsou velmi slibnými modelovými organismy použitelnými v ekotoxikologii, ale třeba i medicíně.

Tato habilitační práce přináši nové poznatky o schopnostech chemické a vizuální komunikace raků, o nových behaviorálních parameterech, stejně jako o interakcích raků mezi sebou a prostředím včetně jejich adaptace nepříznivým vlivům. Kromě toho ukazuje raky jako vhodné organizmy pro posuzování nebezpečných kontaminantů vodního prostředí.

Klíčová slova: rak, biologické invaze, chování, komunikace, interakce, sucho, životní prostředí, znečištění, behaviorální odezva, dynamická expozice.

7. Anglický abstrakt

Buřič, M., 2020. Crayfish behaviour as a tool for the research of invasive species and the changing environmental conditions. Habilitation thesis, University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Vodňany, 166pp.

Habilitation thesis consists of ten scientific publications selected from the publication list of the author to fulfil three thematic units. The first unit comprises two review articles providing a knowledge basis for following two units: „Behavioral adaptations and interactions of crayfish“, and „Crayfish behaviour affected by environmental pollution“.

Crayfish are an interesting group of freshwater animals, where critically endangered species, as well as invasive dangerous species, can be found. Crayfish, if present in the ecosystem, usually constitute a bulk of the biomass of benthic organisms and serve as a prey for wide spectre of predators. Moreover, they act as consumers of all available food sources, and managing the nutrients and energy flow in freshwater ecosystems. Crayfish are therefore often called keystone species of freshwater ecosystems and even ecosystem engineers. The study of intra- and inter-specific interactions of crayfish or not known specifics of their social behaviour and communication are still great challenges for scientific research. It is also obvious, that crayfish are very promising model organisms usable for ecotoxicology, but also in medical research.

Presented habilitation thesis shows new findings about chemical and visual communication in crayfish, about their new behavioural patterns, as well as about their intra- and inter-specific interactions, their interactions with environment, and about their adaptability to the changing environmental conditions. Besides those topics, thesis shows crayfish as usable organisms for an assessment of contaminants of freswaters.

Key words: crayfish, biological invasions, behaviour, communication, interactions, drought, environment, pollution, behavioural response, dynamic exposure.

8. Poděkování

Rád bych prvně poděkoval své rodině za podporu v průběhu mé vědecké práce. Díky samozřejmě z velké části patří všem mým kolegům z Fakulty rybářství a ochrany vod ve Vodňanech, kde bych vypíchl zejména jména Antonína Kouby a Pavla Kozáka. Bez pomoci všech kolegů, ale i studentů bych prezentovaných výsledků dosáhl velmi těžko. Dále bych na tomto místě rád poděkoval i kolegům ze zahraničí, kteří rovněž formovali můj pohled na výzkumnou práci a řešená témata.

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9. Přílohy

Příloha č. 1

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Communication, behaviour, and decision making in crayfish: A review

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ABSTRACT

Animals exhibit behavioural patterns characterized by responses to information received from internal and external stimuli. The ultimate response or decision is based on physical, chemical, or biological factors and their analysis by the nervous system, and may be influenced by previous experiences, stressors, and limitations. This review summarizes behavioural patterns connected with decision making in crayfish, and includes similarities to vertebrates and other invertebrates that may be applied to freshwater crayfish. Concurrently, purpose of this review is introduce behavioural patterns from outside and inside of crustaceans taxa, in which might crayfish have potential exhibit them. Knowledge of patterns influencing behavioural responses and decisions in organisms is necessary for understanding basic biological relationships and for extraction of information applicable to crayfish. Information on sensory and nervous systems, communication, social behaviour, reproductive behaviour, and decision making within the context of species success and adaptations to changing environments will contribute to a model design for future studies of reproductive and social behaviour and behavioural ecology of crayfish. The study of crayfish behavioural patterns can extend knowledge of habitat and reproductive requirements of native crayfish as well as investigate success and impacts of non-native species in environment.

1. Introduction

From the perspective of ethology, studying communication and signal reception is important for understanding behavioural patterns of organisms. Behaviours are dependent on multiple factors, including but not limited to biological and chemical stressors (Moller & Milinski 1998). Communication signals play significant roles in animal societies during important events such as reproduction (sex and maturity recognition, mate choice) and in intra- and inter-specific competition. Animals employ a wide range of signals to share or send information regarding their identity, status, mood, position, and experience of the environment (Bradbury & Vehrencamp 1998). Organisms are able to make decisions and act on preferences for mating, feeding, homing, and survival based on interaction of signals and sensory organs, previously known information, and learning abilities (Atema 1988; Bierbower et al. 2013; Veselovský 2005). Fig. 1.

In this review, we provide an overview of current knowledge about communication processes and their effect on behaviour and decision making in crayfish. Within this topic, it is necessary to integrate the disciplines of neurophysiology, physiology, ecology, behaviour, and even psychology such as personality. Similar integration of disciplines is used in investigations of animals ranging from mammals (Veselovský 2005) to small arachnids (Chapin & Hebets 2016) and worms (Capowiez & Bérard 2006) which have been the subject of studies, with no limitations on behaviours observed.

Crustaceans are widely used as model animals for behavioural studies, largely due to their global distribution (Briffa 2013; Huxley 1880). They are ecologically important mostly benthic macroinvertebrates often regarded as keystone species (Crustacea, Decapoda, Astacida) as well as ecosystem engineers and bioindicators (Fureder & Reynolds 2003; Kouba et al. 2016). Freshwater crayfish, with their high level of social interaction in both the field and the laboratory, offer advantages over many other invertebrate groups (Gherardi 2002). Crayfish are physiologically, ecologically, and behaviourally remarkable animals occurring in a wide range of habitats including lentic and lotic waters such as springs, lakes, ponds, creeks, rivers, alpine and subtropical streams.

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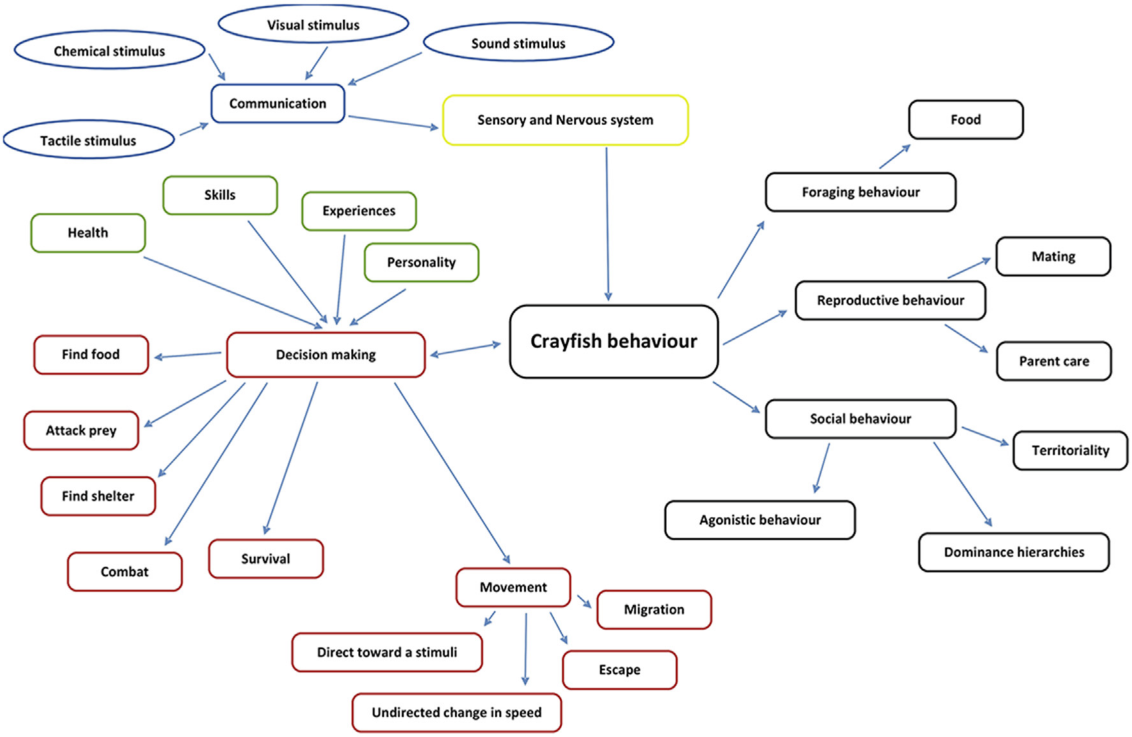

Fig. 1. Illustration of flow map of behaviour in crayfish.

They exhibit diverse life histories, including a broad spectrum of behavioural patterns and show widespread natural distribution patterns that have been further extended via introductions and subsequent spread into new areas (Souty-Grosset et al. 2006). Even their adaptive capacity as long-term survival of Astacidea into brackish water (Holdich et al. 1997) or tolerance of warm-water crayfish (Karplus et al. 1998; Veselý et al. 2015) to cold water in winter period plays role in their success in new areas. Some are significantly invasive, causing damage to freshwater habitats (Crandall & Buhay 2008; Holdich et al. 2009). They are able to dig burrows and can be found in subterranean and semi-terrestrial environments (Holdich 2002; McMahon et al. 2005; Taylor 2002). Crayfish exhibit their highest activity in darkness (McMahon et al. 2005) and possess highly developed sensory organs for movement, orientation, food searching, and communication (Basil & Sandeman 2000). All these features make the crayfish as a suitable model species for ethological studies with broad applicability across research disciplines.

2. Sensory and nervous system

Animals are closely connected to their physical and chemical environment, with which they maintain continuous interaction via their senses. They have adapted sensory organs for obtaining specific information characteristic of the environmental conditions under which they live (Atema 1988). Animals operate in different environments, and their sensory organs and behaviours have unique constraints and opportunities to acquire and filter stimuli from the variety of signals typical of their habitat (Atema 1988; Hu et al. 2003; Palacios et al. 1996).

In crustaceans, as in many other animals, the sensory system comprises interoceptors and exteroceptors that transmit information from the internal and external world to the central nervous system. Interoceptors relay information about the position and motion of the body, while chemo- and baro-receptors monitor the chemical state of body fluids and blood pressure. Exteroceptors include sense organs such as compound eyes, chemoreceptors, and mechanoreceptors that detect stimuli arriving at the surface of the body from a distance: light, scent, sound, and hydrodynamic stimuli (Anger 2001; Brusca & Brusca 2003; Vogt 2002).

The crayfish central nervous system comprises the cerebral ganglion (brain) formed by the fusion of anterior ganglia, together with a ventral nerve cord located below the alimentary tract (Holdich & Reeve 1988; Sandeman et al. 1992). Five paired nerves and two unpaired nerves originate in the crayfish cerebral ganglion. The paired nerves include the oculomotor, antennular, antennary, optic, and tegumentary nerves. The unpaired nerves link the brain with the neural network of the suboesophageal ganglion (Sandeman & Sandeman 2003; Tautz & Muller-Tautz 1983; Vogt 2002). This ganglion splits into nerve roots on each side that proceed to cephalothoracic limbs and continue caudally to connect with muscles of the thorax and the thoracic and abdominal ganglia (Brusca & Brusca 2003; Vogt 2002).

Both groups of nerves serve as conduits for axons of both sensory and motor neurons. The axons terminate in synapses, sites of transmission of information between neurons and motor cells, sensory receptors, and the brain (Anger 2001; Atema 1988; Brusca & Brusca 2003).

3. Bio–communication

Appropriate social, sexual, and defence behaviours depend on mutual understanding. Communication provides location and harmonic coexistence of social and reproductive partners, as well as warning against an enemy (Gherardi 2002; Veselovský 2005). It is a process of information exchange that includes participants or participant groups, a sender and a receiver. The sender creates a signal that contains codes transmitted by specific channels through the environment to the receiver. The selection of the proper channel, whether optic, acoustic, tactile, or olfactory (Table 1), is related to sender ability and the sensory facilities of the intended receiver (Bradbury & Vehrencamp 1998; Hopkins 1988). The receiver‘s sense organs intercept and decode the signal and send the information to the nervous system where it is processed (Vogt 2002). The receiver uses the information in the decision-making process and selects an appropriate response (Dawkins & Krebs 1978). In bio-communication, the transmitted signal generally prepares sender and receiver for new circumstances, which might be manipulation of one or the other or a challenge to mutual cooperation (Schaller 1978). However, reception of signals may vary both among species and among conspecifics. The communicating participants may encounter eavesdroppers such as parasites, predators, potential prey, or rivals (Brandley et al. 2013). A sender may err in the assessment of a new situation and imperfectly encode the signal. However, in bio-communication both sender and receiver should benefit from the information exchange (Bradbury & Vehrencamp 1998).

3.1. *Vision and visual communication*

Crayfish possess a complex visual system including well-developed compound eyes, one on each eyestalk that can be moved by oculomotor muscles (Vogt 2002). The compound eye contains multiple lenses, each associated with a receptor or a cluster typically made up of eight photoreceptors. The typical compound eye contains one hundred lens systems and receptor group units, called ommatidia (Land 2005; Porter et al. 2010) that constitutes part of a composite image that is assembled and transmitted to the brain (Bradbury & Vehrencamp 1998). In the majority of decapod crustaceans (crayfish, lobster, Caridean and penaeid shrimp, and the anomuran galatheids) the ommatidia on the eye surface are square, and the structures beneath are not rigid cylinders, but pyramids of soft jelly (Land 2004; Land 2005).

Advantages of vision are accurate determination of course, rapid dissemination, and detection of movement (Alcock 2001; Veselovský 2005). The transmission of visual signals from sender to receiver over long distances is influenced by terrain barriers, availability and quality of ambient light, and light transmission conditions such as turbidity (Bradbury & Vehrencamp 1998). Visual communication involves cues such as colour or patterning, bioluminescence (Haddock et al. 2010), size and shape of morphological structures (Palmer 2009), and resources (shelters), often in connection with elaborate courtship behaviour (Díaz & Thiel 2004). For example, ground-nesting birds can distinguish a flying predator according to body shape (Manning & Dawkins 2012). Honeybees use visual communication through dancing movements (Dornhaus & Chittka 2004; Wenner 2002). In the absence of light, the frequency of visually-mediated behaviours decreases (Bruski & Dunham 1987).

Exploitation of visual signals has been observed in male *Ocypode* crabs. Pyramids of sand placed in front of burrows are typical for this genus, and males use these readily visible signs to mark their territory and to attract females (Christy et al. 2002; Linsenmair 1965). Many animals possess ornamentation or weapons that may also function as visual signals (Hughes 1996), as in fiddler crab (genus *Uca*) males, which wave their larger claw in species-specific undulations during courtship (Zeil & Zanker 1997). Colour ornaments feature in mate choice of cichlid fish (Baldauf et al. 2010) and in crabs that display prominent colour patterns on the carapace and claw (Cummings et al. 2008) serve for recognition and mates attraction. A similar behaviour is seen in the snapping shrimp (Alpheidae) (Schein 1977). Equally in crayfish, vision plays a role during the initial stages of agonistic behaviour (Bruski & Dunham 1987), facial recognition of previous opponents (Van der Velden et al. 2008), and in selection of a reproductive partner (Aquiloni & Gherardi 2008c; Snedden 1990).

Table 1
Types of signals and general characteristics. Adapted from Alcock (2001).

Properties	Signal			
	visual	acoustic	tactile	chemical
Range	limited	long	short	long
Transmission	rapid	rapid	rapid	slow
Limited by barrier	yes	no	yes	no
Localization	accurate	variable	accurate	variable
Energy expenditure	low	high	low	low

3.2. *Water–borne acoustic communication*

In crayfish, receptors receive acoustic signals or vibrations using the hair pit organ on the chelae, well-suited to measure water velocity (Vogt 2002); statocysts; hair-like setae cells on the body surface; and chordotonal organs associated with antenna joints, legs, and other body appendages. These mechanoreceptors consist of one or more hairs that are associated with sensory cells. Mechanical disturbances from an acoustic signal result in stimulation of the sensory cells (Popper et al. 2001). These sensory cells are often organized tonotopically, *i.e.* different parts are stimulated by different frequencies (Rosenthal & Ryan 2000). Acoustic stimuli can also be sensed by mechanoreceptors that monitor the deformation of the antennal flagella, as well as by the statocysts. The antennae have external and internal joint receptors that react to weak water vibrations, gravity, and possibly to angular acceleration (Vogt 2002).

Similar to light signals, sound signals can be highly degraded when travelling over long distances. High frequencies attenuate and become scattered by obstacles to a greater extent than do low frequencies in all types of habitat including aquatic (Manning & Dawkins 2012). The transmission of a long-distance acoustic signal involves challenges such as attenuation, pattern loss, and ambient noise (Bradbury & Vehrencamp 1998).

Three successive steps in sound production are essential for acoustic communication in an aquatic environment: The production of vibrations carrying specifically coded information, modification of the acoustic vibrations to match biological function such as mechanoreceptors, and finally the coupling of the modified vibrations to the medium in which the sound is propagated (Bradbury & Vehrencamp 1998). In comparison to terrestrial animals, aquatic organisms have an advantage in coupling of sound to medium, as sound waves travel about 5 times faster in water than in air (Hopkins 1988). Acoustic organs of aquatic animals have an impedance sufficiently similar to water that the sounds they produce are transmitted over long distances without the aid of resonant structures (Bradbury & Vehrencamp 1998). Production of acoustic signals by some fish is used predominantly as a tool for attracting females and for mate choice. This pattern has been reported in male plainfin midshipman *Porichtys notatus*, which creates sound using the swimbladder (Brantley & Bass 1994); in gobiid fish *Zosterisessor ophiocephalus* males, which emit sound via head movements and enlargement of the branchial cavity (Malavasi et al. 2003); and in several cichlid species (Longrie et al. 2013). Some cyprinids and silurids also emit acoustic signals using muscular contraction and expansion of the swimbladder (Bradbury & Vehrencamp 1998; Radford et al. 2014). Evidence of acoustic signals is reported, albeit rarely, in crustaceans, such as in snapping shrimp, which strike parts of their claws to make cracking noises

(Versluis et al. 2000). The purpose of snapping shrimp behaviour seems to be to disturb the communication of other animals rather than to communicate (Lohse et al. 2001), whereas semi-terrestrial crabs produce and use sound for communication for social interaction and probably to attract mates (Popper et al. 2001). The acoustic signals of the spiny lobster *Panulirus interruptus* can be used as a startle or aposematic cue to predators (Staaterman et al. 2010).

Sensitivity to hydrodynamic stimuli has been described in the red swamp crayfish *Procambarus clarkii* (Monteclaro et al., 2011) and spiny-cheek crayfish *Orconectes limosus* (Tautz, 1987), but the use of acoustic signals for communication or defence has not been observed.

3.3. *Tactile communication*

Touch plays a prominent role in signalling and discrimination in invertebrates, to distinguish objects, for movement, and orientation, as well as for demonstration of social relationships and to calm aggressive behaviour (Alcock 2001; Bradbury & Vehrencamp 1998). Arthropods obtain information of the near surroundings through the tactile response (Wiese 1993). The major intermediaries for tactile sensory tasks are antennules and antennae. These organs possess both chemosensors and mechanosensors, but antennules primarily have an olfactory function, while the antennae respond chiefly to mechanosensory stimuli (Vogt 2002; Wiese 1993). In crayfish, each antenna consists of basal segments and a long segmented flagellum. In some species, the antennae are as long as the body and extremely flexible. These mechanoreceptive organs assist crayfish in determining the position of objects as well as their own position, posture, and movement by using information from receptors on the flagellum (Vogt 2002; Zeil et al. 1985). Stimulation of sensilla on the flagellum assists in sensing a stimulus (Masters et al. 1982) and this provides information about an object and the distance to it (Zeil et al. 1985). Despite this, evidence of the use of antennae during exploration of new environments is limited. There is no available information about the extent that crayfish explore or navigate in confined spaces or whether mechanosensors are actively involved. On the other hand, in natural aquatic environments, crayfish inhabit areas that contain crevices, stones, and wood debris that restrict or impede movement and force decisions about travel over or around obstacles primarily based on information gained from mechanoreceptors (McMahon et al. 2005).

3.4. *Olfactory receptors and chemical communication*

The chemical signals, pheromones, belong to the oldest communication systems, and are species-specific. Pheromones can have different functions, such as sexual, alarm, and territory-marking, depending on molecular weight (Alcock 2001; Bradbury & Vehrencamp 1998; Breithaupt & Thiel 2011). Some animals, especially aquatic, despite other available senses, primarily use olfactory cues in inter- and intra-specific interactions to decide what they should eat, run from, fight with, or mate with (Breithaupt & Thiel 2011; Hay 2011).

In an aquatic environment, chemical signals influence feeding, habitat, and mating choices and stabilize dominance in hierarchies (Hay 2011). Hence, chemical communication is well developed and widely used by aquatic organisms (Wyatt 2011). Chemical communication is widely exploited in mate attraction, such as reported in blind livebearer *Poecilia mexicana* females, which secrete a substance increasing their sexual attractiveness to males (Tobler et al. 2008). Goldfish and other teleost fish release reproductive pheromones by both females and males (Stacey et al. 1994; Van Den Hurk & Resink 1992). These pheromones not only attract mates, but mediate increasing milt production and enhance fertility in males (Barki et al. 2011). African jewelfish *Hemichromis bimaculatus* use the olfactory system in parental care. Females are able to distinguish their own offspring even at different ages of juveniles (Immelmann et al. 1996). A similar finding of recognition of independent juveniles in crayfish would be interesting. Olfaction is also used in fish including the Atlantic salmon *Salmo salar* (Johnstone et al., 2012; Stabell, 1984) and the European eel *Anguilla anguilla* (Churcher et al,. 2015) for the long-distance migration to their home locations for reproduction (Ueda & Shoji 2002).

Crustaceans, essentially occurring in all environments including aquatic as well as terrestrial habitats, face numerous challenges in communicating with conspecifics (Thiel & Breithaupt 2011). All crustaceans possess a carapace, which impedes diffusion of chemical substances into the environment (Hallberg & Skog 2011). In the aquatic environment, most pheromones are contained in the urine, which is released through the nephropores (Breithaupt & Thiel 2011). Chemoreceptors have evolved for interception of chemical signals in crustaceans (Vogt 2002).

In crayfish, the olfactory system comprises receptor neurons on the antennules, paired olfactory and accessory lobes, and various cell clusters in the brain (Vogt 2002). The chief olfactory detection system is represented by chemoreceptors, the chemosensilla, and the aesthetascs. The chemosensilla are innervated structures of small, often hair-like cuticular organs with sensory cells (See Breithaupt et al. 2016). The sensilla are mainly concentrated on the oral appendages and tips of the pereopods (Breithaupt 2011; Hallberg & Skog 2011). At these sites, chemoreceptors receive signals from chemical sources that are in direct contact with the animal, similar to taste in vertebrates (Hallberg & Skog 2011). The aesthetascs are generally located in the outer flagella on the antennules and are composed of cuticular hairs. Aesthetascs serve to mediate reception of chemical signals such as prey scent or sex pheromones released by conspecifics from a long distance too (Breithaupt 2011; Hallberg & Skog 2011; Vogt 2002). In addition, the major chelae contain necessary chemosensory structures for recognition of female scent and can be an alternative site of reception (Belanger & Moore 2006).

In crustaceans, chemical signals are one of the most significant messengers. They can effectively transmit coded information about specific properties such as sex, sexual receptivity, species identity, health status, motivation to fight, dominance, individual identity, and moult stage and communicate information about important functions throughout life stages (Thiel & Breithaupt 2011). Pheromones are powerful elements in agonistic behaviour and hierarchy establishment (Atema & Steinbach 2007). Lobsters can recognize individuals that they have previously encountered by chemical scent and behave appropriately given their relative dominance (Hay 2011). The hermit crab *Pagurus longicarpus* can distinguish individual conspecifics and their status by chemical scent (Gherardi et al. 2005). Memory of crustaceans in similar cases has been demonstrated to last from one to two weeks (Karavanich & Atema 1998). Chemical scents and signals are used by crustaceans in foraging, especially the food specialists such as the turtle-weed crab *Caphyra rotundifrons* (Hay, 2011).

A major role for pheromones is in mediating reproductive behaviour. The first evidence of a female sex pheromone in a decapod crustacean was demonstrated in the black-spotted swimming crab *Portunus sanguinolentus* (Ryan, 1966). Some male crustaceans, including the green crab *Carcinus maenas* and the American lobster *Homarus americanus*, often guard females through moulting and subsequent egg laying. Mating is restricted to a short time, signalled by sex pheromones. Intermoult females can be rejected by males, since they do not secrete the pheromone indicative of the correct moult stage (Atema & Engstrom 1971;

Hardege et al. 2002). Sex pheromones have also been detected in rock shrimp *Rhynchocinectes typus* (Díaz & Thiel, 2004) and signal crayfish *Pacifastacus leniusculus* (Stebbing et al., 2003).

The use of chemical signals in crayfish is similar to other crustaceans, but in reproduction is not related to the female moulting period (Reynolds & Holdich 2002; Stebbing et al. 2003). Mating in crayfish occurs during the intermoult stage and is dependent on prior agonistic interactions between the sexes (Reynolds & Holdich 2002). Females release chemicals in urine during aggressive acts. Males recognize female receptivity via these urinary components and initiate courtship (Berry & Breithaupt 2010). In dominance competition, importance of chemicals and olfactory receptors of the combatants was observed in red swamp crayfish (Schneider et al. 1999), rusty crayfish *Orconectes rusticus* (Bergman et al., 2003), and narrow-clawed crayfish *Astacus leptodactylus* (Breithaupt, 2011).

4. Social behaviour

In ethology, social behaviour is defined as interactions between two and more individuals, typically within a group of conspecifics (Krebs & Davies 1997; Manning & Dawkins 2012). Examples include coordination of activities between males and females in mating and caring for offspring and the integration of members of group-living species (Bradbury & Vehrencamp 1998; Krebs & Davies 1997). Female crayfish care for eggs while they are attached to the pleopods (Gherardi 2002) using shaking movements, and juveniles are cleaned and fanned to ensure that they receive sufficient oxygen (Vogt 2013). Crayfish males may prey upon juveniles (Mathews 2011). Social behaviour in parental care is demonstrated by recognition and care of conspecific offspring (Beermann et al. 2015).

In addition to providing active assistance and support, social behaviour may be aggressive (Manning & Dawkins 2012). Efforts of an individual to manipulate another member of society to obtain some advantage is an important aspect of social behaviour (Krebs & Davies 1997; Manning & Dawkins 2012). Aggressive behaviour is characteristic of all life stages of crayfish. It is typical of inter- and intra-specific relationships and appears during conflicts for resources such as food and shelter, preferred habitat, and a reproductive partner (Gherardi 2002; Krebs & Davies 1997). Agonistic behaviour is primarily demonstrated by increased aggressiveness between participants of conflict. The use of chemical or visual communication signals to intimidate an opponent can be a substitute for combat and avoid higher costs of physical aggression (Breithaupt & Thiel 2011) (Fig. 2). When pre-fight signals fail, opponents commence the characteristic fight for resources or a mate. This may lead to energy depletion, time lost from foraging or mating, and injury or death of one or both combatants. Opponents are also more prone to predation during conflicts (Jakobsson et al. 1995; Payne 1998). A functioning social hierarchy within the animal group can forestall overt fighting (Gherardi 2002; Manning & Dawkins 2012; Neumann et al. 2011) and ensure the safety of individuals. Generally, hierarchy is based on dominance from the strongest to the weakest, with the dominance rank determined by experience and fitness (Gherardi & Daniels 2003; Manning & Dawkins 2012; Neumann et al. 2011). Studies show dominance hierarchies to be based on strength, size, age category, sex, and ability to manipulate others, as well as ability to recognize individual (Aquiloni et al. 2008; Fero & Moore 2014; Seebacher & Wilson 2007). Even, balance in hierarchy is necessary in social behaviour, high winner effects with low loser effects result in an overall increasing of aggressiveness and lead to decrease of stability of social group (Hock & Huber 2009).

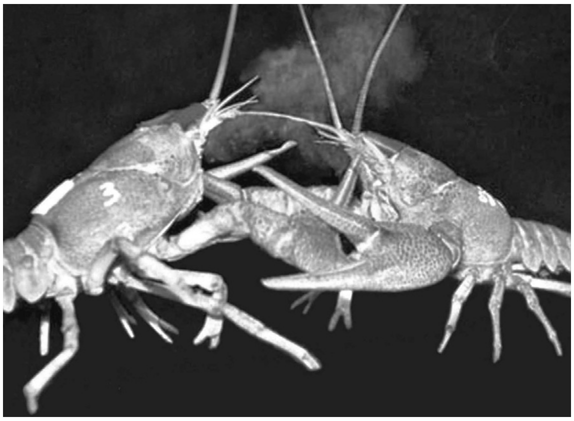

Fig. 2. Chemical communication visualized using Fluorescein dye during aggressive interaction of two males narrow-clawed crayfish (Breithaupt 2011).

Opponents can tolerate only a certain level of direct physical damage in fights. In some cases, fighting may not occur, due to lack of time and physical costs such as loss of body parts, and vulnerability to predation or death. Hence, a potential combatant may decide whether to initiate, persist in, or retreat from combat (Payne 1998).

Agonistic inter- and intraspecific interactions occur when a stranger invades to a crayfish's territory (Bruski & Dunham 1987). In the wild, crayfish are solitary and territorial animals, with rare cases of eusociality (Vogt 2013). Territoriality comes with a hierarchy in which a dominant animal has greater access to food and mates (Delgado-Morales et al. 2004; Fero & Moore 2014). Goessmann et al. (2000) observed that dominant animals and individuals occupying dominant positions became increasingly likely to escalate to higher fight intensity and are less likely to retreat, in the noble crayfish *Astacus astacus*. Manifestation of the agonistic behaviour in crayfish includes a combination of postural displays using the chelipeds, antennae whipping, or walking legs and physical contact in the form of grappling (Kravitz & Huber 2003). Individuals may also use chelipeds to inflict injuries on the opponent (Briffa 2013). Crayfish are able to not only recognize conspecifics, but also the position and characteristics of chelae and the length and thickness of antennae may decide (Delgado-Morales et al. 2004). Red swamp crayfish show the ability to recognize the dominance status of conspecifics (Schneider et al. 1999). Agonistic behaviour is more affected by distribution and availability of shelters and the distance to shelter than is past social experience and current social status (Chibucos et al. 2015). Competition for important resources indirectly increases vulnerability to predation and cannibalism (Figler et al. 1999). Agonistic behaviour and fighting occurs in females as well as males. Female on female competition occurs in establishing dominance and in competition for males (Jennions & Petrie 1997), but female–female combats are not fatalistic and frequent as in males.

Social interactions are not restricted to relationships between conspecifics, but also occur in encounters between native species and invaders. Invasive crayfish species are generally characterized by higher aggressiveness, which enables them to dominate native species. In European region, both signal crayfish and spiny-cheek crayfish are more capable of establishing clear dominance via their greater tendency to continue engagement in high-intensity combat than native species which are losing their habitats (Hudina & Hock 2012). Impact of invasive crayfish interactions is not European problem, it is global. Klocker & Strayer (2004) show in a study of interactions among native and non-native species that elsewhere dominant species spiny-cheek crayfish is oppressed by an even more aggressive rusty crayfish in native habitat.

5. Reproductive behaviour

Reproductive behaviour of animals is a significant manifestation of their life (Alcock 2001; Gherardi 2002; Krebs & Davies 1997) with the exception potential behavioural patterns of species with asexual reproduction such as the marbled crayfish *Procambarus fallax f. virginalis* (Scholtz et al., 2003), where this is still an unexplored field. Most animals are distinguished by sexual differentiation reflecting preferences and requirements of potential mates (Manning & Dawkins 2012). Reproductive behaviour is preceded by courtship, creating of pairs and mechanisms of synchronization and consolidation of relationships between male and female (Alcock 2001; Bradbury & Vehrencamp 1998). In crayfish, reproduction is often tied to factors such as temperature, photoperiod, and season (Gherardi 2002).

In sexual selection, individuals attempt to increase the chances of successful reproduction and the fitness of offspring by choosing the best available mate on the basis of visual (size, coloration), chemical (pheromones, major histocompatibility complex), and behavioural (courtship behaviour, agonistic interactions, dominance status) cues (Alcock 2001; Bakker et al. 1999; Chenoweth & Blows 2006; Partridge 1980; Roberts & Gosling 2003). The right partner most often is of the same population or conhabitant (Lande 1981; Roberts & Gosling 2003; Veselovský 2005). Factors involving sexual history, dominance status, size of body parts, investment in offspring, and fitness provide a decisive advantage in partner finding (Jennions & Petrie 1997). During the selection of a reproductive partner, individuals often engage in agonistic interactions with competitors (Alcock 2001; Hunt et al. 2009). Male competition for a female has been observed in crayfish. This competition is not always decisive, since the female may choose to receive or refuse either male, regardless of the outcome of the battle (Gherardi 2002; Mellan et al. 2014; Villanelli & Gherardi 1998). Males have been observed to remove spermatophores deposited by other males by eating them and subsequently replacing them with their own (Snedden 1990).

An important aspect of successful reproduction is recognition by both partners. Males can distinguish at a distance between receptive and non-receptive females based on pheromone signalling (Breithaupt & Thiel 2011; Gherardi 2002). In crayfish, both sexes are polygamous and mate with multiple partners for higher paternity success, obtaining benefits of greater investment in offspring (Bretman et al. 2009; Reynolds & Holdich 2002) such as larger and stronger juveniles from a larger and dominant partner.

Maternal behaviour is another aspect of social and reproductive behaviour. In crayfish, maternal care is important to egg incubation as well as early developmental stages of offspring (Gherardi 2002; Vogt 2013), with females engaging in extended care to protect juveniles from predation and cannibalism (Mathews 2011). Extent of maternal care in crayfish differs with species, from 1 to 2 weeks in the noble crayfish (Skurdal & Taugbøl 2002) to 5 months in *Paranephrops zealandicus* (Whitmore & Huryn/ 1999). In maternal care, chemical communication is employed for protection of juveniles from stresses (Little 1975; Tricarico 2015; Vogt 2013; Vogt & Tolley 2004), especially the brood pheromone, which attracts juveniles back to the female (Little 1975). This pheromone, possibly species-specific, has not been chemically isolated, but its existence has been described based on behavioural changes in mother and offspring (Aquiloni & Gherardi 2008b; Little 1976; Mathews 2011). Vogt (2013) reports return of juveniles to the mother's pleopods as a response to optical maternal signals. Brood protection is also associated with an increase in territoriality against non-maternal individuals and use of shelters by ovigerous and offspring-carrying females (Figler et al. 2001). Female red swamp crayfish and the Neotropical burrowing crayfish *Parastacus pilimanus* accept and protect offspring of other females without aggressive behaviour (Aquiloni & Gherardi 2008b; Dalosto et al. 2012). Mathews (2011) described a female spiny-cheek crayfish that collected unfamiliar juveniles, heavily overloading her pleopods, although genetic analysis of wild living *Orconectes placidus* has shown that females most often carry their own offspring (Walker et al. 2002). The preservation of own offspring remains paramount, as shown by behaviour after juveniles become independent, when females are likely to cannibalize offspring of other females (Mathews 2011; Tricarico 2015).

6. Decision making

The study of decision making is the core of behavioural ecology (Dill 1987). All living organisms must make decisions throughout life regarding feeding, courtship, sleep, escape, competition. Final decisions should maximize, as much as possible, chances for survival and reproductive success (Krebs & Davies 1997; Manning & Dawkins 2012; Tricarico 2015). In other words, they should represent the optimal strategy at a given time. Hypothetically, optimal behaviour can be compared with actual behaviour and decision making of the animal, to shed light on the evolution of behaviours based on experience, conditions, fitness, motivation, and individual personality (Krebs & Davies 1997; Manning & Dawkins 2012; Mcfarland 1977). Decision making processes in animals are complex and sophisticated, since, for any behaviour directed and coordinated by the brain, nerve impulses, muscle movements, and hormone levels must coordinate in specific ways (Krebs & Davies 1997; Manning & Dawkins 2012).

Basic models of optimal behaviour consider relatively simple problems, such as prey size choice, when the sole objective is maximizing net energy intake rate (Dill 1987; Krebs & Davies 1997). Lima (1998) reviewed decision making on diet selection and preference for certain food types when the animal is at risk of predation. In such situations, crayfish preference for food or shelter may be altered on the basis of environmental variations such as the relative risk of predation compared to the relative reward of resource (Fero & Moore 2014; Jurcak & Moore 2014; Lima 1998). On the other hand, food choices of native and non-native species crayfish may differ. The white clawed crayfish *Austropotamobius pallipes* tends to be selective, while invasive species, represented by signal crayfish, are primarily non-selective and choose larger prey than do natives (Haddaway et al. 2012). Buřič et al. (2016) described an unexpected behavioural pattern in signal crayfish and yabby *Cherax destructor*: both species buried their own exuviae after moulting. An animal behaviour may reflect the expression of evidence of decision making for preference of self-protection or creation of mineral storage (Buřič et al. 2016).

In recent years, animal behaviour is seen to reflect expression of individual personality. This phenomenon in non-human animals aids in understanding of many aspects of their ecology (Gherardi et al. 2012). Personality traits, which are typical for higher animals, are found in crayfish (Tricarico 2015). Pintor et al. (2008) observed positive correlations among activity, aggressiveness, and boldness in populations of signal crayfish. More aggressive behaviour of this species compared to non-aggressive and shy *Pacifastacus fortis* might explain the success of signal crayfish (*P. leniusculus*) invasion. Vainikka et al. (2011) suggested, based on observation of decreased resource holding potential, that boldness can be an individual personality trait. Two test groups of noble crayfish (naïve), upon exposure to predator scent showed differences in behaviour. Vainikka et al. (2011) found individuals with higher levels of boldness repeatedly left shelters during periods of varying risk of predation.

Another manifestation of decision making is choice of reproductive partner. Mate choice can be defined as a response to a pattern of sensory and behavioural properties exhibited by potential mates, grounded in benefits derived from choosing certain mates over others. This leads to higher likelihood of mating with the more attractive members of the opposite sex (Halliday 1983; Jennions & Petrie 1997). Many factors that can be seen as meeting criteria of mate quality play a role in mate choice including potential to mate, degree of investment in offspring, and resource holding potential reflected in the ability to defend resources or shelters from intruders (Bateson 1983; Mellan et al. 2014; Rahman et al. 2004; Tricarico 2015). Preference for conspecifics does not necessarily lead to mating with them. Mate choice may be based on mate quality or sperm competition (Galipaud et al. 2015), and leads to hybridization among the noble crayfish and the narrow-clawed crayfish (Jelić et al. 2013; Maguire et al. 2013).

Penn (2002) reviewed the preference observed in vertebrates for mating with individuals carrying dissimilar genes of the major histocompatibility complex (MHC), which may increase heterozygosity of offspring. The observation of mate choice according to the MHC in the female house mouse, *Mus musculus domesticus* confirms the selection of partners according to ability to produce offspring with greater survival (Ilmonen et al. 2009; Penn 2002). In addition to its role in the immune system, the MHC is responsible for scent secretion. Potential mates with dissimilar MHC loci, which produce a different scent than mates with similar loci, are more attractive for female house mice (Penn 2002; Roberts & Gosling 2003; Veselovský 2005). Evidence for MHC-dependent mating preferences has also been found in humans (Havlicek & Roberts 2009; Ober et al. 1997; Wedekind et al. 1995), the Swedish sand lizard *Lacerta agilis* (Olsson et al., 2003), and the Atlantic salmon (Evans et al. 2012; Landry et al. 2001). Most invertebrates do not possess an MHC system (Dishaw & Litman 2009), but similar polymorphic immune genes with a locus involved in histocompatibility have been described in the tunicate *Botryllus schlosseri* (De Tomaso et al., 2005) and the cnidarian *Hydractinia* (Rosa et al., 2010). Considering the capability for chemical communication in crustaceans, including crayfish, they may possess a similar mechanism enabling recognition of related individuals. Unfortunately, any kind of MHC system is still only speculative in crustacean taxa.

In crustaceans, the evidence for preferences in mate choice is similar to results of studies describing mate selection throughout taxa. The preference for larger sized mates has been reported in amphipods (Beermann et al. 2015; Sutherland et al. 2007), the big-clawed snapping shrimp *Alpheus heterochaelis* (Rahman et al., 2004), hermit crab *Pagurus middendorffii* (Wada et al., 2011), and in the spotted spiny lobster *Panulirus guttatus* (Robertson & Butler, 2013). Similar preference was shown in several species of crayfish, including the white-clawed crayfish *A. pallipes* species complex (Galeotti et al. 2012; Gherardi et al. 2006; Villanelli & Gherardi 1998) and the red swamp crayfish (Aquiloni & Gherardi 2008a, c), in which at least one of sexes spent significantly more time in proximity of larger individuals than smaller. Larger body size is positively correlated with fecundity in crayfish females (Aquiloni & Gherardi 2008c; Rahman et al. 2004). Similarly, females, which produce highly costly gametes in a limited reproductive season, prefer larger males which translates to higher dominance status (Aquiloni et al. 2008). It is positively correlate with size of males (Snedden 1990) which produce of larger quantities of sperm to increase chances of fertilization. Female red swamp crayfish are able to discriminate, and prefer, large-sized as opposed to large-clawed males (Aquiloni & Gherardi 2008c). The hypothesis that larger body size leads to higher investment in offspring is supported by Aquiloni & Gherardi (2008a) who confirmed that females copulating with large males produced larger and heavier juveniles. Larger males also exhibit a significantly higher probability of winning competition for acquiring or defending shelters (Figler et al. 2005; Figler et al. 1999).

Sub-preferences have been observed in mate choice. Aquiloni et al. (2008) and Aquiloni & Gherardi (2008c) described higher preference for victors in male–male competition in red swamp crayfish and signal crayfish. However dominance preference among size-matched males was not shown in naïve females, which had not eavesdropped on male–male competition (Aquiloni & Gherardi 2008c; Berry 2008). After the effect of body size, mate choice may be positively affected by shelter ownership, according to a study of big-claw snapping shrimp, with shelter competition success or occupancy and defence influencing pair formation. When presented with small size-matched males, females paired more frequently with those that occupied a shelter (Rahman et al. 2004).

In some cases, individuals may benefit from information gained from prior reproductive experience to make a mate choice. Previously unmated (virgin) spiny-cheek crayfish of both sexes were chosen more often for mating than mated ones by experienced mates. But virgin animals were choosier than those who had mated in mate choice (Mellan et al. 2014). A similar scenario was described in red swamp crayfish males, which were shown to preferentially discriminate and select virgin females rather than mated (Aquiloni & Gherardi 2008c). Hence, individuals seek unmated mates for highest reproductive success.

The speed of decision making as well as the final choice can be significantly affected by stress factors and predation pressure (Lima 1998). Crowley et al. (1991) reviewed studies of terrestrial and aquatic invertebrates such as the snapping shrimp *Alpheus armatus* (Knowlton, 1980), the katydid *Metaballus* sp. (Gwynne, 1985), and the túngara frog *Physalaemus pustulosus* (Ryan, 1985), all of which showed reduced discrimination in mate selection when under predation risk. We suggest that reduced discrimination may be useful in crayfish females, which are generally selective (Aquiloni et al. 2008).

Infection with pathogens and reduced fitness can also play a significant role in decision making. Beltran-Bech & Richard (2014) reviewed the impact of infection on mate choice with possible application to all animals, including crayfish. Parasites from eukaryotes to bacteria and viruses can reduce fitness and decrease attractiveness or lead to errors in decision making.

Mate choice may not be solely dependent on physical factors. Chemical signals, such as pheromones, can influence decision making by providing chemical cues to signal receptivity, specific phases in the reproductive phase, or mated/unmated status (Johansson & Jones 2007). Stebbing et al. (2003) demonstrated the influence of sex pheromones on male signal crayfish. In the presence of female scent transmitted through air-stones, males initiated seizure, mounting, and deposition of spermatophores on the surface of the air-stone.

7. Conclusions

This review provides an overview of social interactions and decision making, including channels for recognition and processing of environmental stimuli, with the primary focus on freshwater crayfish. We reviewed relationships between the environment and the organism and the basic biology of signal processing. These aspects are important, as every decision and interaction results in consequences for survival and reproduction, *i.e.* at some level of species success.

For ethological studies in aquatic ecosystems, crustaceans, especially crayfish represent very suitable model organism.

Crayfish show advantages over other invertebrates due to their high level of social interaction in both the field and laboratory. They exhibit unique eco-ethological behaviour in diversity of feeding habits, activity cycles, habitat selection, and resource utilization at the level of species, sex, and age (Gherardi 2002). Similar to vertebrates, crayfish demonstrate unique behaviours throughout life. They possess ability to recognize, not only conspecifics, but individuals such as mates and offspring as well.

The study of agonistic behaviour is useful for understanding hierarchy in social communities and resource partitioning, and consequently for creating diverse habitats to reduce conflict and possible losses. Knowledge of individual animal personality can be applied to commercial production and animal welfare (Gherardi et al. 2012).

Chemical stimuli have comparatively high impacts on behaviour of crayfish in mating, kin recognition, and social status. There is opportunity for future research to identify the compounds that influence specific behaviours.

The results of recent studies could be applied in conservation research, captive breeding, and recovery programs as well as to aquaculture. Native crayfish are usually studied to extend knowledge of habitat requirements and mating behaviour (Tricarico 2015), while the invasive crayfish species are investigated for their reproductive behaviour, plasticity, and for developing methods for their control (Gherardi et al. 2011). The study of behaviour in crayfish is currently an open research area in both the field and laboratory.

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REVIEW

Clonal crayfish as biological model: a review on marbled crayfish

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

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

Introduction

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

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

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

Probable origin and current distribution

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

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

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

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

Life cycle

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

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

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

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

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

The maximum reported lifespan of marbled crayfish is 1610 days, while it is typically 2–3 years. Under laboratory conditions, marbled crayfish can complete up to seven reproductive cycles within that lifespan (Vogt 2010). Marbled crayfish begin to reproduce when younger than 6 months, at total body length of c. 40 mm (Vogt 2008c), which is early in comparison with other crayfish species (Table 2). Seitz et al. (2005) reported first spawning at 141–255 days (cephalothorax length of 14.0 to 21.5 mm, weight 0.63–2.0 g) at a water temperature of 20–25 °C. However, the onset of maturity in crayfish can be postponed or interrupted by low hierarchical status or unfavourable conditions (Reynolds et al. 2002). In laboratory condition, moulting was identified as the main cause of death for marbled crayfish. Specimens with lowest life span showed fast growth, early onset of reproduction and short time intervals between reproduction cycles. Females reproduced until high age indicating negligible reproductive senescence and active stem cells in different organs at all ages. Regeneration of appendages did not negatively affect longevity. In contrast, social stress shortened life expectancy considerably (Vogt 2012). Therefore, Vogt (2012) emphasizes the suitability of marbled crayfish for general biogerontology, particularly the investigation of non-genetic aspects of ageing.

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

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

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

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

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

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

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

Ecology and adaptability

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

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

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

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

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

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

Behaviour

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

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

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

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

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

Crayfish plague transmission

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

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

The marbled crayfish as a biological model

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

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

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

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

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

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

Conclusions

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

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

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Compliance with ethical standards

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

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Příloha č. 3

Aquiloni, L., **Buřič, M.**, Gherardi, F., 2008. Crayfish females eavesdrop on fighting males before choosing the dominant. Current Biology 18 (11): 462-463. (IF 2008 = 10,777; SCI = 41) previous experimental evidence of a link between these two traits in birds [10] and our finding in pre-manipulated males that heavier individuals had lower concentrations of androgens. The implication of these results is that androgen concentrations change as a function of an individual's signal expression within a social and reproductive context. Furthermore, such a response might be costly, as darkened males lost mass at a time in the reproductive cycle where individuals typically gain weight after having completed their migration to breeding grounds. The hormonal feedback between signal and physiological traits is a likely mechanism for linking signals appropriately to the expression of sexual or aggressive behavior as both signals and the social environment change.

The existing unidirectional view of how physiology and signals are mechanistically related is not sufficient to explain the bidirectional relationship we uncovered between ventral coloration, androgen concentrations and body mass in male barn swallows. The interactions between the expression of signals, their production processes, and the dynamic qualities (e.g., aspects of health and condition) add important complexity to our understanding of sexual signals. For example, if an animal's androgens change due to social stimuli induced by its own signal, that individual will be more or less likely to perform androgen-associated behaviors, such as mating or aggression. Because physiological parameters in addition to the ones we studied here are temporally variable, we expect our results to apply generally to other animal signals. Signals that are flexible and dynamic themselves (i.e. courtship behavior, vocalizations) may even more strongly affect and respond to an individual's current physiological state. The aim in future studies of the signal–physiology nexus should be to determine the suite of social responses to these flexible signals, including traits that advertise resource defense and parental investment, and their effects on specific physiological processes, such as immune function and stress.

Supplemental data

Supplemental data including experimental procedures are available at http://www.current-biology.com/cgi/content/full/18/11/R461/DC1

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Crayfish females eavesdrop on fighting males before choosing the dominant mate

Laura Aquiloni[1], Miloš Buřič[2] and Francesca Gherardi[1]

Identifying the traits that influence mate choice is a major challenge in behavioral ecology and evolutionary biology. Male dominance often conditions mating decisions by females, but how the hierarchical status of potential mates is assessed remains poorly understood. In crustacean decapods, chemical signals convey information about male sexual responsiveness and quality. At least in the red swamp crayfish *Procambarus clarkii*, however, females appeared unable to recognize dominant mates using smell only. Here, we report the first evidence that a form of social eavesdropping may occur in an invertebrate species.

Since its first formulation in the 1990s [1], social eavesdropping has attracted increasing attention by researchers interested in animal behavior and communication networks. The ability of animals to extract information from signaling interactions between conspecifics [1] and the benefits gained from attending to the behavior of others — instead of gathering the same information on one's own — have stimulated a plethora of studies focused on fish [2], birds [1], and primates [3]. By witnessing interactions between two or more individuals (for example, male–male fights or male–female courtships), bystanders assess the relative or absolute quality of the interactants; such information can be later used in an adaptive fashion when the same individuals are encountered in a social context (for example, territorial aggression or mate choice). The costs of mate choice, for example, may be reduced in female guppies by copying the mating decision of other females [4].

Because of the apparent complexity of the neural machinery involved, social eavesdropping has been assumed until now to be a prerogative of vertebrates. As recently reported [5], the behavior of a crayfish can be affected by observing social interactions between conspecifics. Conversely, the literature gives only hints on the occurrence of social eavesdropping in invertebrates. Females of the crab *Uca deichmanni*, for example, were described as soliciting male fights before selecting the dominant as a mate [6]; however, these observations were not supported by dedicated experiments.

Males of the crayfish *P. clarkii* also often engage in intra-sexual fights during the reproductive season in the presence of females. The winners are expected to be favored by female crayfish, because they are more likely successful at accessing or defending limited resources, such as shelters. Surprisingly, when tested in the laboratory with the simultaneous sight and smell of two seemingly identical males that had previously battled to form a hierarchy, females appeared unable to choose dominants over subordinates [7]. This result falsified our original idea that a putative dominance odor, possibly combined with visual signals, might be used by *P. clarkii* females to select the winner; it also apparently contradicts evidence that, along with conveying information about sex, reproductive condition [8], and size [7], chemicals are involved in recognizing the dominance status of a conspecific [9]. We thus hypothesized that, before making a decision between two apparently identical males, females should be allowed to witness their agonistic interactions.

To test this hypothesis, we compared the responses of bystander (n = 15) and naïve females (n = 15) towards a dyad of males (dominant/subordinate). The experiment was composed of two phases: in the first phase (‘fight phase’) the females were either allowed (bystander) or impeded (naïve) to watch and smell two similarly-sized males fighting; in the second phase (‘choice phase’), both types of female were free to choose between the dominant and the subordinate male (see the Supplemental data available on-line for detailed experimental procedures). Our results are clear in showing that eavesdropping on agonistic interactions improves the female’s ability to recognize the dominant male over the subordinate. In fact, bystander females visited the dominant male more often, remained in his proximity for longer, and engaged him in more numerous pre-copulatory contacts (Table 1). Conversely, naïve females showed no preference for any male. This result is not due to visual signals of dominance released by males during the choice phase: the time spent by them raising the claws, as an index of dominance, did not significantly vary either between dominant/subordinate males ($F_{1,60}$ = 1.685, P = 0.200) or between treatments ($F_{1,60}$ = 1.868, P = 0.177). We thus may conclude that the females cannot recognize dominant males from their posture or from some chemical badges but are instead able to extract information from watching male–male fights without being directly involved in those interactions [1].

By eavesdropping on fighting males, *P. clarkii* females seem to make low-cost, direct comparisons between the two potential mates, obtain information about the quality of the signalers (for example, their hierarchical status), and can then use this information to guide their future decisions. A similar experiment on jumping spiders [10] provided a less clear result, showing that bystander females are unable to choose the winner over the loser, whereas naïve females prefer the loser. Our study offers a first unequivocal proof that social eavesdropping enables invertebrate females to gather information on the dominance status of potential mates. This finding has the potential to open avenues for the future research on the still understudied field of mating systems in invertebrates.

Table 1. Results from the choice phase of the experiment, in which female crayfish, either naïve or bystander, were free to select between a dominant and a subordinate male.

Data	Naïve		Bystander	
	Dominant	Subordinate	Dominant	Subordinate
First male visited	7	8	11	4
Total duration of visits (sec)	254.4 (32.3)	247.6 (31.7)	379.0 (44.28)	180.6 (36.9)
Mean duration of visits (sec)	20.65 (2.18)	20.39 (2.7)	32.8 (3.8)	16.8 (2.1)
Number of contacts	10.1 (1.2)	10.5 (0.9)	14.6 (2.4)	8.4 (1.7)
Statistical results	**Naïve**		**Bystander**	
	G/t/Z	P	*G/t/Z*	P
First target visited	0.065	0.500	3.287	0.059
Total duration of visits (sec)	–0.048	0.963	3.895	**0.002**
Mean duration of visits (sec)	0.151	0.882	4.643	**0**
Number of contacts	–0.22	0.826	2.205	**0.027**

First male visited by females, means (±SE) of the total and mean duration of visits, and numbers of contacts (top). Types of male have been compared using G tests (*G*; df = 1) for the first male visited, Student’s *t*-tests (*t*; df = 14) for the total and mean duration of visits, and Wilcoxon tests (*Z*; n = 15) for the contacts (bottom). Sample sizes are 15 for both naïve and bystander females. Significant values in bold.

Supplemental data

Supplemental data are available at http://www.current-biology.com/cgi/content/full/18/11/R462/DC1

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Příloha č. 4

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The significance of droughts for hyporheic dwellers: evidence from freshwater crayfish

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Freshwater biodiversity is globally threatened by various factors while severe weather events like long-term droughts may be substantially devastating. In order to remain in contact with the water or stay in a sufficiently humid environment at drying localities, the ability to withstand desiccation by dwelling in the hyporheic zone, particularly through vertical burrowing is crucial. We assessed the ability of three European native and five non-native crayfish as models to survive and construct vertical burrows in a humid sandy-clayey substrate under a simulated one-week drought. Three native species (*Astacus astacus*, *A. leptodactylus*, and *Austropotamobius torrentium*) suffered extensive mortalities. Survival of non-native species was substantially higher while all specimens of *Cherax destructor* and *Procambarus clarkii* survived. The native species and *Pacifastacus leniusculus* exhibited no ability to construct vertical burrows. *Procambarus fallax* f. *virginalis* and *P. clarkii* constructed bigger and deeper burrows than *C. destructor* and *Orconectes limosus*. In the context of predicted weather fluctuations, the ability to withstand desiccation through constructing vertical burrows into the hyporheic zone under drought conditions might play a significant role in the success of particular crayfish species, as well as a wide range of further hyporheic-dwelling aquatic organisms in general.

Freshwater ecosystems occupy less than 1% of the Earth's surface, but support approximately one tenth of the world's species and one third of all vertebrates[1]. These systems provide a wide range of valuable services also to human populations. The increased demand on freshwater resources has led to a freshwater crisis in both human and biodiversity perspectives[2]. While the conservation status and distribution of freshwater taxa is less well-known than that of terrestrial species[3], there is growing evidence that freshwater taxa are at greater risk of extinction than those in terrestrial or marine ecosystems[4–6], making freshwater conservation a priority[7].

Freshwater crayfish (Crustacea, Decapoda, Astacida) are considered not only keystone species in freshwaters but also strong ecosystem engineers modifying the environment to suit themselves. Indigenous crayfish species (ICS) have been often designated as e.g., bioindicator, umbrella, or even flagship species in aquatic conservation[7,8]. Three prominent crayfish invaders of North American origin (spiny-cheek crayfish *Orconectes limosus*, signal crayfish *Pacifastacus leniusculus*, and red swamp crayfish *Procambarus clarkii*) have been introduced to Europe between 1890 and the mid-1970s and became particularly widespread across the continent[9]. Thousands of populations of native European astacids have been lost, and many more have been substantially reduced, largely due to direct or indirect effects of the presence of non-indigenous crayfish species (NICS). These not only influence their European counterparts by competition but especially spread the causative pathogen of crayfish plague (an oomycete *Aphanomyces astaci*), causing mass mortalities to crayfish not originating from North America. Apart from imposing strong competitive pressures on native crayfish populations, these invaders possess the ability to alter food webs and entire ecosystems[10]. The main reason for NICS introductions in Europe was initially their expected commercial use (fisheries and aquaculture)[11]. In recent years, however, introductions of further NICS have usually involved escapes or intentional releases of aquarium-bred specimens[12,13], making the situation more inauspicious.

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Besides the expectation of high extinction rates in crayfish in general[7,14], interactions with non-native crayfish are the leading cause of decline of the native counterparts[15,16]. Explanation of these displacements have been generally based on the evaluation of one, or a combination, of four biotic mechanisms: competition, predation, reproductive interference and disease transmission[15], as well as lower environmental requirements in non-native crayfish[11]. However, an array of factors threatening biodiversity and aquatic ecosystems in general is much wider and more complex, including climate change and severe weather events like long-term droughts, unexpected floods, fires, heavy storms etc.[7,17] having far reaching consequences[18,19]. Indeed, the role of abiotic disturbances such as long-term droughts on aquatic biota has remained overlooked and poorly understood for a long time[19–21], and such knowledge is still scarce and fragmentary[22–24]. In the context of ongoing and predicted weather fluctuations[25,26], the ability to withstand desiccation and particularly to be involved in vertical burrowing under severe drought conditions might play a significant role in the success of various crayfish. Similar kinds of adaptations have been documented for unionid mussels, gill-breathing snails[27,28] and a wide range of aquatic insects[19,21].

In this study, we assessed the ability of three European ICS and five NICS as models to survive simulated drought conditions and to construct vertical burrows in a humid sandy-clayey substrate as a protection against drought conditions.

Results

Substantial differences were detected by means of survival analysis among studied species ($\chi^2 = 44.3$, df = 7, $p \leq 10^{-6}$). All specimens of red swamp crayfish, yabby and marbled crayfish survived the simulated one-week drought but certain post-treatment mortality was modeled for marbled crayfish during a one-week observation in aquaria with a final survival of 85.7 ± 13.2% (mean ± SD; Fig. 1). Relatively high survival rates of spiny-cheek crayfish and signal crayfish at the end of the simulated one-week drought (84.6 ± 10.0 and 88.9 ± 10.5%, respectively) were followed by post-treatment mortality resulting in final values of 42.3 ± 21.7 and 59.3 ± 18.5%, respectively. Narrow-clawed crayfish (*Astacus leptodactylus*) and stone crayfish (*Austropotamobius torrentium*) suffered substantial losses during simulated drought (25.0 ± 21.7 and 30.0 ± 23.9% survival, respectively) but the values remained stable thereafter. All noble crayfish (*Astacus astacus*) died within 5 days of simulated drought.

No attempts at vertical burrowing were observed in ICS (noble, stone and narrow-clawed crayfish) and signal crayfish. The remaining species exhibited different degrees of burrowing activity (Fig. 2). Crayfish usually constructed a single burrow in the suggested position ("initial burrow"). One red swamp crayfish and one yabby created two burrows and two other red swamp crayfish, both males, even dug three burrows in different places. The most prominent burrow was always located in the originally suggested depression. Only red swamp crayfish exhibited the ability to close the burrow entrance by means of a mud plug in our experiment. One out of twelve males created the plug but females were more active (n = 7) in doing this ($Z = -2.050$, $p = 0.040$).

Differences in burrowing between the sexes of particular species were detected only in red swamp crayfish (Fig. 2). Although the relative volume was comparable ($t_{24, 22} = -0.670$, $p = 0.491$), females constructed deeper burrows than males ($t_{24, 22} = -2.989$, $p = 0.007$). Marbled and red swamp crayfish constructed bigger ($p < 0.020$

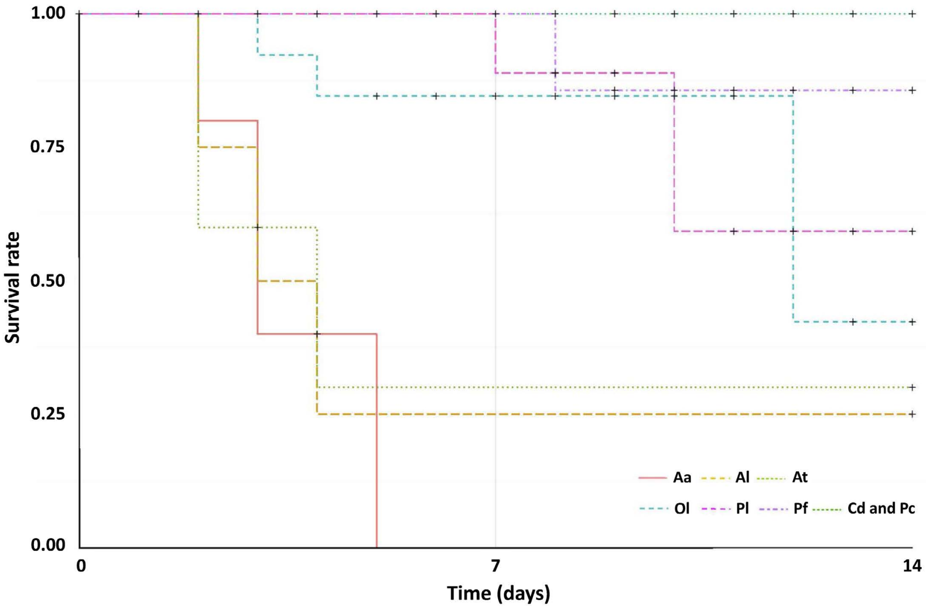

Figure 1. Kaplan-Meier survival analyses of crayfish species involved in the experiment. Legend refers to the particular species as follows: Aa–noble crayfish *Astacus astacus*, Al–narrow-clawed crayfih *Astacus leptodactylus*, At–stone crayfish *Austropotamobius torrentium*, Ol–spiny-cheek crayfish *Orconectes limosus*, Pl–signal crayfish *Pacifastacus leniusculus*, Pf–marbled crayfish *Procambarus fallax* f. *virginalis*, Cd–yabby *Cherax destructor*, and Pc–red swamp crayfish *Procambarus clarkii*.

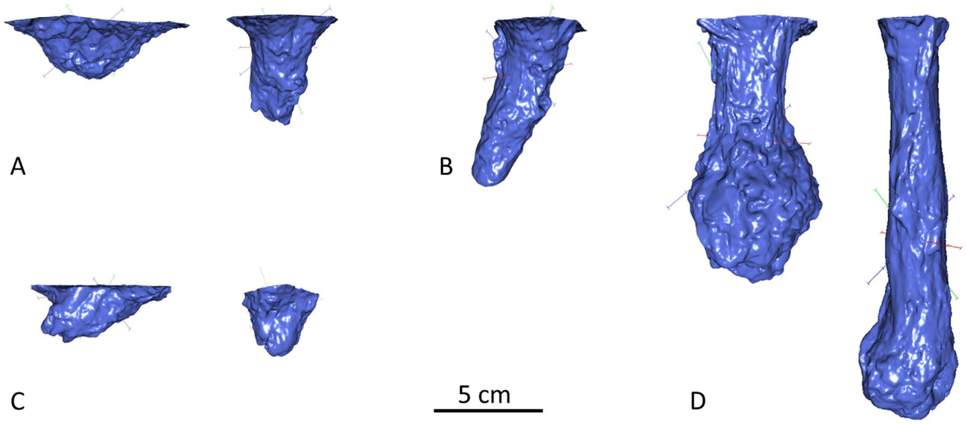

Figure 2. Examples of constructed burrows in yabby (**A**), marbled crayfish (**B**), spiny-cheek crayfish (**C**), and red swamp crayfish (**D**). 3D models of burrows of males (if present) are located on the left side of respective species. Further examples of burrows are available in supplementary materials (Fig. S1).

and $p < 10^{-4}$, respectively) and deeper burrows ($p < 0.002$ and $p < 0.004$, respectively) than did yabby and spiny-cheek crayfish (Fig. 3).

Discussion

We conducted the first comparative study evaluating burrowing activity under drought conditions in both selected native and non-native crayfish species currently present in Europe. Taking the results as a whole, it should be understood that the outlined crayfish desiccation capacities and burrowing abilities possess a degree of simplification and are related to the experimental set up, thus crayfish responses might vary under specific conditions. For instance, crayfish facing desiccation at localities might be exposed to even worse conditions, e.g. in terms of higher temperature and lower air humidity[29,30], the substrate might not be plastic enough for stability of burrows or the presence of coarse particles might prevent burrowing as such, i.e. substrate composition matters[24,31]. Also desiccation capacities and burrowing abilities of small-bodied juvenile animals are expectedly lower compared to sub-adults and young adults. Smaller animals have less water reserves compared to their relatively big body surface, allowing their desiccation; their physical ability to manipulate relatively big substrate particles is lower[29,30]. On the contrary, burrowing capacities of large-bodied specimens are likely also low due to difficulties with movement out of water (among others, the presence of big claws). Nevertheless, we believe it is unexpected that the patterns of desiccation capacities and burrowing abilities presented would change substantially among species under specific conditions. Also, the terminal stages of drought events when free water becomes unavailable at the localities are similar at both lotic and lentic sites, thus some degree of generalization is warranted.

Elevated desiccation capacities under different conditions have been documented among a wide range of NICS[29,32,33]. Our results clearly document substantially reduced survival of European ICS compared to NICS under simulated drought conditions, with absolute resistance in red swamp crayfish and yabby (Fig. 1), both considered to be warm-water species well adapted to conditions even in semiarid and arid regions[34,35]. Marbled crayfish suffered only post-treatment mortality with a final modeled survival of above 80%. The closely related slough crayfish *P. fallax* was found to cope less successfully with drought conditions than the Everglades crayfish *P. alleni*[24,36]; the latter has also been found accidentally released into European waters, although its establishment is considered unlikely[37]. High survival was also achieved by signal crayfish and spiny-cheek crayfish after the simulated drought followed by some post-treatment mortality (Fig. 1).

Resistance to desiccation is a necessary prerequisite for burrowing that mediates successful survival during severe droughts. A certain degree of burrowing is a habit present among crayfish. Less burrowing species just create short, unbranched burrows (or depressions) in the substratum, under stones, logs etc. They may also excavate burrows in the sides of clay banks[38–40]. Nevertheless, such burrowing activity might not be as adequate for survival as vertical burrowing under severe long-term droughts. European ICS species and signal crayfish exhibited no ability to construct vertical burrows. On the other hand, red swamp crayfish and marbled crayfish constructed bigger and deeper burrows than yabby and spiny-cheek crayfish (Fig. 3).

Considering their desiccation resistance and burrowing abilities, the red swamp crayfish is the most tolerant species we compared. It is worth mentioning that red swamp crayfish is also the only species in our experiment exhibiting the closing of the burrow entrance with a mud plug[31,41], particularly in females, which also created deeper burrows than males (Fig. 2). Females frequently use burrows for egg incubation. Females with eggs are not usually submerged in the ground water due to low available levels of dissolved oxygen, and oxygen diffuses directly from the burrow atmosphere while egg are fanned by swimmeret movements[42]. However, deeper burrows constructed by females in our experiment suggest the possible importance of having better access to the water. Even signal crayfish and white-clawed crayfish *Austropotamobius pallipes* eggs artificially stored in a humid environment require incubation in aquatic conditions at least during final stages of embryonic development, likely due to increased metabolic waste excretion[43], thus, at least periodical egg submergence can be expected in crayfish.

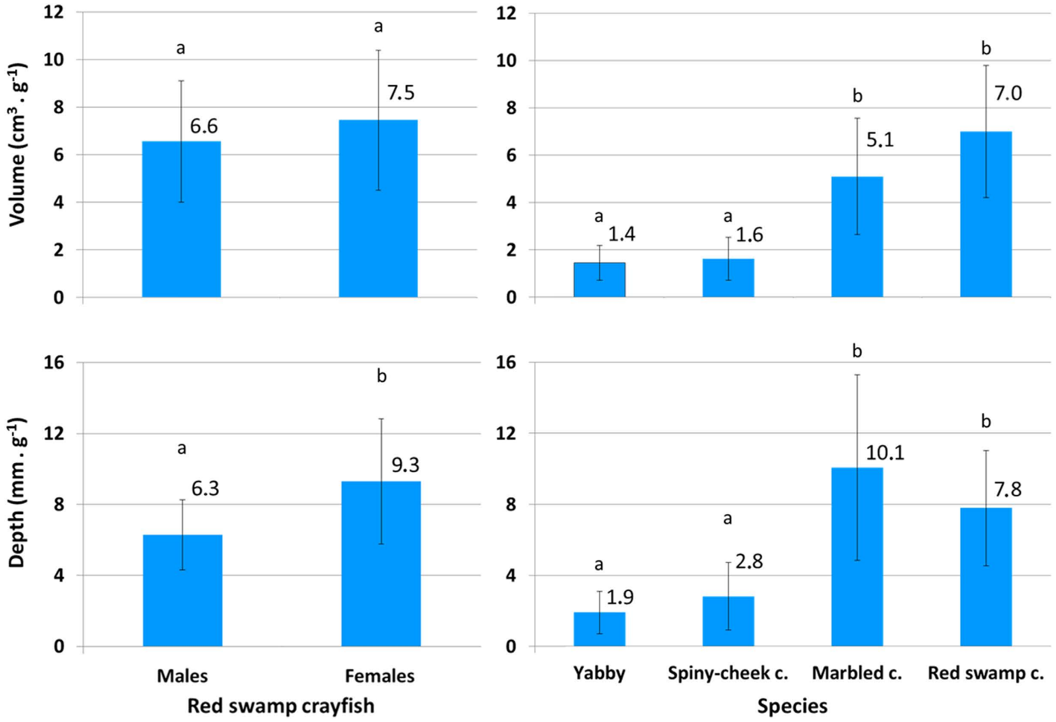

Figure 3. Relative volume and depth of burrows in burrowing crayfish species involved in the experiment. Significant differences between the sexes were detected only in case of red swamp crayfish–left column. Interspecific values are shown in the right column. Data are presented as mean ± SD. Values with differing letters within each graph are significantly different ($P < 0.05$).

Besides red swamp crayfish, further NICS involved in the study exhibit combined strategies focusing on increased desiccation capacity (yabby, marbled crayfish and signal crayfish) and burrowing (marbled crayfish and to a lesser extent also yabby and spiny-cheek crayfish). The lowest success belongs to ICS in particular and signal crayfish in terms of burrowing. We consider desiccation capacity and burrowing as further, still largely overlooked factors[7,11,17], whose importance will rise with ongoing and predicted weather fluctuations in the future[25,26]. Descriptions of current status and projections of droughts in European freshwater habitats together with current and future distributions of crayfish are beyond the scope of this article, however, the first suggestion might be that such events will be particularly pronounced among ICS in the warmer (Mediterranean) regions of the continent. Nevertheless, local extremes cannot be neglected and their importance will likely rise in the future too. For instance, a long-lasting drought hit Europe in 2015. It particularly affected Central and Eastern Europe while in some regions it was the driest (North Slovakia) and in others (Czech Republic and Poland) it was the second driest summer of the last 50 years–following 2003[44]. Expanding from its importance during droughts, burrowing also plays a role in overwintering which might increase the probability of establishment of non-indigenous species[45,46]. Following crayfish as a model group of freshwater organisms, a similar mode of action can be expected in further hyporheic-dwelling aquatic biota e.g. unionid mussels and clams, and a wide range of aquatic insects, as well as crabs and fish.

Methods

Container preparation. To create a suitable test substrate, sixteen kilograms of sand (České štěrkopísky Inc., Čavyně, Czech Republic) with a humidity of 5.2% and 24 kg of WBT clay (Keraclay, Plc., Brník, Czech Republic) with a humidity of 7.1% were thoroughly mixed by hand (=60% clay proportion expressed on a wet weight basis). For size distribution of sand and clay particles see Table S1. Aged tap water was added to get a final humidity of 16.5%. Our preliminary experiment revealed that the clay itself and a mixture with 80% clay proportion are too plastic to facilitate manipulation by crayfish. On the other hand, a substrate with 40% clay proportion was not stable enough for burrowing, which confirms the importance of substrate composition for successful burrowing[24,31]. The resultant humid mixture was used to fill a series of plastic containers (inner diameter = 34.0 cm, height = 44.5 cm) to a depth of ca. 34 cm. To better simulate natural conditions when certain areas with residual water persist at the drying-up localities[47], a shallow "initial burrow" (diameter 2.6 cm, depth 1.3 cm; volume 6.9 cm^3) was created in the margin of the container and 5 mL of water was added to stimulate burrowing in the suggested position. A single crayfish individual (see respective species and numbers below) was placed in the container. Each container was covered by a 0.5 cm thick polystyrene lid in order to prevent acute desiccation of the experimental animal. The air relative humidity (RH) reached at least 99% within an hour after coverage. The coverage was implemented in order to enhance survival of susceptible indigenous crayfish species allowing supposed burrowing. Natural conditions necessarily possess lower air humidity but there is a certain time period before free water becomes unavailable. It opens a space for animals to prepare a burrow, find water pools etc. For comparison, highly tolerant red swamp crayfish exposed to room air of approximately 50% RH showed mortality after 3–7 days' exposure[30] and all animals died at 30% RH and 24 °C within about a day[29]. The experimental temperatures (mean ± SD) of air and the sandy-clayey mixture were 20.2 ± 0.3 and 20.3 ± 0.1 °C, respectively. Temperature was registered hourly using Minikin loggers (Environmental Measuring Systems, Brno, Czech Republic). Fairly similar temperatures are often used in laboratory experiments and are relatively high—representing warm periods of the year when the most pronounced droughts usually occur. Although the temperatures might be even higher during such events[29,30], we considered values close to 20 °C the best compromise, taking the requirements of the most sensitive species involved (the stone crayfish) into account[48,49].

Experimental animals. We selected intact (with all walking legs including well developed chelae) intermoult specimens of three European ICS and five NICS. These were usually adults based on biometry and secondary sexual characters, but a few subadults might also have been involved. The sex ratio was balanced except for marbled crayfish *Procambarus fallax* f. *virginalis* where only females occur. For reasons of conservation and following a lack of vertical burrowing activities (see results above), only limited numbers of ICS ($n = 4$ for each species) were used. Noble crayfish *Astacus astacus* were caught from the pond U Sudu (Těšínov u Protivína, Czech Republic; 49° 20′ N, 14° 28′ E) under permit no. KUJCK 4820/2011 OZZL/4/Ou, Regional Office of South Bohemian Region. Narrow-clawed crayfish *A. leptodactylus* were obtained from the limestone quarry Kosov (Jarov u Berouna, Czech Republic, 49° 56′ N, 14° 3′ E) under permit no. 123564/2012/KUSK, Regional Office of Central Bohemia Region, and stone crayfish *Austropotamobius torrentium* came from Zubřina brook (Havlovice, Czech Republic; 49° 12′ N, 14° 17′ E) based on permit no. ŽP/2450/2011, Regional Office of Plzeň Region. Both signal crayfish *Pacifastacus leniusculus* and spiny-cheek crayfish *Orconectes limosus* ($n = 10$ for both species) were caught from the wild populations in the Vysočina Region and from the Lipno Reservoir, South Bohemian Region, respectively. Marbled crayfish ($n = 12$), yabby *Cherax destructor* ($n = 14$), and red swamp crayfish *P. clarkii* ($n = 24$) were obtained from laboratory cultures. Considering the categorization by Hobbs[50], all crayfish species involved in our experiment belong to the tertiary burrower category. Some members of this group are often incorrectly referred to as non-burrowers[39] but they may respond to habitat drying by excavating shallow simple burrows into the hyporheic zone, although experiencing population declines and local extinctions during severe droughts[22,51–54].

Crayfish were individually acclimatized for three days in a bucket with 8 L of aerated tap water, without feeding. Animal wet weights (to the nearest 0.1 g) and carapace lengths (to the nearest 0.1 mm) were determined and crayfish were placed in the experimental container for a one week period simulating drought conditions (for crayfish biometry see Table S2). Crayfish survival was evaluated daily. After one week's exposure, surviving animals were collected and transferred to aquaria with water for one week to evaluate post-treatment mortality.

All experimental manipulations were conducted according to the principles of the Institutional Animal Care and Use Committee (IACUC) of the University of South Bohemia, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, Vodňany, based on the EU harmonized animal welfare act of Czech Republic. Nevertheless, no specific permissions were required for the locations and activities considering taxa involved in this study.

Creation and measurement of casts. Gypsum casts of any burrows excavated were created after removal of animals. If direct collection of crayfish from burrows was impossible, a small amount of carbonated water was added to the burrow in order to evict animals, which led to success in most cases. If collection of animals was prevented (as occurred only with several specimens of red swamp crayfish), a new independent replication was conducted. Any excess water was removed from the burrow bottom by blotting with absorbent paper. Depth of casts was measured by a digital caliper to the nearest mm. Casts were further scanned by an Artec Spider™ hand-held 3D laser scanner (Artec Group, Luxenbourg) located at the Department of Cybernetics, Faculty of Applied Sciences, University of West Bohemia in Pilsen with a stated resolution of 0.1 mm and accuracy up to 0.03 mm. The scanner is based on the structured light principle and provides a 3D mesh of the object as an output, generated in real world coordinates (mm). The resulting STL (STereoLithography) mesh was imported to the Artec Studio, version 10 (Artec Group, Luxembourg) where the volume of the 3D mesh was calculated. As size naturally varies within and among crayfish species, relative data reflecting weight of respective animals were used in presentation of burrowing activity (volume and depth).

Statistical analysis. Non-parametric Kaplan-Meier survival analyses were performed in the R-statistics software (version 3.2.4, R Development Core Team 2015), with the packages: "KMsurv" and "survival". In addition, for graphical visualisation the packages "GGally" and "ggplot2" were employed. In assessing sex differences, the ability to close the burrow entrance by means of a mud plug was assessed as 1 or 0. Due to lack of normality and homoscedasticity of this dataset (evaluated with Kolmogorov–Smirnov and Levene's tests, respectively; these tests uniformly used further if appropriate for testing assumptions of parametric tests), a non-parametric Mann-Whitney U was applied. Intersex differences in term of relative burrow depth and volume were compared with Student's t-test. Because of heteroscedasticity in data, the non-parametric Kruskal-Wallis test followed by multiple comparisons of mean ranks for all groups was applied for interspecific comparisons (values of both sexes were pooled among species for this purpose). These data were analyzed using Statistica 12.0 (StatSoft, Inc.). The null hypothesis was rejected at $\alpha = 0.05$ in all tests of this study.

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Author Contributions

A.K. conceived the experiment and wrote the core part of the manuscript. J.T., L.V., M.F. and J.Př. conducted the experiment. P.C. analyzed the burrows. J.Pa. and M.B. improved earlier versions of the manuscript.

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SHORT REPORT

Crayfish bury their own exuviae: a newly discovered behavioral pattern in decapods

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Abstract

Invertebrates are a very diverse group of animals, showing a wide spectrum of life strategies and adaptations. They often exhibit very complex behavioural and social patterns. In crayfish, the largest freshwater invertebrates, we found a new behavioural pattern, burying their own exuviae after moulting. Such a pattern may be an as yet unrecognized type of hoarding or caching. The buried exuvia is exhumed after 2 or 3 days (when the crayfish body is no longer as soft) and consumed. This behaviour is probably self-protective (hiding the mark of a helpless prey), as well as having mineral storage reasons. Such complex behavioural patterns in invertebrates present new challenges for future research.

Keywords: Invertebrate, Behaviour, Hoarding, Caching, Crustacean, Crayfish

Background

Caching or hoarding behaviour is well known, particularly in mammals (Prestrud 1991; VanderWall and Jenkins 2003) and birds (Bugnyar and Kotrschal 2002; Emery and Clayton 2001), as a type of appetitive behaviour characterized by foraging and carrying food from the source to a hidden place for a period before it is consumed (Schneider et al. 2013; VanderWall 1990). Such behaviour is usually induced by the need of a food supply for specific unfavourable conditions e.g. winter season, when food sources are not available or considerably limited (Vander Wall 1990). On the other hand, food hoarding can be promoted by food deprivation in some previously classified non-hoarders (Yang et al. 2011). In invertebrates, food storage may be induced by a period of starvation, as in decapod crustaceans (Kim 2010; Wickins et al. 1996), or by specific life traits as in leafcutter ants (Mueller et al. 2011) or spiders (de Crespigny et al. 2001). But burying or caching in invertebrates is at least very sporadic and any report of exuviae caching has never been reported in invertebrates.

During acclimation of two crayfish species (representatives of both northern and southern hemisphere crayfish species) prior to experimental work unexpected evidence was found of undescribed behaviour in invertebrates. Hence, the observed caching behaviour was explored fortuitously without previous hypothesis or expectations. Present work therefore should outline the new hypothesis and challenges in research of invertebrate behavioural patterns.

Methods

Two species were involved in the study; signal crayfish (*Pacifastacus leniusculus* (Dana, 1852), family Astacidae) and yabby (*Cherax destructor* Clark, 1936, family Parastacidae). Signal crayfish specimens were captured in a pond near Velké Meziříčí (Czech Republic; 49°22′42″N, 16°4′53″E) in April 2015. They were placed in a flow-through system with natural ambient light and water temperature conditions. Seventy specimens (young adults and sub-adults; carapace length, CL = 29.9 ± 2.5 mm, weight, w = 7.5 ± 2.0 g) were taken for acclimation for a planned study under experimental conditions (28 May 2015). Crayfish were individually stocked into plastic boxes filled with a sandy layer (200 ml–376 g of sand) and 2000 ml of tempered tap water, and placed to an incubator maintaining the water temperature at 16 °C. Five (CL = 28.3 ± 2.7 mm, w = 6.4 ± 1.7 g) of 70 stocked

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specimens moulted during the 24 h acclimation period. All of these exhibited exuvial burying behaviour and were therefore observed for a further 3 days at 24 h intervals (to prevent disturbance). The time from burying to exhuming of exuviae and time of its consumption was monitored.

Yabby specimens originated from our own aquarium culture reared under stable conditions of approximately 20 °C of water temperature and a light regime of 12 h light and 12 h dark. The first evidence of burying behaviour in yabby, seen in non-standardized conditions of small aquaria with sandy substrata, was discounted as a random event in March 2015. Following similar observations on signal crayfish, ten yabby specimens (CL = 25.9 ± 3.0 mm, w = 5.8 ± 2.6 g) were selected, exhibiting signs of forthcoming moulting (softened carapace) and placed in the same standardized conditions (except for water temperature, which was maintained at 20 °C) as described above for signal crayfish for 24 h. Only two crayfish (CL = 23.7 ± 1.0 mm, w = 4.4 ± 1.0 g) moulted, but both specimens exhibited the same burying pattern.

Results and discussion

We observed unexpected behavioural pattern, caching or burying of own exuviae, in two crayfish species: yabby (*Cherax destructor*, a southern hemisphere crayfish of the family Parastacidae) and signal crayfish (*Pacifastacus leniusculus*, a northern hemisphere crayfish of the family Astacidae) in experimental conditions. Crayfish were found to bury their exuviae after moulting event prior to its later consumption in 2–3 days. This is the first evidence of exuvial caching in invertebrates.

This unexpected behaviour is unusual and surprisingly has escaped attention despite the fact that both species are cultured in many countries of the world (Holdich et al. 2006). The postponed consumption of exuviae, as a source of minerals for carapace re-calcification, is probably because mouthparts are still hardening from internal resources during the post-moult period (Reynolds 2002). During this period lasting 2 or 3 days, when soft shelled specimens are unable to defend themselves, the exuviae are buried in suitable textured substrata. Food hoarding can be promoted by previous food deprivation in some previously classified non-hoarders (Yang et al. 2011). However, the newly described behaviour is probably connected with self-protective activities because the exuviae left beside a shelter can mark an available helpless prey.

The caching ability was not the primary reason of the research, but was observed fortuitously, when yabby and signal crayfish, were placed individually in small aquaria to acclimate for 24 h with a sandy layer prior to experiments designed for different purposes. The observed behavioural patterns have remained undetected over many decades of decapod research, probably due to many issues which could becloud or prevent the observation of this pattern (e.g inappropriate conditions for observation of such events in natural conditions, tanks and aquaria without suitable substrata, use of animals in intermoult period). Another possible reason could be marginalizing of the observed events, as we did at first case with the yabby. In that case, the burying and exhuming of exuviae was marked as an interesting but probably random event caused by digging activity. Despite this, the burying pattern was later confirmed in two other moulted yabbies. In signal crayfish, all five moulted specimens independently buried their exuviae under a mound of sand (Fig. 1), which is not a sign of a random phenomenon. The last reason why this behavioural pattern may have been overlooked is the likely frequent failure of the burying activity, perhaps in response to the presence of potential danger, which can frequently interrupt caching (Bugnyar and Kotrschal 2002).

All observed specimens exhumed and ate their exuviae after 2 or 3 days (Fig. 2). The delay between moulting and eating exuviae cannot be marked as hoarding, because that behaviour is directly connected with food (Vander-Wall 1990), whereas exuviae are not a main or the only food item, despite the fact that it can be utilized as valuable source of minerals for recalcification of exoskeleton (Reynolds 2002). A better designation is caching or, even better, burying, because the event is only completed by burial in a sandy mound.

The explicit reason for such behaviour is unknown, but may be motivated by two main reasons. Firstly, hiding and storing of this easily available source of minerals for re-calcification [internal resources are insufficient to harden the whole exoskeleton (Reynolds 2002)] during the time when crucial feeding structures such as chelae mouthparts and other limbs are hardening from internal resources (particularly from gastroliths) (Greenaway 1985). After this period, mouthparts are able to chew and exuviae can be uncovered and eaten. Secondly, by burying their exuviae, just moulted crayfish hide their own soft body vulnerability, as considerable mortality occurs by predation or cannibalism in this time (Reynolds 2002). Exuviae are hidden from the sight of both conspecifics and heterospecifics and their concealment through burial can limit location of freshly moulted specimens. It must be said that crayfish are unable to guard the hiding place of their exuviae and pilferage can often occur. On the other hand, such pilferage leads only to resource loss, while the builder of the burial mound remains unseen.

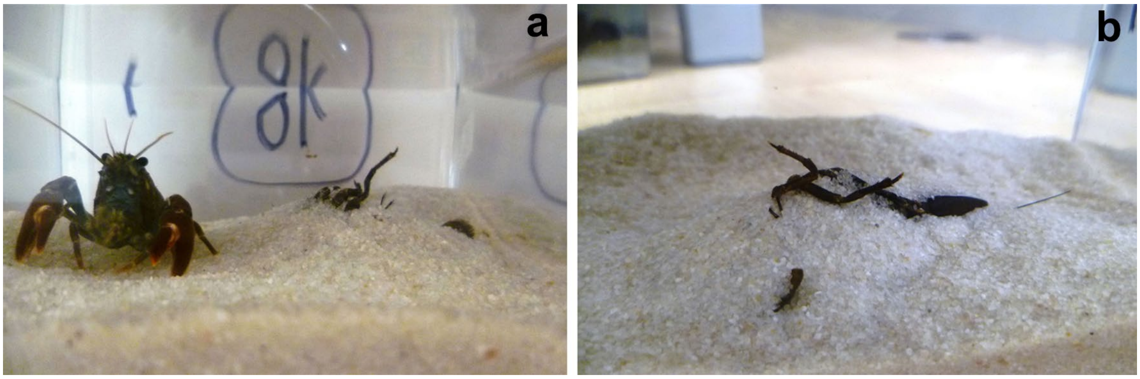

Fig. 1 Signal crayfish (*Pacifastacus leniusculus*), initial carapace length = 32.4 mm, and its burial mound for exuviae located centrally in aquaria (**a**) and a detailed view of exuviae buried in sand (**b**)

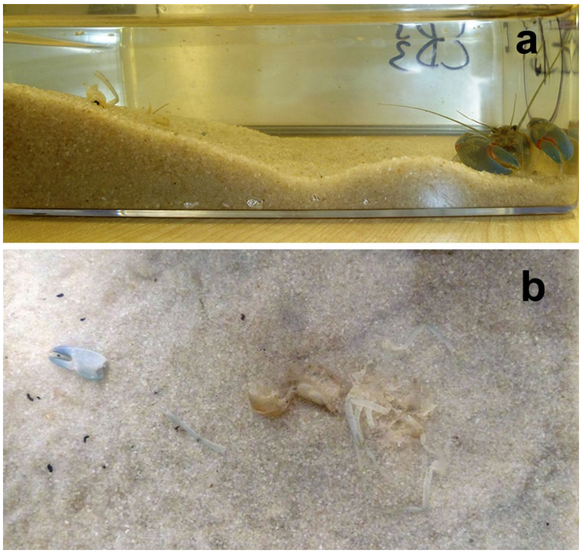

Fig. 2 The burial mound of yabby (*Cherax destructor*), initial carapace length = 24.7 mm, made by heaping sand against the side of the aquarium (**a**) and its excavation during consumption of the exuviae (**b**)

Conclusions

Generally, the described type of caching has not been classified among the known types of hoarding or caching described in other animal taxa (Dally et al. 2006; Vander-Wall 1990). Nevertheless, the controlling mechanisms of such behaviour could be similar and can suggest broader and more complex behavioural patterns in invertebrates so far unknown. Mechanisms inducing or inhibiting the newly observed behaviour remain unidentified, and represent a new challenge for future research as well as presence/absence of this behavioural patterns in other decapods. Present work therefore should outline the new hypothesis and challenges in research of invertebrate behavioural patterns.

Authors' contributions

MBU and AK wrote the main manuscript text. MBU, AK, MF, PK and LV managed experimental work. MBU, MBL and LV prepared figures. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Ethical statement

No specific permissions were required for the locations and activities involved in this study. The study did not involve endangered or protected species. All experimental manipulations (rearing, capture and measurements) were conducted according to the principles of the Ethical Committee for the Protection of Animals in Research of the University of South Bohemia, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, Vodňany, based on the EU harmonized animal welfare act of Czech Republic. The above named Institutional Animal Care and Use Committee (IACUC) specifically approved this study. The principles of laboratory animal care and the national laws 246/1992 and regulations on animal welfare were followed (Ref. number 22761/2009-17210).

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Příloha č. 6

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

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

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

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Introduction

Agonistic interactions (i.e. antagonism and aggression) are the key aspects of animal behaviour (Parker 1974; Manning and Dawkins 2012) contributing to a success of species in the environment. Aggressive behaviour occurs during the entire life span of crayfish, and is associated with an invasion success (Sih et al. 2012). Their importance often arise up when animals forage in overlapping ecological niches and use identical resources, e.g. food and shelter (Wilson 2000; Huntingford 2013). Agonistic behaviour is primarily demonstrated by increased aggressiveness between participants of conflict (Gherardi 2002) and boosts success in coping with novel predators and competitors (Duckworth 2008; Hudina and Hock 2012). The non-native species that persist, establish, and become invasive can cause negative effects on economy, native biota and ecosystem functioning (Kolar and Lodge 2001).

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

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

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

Materials and methods

Experimental animals and measurements

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

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

Acclimation and experimental set-up

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

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

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

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

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

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

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

Event	Definition
Contact	Touch with claws, body (carapace, abdomen) rather than antennae and antennules
Avoidance	Retreat from an approaching crayfish that has not exhibited threatening behaviour
Threat	Approach with signs of warning—mainly raising, waving, and shaking claws
Winner	The individual that did not retreat from the fight or that left the opponent that assumed a body down posture or remained motionless. In the very few cases when both individuals retreated simultaneously no winner was scored
Fight	Crayfish make contact with pushing or slight pinching with claws or direct contact and biting with clear intent to inflict injury
Fight initiator	Crayfish which one start first the fight activity recognize as a fight initiator
Tail flip	Rapid abdominal flexions results powerful backward swimming stroke

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

Ethical statement

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

Statistical analysis

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

Results

Dominance establishment

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

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

Agonistic encounters and contacts

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

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

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

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

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

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

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

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

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

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

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

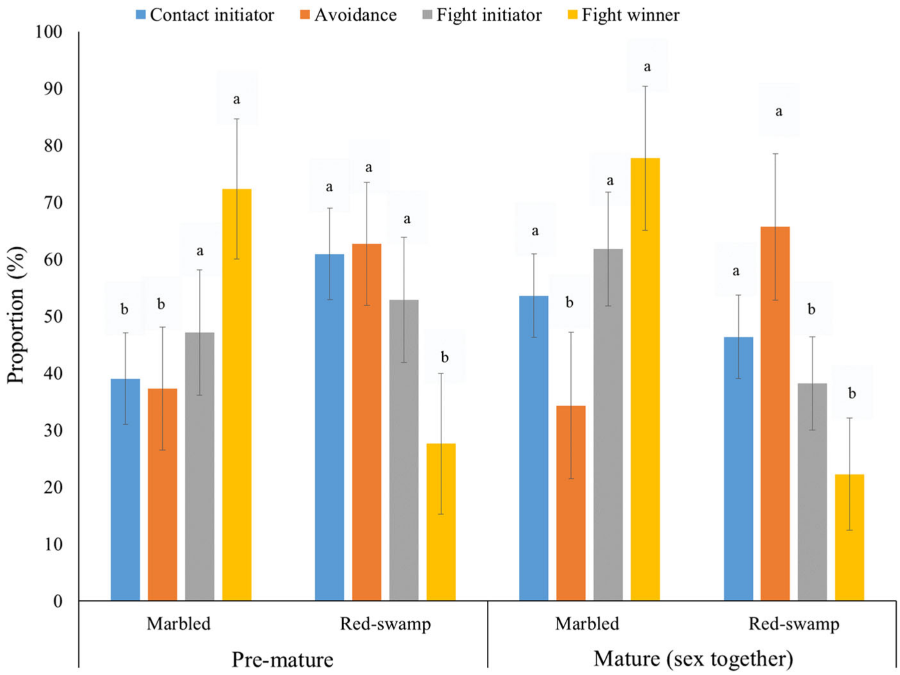

Fig. 1 Comparison of percentage of contact initialization, avoidance events, fight initialization and fight wins between premature and mature *P. virginalis* and *P. clarkii*. Values are presented as mean ± SE. Different superscripts within the column colures (groups apart) highlight the statistically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

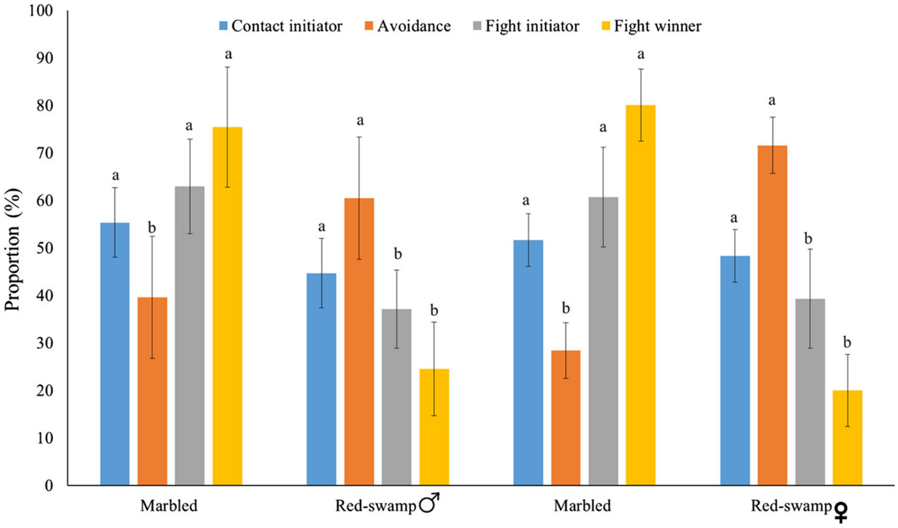

Fig. 2 Comparison of percentage of contact initialization, avoidance events, fight initialization and fight wins between mature *P. virginalis* and *P. clarkii* in respect to sex of *P. clarkii*. Values are presented as mean ± SE. Different superscripts within the column colours (groups apart) indicates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

Discussion

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

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

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

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

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

Conclusion

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

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Příloha č. 7

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PRIMARY RESEARCH PAPER

Females bet on the known: crayfish females recognize and prefer males from familiar population, males are not picky

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Abstract Reproductive behaviour is a central pattern in animals and understanding mate choice is among major challenges in behavioural and ecological studies. Mate choice is often based on recognition of conspecifics, their physiological state and social experiences, which play important roles in decision making. In crayfish, females are choosier than males because of their higher energy costs for reproduction (oogenesis, incubation, parental care). We analysed mate preferences of the spiny-cheek crayfish (*Faxonius limosus*) where the choosing crayfish was faced with two similarly sized mature counterparts or different origin (three geographically separated populations were used). Both sexes were presented either with a single familiar and unfamiliar counterpart or with two unfamiliar ones, reflecting the source population. Our results suggest that females are able to recognize and prefer mates from the familiar population, whereas males do not exhibit any specific preference. Chemical communication is the most possible driver but the mechanism of recognition at population level is not known. The specific population microbiome may be contributing to this pattern.

Keywords Invertebrates · Decapoda · Communication · Mate choice · Population · Chemical ecology

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Introduction

Ever since Darwin's idea of female preferences for male ornaments, research into sexual selection continues to be a rapidly growing field (Danchin & Cézilly, 2008). Evidence of many different traits affecting mate choice has been described from a range of animals, from small invertebrates to large mammals. Identification of particular traits of reproductive behaviour that influence mate choice is a major challenge in behavioural ecology and evolutionary biology (Aquiloni et al., 2008; Danchin & Cézilly, 2008). Andersson (1994) reviewed sexual selection in the animal world as a way to maximize reproductive success. Mating entails costs and potential benefits to both sexes; therefore it is necessary to make optimal choices from among the available potential mates (Mellan et al., 2014).

Females are generally choosier than males, because they invest more energy into production of relatively low number of big gametes. Males producing large numbers of relatively low-cost sperm are usually limited only by the frequency of mating (Aquiloni & Gherardi, 2008c) and sperm maturation (Vogt, 2002). For these reasons, males are less choosy than females in mate choice (Trivers, 1972). Individual selectivity is a specific function of many factors such as mate quality, availability of potential mates, degree of investment into offspring or ability to defend an important resource, e.g. shelter, food (Bateson, 1983; Rahman et al., 2004; Mellan et al., 2014; Tricarico, 2015). A range of preferences that influence decision making in mate choice is known in crustaceans. The studies of Rubolini et al. (2006), Aquiloni & Gherardi (2008b) and Aquiloni & Gherardi (2008c) showed higher preference for mates having larger body size in both sexes. Similarly, crayfish females preferred dominant males when choosing between similarly sized known males (Aquiloni et al., 2008; Aquiloni & Gherardi, 2008b). On the other hand, males are able to recognize and prefer virginity in females (Aquiloni & Gherardi, 2008c). Recognition of functional state of crayfish, or of an individual crayfish, is possible mainly through chemical communication (Simon & Moore, 2007; Berry & Breithaupt, 2010) processed by well-developed sensory organs (Vogt, 2002; Hallberg & Skog, 2011). This is supported by many studies examining the ability of individual recognition among crustaceans, e.g. in the hermit crabs *Pagurus longicarpus* Say, 1817 (Gherardi & Tiedemann, 2004), in the American lobster *Homarus americanus* H. Milne Edwards, 1837 (Karavanich & Atema, 1998b), in the big-clawed snapping shrimp *Alpheus heterochaelis* Say, 1818 (Ward et al., 2004) and in yabby *Cherax destructor* Clark, 1936 (Van der Velden et al., 2008). Aquiloni et al. (2009) also investigated sex identification and recognition in red swamp crayfish *Procambarus clarkii* Girard, 1852 during female–male interactions via chemical cues only.

In the current study, we investigated if crayfish could recognize mate conspecifics from their own (familiar) or geographically distinct (unfamiliar) population. When the study was designed, we supposed that if there is a preference, it would probably be for unfamiliar mates, to increase heterozygosity of the population.

Materials and methods

Collection and maintenance of animals

Crayfish collections were planned and designed to capture on small area (up to 100 m of river bank) at each locality. This methodology would be appropriate to fulfil familiarity (previously met) assumptions. All used crayfish were adults that have travel radius within tens of metres for foraging, social contact and mating. Males and females of the spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817) were captured by hand from three separate localities in the Czech Republic: (1) the Elbe River (50°45′45″N; 14° 23′65″E), (2) Hrejkovický brook where it enters the Orlík water reservoir (49°40′38″N; 14°25′37″E) and (3) the Malše River at its mouth to the Vltava River in České Budějovice (48°97′28″N; 14°46′99″E) (see Fig. 1). All localities belong to the Elbe River catchment and have been highly invaded by spiny-cheek crayfish for more than 10 years (Petrusek et al., 2006). Crayfish were collected from July to August 2015, before the reproductive season. In the laboratory, crayfish were held individually in floating plastic boxes placed in flow-through channels under natural ambient light and temperature conditions for at least 5 weeks until the onset of breeding. Crayfish from all three population can smell one another but any tactile or visual contact was prevented.

Both sexes were examined for sexual maturity before the experiment. Sexual maturity of males was assessed using the shape of gonopods, and only sexually active FI males were used (Hamr, 2002; Buřič et al., 2010b); and by the presence of developed

Fig. 1 Map of the Czech main river network showing source localities of the spiny-cheek crayfish. *E* the Elbe River, *O* the Orlík reservoir, *M* the Malše River

glair glands in females (Hamr, 2002; Buřič et al., 2010a). In all used crayfish, we measured carapace length and length and width of both chelae to the nearest 0.1 mm using Vernier callipers. Wet weight was measured to the nearest 0.1 g with an electronic balance (Kern & Sohn GmbH, Balingen, Germany). Thereafter each measured crayfish was assigned a unique box with identification code. Only crayfish without visible damage (lost or regenerating chelae or other appendages) were selected for experimentation.

Experiment design

The experiment was conducted in the reproductive period between 15th and 28th October 2015. Experimental triads were made from two electors (two males or two females) and one chooser of the opposite sex. Electors were chosen as one familiar and one unfamiliar, or as two unfamiliar individuals (control). On the basis of studies by Aquiloni & Gherardi (2008b) and Aquiloni & Gherardi (2008c), we tried to eliminate size-preference effects. Electors in each single triad were therefore chosen using a size-matching strategy where differences in carapace length and chelae size (both length and width) were always $< 5\%$. Used crayfish males and females were encoded not to be influenced by unconscious biases of the observer. We set up 12 trials with familiar and unfamiliar and 6 trials with two unfamiliars per population and sex; in total we observed 108 trials.

For the experiments, we used the modified experimental apparatus from Aquiloni & Gherardi (2008c) which consisted of an elliptical plastic arena (60 × 40 cm, water level 10 cm). Water for experiments was from the same source as for flow troughs with crayfish. On the opposite sides of the arena were two elector's areas (radius of 15 cm marked by small points) (Fig. 2), where each elector was carefully attached around the thorax (between the 3rd and 4th pair of pereopods) using a fine fishing line, enabling free movement in a defined area. After the electors were attached, the chooser was placed into the middle of the arena and covered by a 15-cm opaque circular perforated container for 10 min of acclimatization, during which only chemical stimuli were possible. After that, the mate choice experiment began with the removal of the container, when the chooser was free to move in the entire arena. The behaviour of the stocked crayfish was then observed for 10 min in each trial. Selected time range is appropriate for animal communication and decision making in mate choice. To avoid pseudo-replication, each chooser crayfish was used only once. Between each trial, the experimental arena was thoroughly washed with clean tap water and new water was provided for a subsequent trial.

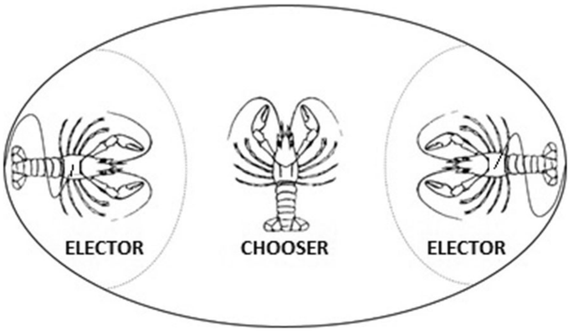

Fig. 2 Illustrated schematic drawing of the experimental arena (dimensions are not to scale). Elector crayfish attached by fishing line; chooser crayfish is freely moving; dotted line—marked elector's area

Collecting of data

Each trial was video-taped using a Sony digital camera (HDR-CX240E). Each record was examined by an experienced observer. During the mate choice experiments, the observer recorded parameters of the chooser's selection such as the first choice, time spent and numbers of pre-copulatory contacts, the last choice and mating. Records were processed using the software program EthoVision XT® 13 (Noldus Information Technology b.v., Netherlands) for time spent in each elector's area.

Statistical analyses

Data were tested for normality using the Kolmogorov–Smirnov test. Variables such as time spent in area (carapace centre of chooser crossed the elector's area border), and spent time and number of contacts (chooser actively grasped the elector by claw), fulfilled assumptions of parametric test, were analysed using two-tailed Student's tests (t) for dependent samples; if not, the Wilcoxon matched paired test (z) was used. Other variables such as first and last choices, and mating were tested using Pearson's contingency table χ^2 test. The null hypothesis was rejected at $\alpha = 0.05$ using the software program Statistica version 13.2 for Windows (StatSoft).

Results

Chooser: female

In general, females visited both males about three times during mate choice period, only in four trials females visited just one male. Females did not show preference for males from any unfamiliar population in time spent in elector's areas, time spent and numbers of pre-copulatory contacts, and first and last choices (Table 1). Female choosers from all three populations spent significantly more time in the familiar elector's area and made more contacts with them. Females preferred familiar males more often than unfamiliar in comparison of time spent in elector's area (Fig. 3), spent time in contact (Fig. 4) and in numbers of contacts (Fig. 5). First and last choices of choosers were without significant preference to familiar or unfamiliar mates (Table 2).

Female choosers copulated in 19 trials (35.2%) out of 54 trials. Females showed preference for copulation with familiar males in 6 trials. In some cases (8 trials), the unfamiliar male grasped a female passing them. As this act was involuntary, these copulations were not included in results as the decisive factor for mate choice. No females were observed copulating with more than one male in each trial.

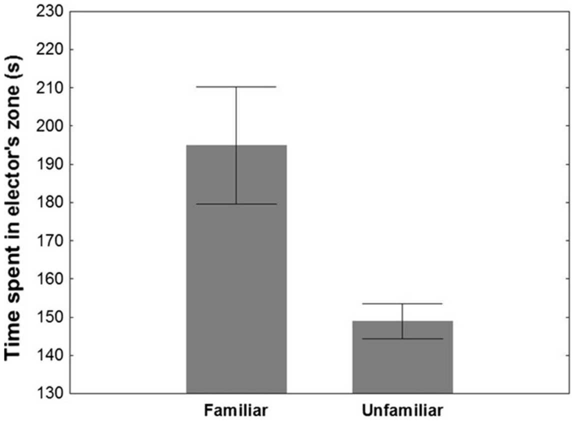

Fig. 3 Average time spent by female chooser in familiar and unfamiliar elector's zone. Data are mean ± SE. Significant difference between familiar and unfamiliar group $P < 0.05$

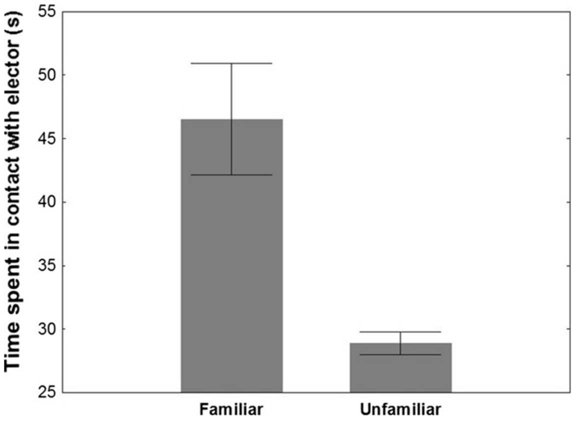

Fig. 4 Average time spent by female chooser in familiar and unfamiliar elector's zone. Data are mean ± SE. Significant difference between familiar and unfamiliar group $P < 0.05$

Table 1 Statistical analysis of female preferences for both unfamiliar electors (M–E–O)

Variable	Population					
	M (ct = 6)		E (ct = 6)		O (ct = 6)	
	Statistic	*P* value	Statistic	*P* value	Statistic	*P* value
First choice (*f*)	$\chi^2 = 0.00$	1.000	$\chi^2 = 1.33$	0.248	$\chi^2 = 1.33$	0.248
Time spent in elector area (s)	$z = 0.94$	0.345	$z = 1.57$	0.116	$z = 0.52$	0.600
Time spent in contact (s)	$z = 1.15$	0.249	$z = 1.57$	0.116	$z = 0.11$	0.916
Number of contacts (*n*)	$z = 0.00$	1.000	$z = 0.13$	0.893	$z = 1.21$	0.225
Last choice (*f*)	$\chi^2 = 1.33$	0.248	$\chi^2 = 1.33$	0.248	$\chi^2 = 1.33$	0.248

M the Malše River, *E* the Elbe River, *O* the Orlík water reservoir, *z* Wilcoxon test, *ct* number of crayfish trials, *f* frequencies, *s* seconds, *n* numbers

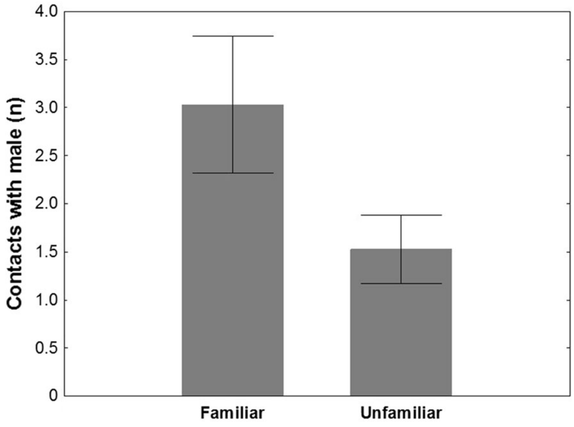

Fig. 5 Average number of female chooser's contacts with familiar and unfamiliar males. Data are mean ± SE. Significant difference between familiar and unfamiliar group $P < 0.05$

Chooser: male

Total number of copulated male choosers was 34 individuals (63.0%) out of 54 trials. Males did not exhibit interest in any specific population, either familiar or unfamiliar. Their choices were independent on origin of females. No significant preferences were observed in time spent in elector's areas, time spent and numbers of pre-copulatory contacts, first and last choices and copulations (Table 3). Similar non-significant results were obtained in comparison of male first choice, times spent and contacts with familiar and unfamiliar females (Table 4). An exception was observed only for the last choice, where males significantly preferred unfamiliar females ($P < 0.05$). No females copulating with more than one male were observed.

Discussion

Recognition of individuals by crayfish has been studied in recent decades. Several studies demonstrate capabilities of individual recognition in crayfish from different families (Seebacher & Wilson, 2007; Aquiloni & Gherardi, 2008a; Aquiloni et al., 2009). However, there are no reports if crayfish are able to distinguish between animals from geographically distinct areas. Previous studies mainly focused on social interactions such as hierarchy levels, mate size, winner and loser cognition, and gender and conspecific recognition (Acquistapace et al., 2002; Aquiloni et al., 2008; Van der Velden et al., 2008).

Our results show that at least females of spiny-cheek crayfish are able to recognize males from their familiar population and prefer them to ones from a distant unfamiliar population. Newly introduced animals can therefore theoretically be discriminated in reproduction in accordance to female choice. On the other hand, males did not show any recognition-preference in the study, which confirms the previous findings of Villanelli & Gherardi (1998). They found males not to be choosy, targeting mainly the quantity of mates rather than quality, despite the described preference for large size or virginity in males (Ameyaw-Akumfi & Hazlett, 1975; Acquistapace et al., 2002; Aquiloni & Gherardi, 2008c). Hence, males are very active in order to find as many mates as possible during the mating season (Gherardi & Barbaresi, 2000; Buřič et al., 2009) especially when they can remove the sperm of a previously mated male, as in Cambaridae (Snedden, 1990) and Astacidae (Villanelli & Gherardi, 1998). In contrast, females consider their choices more because of higher investment in oocyte development and future incubation and parental care (Thiel, 2000, 2003).

Table 2 Statistical analysis results of variables in preferences for familiar or unfamiliar males

Variable	Familiar versus unfamiliar (ct = 36)	
	Statistic	*P* value
First choice (*f*)	$\chi^2 = 0.89$	0.346
Time spent in elector area (s)	$t_{35} = -2.71$	0.010
Time spent in contact (s)	$z = 3.03$	0.002
Number of contacts (*n*)	$z = 2.73$	0.006
Last choice (*f*)	$\chi^2 = 0.00$	1.000
Mating (*f*)	$\chi^2 = 0.09$	0.759

z Wilcoxon paired test, *t* Student's test, *ct* number of crayfish trials, *f* frequencies, *s* seconds, *n* numbers

Table 3 Statistical analysis of male preferences for both unfamiliar electors (M–E–O)

Variable	Population					
	M (ct = 6)		E (ct = 6)		O (ct = 6)	
	Statistic	*P* value	Statistic	*P* value	Statistic	*P* value
First choice (*f*)	$\chi^2 = 1.33$	0.248	$\chi^2 = 1.33$	0.248	$\chi^2 = 1.33$	0.248
Time spent in elector area (s)	$z = 0.52$	0.600	$z = 1.78$	0.075	$z = 0.32$	0.753
Time spent in contact (s)	$z = 0.32$	0.753	$z = 1.78$	0.075	$z = 0.73$	0.463
Number of contacts (*n*)	$z = 0.99$	0.382	$z = 0.51$	0.605	$z = 0.91$	0.412
Last choice (*f*)	$\chi^2 = 1.33$	0.248	$\chi^2 = 1.33$	0.248	$\chi^2 = 0.00$	1.000

M the Malše River, *E* the Elbe River, *O* the Orlík water reservoir, *z* Wilcoxon test, *ct* number of crayfish trials, *f* frequencies, *s* seconds, *n* numbers

Table 4 Statistical analysis results of variables in preferences for familiar or unfamiliar males

Variable	Familiar versus unfamiliar (ct = 36)	
	Statistic	*P* value
First choice (*f*)	$\chi^2 = 3.56$	0.059
Time spent in elector area (s)	$t_{35} = 0.14$	0.889
Time spent in contact (s)	$t_{35} = 0.11$	0.913
Number of contacts (*n*)	$z = 0.01$	0.992
Last choice (*f*)	$\chi^2 = 18.00$	$< 0.10^{-3}$
Mating (*f*)	$\chi^2 = 2.36$	0.125

z Wilcoxon paired test, *t* Student's test, *ct* number of crayfish trials, *f* frequencies, *s* seconds, *n* numbers

However, some questions still persist, e.g. why females prefer, and how do they detect the difference between familiar and unfamiliar individuals. The reason for our original hypothesis was because all spiny-cheek populations in Europe are probably based only on 90 individuals introduced at the end of the 19th century and underwent many bottlenecks during their man-made translocations (Filipová et al., 2011). But crayfish are probably not as capable and resourceful as to manage their population diversity. A more realistic answer as to why to choose between a familiar and unfamiliar mate can be reached from literature where recognition and preference of familiar individuals for shoaling are well documented in many fish species, e.g. Griffiths & Magurran (1997), Brown (2002) and Ward & Hart (2003). Mate choice towards familiars was also detected in other crustaceans (Johnson, 1977; Caldwell, 1992; Karavanich & Atema, 1998a). A familiarity mechanism of natural selection can potentially reduce costly aggressive behaviour as reported in fish (Hojesjo et al., 1998), Seppa et al. (2001) and also discussed in crayfish in light of decreased agonistic behaviour, reduced costs of competition (Bovbjerg, 1953; Daws et al., 2011) and shortening of the courtship period. Such advantages can play an important role in determining why females preferentially select mates from the same locality.

However, the mechanism by which females make their decision is unknown. Due to origins of used experimental animals, we were unable to tell if the effect was based on familiarity rather than kinship (Griffin & West, 2003; Mathews, 2011). Obviously, mate preference is not based on long-term memory or full differentiation of known individuals. Moreover, Van der Velden et al. (2008) described cognition ability of crayfish, although based only on three individuals. In addition, these crayfish were stored individually for at least 5 weeks and to our knowledge there is no report in the literature about such long-term memory in crayfish, regardless of the stimuli (visual or chemical).

It can be assumed that the recognition is rather based on chemical stimuli, as crayfish are aquatic animals mostly dependent on chemical signals from the environment, predators, prey, food or heterospecifics and conspecifics (Gherardi, 2002; Breithaupt, 2011). Olfactory cues are therefore widely used for circumstances of evaluation and decision making, so we can consider specific odours actively or passively produced by males. It seems too unlikely to imagine that males actively report about their place of origin, but the cues may be a function of previous cohabitation at a locality which could lead to a special "odour of the locality". This odour can probably be connected with the similar microbiome of animals living in a similar locality (Ezenwa et al., 2012; Arbuthnott et al., 2016), with minimal impact on metabolic, functional and virulence potential during the change of environmental conditions (Van Bonn et al., 2015). Specific chemical, physical and biological conditions in different localities can lead to significant differentiation of microfloral settlement and therefore to different odour scent (Lize et al., 2013). The concrete mechanism of familiar population recognition and its functioning is a challenge for future research.

Conclusions

This study demonstrated different reproductive strategies of females and males, from observations that females rely on some familiarity of sexual partners as a reliable guarantee for successful mating, to confirmation of a different male reproductive priority for securing successful mating which includes as many mating acts as possible. This study raised the question of the importance of familiarity in mating in mixed wildlife populations where there are significant differences in adult size. Is familiarity of a partner stronger than a larger migrating super-male? And finally, how can females recognize conspecifics from the same populations? Future studies are expected to solve these issues.

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Compliance with ethical standards

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

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Příloha č. 8

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Environmentally relevant concentrations of tramadol and citalopram alter behaviour of an aquatic invertebrate

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ABSTRACT

Environmental pollution by pharmaceutically active compounds, used in quantities similar to those of pesticides and other organic micropollutants, is increasingly recognized as a major threat to the aquatic environment. These compounds are only partly removed from wastewaters and, despite their low concentrations, directly and indirectly affect behaviour of freshwater organisms in natural habitats. The aim of this study was to behaviourally assess the effects of an opioid painkiller (tramadol) and antidepressant drug (citalopram) on behaviour patterns of a clonal model species, marbled crayfish. Animals exposed to environmentally relevant concentrations of both tested compounds ($\sim 1\,\mu g\,l^{-1}$) exhibited significantly lower velocity and shorter distance moved than controls. Crayfish exposed to tramadol spent more time in shelters. Results were obtained by a simple and rapid method recommended as suitable for assessment of behaviour in aquatic organisms exposed to single pollutants and combinations.

1. Introduction

Pharmaceutically active compounds (PhAC) are an important group of pollutants that represent a serious threat to aquatic ecosystems worldwide (Boxall et al., 2012). Freshwater ecosystems are exposed to mixtures of PhACs and their residues (Azuma et al., 2017; Li et al., 2011) originating from municipal wastewater from which they are removed only partially or not at all by sewage treatment (Golovko et al., 2014a; Heberer, 2002; Petrović et al., 2003). Pharmaceutically active compounds are designed to be effective at low concentrations and their residues entering environments through wastewaters can therefore affect non-target organisms (Huerta et al., 2012; Hughes et al., 2012; Santos et al., 2010). Toxic effects of PhACs on mammals are well-known, but information of their ecotoxicity and ecological effects is sparse (Boxall et al., 2012). Antibiotics and hypertension drugs, the most common PhACs found in the environment as well as being those at the highest levels, were the focus of most past studies (Lindberg et al., 2014; Padhye et al., 2014; Petrie et al., 2016). Pharmaceutically active compounds without obvious toxic effects, such as antidepressants, psycholeptics, and opioids have received less attention (Brodin et al., 2013; Brodin et al., 2014).

Psychoactive pharmaceuticals are designed to impact signal pathways in the brain, so they may be expected to affect organism behaviour (Fedorova et al., 2014b; Feighner, 1999; Fong and Ford, 2014; Irvine et al., 2011; Thomas et al., 2014). Some antidepressants bioaccumulate in fish tissues (Du et al., 2012; Gelsleichter and Szabo, 2013; Grabicova et al., 2017; Grabicova et al., 2014) and in the benthos community (Grabicova et al., 2015), which increases the possibility of detectable effects on these organisms.

Similar to fish and aquatic and semi-aquatic insects (Barry, 2013; Jonsson et al., 2014), crayfish are susceptible to behavioural changes induced by extraneous substances in water (Cook and Moore, 2008; Lahman et al., 2015). Exposed aquatic animals often exhibit alterations that affect predation, social interactions, reproduction, and migration (Brodin et al., 2013; Corcoran et al., 2010; Valenti et al., 2012). These effects are not lethal but can indirectly influence entire populations, and thus ecosystem functioning, through disrupted prey/predator relationships and altered defence and reproductive behaviour. Several psychoactive PhACs are still labelled as environmentally safe due to their non-lethal effects, despite their known influence on behaviour of aquatic organisms (Boxall et al., 2012; Fong and Ford, 2014).

Assessment of the effects of PhACs requires a method simple enough to be repeated in precisely-defined conditions as well as a suitable model species. Crayfish show a complex morphology, development, and behaviour, including elaborate social interactions (Gherardi, 2002; Vilpoux et al., 2006). They are considered keystone species in freshwater ecosystems and are strong ecosystem engineers (Creed and Reed, 2004; Dorn and Wojdak, 2004). Hence, pollution impact on native

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crayfish stock can result in ecosystem instability (Creed and Reed, 2004; Momot, 1995; Usio and Townsend, 2004).

The marbled crayfish (*Procambarus virginalis* Lyko 2017) is an ideal model organism for many research areas with the potential to generalise results to other members of Crustacea/Decapoda/Reptantia (Vogt, 2011). They are easily cultured, of suitable size, and exhibit individuality, tolerance to handling, high fertility, a relatively short generation time, and adaptability to a wide spectrum of environmental and nutritional conditions (Kawai et al., 2015; Vogt, 2008). In addition, mother, offspring, and all batch-mates of marbled crayfish are genetically identical (due to reproduction by obligatory apomictic parthenogenesis), which can partly eliminate variability related to genotype (Martin et al., 2007; Vogt, 2008). On the other hand, these clones need not be sensitive enough due to their low demands and high adaptability (Kawai et al., 2015).

Behavioural patterns can be detected using specialised software enabling movement tracking in defined, appropriate conditions for testing PhAC effects. The goal of this study was to compare the behaviour of size matched, genetically uniform, marbled crayfish exposed to environmentally relevant concentrations of an opioid painkiller tramadol and the antidepressant drug citalopram with un-exposed controls.

2. Material and methods

2.1. Chemicals

Tramadol hydrochloride and citalopram hydrobromide were obtained from Sigma-Aldrich (USA). Individual stock solutions were prepared in ultra-pure water (aqua-MAX-Ultra system, Younglin, Kyounggi-do, Korea) at a concentration of 10 mg L^1 and stored at 4 °C. The exposure solutions of 1 μg L^{-1} were prepared by dilution of the stock solution with aged tap water.

Acetonitrile (LC/MS grade purity) was obtained from Merck (Germany), formic acid (for acidification of mobile phases of LC) from Sigma-Aldrich (Germany). Isotopically labelled tramadol (D3) and citalopram (D6) were purchased from Toronto Research Chemicals (Canada) and Lipomed (USA), respectively, and were used as internal standards for LC–MS/MS analyses of water samples.

2.2. Experimental animals

Marbled crayfish were cultured in our laboratory at the Research Institute of Fish Culture and Hydrobiology in Vodňany, FFPW USB, Czech Republic, where all experimental work was conducted. Young mature marbled crayfish specimens were randomly selected from the culture tanks. Carapace length to the nearest 0.1 mm was measured using Vernier callipers and weight to the nearest 0.1 g was obtained with an electronic balance (Kern & Sohn GmbH, Balingen, Germany) after video-recordings. The mean length and weight (Table 1) did not differ between control and exposed groups.

Table 2
Concentration of tramadol and citalopram in marbled crayfish exposure solution and control water at 0 and at 48 h (α = 0.05). Data are presented as mean ± standard deviation.

Tested compound	Group	n	Time 0 (μg L^{-1})	Time 48 (μg L^{-1})	Paired *t*-test	*P*
Tramadol	exposed	3	0.98 ± 0.03	0.81 ± 0.11	1.417	0.391
	control	3	< 0.02	< 0.02	—	—
Citalopram	exposed	4	0.87 ± 0.06	0.89 ± 0.09	−0.457	0.678
	control	4	< 0.02	< 0.02	—	—

2.3. Experimental design

Crayfish were exposed to either tramadol (7 d) or citalopram (21 d) at the concentration of ~1 μg L^{-1} of pure compound (Table 2), a level considered environmentally relevant (Fedorova et al., 2014b; Grabic et al., 2012; Grabicova et al., 2015; Rúa-Gómez and Püttmann, 2012; Thomas et al., 2014). The exposure times were based on the mechanisms of action of the selected compounds. Tramadol acts immediately (peak plasma concentration in humans is observed 1.5 h post-ingestion (www.medscape.com, 8 September 2017), in contrast to citalopram in which a steady-state plasma concentration is achieved in one to two weeks (www.fass.se, 8 September 2017). Animals maintained in pharmaceutical-free aged tap water were used as control and handled in the same way as exposed animals. Animals were stocked individually in clear plastic boxes with capacity of 1.25 L (190 × 140 x 75 mm) containing 0.5 L of exposure solution or aged tap water alone. Water quality parameters were ammonium (NH_4^+) < 0.01 mg L^{-1}, nitrite (NO_2-) < 0.01 mg L^{-1}, nitrate (NO_3-) 4.2 mg L^{-1}, fluoride (F-) 0.6 mg L^{-1}, chloride (Cl-) 8.9 mg L^{-1}, iron (Fe) 0.093 mg L^{-1}, calcium (Ca) 32.0 mg L^{-1}, magnesium (Mg) 8.5 mg L^{-1}, manganese (Mn) < 0.02 mg L^{-1}, conductivity 24.1 mS m^{-1}, hardness 1.16 mmol L^{-1}, and turbidity 0.65 FNU.

The animals were fed ad libitum on commercial feed (Sera Granugreen, Sera, Heinsberg, Germany). Boxes were cleaned during the solution/water change every second day. The control group was cleaned first in order to avoid contamination with tested compounds. Animals that moulted or spawned during the exposure period were removed from the experiment. The water temperature (alcohol thermometer accurate to 0.1 °C) did not differ among exposed and control groups in tramadol and citalopram exposure and reached values of (mean ± standard deviation) 20.0 ± 0.5 °C and 20.0 ± 0.2 °C, respectively.

The real concentration of pharmaceuticals in exposure solutions and water were checked by liquid chromatography tandem mass spectrometry (LC–MS/MS) twice over the exposure period for tramadol and four times for citalopram, before (at time 0, i.e. fresh water or freshly prepared exposure solution) and after changing the water (at time 48 h, i.e. water or exposure solution removed after 48 h). Samples were filtered through a syringe filter of regenerated cellulose, 0.45 μm pores (Labicom, Olomouc, Czech Republic) and stored at −20 °C until the

Table 1
Carapace length (CL) and weight (W) of marbled crayfish specimens used in experimental groups. The number of individuals in each experimental group was n = 20. Data are presented as mean ± standard deviation.

Tested compound	Group	Shelter available	CL (mm)	*t*-test	*P*	W (g)	*t*-test	*P*
Tramadol	exposed	no	21.2 ± 1.9	−1.07	0.290	2.8 ± 0.8	−1.39	0.173
	control	no	20.5 ± 2.4			2.4 ± 0.7		
	exposed	yes	20.7 ± 1.6	−0.47	0.643	2.6 ± 0.6	−0.58	0.567
	control	yes	20.5 ± 1.7			2.4 ± 0.7		
Citalopram	exposed	no	21.3 ± 1.8	0.15	0.884	2.6 ± 0.6	0.52	0.608
	control	no	21.4 ± 2.1			2.7 ± 0.9		
	exposed	yes	21.2 ± 1.8	−0.09	0.925	2.5 ± 0.7	0.16	0.873
	control	yes	21.2 ± 1.5			2.6 ± 0.6		

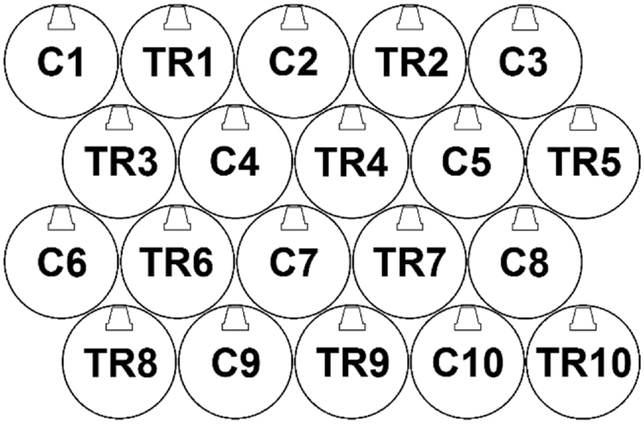

Fig. 1. Schematic of the arrangement of the replicate group of tanks for video-recording of marbled crayfish under treatment (TR1 -T10) and control conditions (C1 - C10) with shelters. Crayfish in all tanks were recorded and analysed simultaneously.

analysis. Water samples were thawed and internal standard was added. The analyses were conducted using a triple stage quadrupole MS/MS TSQ Quantum Ultra Mass Spectrometer coupled with an Accela 1250 and Accela 600 LC pumps (Thermo Fisher Scientific) according to Fedorova et al. (2014a) and Lindberg et al. (2014).

2.4. *Experimental set-up and data acquisition*

Crayfish were placed individually in 280 mm diameter plastic tanks containing 2 L aged tap water and 200 mL fine sand (< 1 mm) (Fig.1). For each tested compound, two groups each comprising 20 crayfish, one group without shelter and the other with shelter consisting of a half a ceramic plant pot, 50 mm depth, 60 mm entry attached to the bottom of the tank. Each group was further separated into 10 exposed and 10 controls. The trial was duplicated.

The crayfish were video-recorded for 4 h using a digital video camera (Sony HDR-CX240, Sony, Japan) attached above arenas. Light was provided as a permanent indirect illumination by fluorescent tubes (day light, 2310 lm). Video recordings were evaluated by an EthoVision® XT 12.0 software (Noldus Information Technology by, Wageningen, Netherlands) using a multiple arena module to detect patterns of crayfish movement. *Distance moved* (cm), *activity* (percentage of time when crayfish locomotion was detected), and *velocity* (cm s^{-1}) were evaluated. When shelters were included, the software was programmed to detect the *percentage of time spent outside the shelter.*

2.5. *Statistical analysis*

Statistical analyses were performed using Statistica 12.0 (StatSoft, Tulsa, OK, USA). A paired *t*-test was used to compare the concentrations of the tested compound at time 0 (newly prepared exposure solution) and time 48 (exposure solution after 48 h). For the comparison of length and weight of exposed and control crayfish, *t*-test for independent samples was used. Nested ANOVA was used to analyse distance moved, velocity, activity, and time spent outside the shelter (replicate groups as a random factor, exposure as a fixed factor), followed by multiple comparisons of means as a post hoc test (Fisher's exact test). The null hypothesis was rejected at $\alpha = 0.05$.

3. Results

3.1. *Analysis of water samples*

The concentrations of tested compounds in analysed water samples at the time 0 and 48 h of exposure did not differ. Water from control group revealed concentration lower than detection limits of the method used (Table 2).

3.2. *Tramadol*

In the set-up without available shelter, exposed crayfish moved significantly shorter distances than control animals ($F_{1,35} = 4.34$, $P = 0.045$). Tramadol-exposed crayfish showed significantly lower velocity than controls ($F_{1,35} = 4.34$, $P = 0.045$). Activity duration was slightly greater in the treated group compared to control, but difference was not significant ($F_{1,35} = 1.65$, $P = 0.21$) (Fig. 2). None of the tests between replicate groups within treatments were significant.

In the crayfish with available shelter, distance moved did not differ significantly between exposed and control ($F_{1,36} = 1.55$, $P = 0.221$). We found no significant differences in velocity between exposed and control animals ($F_{1,36} = 0.57$, $P = 0.455$). Activity duration of exposed and control animals showed no significant differences ($F_{1,36} = 0.06$, $P = 0.810$). There was a trend that exposed crayfish spent less time outside the shelter ($F_{1,36} = 3.99$, $P = 0.053$) and this pattern was similar in both groups ($F_{1,36} = 0.0002$, $P = 0.988$). None of the test between replicate groups within treatments were significant.

3.3. *Citalopram*

In the group without available shelter, crayfish exposed to citalopram moved significantly shorter distances than control animals ($F_{1,35} = 5.91$, $P = 0.020$). Citalopram-exposed crayfish also showed

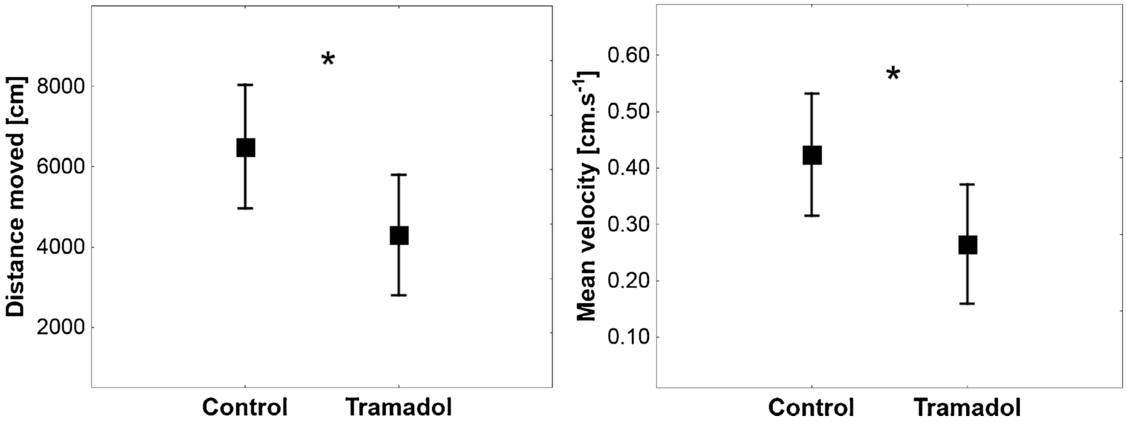

Fig. 2. Total distance moved and mean velocity in marbled crayfish exposed to an environmentally relevant concentration of tramadol and control in the system without available shelter. The number of replicates in both groups (Control and Tramadol) was n = 20. * = Significant differences ($\alpha = 0.05$) between exposed and control crayfish. Data are presented as mean ± standard error of mean.

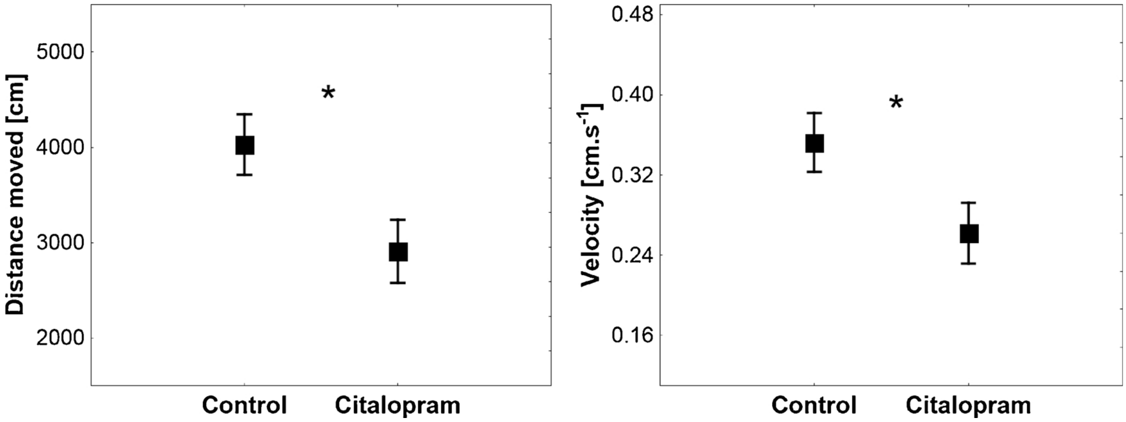

Fig. 3. Total distance moved and mean velocity in marbled crayfish exposed to environmentally relevant concentration of citalopram and control in the system without available shelter. The number of replicates in both groups (Control and Tramadol) was n = 20.* = significant differences (α = 0.05) between exposed and control crayfish. Data are presented as mean ± standard error of mean.

significantly lower velocity than control ($F_{1,35} = 4.61$, $P = 0.039$). Activity of treated and control animals did not differ ($F_{1,35} = 0.49$, $P = 0.488$) (Fig. 3). None of the test between replicate groups within treatments were significant.

When shelter was available, the distance moved did not differ significantly among exposed and control animals ($F_{1,36} = 0.07$, $P = 0.797$). The difference in velocity of treated and control animals was not significant, either ($F_{1,36} = 1.01$, $P = 0.321$). Activity of exposed and control crayfish did not differ ($F_{1,36} = 2.70$, $P = 0.109$), but the pattern was visible in both replicate groups. Exposed and control animals spent similar proportion of time outside the shelter ($F_{1,36} = 0.21$, $P = 0.647$). None of the test between replicate groups within treatments were significant.

4. Discussion

The quality of surface waters is crucial for ecosystem function and biodiversity as well as for human use for domestic water and agriculture (National Intelligence Council, 2012). Research into the effects of PhACs on non-target aquatic organisms has recently expanded from investigations of the most commonly occurring compounds (antibiotics and hypertensive drugs) to micropollutants that are present at lower concentrations (e.g. antidepressants, psycholeptics, and opioids) (Brodin et al., 2014; Fong and Ford, 2014; Klaminder et al., 2014), also entering surface waters through sewage treatment plant effluent (Golovko et al., 2014a, b; Lajeunesse et al., 2012). At least some have been shown to induce behavioural changes in aquatic organisms at low (Barry, 2013; Berninger et al., 2011; Brodin et al., 2014) and environmental concentrations (Brodin et al., 2013; Fong and Ford, 2014; Kellner et al., 2016).

Alterations in basic behavioural patterns such as activity level, boldness, and sociability, especially of predatory and omnivorous organisms, can change functioning of entire ecosystems (Krebs and Davies, 1997; Manning and Dawkins, 2012; Schmitz, 2007). Increased activity, aggressiveness, and changed foraging behaviour can rapidly impact resources and lead to ecosystem instability, disrupting relationships in the food chain and affecting biodiversity (Duffy et al., 2007; Schmitz, 2007). On the other hand, decreased activity, slower reactions, and reduced foraging can render the animal more prone in predatory or limit successful reproduction (Gherardi, 2002; Krebs and Davies, 1997; Manning and Dawkins, 2012). Evidence of the effects of PhACs at environmentally relevant concentrations is scarce (Brodin et al., 2014; Fong and Ford, 2014; Klaminder et al., 2014), and information of their ecological impact in aquatic ecosystems is therefore insufficient (Boxall et al., 2012; Corcoran et al., 2010; Hughes et al., 2012). The development of practical methods to detect ecologically important behaviours of animals exposed to environmentally relevant concentrations is an important step toward realistic risk assessment of PhACs (Brodin et al., 2014; Klaminder et al., 2014).

Studies of chronic exposure to PhACs or other pollutants often use observation of a wide spectrum of behavioural patterns in exposed animals compared to controls (Brodin et al., 2014). These studies, as opposed to those assessing acute toxicity, development, or reproduction as endpoints, are suggested as appropriate tools for evaluating the effects of environmental contaminants (Melvin and Wilson, 2013). Studies have confirmed that some PhACs induce behavioural alterations with drug- and species-specific effects (Backhaus, 2014; Fong and Ford, 2014). For example, changes in activity and feeding rate are reported as effects of antidepressants or psychiatric drugs (Backhaus, 2014; Brodin et al., 2014). Generally, methodology should be standardized and as simple as possible to make the study replicable and should allow observation of control and exposed animals simultaneously. We used video recording of individual animals in a defined space and conditions (unknown novel ambience for all tested animals), enabling evaluation of 10 control and 10 treated animals simultaneously, limiting the potential of differing conditions between control and treated individuals. Data analysis by automatic software also eliminated any bias of subjective evaluation.

We found wide variation in the data. Both control and exposed crayfish showed individuality in activity, distance moved, velocity, and time spent outside the shelter, with wide differences in exploratory behaviour in novel conditions. This suggests potential for personality, individual variation in behaviour that is consistent across time or context, e.g. tendency to exploration in new environments, level of boldness, activity levels in a non-novel environment, aggressiveness, and non-agonistic sociability (Réale et al., 2007). Personality traits have been confirmed and studied in numerous vertebrate and invertebrate taxa (Cote et al., 2014; Cote et al., 2013; Sih et al., 2012), including crayfish and other aquatic animals (Pintor et al., 2008; Vainikka et al., 2011; Zhao and Feng, 2015). Here we confirmed differences in tendency to explore a new environment in genetically identical animals, in which lower variability could be expected. The effect of the individualistic nature of the chosen model species could be counteracted by reasonable number of replications.

The groups always showed similar levels of variation and similar behaviour patterns with the compounds tested. Despite the high individual variation, significant differences were detected between control and exposed stocks in both tested compounds. Tramadol was associated with lower velocity, resulting in shorter distance travelled in crayfish without shelter. On the other hand, crayfish exposed to tramadol were slightly more active without available shelter. This pattern disappeared in the group with available shelter, no effect of Tramadol could be detected.

Shelter availability, an important resource for crayfish (Gherardi, 2002; Longshaw and Stebbing, 2016), led to the elimination of differences between treatments in all observed behaviours. Differences in time exposed and control animals spent outside the shelter were borderline significant ($P = 0.053$). Tramadol exposed animals spent less time outside the shelter, which can reflect higher shyness or reduced mobility.

Similar results were obtained in the animals exposed to citalopram. Movement of experimental animals was significantly slower, resulting in shorter distances moved in the absence of shelter. No differences were observed between groups when shelters were available.

The observed behavioural differences may be related to the mode of action of the opioid painkiller with analgesic properties on the central nervous system. Tramadol is reported to cause decreased alertness, drowsiness, dizziness, loss of appetite, and impaired sensitivity to environmental cues (Langley et al., 2010; National Center for Biotechnology Information, 2017a, b). Citalopram is described as reducing aggressive and impulsive behaviour (Armenteros and Lewis, 2002; Reist et al., 2003) and decreasing desire for the reward (Tiihonen et al., 1996).

Most studies of PhACs on behavioural patterns of aquatic organisms have used concentrations thousands or tens of thousands times higher than those reported in surface waters, or tested chemicals administered by injection into the circulatory system (Brodin et al., 2014; Fong and Ford, 2014; Klaminder et al., 2014). Research of effects on aquatic animals of PhACs that act via serotonin and norepinephrine reuptake transporters and interaction with the serotonin system (Brodin et al., 2014; Lillesaar, 2011) provides clear information of their influence on physiology and behaviour (Brodin et al., 2013; Gunnarsson et al., 2008) and their crucial role in activity, locomotion, territoriality, aggression, and reproductive behaviour (Brodin et al., 2014; Fong and Ford, 2014; Kohlert et al., 2012; Lillesaar, 2011).

Several fish species respond to very low concentrations of Citalopram, e.g. increased locomotor activity was observed in three-spined stickleback *Gasterosteus aculeatus* at $1.5\,\mu g\,L^{-1}$ (Kellner et al., 2016). Conversely, no effect on rainbow trout *Oncorhynchus mykkis* was reported even at concentrations hundreds- or thousands-fold higher (Holmberg et al., 2011). Crayfish, a representative of freshwater invertebrates, showed in our experimental work behavioural changes at environmentally relevant concentration ($1\,\mu g\,L^{-1}$). Hence, the response of tested animals to Citalopram seems to be species-specific as reviewed Brodin et al. (2014).

The effects of environmentally relevant concentrations of tested compounds areclear. Concentrations of $\leq 1\,\mu g\,L^{1}$ of these widely used PhACs have shown impact on basic behavioural patterns of freshwater biota, here represented by the crayfish. The use of behaviour as a criterium has the potential to allow generalization of behavioural effects across species within classes of pharmaceuticals and for monitoring key behaviours when assessing the risk of ecological effects of PhACs (Backhaus, 2014; Brodin et al., 2014; Huerta et al., 2012; Klaminder et al., 2014). The knowledge of how a particular species is affected by particular PhAC compounds or their mixtures is of key importance for future research. The manifestation of behavioural changes of organisms in ecosystems and their impact on ecosystem properties and functioning are of the most concern (Backhaus, 2014; Melvin and Wilson, 2013). Species-specific effects of PhACs are suggested as possible drivers disrupting or altering food-web structure and ecosystem functioning (Brodin et al., 2013; Brodin et al., 2014). In our study, exposed animals that did not have access to shelter showed less tendency to explore the new environment, and their movements slowed down. Under natural conditions this may result in reduced foraging and greater vulnerability to predation when leaving the shelter. Shelter availability removes the difference between control and exposed groups, although the tramadol-exposed crayfish tended to spend more time in the shelter than did controls. The target behaviours are reported as crucial determinants of fitness and are connected to growth, reproduction, and survival (Brodin and Johansson, 2004; Longshaw and Stebbing, 2016). Reduced nutrition resulting from lethargy may lead to higher vulnerability to environmental changes, reduced competitiveness, and, ultimately, lower survival (Reynolds, 2002). Lower success in mate searching, couple forming, and mating may affect successful reproduction (Gherardi, 2002; Krebs and Davies, 1997). Impact on native crayfish species, which play an important ecological role, may lead to a reduction in ecosystem stability (Creed and Reed, 2004; Momot, 1995; Usio and Townsend, 2004).

The tested PhACs can significantly alter behaviour of large aquatic invertebrates at dilute concentrations similar to those detected in environmental conditions (Grabic et al., 2012; Grabicova et al., 2015; Grabicova et al., 2017). The impacts of environmentally relevant concentrations of PhACs remain largely overlooked, despite their widespread presence worldwide (Ebele et al., 2017; Luo et al., 2014; Petrie et al., 2016). Increasing concentrations of pharmaceutical residues in aquatic systems can be expected, as pharmaceutical use is projected to increase (National Intelligence Council, 2012).

5. Conclusions

Our results highlight the ecological importance of PhACs in aquatic ecosystems, and call for new test protocols to examine the full environmental impact of PhAC residues. Our research also presents a relatively simple method for assessing effects on organisms of compounds commonly present in surface waters.

Aquatic organisms living in contaminated environments are exposed not only to single contaminants but to combinations of PhACs that could lead to antagonistic, additive, or magnified effects (Backhaus, 2014; Schoenfuss et al., 2016). Hence, research is needed to assess effects of particular pharmaceuticals along with studies of effects of PhAC combinations to evaluate the real ecological impact on affected ecosystems.

Declarations of interest

None.

Contributors

MB, JK, KG, AK, IK, TR, and PK participated in research preparation. MB, JK, KG, and RG participated in data analysis, MB, AK, KG, RG, and TR participated in article preparation. All authors have approved the final article prior submission.

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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Chemicals

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

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

2.2. Experimental animals

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

2.3. Experimental design

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

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

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

2.4. Behavior observations and data acquisition

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

Table 1

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

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

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

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

2.5. Statistical analysis

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

3. Results

3.1. Analysis of water samples

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

3.2. Methamphetamine

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

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

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

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

3.3. Sertraline

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

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

3.4. Life history traits

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

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

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

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

4. Discussion

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

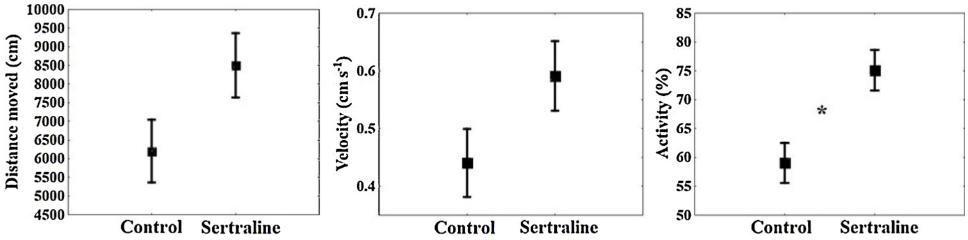

Fig. 1. Total distance moved (cm), velocity (cm s^{-1}), and activity (%) of *Procambarus virginalis* exposed to an environmentally relevant concentration (~1 μg L^{-1}) of sertraline and control without shelter available. The number of replicates in both groups was 27. * = significant differences (α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

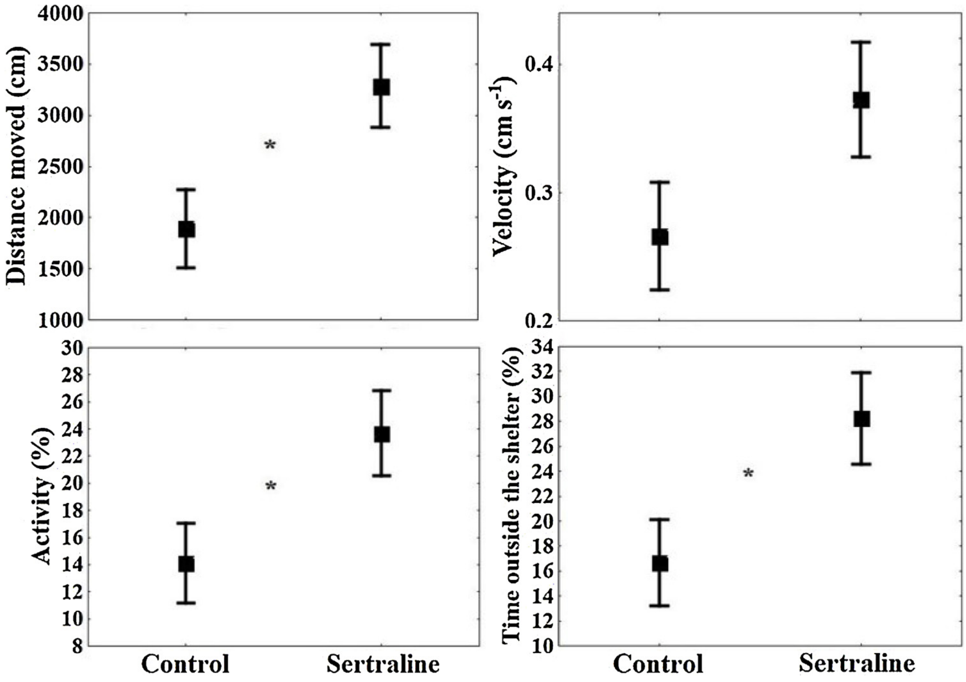

Fig. 2. Total distance moved (cm), velocity (cm s^{-1}), activity (%), and time spent outside the shelter (%) of *Procambarus virginalis* exposed to environmentally relevant concentration (~1 μg L^{-1}) of sertraline and control with available shelter. The number of replicates was 25 and 24 for control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

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

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

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

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

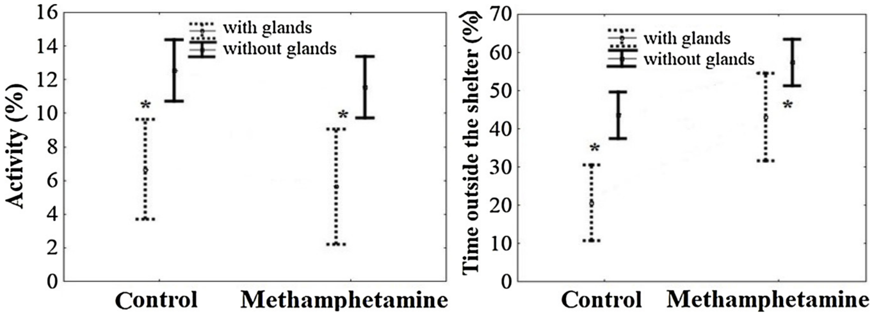

Fig. 3. Activity (%) and time spent outside the shelter (%) in *Procambarus virginalis* exposed to an environmentally relevant concentration (~1 μg L^{-1}) of methamphetamine and control in systems with shelter with respect to glair gland status. The number of replicates was 30 (9 with gland and 21 without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

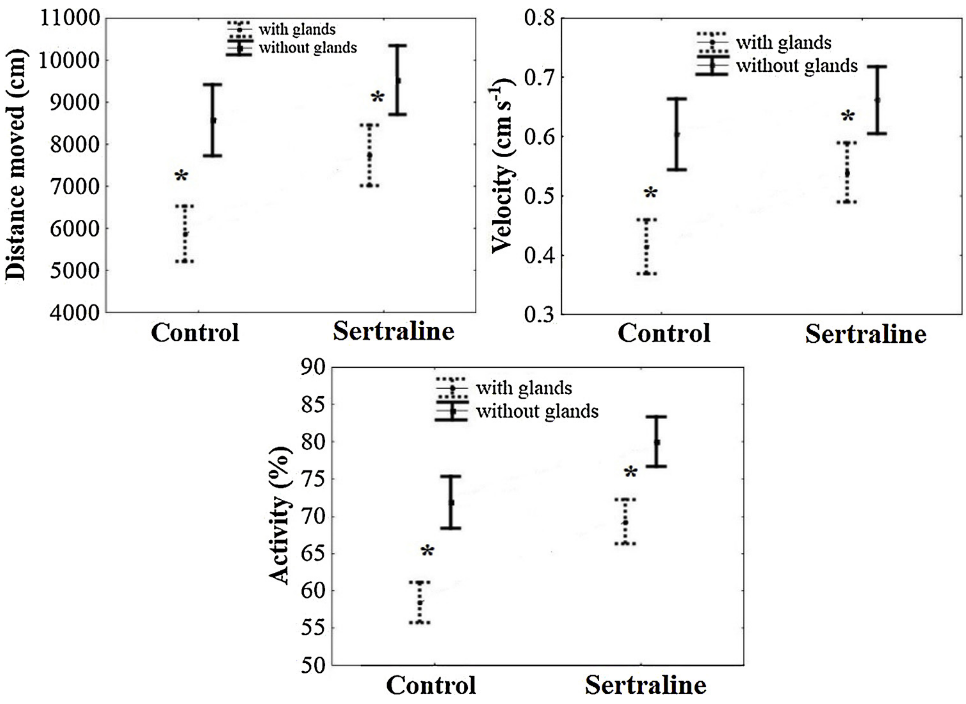

Fig. 4. Total distance moved (cm), velocity (cm s^{-1}) and activity (%) of *Procambarus virginalis*, with and without glair glands, exposed to an environmentally relevant concentration (~1 μg L^{-1}) of sertraline and control in systems without available shelter. The number of replicates was 27 for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

Table 3

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

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

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

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

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

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

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

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

5. Conclusions

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

Author contribution

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

Declaration of Competing Interest

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Appendix A. Supplementary data

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

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Exposure paradigm of fluoxetine impacted the *Faxonius virilis* agonistic behavior differently

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HIGHLIGHTS

- Effects of aquatic pollutants on organisms behavior are not as simple as standard toxicological tests would indicate.
- Chemical concentrations in a plume vary in dynamic systems which impacts organisms more than standard test's uniform concentrations.
- The agonistic behavior of crayfish was affected by fluoxetine concentrations and exposure paradigms.

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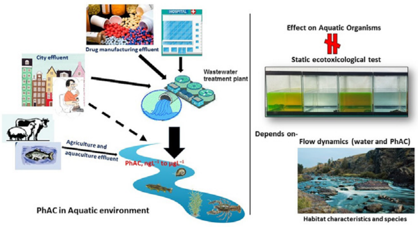
GRAPHICAL ABSTRACT



ABSTRACT

Pharmaceutically active compounds are major contaminants of aquatic environments. The effects on aquatic organisms have been assessed mostly through eco-toxicological tests performed using static exposure systems or flow through systems with constant concentrations. Yet, constant concentration exposures ignore the spatio-temporal dynamics of chemicals in flowing environments. In dynamic systems, a chemical's effect on an organism will vary due to fluctuations in the frequency, magnitude, and duration of the chemical concentration within the plume, which develops due to turbulence interacting with the geomorphology of habitat. The aim of this study was to analyze how different exposure dynamics to the antidepressant fluoxetine might alter the agonistic behavior of aquatic organisms. Male crayfishes, *Faxonius virilis*, were subjected to 23 h exposures at different concentrations of fluoxetine (control, 0.05, 0.5, 1, 10 and 100 µg/l) in both static and dynamic mesocosm systems. After exposure, size-matched crayfishes, from the same exposure system and fluoxetine concentration, underwent a fifteen minute fight trial. The aggressive intensities and duration of agonistic interactions were quantified. The time spent performing a tailflip was significantly longer for fluoxetine concentrations of 1, 10, 100 µg/l in the static exposure than in the dynamic exposure. On other hand, the time spent at higher intensities and the time to escalate to the highest intensity of interactions in control treatments were significantly lower in the dynamic exposure than in the static exposure. Whereas, in elevated fluoxetine concentrations, these times were significantly higher in the dynamic than in static treatments. Therefore, we could conclude that the fight dynamics and duration of agonistic behavior in crayfish were affected by static and dynamic exposure paradigms differently. Despite these behavioral changes, serotonin levels in fluoxetine-exposed crayfish did not differ significantly between exposure paradigms.

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Future research should incorporate different exposure methods that more accurately represent chemical exposure in natural habitats.

1. Introduction

Pharmaceuticals, extensively used in human and veterinary prescription, were invisible pollutants in the aquatic environment until the 1960s. Within the last twenty years, these chemicals have been increasingly detected in aquatic environments, especially in surface water samples, and have gained environmental attention (Burkina et al., 2015). Due to their increasing presence and concentrations in aquatic habitats, pharmaceuticals are considered an emerging contaminant (Ebele et al., 2017). Pharmaceuticals from human or animal excretion, industrial waste, runoff from hospitals, or improper disposal (Chang et al., 2007; Escher et al., 2011; Loos et al., 2013; Collado et al., 2014; Frédéric and Yves, 2014) find their way to surface waters via the effluent of sewage treatment plants (STPs) (Heberer, 2002; Blair et al., 2013; Golovko et al., 2014). Nonsteroidal anti-inflammatory drugs (NSAIDs), fibrates, β-blockers, selective serotonin reuptake inhibitors (SSRIs), azoles, and antibiotics (Loos et al., 2013) are the most common mixture of pharmaceuticals residues in effluents from STPs, surface waters, groundwater, and sediments. Pharmaceuticals are designed to adjust the physiology and subsequent behavior of their intended target organisms without creating detrimental effects (Boxall et al., 2012; Meredith-Williams et al., 2012). Residues of these compounds that enter into the aquatic ecosystems could also have non-lethal effects on non-target organisms which could subsequently alter ecosystem functioning as well (Boxall et al., 2012).

The frequent detection of antidepressants in aquatic environments across the world make these chemicals an important class of pharmaceuticals to examine from an ecotoxicological perspective (Mazurova et al., 2008; Fong and Ford, 2014; Hamilton et al., 2016). Standard acute toxicity tests of a number of pharmaceuticals, including antidepressants have shown no lethal acute toxicity to aquatic organisms at low environmental concentrations (10–1000 ng.l^{-1}) (Fent et al., 2006). However, antidepressants, such as selective serotonin re-uptake inhibitors (SSRI), are of particular interest as these chemicals are designed to alter the behavior and neurophysiology of the target organism (Tain et al., 2006; Valenti et al., 2012; Brodin et al., 2014; Hamilton et al., 2016; Woodman et al., 2016). Thus, it is possible that these compounds can affect organisms even at these environmentally-low concentrations (Mazurova et al., 2008; Valenti et al., 2009; Guler and Ford, 2010; Kaushik et al., 2016). Behavioral effects could be exaggerated due to bioaccumulation rates in tissues and in the nervous system of fish, crayfish, or other benthic organism (Fong and Ford, 2014; Grabicova et al., 2014; Grabicova et al., 2015; Grabicova et al., 2017).

Fluoxetine, a compound of the SSRIs class, is a widely prescribed antidepressant around the world. As such, concentrations have been detected in surface waters at levels up to 0.54 to 0.93 μg/l, in ground water at 0.056 μg/l (Silva et al., 2012), and in seawater at 0.0026 μg/l at Chesapeake Bay, USA (Pait et al., 2006). Diverse sets of effects of fluoxetine or SSRIs on aquatic life have been reported and are exact changes on behavior depend upon the exposure scenarios, exposure duration, as well as concentration used (Brooks et al., 2003). Fluoxetine has negatively impacted the growth, reproduction, and behavior of invertebrates and vertebrates (Santos et al., 2010; Bossus et al., 2014; Tierney, 2016; Ding et al., 2017). In toxicological studies, animals have commonly been exposed to antidepressant either by direct injection, infusion, or through an aqueous immersion. Static exposures and constant concentration flow-through exposure systems are usually applied to assess lethal toxicity endpoints (LC_{50}: concentrations at which 50% of the test animals die from exposure within a certain time period) (Stephan, 1977). These same paradigms have been used to investigate sub-lethal effects. However, the introduction of anthropogenic chemicals to natural systems (rivers and lakes) is typically not a steady-state function as turbulent plumes are chaotic in nature in both lakes and rivers. So, these uniform concentration paradigms do not reflect the dynamic nature of exposure in flowing systems (Harrigan and Moore, 2017). The spatial and temporal fluctuations of chemicals are dependent on seasonal fluctuations in the hydrological cycle, the physicochemical properties of the compounds, removal rates in the STPs, stream/river dilution factors, turbulence as well as habitat structures of streams (Harrigan and Moore, 2017). Therefore, for the effective quantification of chemical impacts, it is necessary to consider the spatial and temporal variability of chemical (toxicant) fluctuations in natural systems. Given the wide fluctuations in concentrations under turbulent dispersal, the effects of antidepressants in dynamic systems should be more variable than those assessed in static systems (Neal and Moore, 2017; Steele et al., 2018). This conclusion doesn't even consider the exacerbation of the chemical effect due to bioaccumulation (Grabicova et al., 2017).

In the present study, the crayfish *F. virilis* was used as a model organism because of its well-studied morphology, development, and behavior. The latter includes elaborate social interactions (Vilpoux et al., 2006; Gherardi, 2002). Along with these characteristics, crayfish are keystone species as well as ecosystem engineers (Creed and Reed, 2004; Dorn and Wojdak, 2004). Thus, they are an excellent candidate species to be used as a bioindicators to measure overall aquatic ecosystem health. Presence or absence of crayfish in particular habitats, or even changes in particular behaviors such as foraging or aggression, could have large effects on ecosystem services. Agonistic behavior is an ecologically important behavior for crayfish as these intraspecific interactions determine social hierarchies (Souty-Grosset et al., 2006) as well as the acquisition of food, shelters and mates (Gherardi, 2002).

As a principle, SSRIs are designed to block the functioning of serotonin reuptake transporters which can alter behavioral states by changing the concentrations of the neurotransmitter, serotonin, within synaptic clefts (Hyttel, 1994; Fedorova et al., 2014). Serotonin functions as a modulator of anxiety-like and social (agonistic) behaviors in crayfish (Fossat et al., 2014; Fossat et al., 2015). The formation of social hierarchies heavily depends on serotonin levels (Huber and Delago, 1998; Sneddon et al., 2000) and elevated levels of serotonin in an animal's nervous system can increase the likelihoods of subsequent winning in combat (Bergman et al., 2003). In the present study, crayfish were exposed to different concentrations of fluoxetine in both static and dynamic exposure systems to assess the changes of agonistic behavior due to different exposure paradigms. We hypothesized that crayfish behavior and serotonin level would be impacted more in the dynamic exposure due to the large scale fluctuations in fluoxetine concentration compared to a static exposure paradigm.

2. Materials and methods

2.1. Experimental animals and care

Male *F. virilis* (3.60 ± 0.39 cm, mean carapace length ± SD) were collected from Maple Bay of Burt Lake located in Cheboygan County, Michigan, USA (45.4873°N, 84.7065°W) by the use of metal hand nets. After collection, crayfish were sorted according to size, sex, and health conditions. Only crayfish with all of their appendages intact were selected for the study. All animals were kept isolated mechanically and visually in plastic containers (15 × 10 × 10 cm) which were housed in a flow-through artificial stream. The stream was constructed using cinder blocks (20.3 × 20.3 × 40.6 cm) and polyethylene sheeting (0.01 mm thickness) and unfiltered water from the Maple River which was fed into one end of the stream. All research and housing took place at the Stream Research facility of University of Michigan Biological Station, Pellston, Michigan, USA. Crayfish were kept isolated for at least seven days to remove any previous social status that may have been established in their natural settings (Bergman et al., 2003; Hudina et al., 2016). In the holding streams, unfiltered Maple River water delivered naturally produced detritus which allowed the crayfish to feed. During this holding time, crayfish were exposed to ambient water temperature (~20 °C) and a natural light: dark cycle (15:9 h).

2.2. Experimental design

The experiment consisted of a 2 × 6 fully factorial design with two factors. The first factor was fluoxetine concentration and had levels 0 (control), 0.05, 0.5, 1, 10 and 100 µg/l measured at the location of the animal (see below). The second factor was the exposure paradigm which consisted of a static or dynamic exposure system. All animals were used only once throughout the experiment either in static or dynamic exposure systems. A total 129 trails (258 male crayfish) were analyzed in this study (Table 1).

2.3. Exposure paradigms

2.3.1. Dynamic exposure arena

Four artificial streams consisting of cinder blocks and 0.01 mm polyethylene sheeting were constructed for the dynamic mesocosms. The streams were 160 × 40.5 × 40.5 cm (interior L × W × H, Fig. 1). Water from the east branch of the Maple River was pumped into reservoir head tanks and delivered to each stream via two garden hoses (1.72 ± 0.2 cm, mean ID± SEM) at a constant flow of 0.22 ± 0.003 l/s (mean flow ± SEM). The water was filtered through nylon mesh (0.01 cm^2 holes) to remove large debris and macroinvertebrates. To reduce the turbulence and to increase replication across the streams, collimators made of acrylic egg crating (1.7 cm^2 square holes) covered with fiberglass window screening (0.1 cm^2 square holes) were placed 20 cm downstream of the inflow. The bottom of each stream was filled with sand substrate to a depth of approximately 3 cm to approximate local river conditions. The water depth of the artificial streams and outflow of water were controlled through the outflow blocks at the downstream end. Outflow blocks constructed of a single cinder block turned on its size so that the holes were horizontal and window screening attached to middle holes of the cinder block. The water level of each stream was maintained at a depth of 27 ± 0.2 cm (mean water depth ± SEM). Crayfish movement within the exposure stream was limited by attaching a piece of Velcro® to the carapace via superglue. The opposite Velcro® square was attached to a glazed tile via 10 cm fishing line. The crayfish/tile/fishing line combination was placed within each stream at the same distance downstream from the chemical delivery source. This setup allowed the animal to move freely within a circular area, at relatively constant distance downstream from the chemical source (Ludington and Moore, 2017; Neal and Moore, 2017). Input tubing of fluoxetine was placed 49 cm upstream of animal location at the ground water according to Steele et al. (2018) (Fig. 1). Flow of fluoxetine solutions into the artificial streams was 0.97 ± 0.02 ml/s (mean ± SEM).

Table 1
Number of crayfish fight trials after exposure to different graded concentrations of fluoxetine under variable exposure systems.

Concentration (µg/l)	Exposure type	Number of trials
Control (0)	Static	11
	Dynamic	08
0.05	Static	11
	Dynamic	11
0.5	Static	10
	Dynamic	12
1	Static	12
	Dynamic	11
10	Static	10
	Dynamic	10
100	Static	10
	Dynamic	12

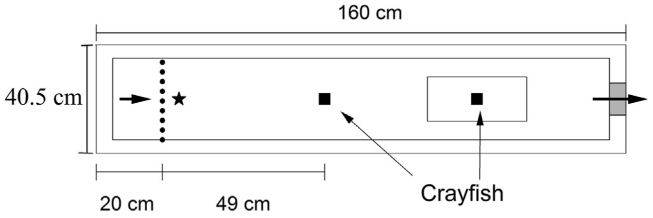

Fig. 1. Diagram of artificial dynamic and static exposure arenas drawn to scale. Fluoxetine source input at upstream region is marked by the star. Solid black squares indicate locations of crayfish and the thin line around the downstream crayfish represents the static-exposure aquarium. Flow is in the direction of animals. The dotted line represents the collimator used to reduce turbulence and the exit cinder block is marked by the grey section.

Fluoxetine was delivered into streams using a gravity system connected to a constant head tank. The design allowed for a constant and consistent delivery of fluoxetine into the dynamic exposure streams throughout the exposure period. The constant head tank consisted of a reservoir (75.7 l) that housed the stock solution and a delivery tank (1.33 l) that allowed for the gravity feed. The delivery tank was constructed out of 7.62 cm ID PVC tube (height = 29.2 cm) with an end cap fitted with 12 cm of 0.6 mm ID tubing connected to 0.5 cm ID aquarium tubing for output of fluoxetine solution. A Hydor pump (model: Seltz l30; 700 l/h) continually replenished the fluoxetine solution in the delivery tank from the reservoir. A 7.62 × 7.62 × 5.08 cm PVC Y connected to a 5.08 cm ID PVC (length = 36.8 cm) diverted the overflow fluoxetine solution from the delivery tank back into reservoir. The fluoxetine tube was buried beneath the substrate to mimic a groundwater contaminant source (Edwards and Moore, 2014; Steele et al., 2018). Fluoxetine reservoir and delivery tank were covered to reduce contamination and dilution from precipitation and photolytic degradation of fluoxetine. Flow of fluoxetine solution was controlled by powering the constant head tank aquarium pumps with Woods 50015WD Outdoor 7-Day Heavy Duty Digital Plug-in Timers.

While flow-through systems are used in ecotoxicological studies, those systems often present chemicals at a steady or constant concentration. Yet, in natural flow systems, chemicals are dispersed by flow which create turbulent odor plumes where the concentrations are heterogeneous in space and time (Edwards and

Moore, 2014; Ludington and Moore, 2017). The dynamic fluctuations of chemicals downstream have very different effects than flow-through constant concentration exposure paradigms (Neal and Moore, 2017). Thus, the mesocosm system used in this study mimics natural exposures because of the development of a turbulent odor plume. In addition, our ability to quantify the overall average exposure at the location of the animal still allows for comparison of average concentration effects in steady state exposures.

2.3.2. Electrochemical measurement of dilution

The dilution factor for fluoxetine at the location of the crayfish within the dynamic exposure streams was calculated using an EmStat3+ Blue electrochemical detection system (Bioanalytical Systems, West Lafayette, IN). The EmStat system was connected to a 30-μm carbon fiber electrode to measure oxidation-reduction reactions within the water column 5 cm above the sand substrate (Edwards and Moore, 2014; Harrigan and Moore, 2017; Ludington and Moore, 2017). A chemical tracer, dopamine, was used as the detection chemical in calculating the dilution factor. Dopamine has the appropriate molecular weight for similar diffusion and transport mechanism to that of fluoxetine and has been used in the past for this type of procedure (Edwards and Moore, 2014: Ludington and Moore, 2017). The Péclet number for this flowing artificial stream system shows that advection is more important for determining the fine scale distribution of the chemical than diffusion (Denny, 1993). The EmStat system sampled, at the crayfish's location, 50 cm downstream of the chemical source at a sampling frequency of 20 Hz for 5 min. Prior to measuring the dopamine distribution in the streams, the electrodes were calibrated with known concentrations of dopamine. The stock solution of dopamine was delivered to the artificial stream using the same constant head tank used in the exposure studies. All test animals were placed in locations where electrochemical measurements were recorded. Appropriate stock solutions of the fluoxetine were prepared in accordance to the calculated dilution factor of 34 to create the intended concentration at the animal's location.

2.3.3. Static exposure arena

Static trails were done in aquaria (20 *l*) filled with a 3 cm sand layer and 13 l of the same Maple River water used in the dynamic trials. The aquaria were placed at the downstream end of each dynamic exposure streams to maintain similar water temperature and natural light dark cycles experienced by the dynamic exposure crayfish. Each aquarium was fitted with an aerator pump to oxygenate the water. The upstream aquarium wall was painted black to visually isolate the dynamic and static exposed crayfish.

Final concentrations of fluoxetine hydrochloride (Sigma-Aldrich) were calculation dilutions from a stock solution of 1 g/l. All final concentrations of stock solution were verified using LC/MS techniques in the UMBS analytical chemistry laboratory using methods described by Chu and Metcalfe (2007). Samples were extracted by solid phase extraction (SPE) with Oasis HLB cartridges (Waters Inc., Milford, MA, USA), and the analytes were then eluted from the cartridges with methanol Chu and Metcalfe (2007). Liquid chromatography was done using an Agilent Series 1100 HPLC system (Agilent Technologies Canada, Mississauga, ON, Canada) with a Genesis C18 column (150 × 2.1 mm i.d., 4 μm particle size).

2.4. Exposure of experimental animals

Crayfish were exposed to fluoxetine solutions of different concentrations in the dynamic artificial streams or in static aquaria for 23 h. The 23 hour-periods was chosen to allow 1 h to reset the exposure systems for the next treatment such that each set of trials began at the same time of day. Artificial streams were allowed to flush for 1 h before the next treatments and static aquaria where manually cleaned to remove any effects of the previous treatment. In both systems, crayfish were restricted to one location (Fig. 1) by use of tethers consisting of a 0.64 cm^2 Velcro ® square attached to a tile weight via fishing line. The opposite Velcro ® piece was fastened to the carapace of each animal using superglue to secure the animal's position in exposure arenas (Neal and Moore, 2017; Steele et al., 2018). Crayfish were subjected to a natural light: dark cycle of approximately 15:9 h and water temperature of ~20 °C during the 23 h exposure period.

2.5. Behavioral analysis

Immediately following the removal of the crayfish from the exposure arena, dyad fight trials were performed for each exposure treatment. Fight trails were performed between two similarly exposed crayfish, i.e. static with static and dynamic with dynamic. Opponents were sized matched with a maximum of 10% difference in carapace length. Fights occurred in an opaque Plexiglas fight arena (39 × 39 × 14 cm, L × W × H) filled with 13.8 l of water from the East Branch Maple River and divided into four equal quadrants by retractable Plexiglas walls. The fight tank design allowed for animals to be mechanically, visually, and chemically isolated until start of fight trial. Crayfish were initially separated in each

Table 2
Ethogram of crayfish agonistic behaviors adapted from Wofford et al. (2015) and Steele et al. (2018) used in video analysis. Numerical intensities listed represent the intensity of behavior displayed.

Behavior name	Intensity level	Behavior description
Tailflip	−2	Rapid retreat from opponent made by quickly flicking tail in reverse direction
Slow Retreat	−1	Low intensity, slow walking away from opponent
Slow Approach	1	Low intensity, slow walking toward opponent without threat display
Rapid Approach	2	Quick high intensity approach toward opponent with major chelae raised in meral spread threat display
Closed Chelae Touch	3	Non-forceful contact made with opponent using closed major chelae. Chelae are not used to push or box with opponent or grab or hold opponent
Antennal Whipping	4	Rapid flicking of antennae used to hit opponent
Stiff Arm	5	Holding opponent away by presenting closed major chelae straight out in front of body
Closed Claw Boxing	6	Forcefully pushing opponent using closed major chelae. Chelae are not used to grab or hold opponent
Open Claw Touching	7	Non-forceful contact made with opponent using open major chelae. Chelae are not used to push or box with opponent or grab or hold opponent
Open Claw Boxing	8	Forcefully pushing opponent using open major chelae. Chelae do not close and are not used to grab or hold opponent
Grabbing at Appendages	9	Major chelae grasp by closing chelae on appendages of opponent. Force is not used to pull or rip appendages of opponent
Tearing of Appendages	10	Major chelae used to grasp appendages of opponent while forcefully pulling or tearing opponent's appendages
Inversion	11	Major chelae grasp appendages of opponent and with forcefully pulling, tearing, or manipulation invert body of opponent

quadrant and allowed to acclimate for 15 min. After acclimation period, the retractable wall of two quadrants was removed and crayfish were allowed to interact for 15 min (Wofford et al., 2015). Each fight trial was video recorded using a Panasonic (Model # HDC-HS250) or Sony (Model # HDR-CX405) handheld camera. Duration, intensity (escalated or nonescalated), time to reach each intensity level, and time spent at each intensity level were analyzed. Intensity levels were determined using a pre-established ethogram adapted from Steele et al. (2018) (Table 2). Initiation of a fight was defined as the time at which a crayfish aggressive contest begins (i.e., when one animal approaches the other within one body length). End of a fight was defined as opponents remaining approximately two body lengths away for a minimum of 10 s. Duration was defined as difference in seconds between the time at which a fight ended and the time at which it was initiated.

2.6. Quantification of hemolymph serotonin content

After the dyadic fight trials, hemolymph samples were collected from ventral side of crayfish abdomen from the space between particular myosepta using insulin syringes. Samples were collected from at least five pairs of crayfish from each treatment. Approximately 700 μl of hemolymph were collected in Eppendorf tubes from each pair, quickly frozen in liquid nitrogen, and stored in refrigerator at −20 °C until lyophilisation. Lyophilized hemolymph samples were extracted 3× in 0.75 ml 10% sodium bicarbonate in an ultrasonic bath. Following sonication for 15 min, the vials were centrifuged and the supernatant collected. The composite extracts were brought to 5 ml total volume with 10% sodium bicarbonate. In a fume hood, 0.5 ml acetic anhydride was added at ambient temperature with constant stirring. Once completed, this reaction yielded the *n*-acetyl serotonin derivative (and *n*-acetyl dopamine, *n*-acetyl octopamine). The final pH of this solution was 3.5, optimal for extraction of acidic compounds into organic solvents. The solution was extracted 3× with 325 μl of methylene chloride, and concentrated to dryness at 45 °C in a vacufuge. 10 ng of Ibuprofen (internal standard) were added after the vacufuge. The residue was reacted with 15 μl MSTFA at 80 °C for 30 min and then analyzed by LC/MS in selection ion monitoring (SIM) mode (Woodman et al., 2016). With a 1:50 split, the detection limit was 0.01 ng serotonin on-column.

2.7. Statistical analysis

Changes in agonistic behavior dynamics as a result of fluoxetine concentration and exposure paradigm were analyzed by a nonlinear mixed model in R (Bates et al., 2015; R Core Team, 2018). The difference in time to different behavioral intensities as well as the time spent at different behavioral intensities was the independent variables within the mixed model. The model was run with the full interactions between fluoxetine concentration and exposure paradigm using the lmer function. In addition, stream number was included in the model as a random factor to account for any potential differences between exposure streams/aquaria. When significant differences were found with the interaction terms, pairwise comparisons were made using the emmeans function with a Tukey-hsd correction factor (R Core Team, 2018; Russell, 2018).

3. Results

3.1. Time spent at different intensities

3.1.1. Time spent at −2 (tailflip)

The mixed model exhibited a significant difference of time spent at −2 between the static and dynamic exposure conditions ($F_{(1,128,0.05)} = 4.97$, $p = 0.027$), but no difference between concentrations ($p = 0.66$) or interaction between concentrations and exposure type. A pairwise Tukey post-hoc comparison showed that the difference between static and dynamic exposure was only apparent at the highest three concentrations (1, 10, and 100 μg/l). At these three concentrations, crayfish in the static condition spent more time tailflipping from their opponents than the crayfish in the dynamic exposure condition (Fig. 2).

3.1.2. Time spent at −1 (retreat)

The mixed model revealed a significant difference of time spent at −1 with the interaction between exposure condition (static and dynamic) and the exposure concentration ($F_{(1,128,0.05)} = 6.62$, $p = 0.027$). A subsequent pairwise Tukey post-hoc comparison showed that the statistical difference between static and dynamic exposure and between concentrations for time spent at −1 was only evident at the highest concentration (100 μg/l). At this concentration, crayfish in the static condition spent nearly four times the amount of time in retreat than those animals in the dynamic exposure condition or than those animals at the other fluoxetine concentrations (Fig. 3).

3.1.3. Time spent at 11 (highest intensity)

The mixed model revealed a significant difference of time spent at the most intensive level of fighting with the interaction between the exposure condition and the exposure concentration ($F_{(1,128,0.05)} = 7.02$, $p = 0.022$). A subsequent pairwise Tukey post-hoc comparison showed a statistical difference between static and dynamic exposure for time spent at 11 for all concentrations, including controls ($p < 0.05$). For control crayfish, the time spent at 11in the static condition was longer than in the dynamic condition. However, this outcome was reversed at all fluoxetine concentrations ($p < 0.05$). In addition, there was no significant difference in the time spent at intensity level 11 between different fluoxetine concentrations under the dynamic exposure condition, but the time was significantly longer in all of these treatments than in con-

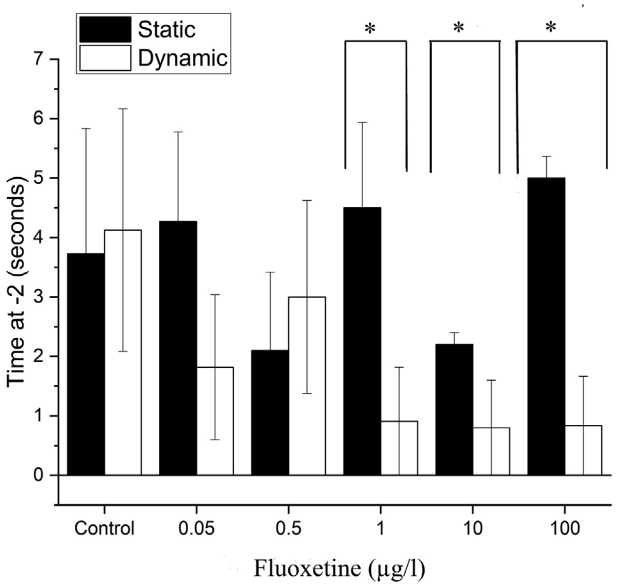

Fig. 2. Mean (±SEM) time spent at intensity level −2 (tailflip) for crayfish exposed to different concentrations of fluoxetine under static (solid black squares) and dynamic (open black circles) exposure conditions. Asterisks indicate a significant difference between the two exposure conditions (static and dynamic) within a single concentration (mixed model followed by Tukey-HSD post-hoc, $p < 0.05$). No significant differences between concentrations within an exposure paradigm were recorded.

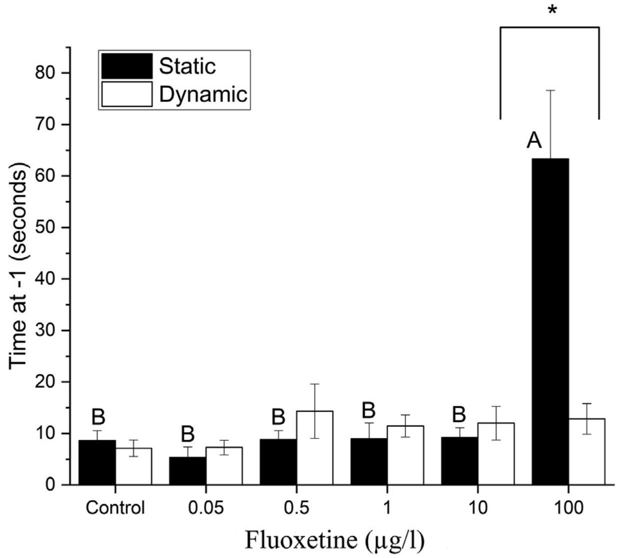

Fig. 3. Mean (±SEM) time spent at intensity level −1 (retreat) for crayfish exposure to different concentrations of fluoxetine under static (solid black squares) and dynamic (open black circles) exposure conditions. Asterisks indicate a significant difference between the two exposure conditions within a single concentration (mixed model followed by Tukey-HSD post-hoc, $p < 0.05$). Different capital uppercase letters indicate significant difference between concentrations within the static exposure paradigm ($p < 0.05$).

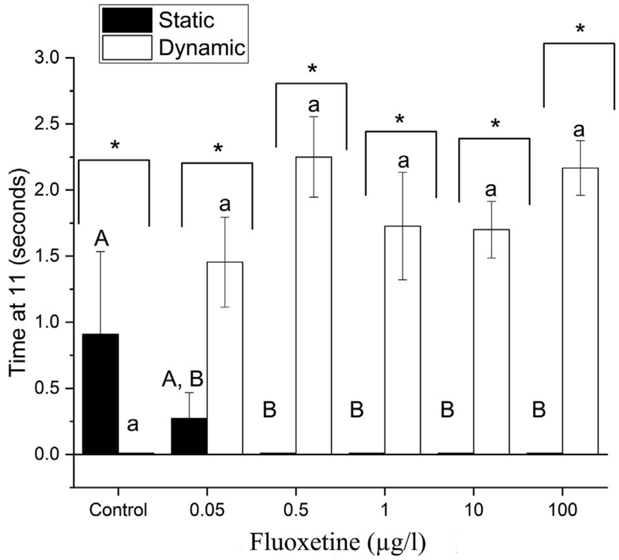

Fig. 4. Mean (±SEM) time spent at intensity level 11 (highest intensity) for crayfish exposed to different concentrations of fluoxetine under static (solid black squares) and dynamic (open black circles) exposure conditions. Asterisks indicate a significant difference between the two exposure conditions within a single concentration (mixed model followed by Tukey-HSD post-hoc, $p < 0.05$). Different capital uppercase letters indicate significant difference between concentrations within the static exposure paradigm. Different lowercase letters indicate significant difference between concentrations within the dynamic exposure paradigm ($p < 0.05$).

trols. This situation was reversed for the statically exposed crayfish which had lower levels of time spent at the highest intensity as fluoxetine concentration was increased ($p < 0.05$) (Fig. 4).

3.2. Time to reach a specific intensity

3.2.1. Time to 10 (tearing of appendages)

The mixed model revealed a significant difference of time to intensity level 10 between the static and dynamic exposure conditions ($F_{(1,\ 128,\ 0.05)} = 4.97$, $p = 0.027$). A pairwise Tukey post-hoc

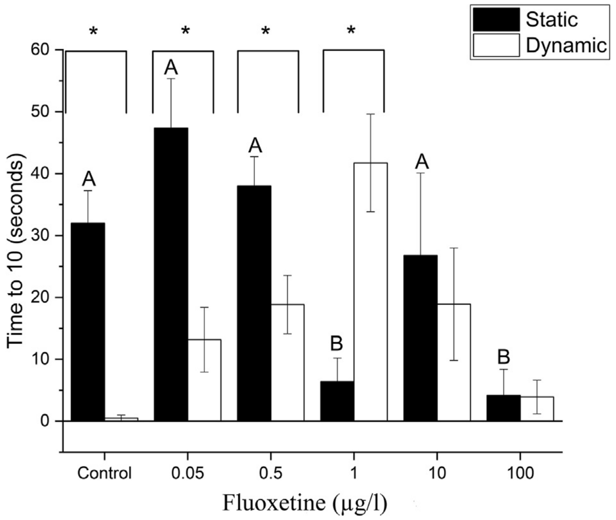

Fig. 5. Mean (±SEM) time spent to reach intensity level 10 (tearing of appendages) for crayfish exposed to different concentrations of fluoxetine under static (solid black squares) and dynamic (open black circles) exposure conditions. Asterisks indicate a significant difference between the two exposure conditions within a single concentration (mixed model followed by Tukey-HSD post-hoc, $p < 0.05$). Different capital uppercase letters indicate significant difference between concentrations within the static exposure paradigm ($p < 0.05$).

comparison showed that the difference between static and dynamic exposure was only apparent at the lowest three concentrations and the control. At 0, 0.05 and 0.5 µg/l crayfish in the static condition took significantly longer to reach intensity level 10 than animals in the dynamic condition, whereas at 1 µg/l they took less time to escalate fights. In addition, an effect of fluoxetine concentration was observed for static exposed crayfish, in which animals exposed to 1 and 100 µg/l took less time to escalate fights than in the other treatments (Fig. 5).

3.2.2. Time to 11 (highest intensity)

The mixed model revealed a significant difference of time to the most intensive level of fighting with the interaction between the exposure condition and the exposure concentration ($F_{(1,128,0.05)} = 8.15$, $p < 0.05$). A subsequent pairwise Tukey post-hoc comparison showed a statistical difference between static and dynamic exposure for time to 11 for all concentrations, including controls ($p < 0.05$). The control crayfish and the crayfish exposed to the lowest concentration (0.05 µg/l) in the dynamic condition escalated fights to intensity 11 much quicker than crayfish exposed to similar concentrations but under static condition. This outcome was reversed at higher concentrations ($p < 0.05$). In addition, there was a significant difference in the time to intensity level 11 for the dynamic exposure condition as concentration of fluoxetine was increased, with longer times to reach the highest intensity at higher concentrations ($p < 0.05$) (Fig. 6).

3.3. Serotonin level

Serotonin levels in the hemolymph of tested crayfish differed significantly with respect to fluoxetine concentration ($F_{(5,76,0.05)} = 4.24$, $p < 0.001$), but no effect due to exposure paradigm or interaction between concentration and exposure paradigm were found. Serotonin levels in crayfish from all exposure concentrations higher than 0.05 µg/l where significantly lower than in controls (figure not shown).

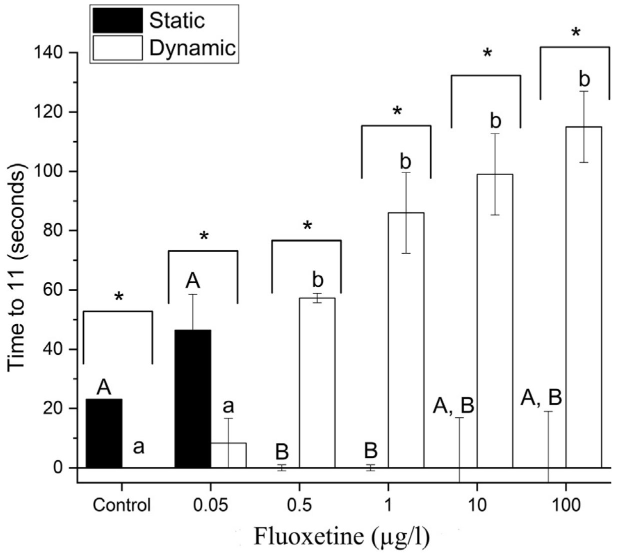

Fig. 6. Mean (±SEM) time spent to reach intensity level 11 (highest intensity) for crayfish exposed to different concentrations of fluoxetine under static (solid black squares) and dynamic (open black circles) exposure conditions. Asterisks indicate a significant difference between the two exposure conditions within a single concentration (mixed model followed by Tukey-HSD post-hoc, $p < 0.05$). Different uppercase letters indicate significant difference between concentrations within the static exposure paradigm. Different lowercase letters indicate significant difference between concentrations within the dynamic exposure paradigm ($p < 0.05$).

4. Discussion

In the present study, crayfish, *F. virilis*, were subjected to chemical exposure under graded fluoxetine concentrations and variable exposure systems (static and dynamic). As a result of these exposures, crayfish spent significantly more time in tailflip and retreat behaviors at the highest concentrations of fluoxetine (1, 10, 100 μg/l for tailflip; 100 μg/l for retreat) in static exposure compared to identical concentrations under dynamic exposure (Figs. 2, 3). Significant differences between static and dynamic exposure conditions were also observed for the highest level of fighting intensity. Time spent at and time to escalate the highest fight intensity were significantly lower in dynamic controls than static controls (Figs. 4, 5, 6). However, in high fluoxetine concentrations, these two parameters were significantly flipped (Figs. 4, 6). All of these results indicate that crayfish exposed to fluoxetine have significantly altered social behavior and that dynamic exposure paradigms exacerbates significantly these behavior.

In previous studies, the antidepressant fluoxetine has shown variable effects on aquatic invertebrates with respect to the concentrations tested, species exposed, experimental design, or duration of exposure (Backhaus, 2014; Fong and Ford, 2014). For example, at 20 μg/l and 40–345 μg/l of fluoxetine, movement and burrowing behavior of freshwater mussels have been altered (Hazelton et al., 2014) and locomotion in marine snails has been retarded (Fong and Molnar, 2013; Fong et al., 2015). Tierney (2016) and Buřič et al. (2018) observed reduced locomotion in crayfish exposed to fluoxetine and citalopram, respectively, at ~1 μg/l. On the other hand, Bossus et al. (2014) reported that swimming speed was significantly increased in the amphipod *Echinogammarus marinus* Leach 1815 at very low concentrations (0.01 μg/l) of fluoxetine and sertraline (another SSRI) after a one-day exposure but not after an eight-day exposure. Whereas, Hossain et al. (2019) observed higher distance moved, higher activity, and more time spent outside of shelter in sertraline (~1 μg/l) exposed marbled crayfish after 21 days of exposure. Guler and Ford (2010) found significant differences in phototaxis behavior of *E. marinus* males after three weeks exposure to fluoxetine at 10 ng/l–10 μg /l. All the above studies and currently most of the eco-toxicological investigations are based on a time-averaged uniform concentration which is a chemically-based measure of concentration (e.g., molarity, ppt/ppb) over a defined time period (e.g., 24, 48, 72 h) within a homogenous testing environment. These models do not appropriately represent the spatial and temporal fluctuations of the fine scale chemical dynamics found within the turbulent plumes of natural aquatic ecosystems (Edwards and Moore, 2014; Harrigan and Moore, 2017).

The spatial and temporal variations in chemical (toxicant) concentrations could create variable responses in exposed animals. In addition, the peak-to-mean ratio in turbulent systems may result in exposure to very high, albeit short, concentrations of chemical. The impact of these dynamic exposures is demonstrated in this study and other previous studies using dynamic exposure paradigms (Bergman et al., 2003; Harrigan and Moore, 2017; Ludington and Moore, 2017; Neal and Moore, 2017; Steele et al., 2018). In this study, the exposure that mimicked natural exposure paradigms produced the greatest changes in the social behavior of crayfish, as evidenced by reduced tailflip and increased time spent in high intensity fight. In static exposures, the opposite effects were observed. This paper is the first report of dynamic exposure effect on benthic crustacean using an antidepressant.

Recent ecotoxicology work has also shown that exposure paradigms impact agonistic behaviors of crayfish differently (Diamond et al., 2006; Cook and Moore, 2008; Welsh et al., 2008; Angel et al., 2015). These variable effects of exposure are primarily due to turbulent dispersion, which creates changes in chemical (toxicant) pulse length, frequency, and magnitude of concentration in natural systems. Previous research has demonstrated that habitat characteristics (e.g., flow rate, roughness, sediment elements, etc.) create different spatial maps of chemical fluctuations (Edwards and Moore, 2014; Lahman et al., 2015; Ludington and Moore, 2017) and that the mode of delivery (Steele et al., 2018) also impacts the dynamic chemical dispersion/exposure landscape. All of these studies point to the fact that subtle variations in the temporal fluctuation in chemical concentration determine the impacts on organisms. Here, non-linear and non-dose dependent results are most likely a result of the variability created in dynamic exposure. Even organisms living in the same spatial region of lotic water, but facing different hydrodynamic regimes, will be exposed to differing fluctuations in chemical concentrations (Harrigan and Moore, 2017; Steele et al., 2018). Finally, the position of organism within the flowing system, upstream or downstream of obstacles, has impacted the behavioral changes due to chemical exposure (Ludington and Moore, 2017). Therefore, future research must consider temporal variation in toxicant concentrations to quantify the real consequences of exposure in natural systems.

The toxicity of certain chemicals is less noticeable when presented as pulse exposure paradigms (Naddy and Klaine, 2001). Yet, in the present study, the effects of fluoxetine on crayfish were more pronounced in dynamic exposure systems. For example, crayfish at the dynamic exposure took longer to escalate fights to highest intensity level and also spent more time at these levels than statically-exposed crayfish. These results are possibly due to the greater peak-to-mean fluctuations of chemicals in flowing water compared to static conditions. In dynamic systems, organisms exposed to frequent high pulse/peak of chemical plumes are potentially prevented from recovery from a previous chemical pulse or accumulate hazardous levels of chemicals (Neal and Moore, 2017). In addition, the peak-to-mean ratio may be what is driving the effects of exposure rather than simply the mean concentration. Chronic uniform concentration effects on organism may be less pronounced because of the lack of the extremely high but transient concentrations achieved in dynamic systems. In our study, we also observed that certain behavioral impacts were reversed as fluoxetine concentration increased in both static and dynamic systems. This may be a result of to a sort of desensitization effect on the behavioral or physiological system during static exposure (Bossus et al., 2014). In mammals responsiveness to fluoxetine decreases after chronic exposure due to a critical decrease of the precursor of serotonin, tryptophan (Delgado et al., 1999). Animals in static exposures may have different physiological responses because of the constant presence of the toxincant/chemical.

The results from this study demonstrate that fluoxetine can induce behavioral changes (agonistic behavior) in benthic crustaceans at concentrations that are present in natural systems and are considered as environmentally safe levels (Alonso et al., 2010; Metcalfe et al., 2010; Kostich et al., 2014). From an ecological point of view, agonistic behaviors (retreat, tailflip and fight intensity are more noticeable and important behavior in crayfish sociality) are important for potential resources acquisition, such as shelter, food, and mates between contestants (Parker, 1974; Martin and Moore, 2010; Fero and Moore, 2014). Ultimately, behavior of animals arise from underlying neurological and physiological processes. As chemicals alter aspects of neurological functioning (serotonin reuptake), behavior should also change. For SSRIs, the antidepressants affect brain function by maintaining the neurotransmitter serotonin within the synaptic cleft (Hyttel, 1994; Kish, 2008). As a result of blocking uptake mechanisms, the physiology and behavior of vertebrates and invertebrates is altered (Fingerman et al., 1994; Frazer and Hensler, 1999; Fong and Ford, 2014). This change in serotonin function can also alter the regulation of hormonal and neural signals (Santos et al., 2010). In our experiment, we found that the exposure paradigm and the interaction of exposure paradigm and concentration had no significant effect on hemolymph serotonin levels of tested crayfish. Yet, behaviors were altered. It is possible that the collection process performed in these studies did not capture the faster temporal fluctuations in reuptake modulation. Still, these results demonstrate that physiological alterations are dependent on concentrations, but not on the exposure paradigm. However, the agonistic behavior of crayfish is influenced by both concentrations and exposure paradigm. Alterations in the social behavior of crayfish (or even in physiological processes) can lead to the disruption of other related behaviors such as foraging or burrowing. Crayfish are ecosystem engineers and play an important role in structuring stream communities. Changes in their behavioral repertoire can have profound effects at the ecosystem level, such as plant community diversity and abundance (Brodin et al., 2014).

5. Conclusions

Certainly, crayfish under a more realistic exposure (dynamic) system show different behavioral effects than those exposed to the same overall concentration but within a static framework. Thus, using static exposure paradigms might be more cost effective, but may underestimate the full impact of chemical pollutants on ecosystem functioning. Finally, we could conclude that these behavioral assessments even under this one type of dynamic exposure are not enough work for a set of straight forward concluding remarks. Future investigation are warranted, but should include a focus on exposure paradigms that alter duration, exposure technique, habitat structure, and species tested in a more realistic and natural design.

Author contributions

Conceptualization, MSH, PAM; data curation, MSH; formal analysis, MSH, PAM; funding acquisition, MSH, PAM; investigation, MSH; methodology, MSH, PAM; supervision, PAM; validation, PAM; visualization, MSH; writing – original draft, MSH, PAM; writing – review and editing, MSH, MB, and PAM.

Declarations of competing interest

None.

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