Fakulta rybářství
a ochrany vod
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Jihočeská univerzita
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Crayfish cardiac and locomotor activity as a tool for study
of pharmaceutically active compounds effect

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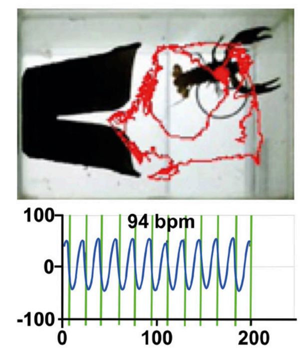

Filip Ložek

Czech Republic, Vodňany, 2020

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

Emergent environmental contamination

Nowadays, in order to increase quality of human wellbeing a wide variety of synthetic chemical substances are used. They are massively produced for industrial, agricultural and domestic use (Bernhardt et al., 2017; Carvalho, 2017; Fang et al., 2019). Large number of them (pesticides, hormones, pharmaceuticals, personal care products, etc.) are generally considered as endocrine disruptors (Ebele et al., 2017; Kümmerer, 2009). Pharmaceutically active compounds (PhACs) represent a large and diverse group of both human and veterinary medicinal products which have been used in significant quantities worldwide (Biel-Maeso et al., 2018). These compounds are produced to cure illnesses and are designed to be highly biologically active in low therapeutic dosages while safe for target organism. Increasing consumption is compensated by mass production which generate waste – manufacturing residues (Heberer et al., 2002; Larsson et al., 2014; Reddersen et al., 2002) transported through different pathways into different environmental media (Figure 1). Residues of PhACs enter aquatic environment (Beek et al., 2016; Boleda et al., 2009; Boleda et al., 2011; Bottoni et al., 2010) mainly by i) human and animal excretion in their native form and/or as metabolites ii) improper waste disposal from the household and manufacturing and iii) limited/insufficient removal efficiency of conventional waste water treatment plants due to their chemical and physical properties (Bound and Voulvoulis, 2006; Du et al., 2015). According to the experts, more than 700 organic chemical substances have been detected in the European aquatic environment (NORMAN).

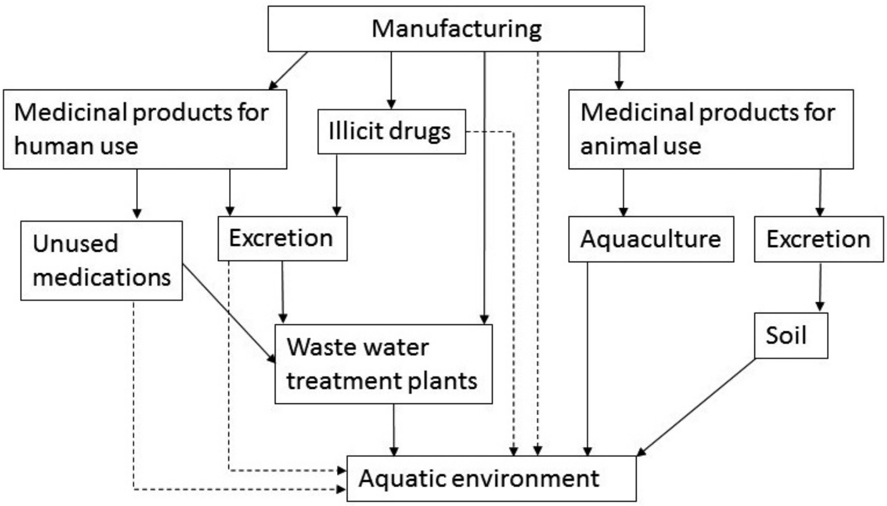

Figure 1. *Schematic overview of potential sources of PhACs and main possible pathways to enter to aquatic environment.*

The most commonly used PhACS such as analgesics, antibiotics, anticancer drugs, anticonvulsives, antihistamines, antihypertensives, lipid regulators, psychoactive drugs and even illicit recreational drugs as well as their active metabolites are frequently found in different environment compartments at relatively high concentration levels, normally in the nanogram or microgram per liter (Beek et al., 2016; Lindberg et al., 2014; Padhye et al., 2014; Petrie et al., 2016). Generally, effects of PhACs on mammals are well known, but the complex information available on environmental and ecological impacts of these compounds is still relatively scarce and standard parameters monitored in conventional toxicity tests do not detect the mode of action of PhACs. Continual input of PhACs, their metabolites and transformation products into the aquatic environment may create negative effects to the biota and the whole ecosystem (Brodin et al., 2013; Brodin et al., 2014; Corcoran et al., 2010; Nilsen et al., 2019; Rosi-Marshall et al., 2015).

Special attention should be given to psychoactive compounds, illicit drugs and their metabolites (Schluesener et al., 2015; Van Nuijs et al., 2018), because they are frequently found in different types of waters due to their increasing production and consumption. According to the Organization for Economic Cooperation and Development (OECD), the consumption of antidepressants (especially selective serotonin reuptake inhibitors e.g. fluoxetine, sertraline, citalopram) has shown increasing trends in most countries since the year 2000 (Health at a Glance 2015: (OECD) Indicators). A similar trend is observed for tramadol, an analgesic used for treatment of acute and chronic pain with a multimode of action on serotonergic and noradrenergic nociception (Vazzana et al., 2015).

Illicit drugs and their metabolites represent an equally large group of emerging pollutants (Pal et al., 2013). Amphetamine type stimulants (e.g. methamphetamine) belonging to the most consumed illicit drugs in Europe probably due to the easy availability of their precursor chemicals and ease of manufacturing (EMCDDA, 2019; Mackuľak et al., 2016). Most psychoactive compounds are hydrophobic (they penetrate through the blood-brain barrier in humans/vertebrates), but on the other hand in aquatic environments they can be easily integrated into aquatic organisms and affect their biological function and behaviour (Rosi-Marshall et al., 2015).

Effects of psychoactive compounds

Physiological systems are also regulated by two biogenic monoamines, serotonin and dopamine. The most commonly used antidepressants act as serotonin reuptake inhibitors (SSRIs). In addition, some analgesics such as tramadol or illicit drugs like methamphetamine have SSRIs effects as well as other effects. They work by modulation of serotogenic neurotransmission, by binding to and inhibiting pre-synaptic reuptake transport proteins. Levels of serotonin influence both physiology and behaviour in all animals and affect activity, aggression and reproductive behaviour (Huber and Delago, 1998; Huber et al., 1997; Sneddon et al., 2000). In crustaceans, the level of serotonin, beside dopamine and octopamine and their ratio, controls many biological functions such as reproduction, development, metabolism, immunological reaction, moulting and behaviour (Fingerman, 1997; Cheng et al., 2005; Li et al., 2005; Sarojini et al., 1995). In the freshwater crayfish *Procambarus clarki* progressive levels of injected serotonin (100 nM to 10 µM) increase crayfish heart rate for hours (Listerman et al., 2000). Increased ovary development and fecundity (hatching rate and the amount of *nauplii*) were reported in various species of decapods e.g. the tiger shrimp *Penaeus monodon*, white Pacific shrimp *Litopanaeus vannamei*, the freshwater giant prawn *Macrobrachium rosenbergii*, the fiddler crab *Uca pugilator* and the crayfish *P. clarkii*, following injection of 5-Hydroxytryptamine (Cheng et al., 2005; Sarojini et al., 1995; Vaca and Alfaro, 2000; Wongprasert et al., 2006). Contrary to serotonin, dopamine inhibits ovary and testicular maturation (Chen et al., 2003; Sarojini et al., 1995).

Psychoactive pharmaceuticals (antidepressants) and illicit drugs i.e. amphetamine type stimulants are designed with the aim of affecting the receptors at the lowest possible dose, and therefore have considerable pharmacological impact on non-target aquatic organisms that share same receptors and enzyme homology with humans. This is based on withdrawal-like behaviour in planarians (Kusayama and Watanabe, 2000; Sacavage et al., 2008) and crayfish

(Alcaro et al., 2011; Imeh-Nathaniel et al., 2016; Imeh-Nathaniel et al., 2017; Nathaniel et al., 2012; Shipley et al., 2017; van Staaden and Huber, 2018) which showed a reinforcing effect to serotonergic or dopaminergic drugs like in mammalian target organisms. Any psychoactive compound in the environment with the ability to modulate level of neurotransmitters has the ability to disrupt the normal endocrine and biological function what is reflected in physiological alterations and the potential of behavioural changes in unintentionally exposed organisms.

Pharmaceutical effects of psychoactive compounds on invertebrates

A wide range of aquatic organisms, including bacteria, algae, invertebrates – molluscs, insects, crustaceans – and vertebrates (fish, amphibians), is facing continuous increase in exposure to PhACs (Brodin et al., 2013; Carfagno and Fong, 2014; Fong and Ford, 2014; Hedgespeth, 2015; Meredith-Williams et al., 2012; Santos et al., 2020). Concentrations of psychoactive compounds in the aquatic environment (from ng to µg L^{-1}) are not considered as acutely toxic. However, physiological alterations in unintentionally exposed organisms leading to behavioural changes with potential disruption of life history of species are reported (Minguez et al., 2015), resulting in loss of biodiversity and impacts on the whole ecosystems (Brodin et al., 2014; Duffy et al., 2007). Multiple physiological alterations leading to disruption of natural behaviour including e.g. phototaxy, boldness, locomotion (Brodin et al., 2013; Saaristo et al., 2019), have impacted predator-prey relationships, social traits, defence strategies, reproduction, adaptation to abiotic factors and migration of aquatic organisms (Bláha et al., 2019; Brodin et al., 2013; Corcoran et al., 2010; Guo et al., 2019) (Table 2).

Among reported physiological impacts of psychoactive compounds caused by modulating serotonin levels, is a reduction of zebra mussel male and female gonads exposed to environmentally relevant concentrations (20 ng L^{-1} and 200 ng L^{-1}) of fluoxetine while higher concentration led to a significant increase in esterified estradiol (Lazzara et al., 2012). Effects of fluoxetine on the reproduction of the snail *Potamopyrgus antipodarum* at a concentration of 10 µg L^{-1} and on the length of daphniid *Daphnia magna* 8.6 µg L^{-1} and on the amphipod *Hyalella azteca* (33 µg L^{-1}) are also reported Pery et al. (2008). Juvenile crayfish *Orconectes rusticus* exposed to fluoxetine in concentrations of 2 and 500 µg L^{-1} displayed significantly reduced locomotion, while juveniles exposed to 500 µg L^{-1} fluoxetine showed also enhanced growth (Tierney et al., 2016) (Table 2).

Impact of physiological changes on specific behaviour caused by exposure to psychoactive compounds is reported by Hazelton et al. (2014) where fluoxetine exposed freshwater mussels *Lampsilis fasciola* increased lure display rates in concentrations of 2.5 and 22.3 µg L^{-1}. In addition, exposure to a concentration 22.3 µg L^{-1} caused earlier time-to-movement, greater total movement, and earlier initiation of burrowing than in control animals. Exposure of psychoactive compounds seem to have not only a dose specific effect, but also species specific. Fong and Hoy (2012) reported significant foot detachment from the substrate in two freshwater snails caused by venlafaxine at a concentration of 313 pg L^{-1} in *Leptoxis carinata* and 31.3 ng L^{-1} in *Stagnicola elodes*, and by citalopram at a concentration of 405 pg L^{-1} in *L. carinata* and 4.05 µg L^{-1} in *S. elodes*. Other species of molluscs like *Urosalpinx cinerea* and *Liphopoma americanum* showed reduced locomotion after exposure to fluoxetine, while exposure to venlafaxine caused an adverse effect on locomotion and at the concentration of 313 µg L^{-1} a need to reach the air/water interface (Fong et al., 2015). In addition, some organisms such as larvae of the chironomids *Chironomus riparius* show high tolerance to effect of fluoxetine in concentrations up to 59 mg L^{-1} (Pery et al., 2008). Serotonin is important for controlling appetite (Halford et al., 2005), suggesting that SSRI exposure could lead to changed feeding behaviour. Thus, it has been shown that fluoxetine reduces feeding rate of young cuttlefish *Sepia officinalis* exposed to environmental concentrations of fluoxetine through affected prey-striking (Di Poi et al., 2013). Also, fluoxetine exposure altered cryptic behaviour of cuttlefish as reported (Di Poi et al., 2014), potentially making them more visible to predators in nature (table 2).

In a study where marine amphipods (*Echinogammarus marinus*) were exposed to environmentally relevant concentrations of fluoxetine (0.001 to 1 μg L^{-1}), increased velocity of amphipods was observed after 1 h exposure to sertraline at 0.01 μg L^{-1} and after 1 day exposure to 0.001 μg L^{-1} fluoxetine. The most predominant effect of drugs on velocity was recorded after 1 day exposure to 0.1 and 0.01 μg L^{-1} concentrations of fluoxetine and sertraline, respectively (Bossus et al., 2014). Fluoxetine significantly altered phototaxis and geotaxis activity at 100 ng L^{-1}; exposed specimens spent more time within the light and occurring higher in the water column (Guler and Ford, 2010). In another study, fluoxetine in a concentration of 100 ng L^{-1} increased swimming velocity of *Gammarus pulex* (De Castro-Català et al., 2017). Exposed *G. pulex* increased ventilation rate at low concentrations of fluoxetine (10–100 ng L^{-1}), while increased locomotion was observed at higher concentrations (1 μg L^{-1}–1 mg L^{-1}) (De Lange et al., 2006; De Lange et al., 2009) (table 2).

Exposure to waste water treatment plant (WWTP, Vodňany municipality, Czechia) effluent water significantly increased feeding rates of dragonfly larvae (*Aeshna cyanea)*, while in tramadol and citalopram (~1 μg L^{-1}) exposure, feeding rates of larvae were significantly lower than the unexposed control group (Bláha et al., 2019). The crayfish species *Orconectes obscurus* and *Procambarus clarkii* exposed to 8 μg L^{-1} of the metabolite O-Desmethylvenlafaxine showed increased aggression and attacked their opponents more consistently (Stropnicky, 2017). In a long-term exposure experiment, marbled crayfish *Procambarus virginalis* exposed to environmental concentrations of venlafaxine (~1 μg L^{-1}) showed no behavioural alterations, while in exposure to the same concentration of benzodiazepine oxazepam, it moved longer distances and showed higher activity when no shelter was available. In addition, oxazepam exposed crayfish showed no difference in behaviour between individuals with or without glair glands as was evident in control animals (Kubec et al., 2019). Sertraline-exposed (~1 μg L^{-1}) marbled crayfish increased activity, regardless of available shelter, and moved greater distances when shelter was available (Hossain et al., 2019). Conversely no effect of decreased activity was observed as a response to predation risk of snail *Radix baltica* exposed to sertraline in concentrations of 0.4, 40 ng L^{-1} and 40 μg L^{-1} (Hedgespeth et al., 2018). Buřič et al. (2018) reported lower velocity and shorter distance covered of marbled crayfish after exposure to environmentally relevant concentrations (~1 μg L^{-1}) of opioid analgesic tramadol and antidepressant citalopram, in addition crayfish exposed to tramadol spent more time in shelters. Decreased locomotor activity and increased cardiac activity after exposure to tramadol (~1 μg L^{-1}) was reported in signal crayfish *P. leniusculus* after exposure to injured conspecific scent (Ložek et al., 2019) (table 2).

Illicit drugs are interesting group of psychoactive compounds, many of them alkaloids or their synthetic derivates serving to plants as a protection against invertebrates. They are often highly addictive to mammals and some studies proved similar effects in crayfish (Imeh-Nathaniel et al., 2017; van Staaden and Huber, 2018). Thus, some authors consider crayfish as possible model for neurobehavioural study of addiction (Jackson and van Staaden, 2019). However, despite the presence of illicit drugs in aquatic environments and their evident effect on non-target organisms, there is only a limited number of studies investigating their effect in environmental concentrations on invertebrate non-target organisms. There is also evidence that exposure to some serotonergic illicit drugs e.g., cocaine and its metabolite benzoylecgonine, has cytogenetic effects (protein profile alteration – oxidative stress) in the freshwater mussel *Dreissena polymorpha* (Binelli et al., 2013; Binelli et al., 2012) with sublethal effects (Parolini et al., 2013). In addition, Parolini et al. (2015) reported higher protein profile alterations of *D. polymorpha* exposed to realistic mixtures of llicit drugs (cocaine 50 ng L^{-1}), benzoylecgonine (300 ng L^{-1}), amphetamine (300 ng L^{-1}), morphine (100 ng L^{-1}) and 3,4-methylenedioxymethamphetamine (50 ng L^{-1}) compared to effects of similar concentrations of each drug tested individually. With methamphetamines, whose effect on marbled crayfish *P. virginalis* was also studied within environmentally relevant concentrations (~1 µg L^{-1}), exposed crayfish spent more time out of shelter (Hossain et al., 2019). Females of red swamp crayfish exposed to methamphetamine at ~1 µg L^{-1} constructed burrows of lower depth and volume relative to individual weight than did control crayfish (Guo et al., 2020). Signal crayfish exposed to environmental concentrations (~1 µg L^{-1}) of methamphetamine showed a weaker cardiac response to stress and lower locomotor reaction post stressor application than that observed in controls (Ložek et al., 2020) (Table 2).

Table 2. *Studies of pharmaceutical effect on physiology or behaviour of invertebrates, including type of pharmaceutical substance, study species, type of behaviour studied (endpoint), concentration at which effects were observed and the reference. Concentrations are given in µg l^{-1}. Species names: A. cyanea, Aeshna cyanea (dragonfly); C. riparius, Chironomus riparius (harlequin fly); D. magna, Daphnia magna (planktonic crustacean); D. polymorpha, Dreissena polymorpha (zebra mussel); E. marinus, Echinogammarus marinus (marine amphipod); G. pulex, Gammarus pulex (freshwater amphipod); H. azteca, Hyalella azteca (amphipod crustacean); L. fasciola, Lampsilis fasciola (freshwater mussel); L. americanum, Lithopoma americanum (starsnail); L. carinata, Leptoxis carinata (freshwater snail); O. obscurus, Orconectes obscurus (crayfish); O. rusticus, Orconectes rusticus (crayfish); P. leniusculus, Pacifastacus leniusculus (signal crayfish); P. antipodarum, Potamopyrgus antipodarum (New Zealand mud snail); P. clarkii, Procambarus clarkii (redswamp crayfish); P. virginalis, Procambarus virginalis (marbled crayfish); S. officinalis, Sepia officinalis (common cuttlefish); U. cinerea, Urosalpinx cinerea (oyster drill). Substances: o, O-desmethylvenlafaxine; meth, methamphetamine; c, cocaine; b, benzoylecgonine.*

	Species	Endpoint	concentration µg l^{-1}	reference
fluoxetine	*D. magna*	life cycle	10	Péry et al. (2008)
	E. marinus	swimming behaviour, transcriptional mapping	0,001–1	Bossus et al. (2014)
	E. marinus	phototaxis, geotaxis	0,01–10	Guler and Ford (2010)
	G. pulex	activity	0,1	De Castro-Català et al. (2017)
	G. pulex	ventilation, locomotor activity	0.001–0.1; 1–1000	De Lange et al. (2009)
	H. azteca	life cycle	33	Péry et al. (2008)
	O. rusticus	growth, behaviour	2; 200; 500	Tierney et al. (2016)
	C. riparius	life cycle	59500	Péry et al. (2008)
	D. polymorpha	gonads development, spawning, estradiol level	0.02; 0.2	Lazzara et al. (2012)
	L. americanum	locomotor activity, reaching air water interface	345	Fong et al. (2015)
	L. fasciola	activity, burrowing	0.5; 2.5; 22.3	Hazelton et al. (2014)
	U. cinerea	locomotor activity	31.3	Fong et al. (2015)
	S. officinalis	cryptic behaviour, feeding, cognitive abilities	0.001–0.1	Di Poi et al. (2013; 2014)
	P. antipodarum	life cycle	10	Péry et al. (2008)

	Species	Endpoint	concentration µg l^{-1}	reference
venlafaxine	*O. obscurus*	aggression	1; 8	Stropnicky et al. (2017)
	P. clarkii	aggression	1; 8	Stropnicky et al. (2017)
	P. virginalis	locomotor activity, sheltering	1	Kubec et al. (2019)
	L. americanum	locomotor activity/reaching air-water interface	31.3	Fong et al. (2015)
	L. carinata	foot detachment	0.000313	Fong and Hoy (2012)
	S. elodes	foot detachment	0.0313	Fong and Hoy (2012)
	U. cinerea	locomotor activity	31.3	Fong et al. (2015)
sertraline	*E. marinus*	swimming behaviour, transcriptional mapping	0,001–1	Bossus et al. (2014)
	P. virginalis	locomotor activity, sheltering	1	Hossain et al. (2019)
	R. balthica	activity, boldness	0.00004; 0.04; 40	Hedgesphett et al. (2018)
tramadol	*P. leniusculus*	cardiac, locomotor activity	1	Ložek et al. (2019)
	P. clarkii	burrowing	1	Guo et al. (2020)
	P. virginalis	locomotor activity	1	Buřič et al. (2018)
	A. cyanea	feeding rate/predation	1	Bláha et al. (2019)
o	*P. virginalis*	locomotor activity	1	Kubec et al. (2019)
citalopram	*P. virginalis*	locomotor activity	1	Buřič et al. (2018)
	A. cyanea	feeding rate/predation	1	Bláha et al. (2019)
	L. carinata	foot detachment behaviour	0.000405	Fong and Hoy (2012)
	S. elodes	foot detachment behaviour	4.05	Fong and Hoy (2012)
meth	*P. leniusculus*	cardiac, locomotor activity	1	Ložek et al. (2020)
	P. clarkii	burrowing	1	Guo et al. (2020)
	P. virginalis	locomotor activity, sheltering	1	Hossain et al. (2019)
c	*D. polymorpha*	cytophysiology	0.04; 0.22; 0.5; 1; 10	Bineli et al. (2012; 2013)
b	*D. polymorpha*	cytophysiology	0.5; 1	Parolini et al. (2013)

Tools for study of effects of psychoactive compounds

Aquatic organisms respond to a variety of chemicals including psychoactive compounds through changes of their biological parameters (Derby and Schmidt, 2017). By various techniques we are able to identify and quantify these responses in different model organism in laboratory conditions. Standard ecotoxicology tests on daphniids, provide basic information about acute lethal concentration (mortality rate) or lowest observed effect (immobility) which are still considered as a basic form of data used in the risk assessment of chemicals (Minguez et al., 2018; Minguez et al., 2015). However, to identify effect of sublethal concentrations which are occurring in the environment, it is useful to observe changes in behaviour (Buřič et al., 2018; Kubec et al., 2019), cardiac or respiratory rates (Dzialowski et al., 2006), biochemical parameters of haemolymph, histological, or their combinations (Bierbower and Cooper, 2009; Cooper et al., 2011; Kuklina et al., 2019; Kuznetsova et al., 2010). For detection of physiological activity i.e. gill rate, heart rate invasive/non-invasive sensors were used (Bini and Chelazzi, 2006; Kholodkevich et al., 2009; Listerman et al., 2000). Behavioural patterns can be detected from video recordings by using specialised image analysis software i.e. Toxtrac (Rodriguez et al., 2018) or Ethovision (Noldus et al., 2001). Crayfish are considered a suitable model organism as they comply to the following parameters: 1. sufficient sensitivity – crayfish are widely accepted as sensitive and rapid bio-indicators of water quality changes (Malinovska et al., 2020; Reynolds and Souty-Grosset, 2011); 2. clearly observable ethophysiological patterns – crayfish show a complex morphology, development, and behaviour, including elaborate social interactions (Gherardi and Daniels, 2003; Vilpoux et al., 2006); 3. clear environmental impact – they are considered keystone species (Longshaw and Stebbing, 2016) in freshwater ecosystems with relations to many other species and are important ecosystem engineers (Creed and Reed, 2004). In addition, their wide geographical diversity make them available for laboratories all around the world. By this we highlight that the biological parameters of crayfish make them potentially suitable for study of effects of PhACs even in environmentally relevant concentrations, ideally measured by non-invasive methods.

Objectives of the thesis:

- Standardisation and description of noninvasive methods for continual recording of crayfish biological functions (cardiac and locomotor activity) and potentially of other aquatic macro invertebrates.
- Characterisation of intensity of crayfish cardiac and locomotor reactions to stimuli from different chemicals.
- Identification of changes in crayfish biological parameters caused by widely used psychoactive compounds, analgesic tramadol (synthetic opioid) and the illicit drug methamphetamine (CNS stimulant) in environmentally relevant concentrations.
- Evaluation of possible impact of psychoactive compounds to the ecology of aquatic invertebrates.

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CHAPTER 2

CONTINUOUS NONINVASIVE MEASURING OF CRAYFISH CARDIAC AND BEHAVIOURAL ACTIVITIES

Kuklina, I., Ložek, F., Císař, P., Pautsina, A., Buřič, M., Kozák, P., 2019. Continuous noninvasive measuring of crayfish cardiac and behavioral activities. Journal of Visualised Experiments 144, e58555.

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My share of this work was about 30%.

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Video Article

Continuous Noninvasive Measuring of Crayfish Cardiac and Behavioral Activities

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Abstract

A crayfish is a pivotal aquatic organism that serves both as a practical biological model for behavioral and physiological studies of invertebrates and as a useful biological indicator of water quality. Even though crayfish cannot directly specify the substances that cause water quality deterioration, they can immediately (within a few seconds) warn humans of water quality deterioration via acute changes in their cardiac and behavioral activities.

In this study, we present a noninvasive method that is simple enough to be implemented under various conditions due to a combination of simplicity and reliability in one model.

This approach, in which the biological organisms are implemented into environmental evaluation processes, provides a reliable and timely alarm for warning of and preventing acute water deterioration in an ambient environment. Therefore, this noninvasive system based on crayfish physiological and ethological parameter recordings was investigated for the detection of changes in an aquatic environment. This system is now applied at a local brewery for controlling quality of the water used for beverage production, but it can be used at any water treatment and supply facility for continuous, real-time water quality evaluation and for regular laboratory investigations of crayfish cardiac physiology and behavior.

Video Link

The video component of this article can be found at https://www.jove.com/video/58555/

Introduction

The subject of aquatic organisms' applications, both as model organisms for various laboratory investigations[1,2] and as tools for monitoring industrial and natural/environmental water quality[3,4], appears to be well studied. Nevertheless, this topic is still of noteworthy interest for humans, irrespective of whether they belong to the scientific community or to other occupations. In spite of the existence of a number of advanced methods for monitoring certain parameters (so-called "biomarkers")[5,6,7,8], the most important requirements for selecting an indicator consist of three simple factors: (i) simplicity, (ii) reliability, and (iii) general availability.

Crayfish, as an essential representative of freshwater fauna, distinguishes itself because it is found worldwide, is widespread, and, in most cases[9], has a sufficiently large and hard carapace suitable for manipulation. This crustacean belongs to the group of higher invertebrates that provide sufficient development of vital physiological systems and respective organs while, at the same time, maintaining a relatively simple organization[10].

Methods based on the assessment of the range of crayfishes' biological and/or behavioral parameters, as described in the scientific literature, have significantly contributed to the development of biomonitoring and crayfish studies in general. Most of the currently available invasive methods for crayfish heart rate measurements are based on electrocardiogram recordings that require a complex and precise surgical procedure[11,12,13]; such manipulations can cause significant stress to and may require prolonged adaptation by the crayfish. Also, it is not known how long a crayfish can carry such electrodes and whether it will successfully molt while carrying such an attachment. The described noninvasive methods are based on plethysmographic recordings, which are complicated by hardware complexity and require a conditioning circuit for signal filtering[14] and an amplification or precise and expensive optic components[15,16].

In this study, we described an approach that contributes to existing results and offers new alternatives for improving current crayfish heart rate measurement procedures. Among the advantages, there are (i) a fast and noninvasive attachment that does not require a prolonged physiological adaptation; (ii) crayfishes' capability to carry the sensor within a period of a few months from molting to molting; (iii) the software capable of monitoring real-time cardiac and behavioral activities and the evaluation of data obtained concurrently from multiple crayfish; (iv) a low manufacturing price and simplicity. The biomonitoring system that we describe permits the noninvasive and continuous monitoring of crayfish cardiac and locomotor activities based on changes in crayfishes' etho-physiological characteristics. This system can easily be applied in laboratory examinations of the crayfish cardiac physiology and/or ethology, in addition to industrial implementations for controlling water quality at water treatment and supply facilities.

Protocol

1. Crayfish Selection

1. In order to successfully apply the current approach to crayfish, select the respective adult specimens with sufficient carapace sizes (which is a carapace length of at least 30 mm) for sensor attachment, visually examine it for the absence of diseases, and check whether it lifts both chelae when it is touched. The above-mentioned parameters indicate an eligible state of crayfish health.
NOTE: If several crayfish are expected to be used in the trial and are exposed to the same conditions, the experimental group should be formed based on several parameters: (i) similar weight and length; (ii) comparable heart rate; (iii) pronounced nocturnal activity; (iv) regular food consumption; (v) inter-molting period[17]. Sometimes, it is hard to define whether a crayfish is near to molting by the heart rate measurements or visual or tactile examinations only; therefore, the analyses of the crayfish's hemolymph total protein content can be helpful. Protein content is expected to be higher when the crayfish is closer to molting than in the inter-molting state[18].

2. Recording of Crayfish Cardiac Activity and Behavior

1. **In order to noninvasively measure crayfish heart rates, preliminarily prepare the sensor for this procedure. Before this, put a crayfish into the tank with water and let it acclimate there for a few days as the preparation of the sensor[19] will also take a few days.**
    1. Axially couple an IR light-emitting diode (LED) with a phototransistor. Attach the optical sensor circuit onto a board; it will require a power supply of 5 V. For the LED connection, place a 200 Ω resistor on the IR sensor board; in order to connect the phototransistor, place a 220 Ω resistor on the board.
    2. When attached to the crayfish, the sensor output is modulated by the amount of hemolymph filling the crayfish cardiac muscle and scatters an incident light from the LED. In order to avoid reciprocal interference of the illuminated IR light by the LED and the reflected IR light from the crayfish heart, which is received by the phototransistor, place a small wall (0.5 x 1.5 x 4 mm, thickness x height x width) made of black antistatic plastic between the LED and the phototransistor.
    3. Place the LED in a waterproof package, and cover the surface of the sensor with the waterproof dielectric gel from the side adjacent to the carapace for the protection of the electronic components from potential damage (**Figure 1**). Let the gel dry for 3 days in order to gain its best protective properties.
    4. For an analog signal, attach thin flexible cables (about 3 m long) to the sensor and connect to the analogue-to-digital converter (ADC); from this, a digitized signal will be transferred to a personal computer over a USB interface, at which point the information about the crayfish cardiac activity is saved, analyzed in real-time with special software (see **Table of Materials**), and stored for further detailed analyses.

2. As soon as the sensor is prepared, attach it to the crayfish. In order to do this, switch the computer on and run the software. Determine the number of crayfish to be fixed to the sensors and recorded heart rate to be saved to the date file.
3. Remove the crayfish from the water and wipe its dorsal carapace side with a paper towel. Wrap the chelae and abdomen of the crayfish in the paper towel in order to avoid any damages by human hand and to eliminate additional stress on the crayfish caused by warm human hands.
NOTE: Do not use a previous cooling of the crayfish on ice or in the freezer for its immobilization before manipulations with the sensor attachment. The difference in temperatures leads to crayfish dorsal surface weeping which, in turn, leads to unreliable sensor fastening and quick adhesive detachment from the crayfish's carapace.
4. Prepare a surface (i.e., take a small flat piece of plastic or tear a piece of sticky tape and fix it to a table) and a stick for mixing the glue. Press out two small drops (of a diameter of about 0.5 cm) from tubes A and B containing epoxy glue and quickly mix them.
5. Attach the sensor to the crayfish dorsal carapace and try to find the place in which the cardiac signal amplitude would be maximal. Hold the crayfish with the sensor in one hand and, using the other free hand, put a drop of mixed glue on each of the four auxiliary wires located on the sensor (fix them in between steps 2.1.1 and 2.1.4.). Do not move the sensor at least 5 min until the glue hardens (the glue hardening depends on the ambient temperature and humidity).
NOTE: When fixing the sensor to the crayfish carapace, examine thoroughly the whole cardiac area from the carapace side in order to define the area with the best (maximal) cardiac signal amplitude. That will help the software to provide more precise heart rate calculations.
6. Touch the glue using a free hand, and if it is not sticky, put the unwrapped crayfish with the attached sensor (**Figure 2**) to the box without water for few more minutes until the glue is completely dry.
NOTE: An optimal temperature for crayfish and glue manipulation varies from 18 to 22 °C. At these temperatures, the glue hardens within 5 to 7 min and is completely dry within 8 to 10 min. At lower temperatures, the stress in the crayfish is less pronounced; however, the glue needs more time to harden, about 15 and 20 min under 15 °C and 10 °C, respectively. At higher temperatures, particularly above 25 °C, the glue hardens within 3 min, but the crayfish undergoes much more stress; therefore, try to minimize the exposure of the crustacean to extreme conditions without water.
7. Before moving the crayfish back into the tank, dip its cephalothorax into the water several times with short intervals of a few seconds in order to allow a discharge of the air that has accumulated in the gills, and leave the crayfish in the water for approximately 1 h to remove any excess chemicals. After this process is complete, release the crayfish into the water and allow it to acclimate for one to two weeks under experimental conditions, depending on observed physiological indices. Optimal water exchange during the acclimation periods is every other day.
NOTE: Characteristics of crayfish that have acclimated and are in a healthy state include pronounced circadian cardiac and locomotor activities, regular food consumption, and spending most daylight in a specialized shelter (if provided).

3. Camera and Software Setup

1. Start the software; the video camera will automatically switch on.
2. Select an option of movement detection, thoroughly detect area of each tank on the screen and the software will start tracking the behavior and linking it with the cardiac activity recordings.
   NOTE: A crayfish motion detection module consists of a video camera that tracks crayfish behavior from below the tank and the software that combines the behavior with cardiac activity. The data from the module are used to facilitate more precise cardiac activity data processing by eliminating periods in which the crayfish demonstrates high locomotive activity. Sudden crayfish movements (i.e., an escape reaction or feeding initiation) can result in fluctuations or short-time spikes in cardiac signals that may reduce the precision of cardiac interval calculations.

Representative Results

As a result, we obtained a combination of crayfish cardiac and behavioral activities, recorded and saved in a txt-format file (**Figure 3**). Besides the number of experimental crayfish, the date, and the sampling rate, the file consists of three columns: (1) the continual time in hh:mm:ss format; (2) the heart rate automatically calculated in beats per minute; (3) the locomotion registered as absence (0) or presence (1) of any movement. When the crayfish was inactive, zero was assigned to the cell responsible for movement, and when it moved, then number one appeared in the respective cell. When continuously recording, the data file was automatically created every day at 00:00 hours (12:00 AM). It was crucial to include locomotion since it could have caused changes in the heart rate (**Figure 4**). After 10 s, a food odor (milled, filtered, and diluted Chironomidae larvae) was delivered into the tank containing the crayfish, using a peristaltic pump. At 14 s, the crayfish recognized the stimulus, and its heart rate slightly decreased due to the so-called orienting response. After 20 s, the heart rate increased, thus resulting in a decrease in cardiac intervals. At 26 s, the crayfish moved toward the stimulus source, and both the physiological excitation caused by the food odor and the locomotion initiation resulted in a substantial heart rate increase. At 37 s, there was also evidence of abrupt crayfish motion. Additionally, locomotion could have substantially contributed to the heart rate growth during the crayfish's reactions to certain stimuli (**Figure 5**). A disturbed crayfish typically has an increase in heart rate, as seen during the 30- to 40-min interval with occasional locomotion. However, during the 45 to 50 min interval, the locomotion is much more pronounced. This locomotion contributed to a heart rate that is significantly higher than that seen during the period with decreased locomotion. If the data from the file is transferred to another application or the above programming algorithm is used, the data containing just the cardiac activity of the crayfish could be obtained and subsequently processed if necessary (**Figure 6**). The heart rate of undisturbed crayfish is characterized by a monotonic amplitude of the heartbeat curve and by approximately equal cardiac intervals between each cardiac peak.

In order to analyze crayfish behavioral patterns (such as passed distance, preference of a certain area in the tank or arena, and locomotion velocity), it would be possible to exchange the current camera with a standard video camera with a flat wide-angle lens, as the currently used camera does not make a recording but just tracks locomotion. Alternatively, a recording with any of the online applications for catching a video from the screen could be used.

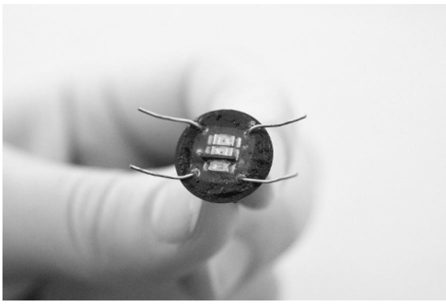
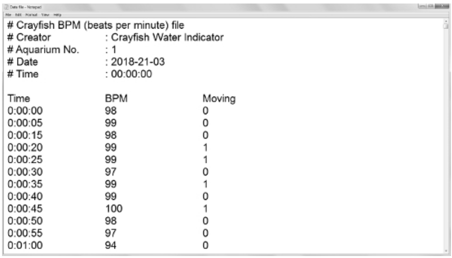


Figure 1: Noninvasive infrared optoelectronic sensor. Please click here to view a larger version of this figure.

Figure 2: Signal crayfish, *Pacifastacus leniusculus*, holding the sensor on its carapace. Please click here to view a larger version of this figure.



Crayfish BPM (beats per minute) file
Creator : Crayfish Water Indicator
Aquarium No. : 1
Date : 2018-21-03
Time : 00:00:00

Time	BPM	Moving
0:00:00	98	0
0:00:05	99	0
0:00:15	98	0
0:00:20	99	1
0:00:25	99	1
0:00:30	97	0
0:00:35	99	1
0:00:40	99	0
0:00:45	100	1
0:00:50	98	0
0:00:55	97	0
0:01:00	94	0

Figure 3: An example of the data file. Please click here to view a larger version of this figure.

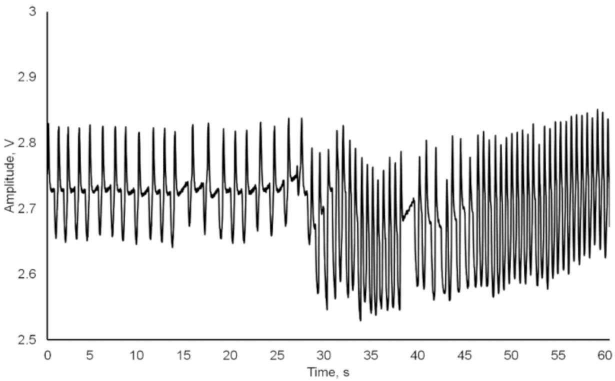


Figure 4: Crayfish heartbeat during the change from normal to disturbed conditions when exposed to food odors. Please click here to view a larger version of this figure.

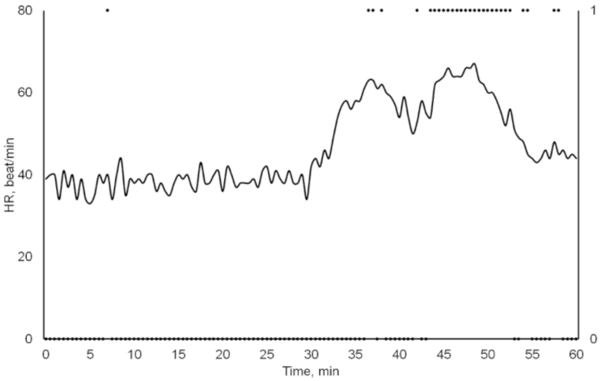


Figure 5: Heart rate and locomotion activities of a crayfish in undisturbed (0–30 min) and disturbed (30–60 min) conditions. Please click here to view a larger version of this figure.

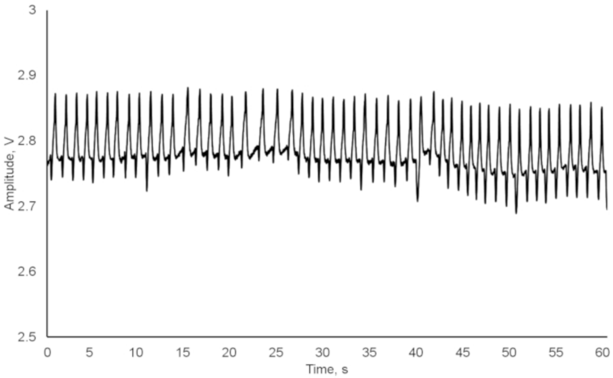


Figure 6: Undisturbed crayfish heart rate. Please click here to view a larger version of this figure.

Discussion

It has been widely suggested that the measurement of certain physiological parameters (such as heart or ventilation rate or both) is a more reliable method for recording crayfish reactions than the evaluation of behavioral responses that do not always occur immediately[11]. However, it is evident that the most efficient approach for assessing real crayfish reactions to environmental changes is the combination of cardiac activity and behavior recordings since that makes it possible to see the reason(s) for the crayfish heartbeat changes and whether or not they occur as a result of chemical alterations in the ambient environment or because of locomotion initiation. During water quality monitoring, it is crucial to eliminate all outside influences on the changes in crayfish physiological markers, including abrupt movements that have increasing effects on the heart rate but do not present an alarm for the biomonitoring system.

Another possibility for facilitating a more precise and informative heartbeat evaluation are the chronotropic and inotropic parameter analyses of crayfish cardiac activities mainly related to specific shapes in crayfish cardiac signals[19]. Such analyses confirmed that even when the heartbeat changed only a few beats per minute, some of the secondary parameters can indicate significant changes in crayfish cardiac activities[19].

Despite the number of benefits in using the described approach, research around monitoring crayfish has moved toward an absolute minimization of tactile crayfish manipulations. In the recently developed contactless system[20], the elimination of sensors and their respective wires means that crayfish of any size can be used for the monitoring procedure. It is also possible to keep multiple crayfish in one experimental area since the absence of any wires prevents wire tangling and crayfish movement restrictions. The crayfish will carry just two tiny pieces of a highly reflective tape that indicates its cardiac area. These pieces of tape can be attached to the crayfish even after a few post-molting days.

Crayfish cardiac activities and behaviors are recorded by the video camera and analyzed in real-time by the coordinating software. Along with other technical advances, the modified approach will cause a significant decrease in the price of the monitoring system due to limited hardware.

Disclosures

The authors have nothing to disclose.

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CHAPTER 3

CRAYFISH CAN DISTINGUISH BETWEEN NATURAL AND CHEMICAL STIMULI AS ASSESSED BY CARDIAC AND LOCOMOTOR REACTIONS

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RESEARCH ARTICLE

Crayfish can distinguish between natural and chemical stimuli as assessed by cardiac and locomotor reactions

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Abstract

In this study, cardiac and locomotor activities of signal crayfish *Pacifastacus leniusculus* were investigated under exposure to a range of natural (i.e., odors of conspecific crayfish, predatory fish, food, and injured conspecific) and one chemical (i.e., disinfectant chloramine-T) stimuli. Crayfish locomotion was simultaneously initiated with an increase in heart rate only when affected by chloramine-T, while locomotor response was delayed in all cases (or was not manifested at all by some specimens) when disturbed by the natural stressors. The heart rate differences measured before and during the stimulation were arranged as follows: odor of conspecific crayfish (9.2 ± 7.1%) < predator (18.4 ± 13%) < food (33.5 ± 15.7%) < chloramine-T (41.1 ± 14.7%) < injured conspecific (51.8 ± 28.4%). Analysis of the peculiarities of crayfish heartbeat under exposure to the tested stimuli revealed complex cardiac responses as was previously observed by an electrocardiography approach, that is, a slowed heart rate followed by a delayed increase. Evaluation of the intrinsic parameters of crayfish bioindicators remains essential due to the possibility of detection of the substantial ethological responses even in motionless animals. The role and appropriateness of signal crayfish as a bioindicator of water quality is discussed; they seem to be an applicable species for this task due to their sufficient sensitivity and broad availability. In addition to providing a better understanding of stereotypic crayfish behaviors induced by common and chemical stressors, the results of this study may serve as reference data for the evaluation of crayfish suitability for water quality tests.

Keywords Bioindicator · Biomonitoring · Heart rate · Invasive crayfish species (ICS) · Movement · *Pacifastacus leniusculus*

Introduction

Aquatic animals can detect, discriminate, and respond to a variety of natural cues that are constantly present in water, including polluting substances (Derby and Sorensen 2008). Therefore, using crayfish (and other hard shell macroinvertebrates) as a bioindicator is among the top approaches in current ecobiological studies. Behavioral and physiological responses are a function of crayfish environmental adaptation (Burmistrov and Shuranova 1996). In order to learn more about crayfish etho-physiological capacity and limits in relation to the ambient stress, such responses should be investigated under the influence of various stimuli. In a broader sense, when ambient conditions change negatively, the crayfish tend to adapt, demonstrating alterations of locomotor, cardiac, and ventilatory functions conditionally divided into physiological and ethological responses (Burmistrov and Shuranova 2010; Schapker et al. 2002; Shuranova and Burmistrov 2010).

Red swamp crayfish *Procambarus clarkii* were more responsive to an alarm odor of conspecifics rather than to the potential predator of the same geographical origin (largemouth bass *Micropterus salmoides*), which was reflected in feeding, locomotion, and defensive crayfish behaviors (Gherardi et al. 2011). Likewise, movement rates of virile crayfish *Orconectes virilis* were not altered at all under exposure to odors from a range of predatory fish (i.e., rock bass *Ambloplites rupestris*, yellow perch *Perca flavescens*, darters *Etheostoma nigrum*, and *Etheostoma exile*), despite that all species were common to the

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sampling area (Keller and Moore 1999). Evidently, visible behavioral changes often may be not manifested even when crayfish are disturbed significantly (Li et al. 2000; Schapker et al. 2002). Thus, observation of ethological reactions can be insufficient for evaluation of the ambient conditions, while measurement of certain physiological parameters (i.e., heart or breath rate) along with behavior might help to reflect the real crayfish state under extrinsic exposure.

Up to now, crayfish cardiac responses were investigated under exposure to numerous physical and chemical stressors (Bierbower and Cooper 2010; Bini and Chelazzi 2006; Bloxham et al. 1999; Chung et al. 2012; Kozák et al. 2011; Sladkova and Kholodkevich 2011; Udalova et al. 2012; Villarreal 1990); however, very few experiments have been done to evaluate both cardiac and behavioral responses of stressed crayfish (Bojsen et al. 1998; Li et al. 2000), and no studies have involved the comparison of etho-physiological crayfish responses under exposure to both natural and chemical stressors.

This study seeks to examine the ethological and physiological effects of natural odors and chemical stimulus in order to prove the possibility of using crayfish for water quality biological monitoring, a critical issue of aquatic ecology. We hypothesize that crayfish will be more sensitive (in terms of heart and locomotor activity) when exposed to chemical stimulus than to natural cues, although at least cardiac responses of crayfish to exposure of natural odors are also expected.

Materials and methods

Experimental animals

Signal crayfish *Pacifastacus leniusculus* (Dana 1852) adults were caught in baited traps in early June, 2013, in the Vysočina region, Czech Republic. The experimental group comprised 12 adult individuals of both sexes (six males, six females) with the following biometry: weight, 56 ± 6.1 g; carapace length, 54 ± 2 mm; and total length, 108 ± 4 mm (mean ± standard deviation, s.d.). Only intact animals with both claws present and the intermolt period of growth were selected. Visual examination for absence of diseases as well as manifestation of defensive reaction (rising both chelae when attempting to touch by hand) was also suggested as a parameter that indicated appropriate health and physical status.

Experimental conditions

Our own experiment was conducted in August, 2013. For acclimatization, crayfish were maintained during 2 weeks in individual 10-L opaque glass tanks, each equipped and positioned in the same corner of each tank shelter glued to its bottom and constructed of a 12-cm-long and 7.5-cm-diameter piece of a plastic tube, with a narrow oblong opening cut in its upper part to ensure crayfish carrying sensor unimpeded entrance to the shelter. Prior to manipulations, selected crayfish were marked by a permanent water-resistant marker with individual numbers to avoid mixing or repeated exposure of animals. A week prior to recordings, the noninvasive sensors were fastened to the dorsal side of the crayfish carapace directly over the heart using a two-component fast-fastening epoxy adhesive. Throughout the experimental period, water quality parameters were as follows: temperature 19–21 °C, pH 7.2–7.4, and dissolved oxygen higher than 6.5 mg L^{-1}. To avoid disruption of the crayfish nocturnal periodicity, the illumination regime was set and automatically maintained at 12 h of light and 12 h of dark. Crayfish were fed twice a week on chironomid larvae during the acclimation. The remaining food and waste were removed during the water exchange the next day after feeding. During the exposure tests, animals were fed on the test day a few hours after the experimental trial was finished. Tanks were cleaned the next morning, and the water was exchanged.

Experimental treatments

The effect of the following odors on signal crayfish was investigated: conspecific crayfish, predatory fish, food odor, injured conspecific crayfish, and chloramine-T (Cl-T): (i) odor of conspecific crayfish was obtained from additional three male and three female crayfish of the same species and similar sizes that were not used in the HR measurement; males and females were separately kept in two 50-L shared tanks with still water for 24 h, after which water was collected just before the trial and used for treatment; (ii) predator odor was obtained from adult European perch *Perca fluviatilis* (about 3 years in age and about 25 cm length) reared at the Experimental Fish Culture and Facility of the Research Institute of Fish Culture and Hydrobiology. The perch was placed into an isolated tank with 100 L of still water and kept there for 24 h; just prior to the experimental trial, fish water was collected and used; (iii) odor of food was prepared from milled chironomid larvae. For this purpose, 1 g of defrosted chironomids was milled and mixed with 1 L of distilled water, filtered, and then water containing food odor was used; (iiii) odor of injured conspecific was prepared by placing one crayfish into a separate aquarium filled with 2 L of water; after that, the last thoracic leg was ablated, and the crayfish that was without the leg was left in an aquarium for 1 h. Then, water was collected, and the experimental trial was started immediately; (iiiii) chemical odor was simulated by Cl-T, industrial common disinfectant and parasiticide, which was predissolved in the distilled water 1 h prior to exposure, covered, and left in laboratory glass until the trial. During the treatment, the final Cl-T concentration in each aquarium was 10 mg L^{-1}. Approximate concentrations of nonchemical odors were selected empirically since similar tests were not previously described in the literature. Concentration of Cl-T chemical was selected based on a previous study (Kuklina et al. 2014).

During each exposure, three control crayfish were simultaneously treated with the settled tap water of the same temperature as in the experimental tanks to prove that there was no effect of using the peristaltic pump on crayfish heartbeat and locomotion.

Each treatment odor was applied to the same crayfish every third day after the previous exposure in the following sequence: conspecific odor, predator odor, food odor, injured conspecific odor, and Cl-T. Since the crayfish were exposed to the treatment in relatively low concentrations and for relatively short periods, it was suggested that crayfish would not be affected by the previous treatment. Considering the order mentioned above, crayfish were not used in any experimental trial on natural stimuli after chemical exposure.

For each exposure, treatment solutions or clean water in an amount of 50 mL were simultaneously pumped into all aquariums via Watson-Marlow peristaltic pump (Watson-Marlow Pumps, England) set at a flow rate of 50 mL min^{-1}. After finishing each recording, the water in aquariums was exchanged, and a recirculating system was run. No mortality occurred during the experimental period and within a week following the chemical exposure.

Etho-physiological assay

Crayfish heart rate (HR) was registered using the noninvasive crayfish cardiac and behavioral activities monitoring (NICCBAM) system (Pautsina 2015), which is capable of supervising up to 12 crayfish simultaneously. The principle and the process of registration of the HR are described in detail in Pautsina et al. (2014). Within each test, the HRs of crayfish were registered simultaneously with locomotion. For registration of the locomotor activity, the monitoring system utilizes two standard internet protocol video cameras (Vivotek IP8161, 2 MP, H.264, day and night, Lien-Cheng Rd., Chung-Ho, Taipei County, Taiwan). Each camera is fixed in such a position and orientation that the camera can simultaneously operate at the bottom of six tanks. The system enables to mark manually the rectangular area of each tank bottom on the computer screen and then connects it with the recording of cardiac activity using special software. Video tracking is processed in real time, and the movement of every single crayfish is detected. An image of each aquarium is independently analyzed by the software with sensitivity from a single leg up to the total change of crayfish position. Simultaneously, video recording provides information to the system of cardiac activity analysis. Thus, it is possible to identify those periods where either only the heartbeat was disturbed or where both cardiac and locomotor activities were affected (Pautsina 2015).

Each experimental trial lasted 60 min, during which crayfish were monitored for 30 min before and 30 min during the exposure (after the moment of exposure initiation). Such a relatively short period of recording was chosen to examine the crayfish primary reactions to selected stimuli. Additionally, preceding visual observations and pre-evaluated heartbeats showed crayfish became responsive to either solution within 5 min to the maximum, depending on stimulus intensity. Such time period is in agreement with the experiment of Breithaupt (2001), who reported that crayfish create water flows toward the head region with velocities between 0.1 and 0.5 cm s^{-1}. The length of experimental aquariums was 35 cm; therefore, the expected time of recognition of the stimulus by crayfish was between 1.2 and 5.8 min. Therefore, a period of 30 min was sufficiently long to observe entire crayfish reaction.

Statistical analyses

Experimental data were evaluated using STATISTICA.10 software for Windows (StatSoft, Czech Republic). Weight, total length, and carapace length of male and female crayfish were compared with the Kruskal-Wallis test. Dependence of locomotion initiation and crayfish sex (initiation of movement or remaining motionless) was evaluated by Pearson's chi-square test. The Mann-Whitney test was used to compare pretreatment and post-treatment HR of male and female crayfish. The Wilcoxon test was used to evaluate whether crayfish HR significantly increased when exposed to each treatment. The Wilcoxon test was also used in order to show whether the peristaltic pump had an effect on the HR of control crayfish. The Kruskal-Wallis test followed by multiple comparisons of mean ranks of all groups as a post hoc test was used to compare crayfish HR after stimulation among all treated groups as well as to compare the initial HR values in the pretreatment state. Nonparametric tests were applied because of non-normality of the data distribution and heterogeneity of the variances. All results were considered to be significant when $\alpha < 0.05$.

Results

Crayfish cardiac and locomotor responses

The locomotion was initiated irrespective of whether it was male or female crayfish: $\chi2 = 0.351$, $P = 0.551$ ($\chi2_{max} = 9.050$). The mean HR of male crayfish did not differ from the HR of female crayfish in the pretreatment state (conspecific: $MW\text{-}U_{1,\,8} = 7.00$, $P = 0.70$; predator: $MW\text{-}U_{1,\,8} = 6.00$, $P = 0.52$; food: $MW\text{-}U_{1,\,8} = 7.50$, $P = 0.80$; injured conspecific: $MW\text{-}U_{1,\,8} = 8.00$, $P = 0.90$). Because of an approximately equal increase of the HR in both sexes during each treatment, the HR that corresponded to the posttreatment state did not differ significantly for males and females (conspecific MW-$U_{1,\ 8} = 8.00$, $P = 0.90$; predator MW-$U_{1,\ 8} = 9.00$, $P = 1.00$; food MW-$U_{1,\ 8} = 5.50$, $P = 0.44$; injured conspecific MW-$U_{1,\ 8} = 8.00$, $P = 0.90$). Therefore, data for both sexes were merged into one group comprising nine crayfish, and the effect of each odor was evaluated. Thus, the intensity of crayfish cardiac reactions to tested odors was arranged in the following order: conspecific crayfish < predatory fish < food < Cl-T < injured conspecific (KW-$H_{5,\ 57} = 39.8368$, $P = 0.0000$), as shown in Fig. 1. More specifically, crayfish HR increased significantly during exposure to each treatment (conspecific: W-$Z_{1,\ 9} = 2.3105$, $P = 0.0209$; predator: W-$Z_{1,\ 9} = 2.4201$, $P = 0.0185$; food: W-$Z_{1,\ 9} = 2.6656$, $P = 0077$; injured conspecific: W-$Z_{1,\ 9} = 2.6657$, $P = 0.0078$; chloramine: W-$Z_{1,\ 6} = 2.2014$, $P = 0.0277$), except for the control crayfish (W-$Z_{1,\ 15} = 0.1256$, $P = 0.9001$) treated with clean water during each trial. The initiation of movement of several individuals was noticed during each test; however, locomotion activity was not manifested at once with the HR increase (Fig. 2), except for Cl-T (Fig. 3), when an increase of the HR coincided with initiation of movement (Table 1).

Heartbeat peculiarities

When analyzing the heartbeat, it was noted that the crayfish had decelerating heartbeats during the first minute of reaction manifestation (Fig. 4a) that was already visible on the raw heartbeat curve, where increase of cardiac intervals was marked after stimulus introduction (Fig. 4b). Generally, the initial cardiac reaction of the crayfish could be divided into three phases according to our observations: (1) awaiting cardiac response (time after stimulus introduction till reaction manifestation); (2) decrease of HR within several seconds; (3) delayed HR acceleration till undisturbed level merged into the pronounced HR increase (Fig. 4a).

Discussion

The evidence that crayfish HR disturbance was often accompanied by locomotion highlights the tight relation between behavioral and cardiac activity. We can also conclude that chemical exposure produced a stronger stimulating effect, since crayfish manifested locomotor reactions immediately following the exposure only in that case. However, there is no evidence for having higher HR under chemical exposure as compared to "natural" odors. Thus, food and injured crayfish odors were more intensive as compared with Cl-T, while predator and conspecific odors were much less disturbing in comparison with the chemical one. This could be explained from several points of view. First, the treatment dose should be considered. A concentration of 10 mg L^{-1} of Cl-T is not reported to be high and is even recommended for treatment of drinking water within 1 h (Haneke 2002). For crayfish, even this relatively low concentration was enough to be recognized in the water and to signal its presence by heartbeat and motion alterations. Second, the experimental period should be considered. For testing the conspecific odor, the water was obtained separately from both males and females. However, when these odors were applied independently to both sexes, there was no difference between reaction intensity. August, when the current experiment was conducted, is not the period for signal

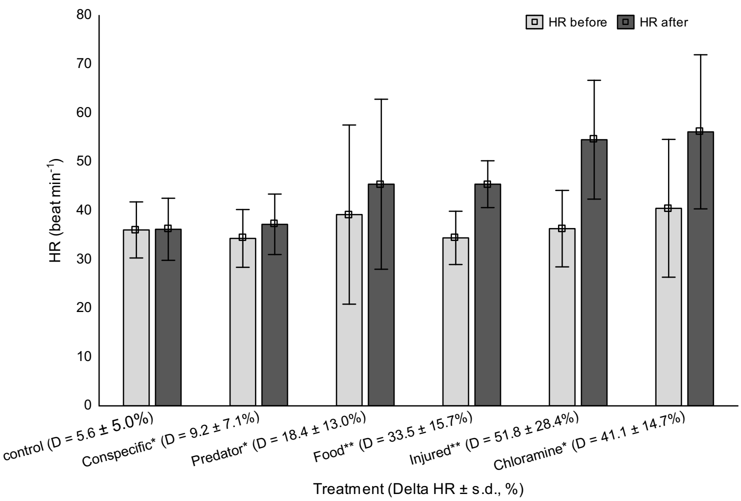

Fig. 1 Comparison of crayfish reactions to all tested stimuli. Light and dark gray columns show the HR of crayfish in the pretreatment and in the treatment states, respectively, the column top corresponds to the mean HR value; whiskers denote s.d. of the HR. D—difference of the HR in percent before/after treatment ± s.d. The asterisks denote cases when difference before/after treatment (evaluated by the Kruskal-Wallis test) was significant: *$P < 0.05$, **$P < 0.01$

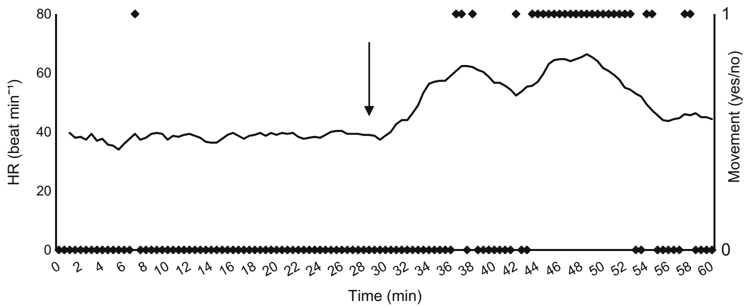

Fig. 2 Example of crayfish cardiac and locomotor activity under impact of the food odor. The solid line shows crayfish heartbeat, and diamonds show crayfish movement: 1—initiated, 0—no movement. Time of exposure initiation is 30 min (denoted by vertical arrow)

crayfish reproduction (Abrahamsson 1971; Söderbäck 1995); therefore, the conspecific odor was evidently not very attractive for experimental crayfish. Food odor was among the most intensive stimuli, as was reflected in the HR increase (Fig. 1), since the need for feeding is naturally one of the most important needs for living organisms. On the contrary, such pronounced reaction to odor of injured conspecific can be partially caused by the same reason as reaction to food odor. When the walking leg of the crayfish was ablated, some amount of hemolymph was released into the water along with urine containing other smells excreted by the injured conspecific, so it could be perceived as the mixture of food and stress odors. The crayfish are known to be carnivorous or omnivorous (Bondar et al. 2005), but often they can be cannibalistic: lost chelae were reported in signal crayfish to be the reason for being cannibalized by specimens with unbroken chelae (Mason 1979). This can likely explain such significant HR elevation under exposure to injured conspecific odor, although crayfish have not manifested feeding behaviors characterized by gathering movements of the first thoracic legs as well as by active moving of maxillipeds, as was observed under exposure to the food odor. An increase of the locomotor activity under the impact of a stronger (primarily chemical) stressor is likely the demonstration of an avoidance reaction of crayfish whose movement is restricted by aquarium size. The cases when locomotion was not manifested in part could be caused by shelter use. The last could make the crayfish feel somewhat protected against coming stimuli. This is a typical hiding behavior that is demonstrated, for example, in the presence of a predator or stronger conspecifics (Gherardi et al. 1999).

Crayfish responded to the European perch odor with a quite weak increase in HR in our experiments. This is worth noting since the presence of fish predators was reported to be a leading cause of the increased use of shelters and reduced locomotor activity in juvenile signal crayfish, particularly when the fish could be seen (Blake and Hart 1993). We can suppose that juvenile crayfish possess smaller body size and chelae, which makes their defense more difficult in comparison with that of adults. Therefore, smaller crayfish exhibited avoidance behavior rather than defensive behavior. Since our knowledge on crayfish HR dynamics in case of only visual observations is limited, it is hard to discuss the level of crayfish stress in the shelter, particularly with reduced locomotor activity, as was reported by Blake and Hart (1993). On the contrary, absence of locomotor activity of the signal crayfish in a shelter when the odor of a predator got into the tank accompanied by HR increase is direct evidence of the crayfish reaction shown in the current study. However, Keller and Moore (1999) suggest that an absence of response can be associated with the fact that crayfish already have an experience of meeting certain predators in nature and therefore does not cause a stress reaction. Signal crayfish are not native to Europe, but they have coexisted for almost 40 years with European perch in the Czech waters (Holdich et al. 2009).

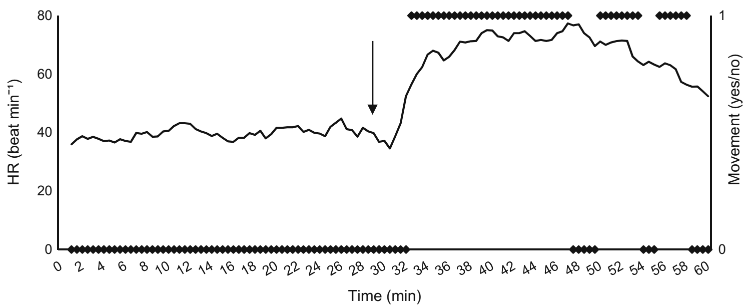

Fig. 3 Example of crayfish cardiac and locomotor responses under exposure to Cl-T. Solid line indicates crayfish heartbeat; diamonds show locomotor activity: 1—movement detected, 0—no movement. Time of exposure initiation is 30 min (denoted by vertical arrow)

Table 1 Responses of crayfish to examined odors

Stimulus	*n*	f_{LA}	HR vs LA
Control	15	2/15	Spontaneous
Conspecific	9	2/9	Non-coincident
Predator	9	3/9	Non-coincident
Food	9	5/9	Non-coincident
Injured	9	4/9	Non-coincident
Chloramine-T	6	6/6	Coincident

n, number of crayfish recordings; f_{LA}, frequency of the locomotor activity initiation (2/15, i.e., 2 out of 15 crayfish moved, etc.); *HR vs LA*, coincident or non-coincident crayfish locomotion initiation and heart rate increase, or spontaneous movement only

It is believed that in nature male crayfish are able to protect themselves better than females against predators and competitors due to their bigger chelae (Gherardi et al. 1999, 2000), and therefore they do not respond to the threat as acutely as female crayfish do. However, particular laboratory investigations show that crayfish are very individualistic and can sometimes manifest very specific behavior, particularly when a source of potential danger is invisible, and crayfish are hidden in the shelter at the moment. Thus, in our experiment, the HR of male and female crayfish did not differ significantly when they were exposed to natural odors. Evidently, that meeting certain unusual agents in the water, crayfish can just "accept" (manifest tolerance) it or "not accept" (manifest excitation) it. This is shown as increases to the HR on the lower or higher level or can be shown by the manifestation of avoidance behavior (or even remaining motionless). On one hand, we could assume that the presence of shelters influenced crayfish behavior, that is, they felt safer when covered. On the other hand, elevation of both HR and locomotion even in crayfish staying inside the shelter during the whole trial is evidence of fear presence, and individual movements out of the shelters confirm disturbance of crayfish as well.

The way that signal crayfish reacted to the chemical stimulus is not only the evidence of the stronger irritating effect of Cl-T, but it also confirms that this species is appropriate for water quality bioindication. The results of the present study are well matched with our earlier data presented in Kuklina et al. (2014), where native narrow-clawed crayfish *Astacus leptodactylus* showed a very similar cardiac reaction to the same concentration of Cl-T in water. This means that signal crayfish, despite being invasive in European waters, can still serve as reliable bioindicators, at least for monitoring chemical pollutants in the water.

Depending on the intensity of the stimulus, crayfish demonstrated a decreasing heartbeat within approximately 60 s followed by the slightly delayed HR increase (Fig. 4a, b). It

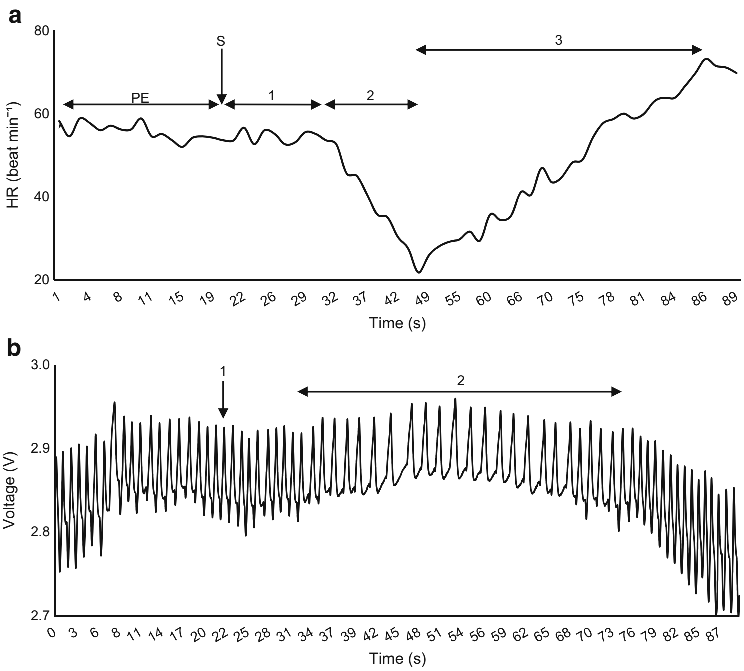

Fig. 4 **a** Example of crayfish cardiac reaction to introduced food stimulus. Period PE corresponds to pre-exposed state. Period 1 corresponds to cardiac response await. Period 2 shows HR deceleration. Period 3 describes subsequent HR acceleration. Vertical arrow S denotes stimulus initiation (20 s). **b** Typical cardiac reaction of crayfish (Y, raw heartbeat curve) to introduced food stimulus. Decrease of the HR described in the text corresponds to increased cardiac intervals on the figure (horizontal arrow 2). Vertical arrow 1 denotes stimulus introduction (20 s)

should be mentioned that such peculiarity of crayfish cardiac reaction in the presence of disturbance has been previously reported from analysis of electrocardiograms (ECG). Thus, the initial cardiac reaction of crayfish characterized by slowing HR was previously observed in the red swamp crayfish ECG by Burmistrov and Shuranova (1996). The similar reaction to outward stimulus was earlier described in slowing ECG of higher vertebrates by Graham and Clifton (1966), which was followed by delayed acceleration in the ECG. It was later called the orienting response (its role consists of an increase of the sensory input to the organism) and defensive response, respectively (Graham 1979). Graham (1979) also notes that in some cases, the orienting response can be absent or can overlap with the defensive one. Nevertheless, in our experiments, all phases of cardiac reaction were clearly observable, which additionally confirms the appropriateness of the methodology based on comparison with such complex approach as ECG. Cessation of scaphognathites beating along with cardiac arrests for up to 20 min was reported by McMahon and Wilkens (1972) in the American lobster *Homarus americanus* and was associated with a way to conceal from prey or predators. According to the observations of the present study, cardiac arrest did not last for as long as that in the American lobster; however, it is in agreement with the differentiation of the cardiac response by Graham (1979), since both orienting and defensive responses were well pronounced and seemed to correspond to crayfish behavior.

It is worthwhile to note that crayfish movement can affect their cardiac activity as well. For comparison, in hermit crabs *Dardanus arrosor*, the cardiac arrests accompanied by periods of bradycardia and followed by the prolonged HR increase were recorded in ECG of the moving animals (Cuadras 1980). In the current study, crayfish exposed to natural stimuli manifested rather motionless behavior. In that case, we can speak about a “clean” HR increase. To the contrary, crayfish exposed to the chemical stimulus will likely have both their HR increased and locomotor activity pronounced. We assume that cardiac activity, if disturbed alone, is characterized by lower HR in comparison with motile crayfish, in case of being disturbed by the same stimulus, which will also have increased HR. That makes cardiac reaction to the natural stimuli and to the chemicals hardly comparable in terms of the HR; however, qualitative comparison remains reasonable and informative.

In conclusion, the study of crayfish reactions to various stressors is critical as it can help (i) to obtain more complex insight into the ethological and physiological state of crayfish in different environmental and laboratory conditions and (ii) to investigate species-specific crayfish sensitivity to stressors for establishing HR threshold levels prior to using crayfish for continuous water quality monitoring. Therefore, there is a remaining need to set up thresholds of crayfish cardiac reactions, which would enable to conclude with confidence whether the manifested reaction was evidence of a high disturbance or was yet acceptable. These points together can help to implement more efficient control and prevent, in time, water quality deterioration.

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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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CHAPTER 4

BEHAVIOUR AND CARDIAC RESPONSE TO STRESS IN SIGNAL CRAYFISH EXPOSED TO ENVIRONMENTAL CONCENTRATIONS OF TRAMADOL

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Behaviour and cardiac response to stress in signal crayfish exposed to environmental concentrations of tramadol

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ABSTRACT

Evidence of the ecological and biological impact of pharmaceuticals in surface waters on aquatic organisms is increasing. Tramadol is a synthetic opioid analgesic used to treat chronic and acute pain. To investigate its long-term effects at environmentally relevant levels, we evaluated heart rate (HR) and locomotion of signal crayfish *Pacifastacus leniusculus* during a 21-day exposure to 1 μg L-1 tramadol followed by 14 days depuration. Locomotion and HR were recorded over a period 30 min before and 30 min after exposure to physiological fluids of an injured conspecific, a natural stressor, four times during the tramadol exposure and four times during depuration. A significant increase in HR following stress induction was found in the majority of tramadol-exposed and control crayfish, as well as significant group-specific HR changes between both groups. Locomotor activity during tramadol treatment differed from that during depuration, in general showing less time spent in locomotion and lower distance moved. The tramadol exposed crayfish exhibited higher velocity during depuration than during the exposure period. Results may suggest a potential shift in prey-predator relationships.

1. Introduction

Pharmaceutically active compounds (PhAC) are an important group of pollutants threatening aquatic ecosystems worldwide (Boxall et al., 2012; Sehonova et al., 2018, 2017) through contamination by mixtures of their residues in treated sewage water (Azuma et al., 2017; Li et al., 2011). Depending on their molecular properties, PhACs are not removed, or only partially removed, by waste water treatment plants (Golovko et al., 2014; Heberer, 2002; Petrović et al., 2003). The presence of PhACs in the aquatic environment is not associated with acute toxicity but can impact physiology, resulting in behaviour alterations in non-target organisms (Brodin et al., 2013; Burgos-Aceves et al., 2018a, 2018b; Buřič et al., 2018; Valenti et al., 2012).

Their chemical and physical properties give some antidepressants the potential to bioaccumulate in fish tissues (Du et al., 2012; Gelsleichter and Szabo, 2013; Grabicova et al., 2017) and to accumulate in sediments and induce adverse effects on benthic organisms (Díaz-Cruz et al., 2003; Grabicova et al., 2015). Pharmaceutically active compounds are designed to produce a biological effect in target organisms at low concentrations, so their residues are present in the aquatic environment at relatively low concentrations, but continual input can induce neurohormonal impacts leading to a variety of physiological and behaviour alterations in non-target vertebrates and invertebrates including fish (Barry, 2014), amphibians (Weinberger and Klaper, 2014), molluscs (Fong and Hoy, 2012), and crustaceans (De Lange et al., 2009, 2006).

Pharmaceuticals can act as selective serotonin reuptake inhibitors (SSRI). Serotonin in crustaceans, as in other animals, affects physiology (Cheng et al., 2005; Kulkarni et al., 1992; Vaca and Alfaro, 2000; Wongprasert et al., 2006) and social behaviour (Huber et al., 1997; Huber and Delago, 1998). Among freshwater crustaceans, crayfish are particularly susceptible to PhACs (Buřič et al., 2018; Kubec et al., 2019; Tierney et al., 2016). They possess a complex physiology and exhibit complex intraspecific interactions (Gherardi et al., 2010; Kubec et al., 2019; Vilpoux et al., 2006), and are keystone species in freshwater ecosystems, functioning as strong ecosystem engineers (Creed and Reed, 2004; Dorn and Wojdak, 2004). The Signal crayfish *Pacifastacus leniusculus*, Dana 1852 is a suitable species for physiological and behavioural studies due to its sensitivity and availability and displays alterations in cardiac activity in response to naturally occurring and anthropogenic chemicals (Kuklina et al., 2018).

The objectives of this study were to determine effects of exposure to environmentally relevant concentrations of the opioid painkiller tramadol on cardiac activity and behaviour of signal crayfish from which to deduce potential ecological impact of pharmaceutical pollution in the aquatic environment on predator-prey relationships.

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2. Materials and methods

2.1. Chemicals

Tramadol hydrochloride and isotopically labelled tramadol (D_3) were obtained from Sigma-Aldrich (USA) and Lipomed (USA), respectively. A stock solution of tramadol was prepared in ultra-pure water (aqua-MAX-Ultra system, Younglin, Kyounggi-do, Republic of Korea) at a concentration of 10 mg L^{-1} and stored at 4 °C. The exposure solution of ~1 μg L^{-1} of pure compound was prepared by dilution of the stock solution with settled tap water immediately prior to use. The concentration was considered environmentally relevant based on reports in the literature (Fedorova et al., 2014; Grabic et al., 2012; Grabicova et al., 2015; Rúa-Gómez and Püttmann, 2012; Thomas et al., 2014)

A solution of tramadol-D_3 was prepared in methanol at 1 μg mL^{-1} and used as internal standard for analysis. Methanol and acetonitrile (both LC/MS grade) as well as formic acid used for acidification of mobile phases in liquid chromatography were obtained from Sigma-Aldrich.

2.2. Animals

Signal crayfish were collected by hand in summer (2017) from non-polluted Křesanovský brook (49°03′35.2″N 13°45′33.8″E) near Šumava National Park, Czech Republic and transferred for acclimatization to a recirculation system at the Faculty of Fisheries and Protection of Waters USB, Czech Republic. Twelve specimens in intermoult stage were sex (1:1) and size selected (Cl = 42.8 ± 0.74 mm) from acclimated stock.

2.3. Experimental setup and data acquisition

Six specimens for tramadol exposure and six controls were immobilised using a folded paper towel. Non-invasive sensors of cardiac activity were attached with non-toxic epoxy resin to the dorsal carapace over the cardiac muscle location (Kuklina et al., 2019). When glue was sufficiently hardened, crayfish were placed in separate tanks filled with 10 L aged tap water. The tanks contained shelters made from half of a plastic plant pot with a narrow oblong opening cut in the upper part to ensure unimpeded entrance by the crayfish carrying sensors. Crayfish were held for 14 days acclimatization prior to experimentation. Photoperiod was automatically maintained at 12:12 L:D. Crayfish were fed twice weekly on chironomid larvae. Uneaten food and waste were removed during water exchange the following day. During exposure, animals were fed on stress test days a few hours after completion of the test. Tanks were cleaned the following morning. Water was exchanged every 48 h using aged tap water and freshly prepared tramadol solution. Crayfish were exposed to tramadol for 21 days followed by fourteen days depuration in pharmaceutical-free water.

The real concentration of pharmaceuticals in aquarium water was measured by liquid chromatography with tandem mass spectrometry (LC–MS/MS, Thermo Fisher Scientific, USA) four times during the exposure period. Grab water samples were collected from renewed solution (time 0 h) and from removed solution (48 h), filtered through a regenerated cellulose filter (0.20 μm, Labicom, Czech Republic) and stored at −20 °C until analysis. The LC–MS/MS method was adapted from Grabic et al. (2012).

On days 0, 1, 7, 14, and 21 of drug exposure and on days 1, 2, 7, and 14 of depuration, 50 mL of a stressor (Table 1) was administered using individual peristaltic pumps via small tubes fixed to the edge of the tank to avoid undue disturbance and air discharge. The stressor was haemolymph and other fluids obtained from a crayfish anesthetised on ice and cut in a sagittal plane while held over a 2 L of dechlorinated tap water of the same temperature as that in the experimental tanks. Fluid was stirred into the water with a glass rod and used immediately. The control crayfish kept in pharmaceutical-free water were managed in the same way as the tramadol-exposed.

Cardiac activity and locomotion were continuously recorded from 0.5 h before to 0.5 h after stressor administration (12 samples min^{-1}). This relatively short period of recording was chosen to determine crayfish immediate reactions to the stimulus as previously described (Fossat et al., 2014).

Locomotion was recorded using a Microsoft Kinect Sensor (Microsoft Corporation, Redmond, Washington, USA) situated under the tanks. Video recordings were evaluated by EthoVision XT 13.0 software (Noldus Information Technology, Wageningen, Netherlands) using a multiple-arena module to detect patterns of crayfish movement. Distance moved (cm), time spent in motion (s), and velocity (cm s^{-1}) were evaluated.

Cardiac activity was recorded as number of beats per minute (HR) using software developed by Pautsina et al. (2014) with sampling frequency 0.2 Hz and pre-evaluated in Microsoft Excel for statistical analysis.

2.4. Statistical analysis

Data were evaluated using Statistica 13 (StatSoft Inc., Tulsa, USA). Crayfish heart rate pre- and post-stimulus were compared using a paired *t*-test for dependent samples. Heart rate differences in control and tramadol treated crayfish after stress application were evaluated using a *t*-test for independent samples. Distance, time spent in locomotion, and velocity were compared by non-parametric Mann-Whitney and Kruskal-Wallis tests before and after stress initiation as well as between control and tramadol treated groups during exposure and depuration. Nonparametric tests were applied because of non-normality of the data distribution and heterogeneity of variances. Results were considered significant when $p < 0.05$.

3. Results

3.1. Water sampling

The concentration of tramadol exposure solution in analysed water samples (n = 4) was 0.94 ± 0.14 μg·L^{-1} at time 0 and 0.9 ± 0.13 μg·L^{-1} at 48 h. Water from control group showed concentration lower than the limit of quantification (< 0.039 μg L^{-1}).

3.2. Cardiac activity

During the exposure and depuration periods, mean HR of tramadol-exposed crayfish demonstrated greater increase post-stressor than seen in controls. Only on day 1 of exposure, did mean change in HR of exposed and control crayfish not differ ($p > 0.05$), and on day 14 of exposure and day 7 of depuration, mean HR change post-stress of the control crayfish was significantly higher than that of exposed group (Fig. 1).

Table 1
Days of tramadol exposure (Day) and depuration (Dday) of stimulus application and data collection; tram = tramadol solution, tap = aged tap water.

Stimulus/data collecting	Day 0	Day 1	Day 7	Day 14	Day 21	Dday 1	Dday 2	Dday 7	Dday 14
Exposure	tap	tram	tram	tram	tram	tap	tap	tap	tap
Control	tap	tap	tap	tap	tap	tap	tap	tap	tap

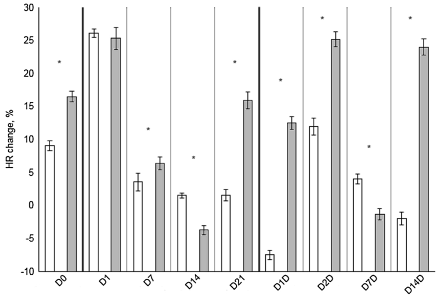

Fig. 1. Mean heart rate change in control (white) and tramadol-exposed (grey) crayfish (n = 6) pre- and post-stress initiation at each sampling day (D0-D14 days of exposure, D1D-D14D days of depuration. *indicates significant difference. Columns correspond to the mean value and whiskers correspond to standard error.

3.2.1. Exposure

In 667% of 24 samplings, HR of individual specimens during the exposure period significantly increased after stressor application in both tramadol-exposed and control crayfish (Table 2). 25% of tramadol-exposed crayfish and 16.7% of control crayfish demonstrated significantly decreased HR after stress initiation. Besides that, 8.3% of exposed and 16.7% of controls showed no significant cardiac response to stress-odour application.

3.2.2. Depuration

Significant increase of cardiac activity after stressor application was detected in 79.2% of 24 samples in tramadol exposed crayfish and in 45.8% of 24 samples of controls during depuration period (Table 3). In 20.8% of exposed and 37.5% of control crayfish demonstrated a significant decrease in HR after stress initiation. No significant cardiac response to stress stimulus was detected in 8.3% of control crayfish. A single moulted crayfish in the control group was not included in analysis of the two final sampling days.

3.3. Locomotion

Although locomotor activity of tramadol-treated crayfish did not significantly differ from that of controls ($p > 0.05$), there were differences observed in locomotor activity in response to stress throughout the trial.

Tramadol-exposed crayfish exhibited lower velocity compared to controls (Fig. 3). During depuration, tramadol-exposed crayfish exhibited velocity twice that of the exposure phase, while locomotor velocity of control crayfish remained at a similar rate (Fig. 3).

Conversely, tramadol-exposed crayfish demonstrated reduction in distance moved (Fig. 2) and time spent in locomotion upon stress-stimulus (Fig. 4) but an increase in velocity of movement (Fig. 3). Controls demonstrated increased locomotion upon stimulus compared to pre-stimulus that was reflected in greater distance moved (Fig. 2), increased velocity (Fig. 3), and greater time spent in locomotion (Fig. 4).

4. Discussion

Previous studies of effects of PhACs on aquatic organisms at environmentally relevant concentrations have been primarily focused on behaviour (Brodin et al., 2014; Buřič et al., 2018; Fong and Ford, 2014; Péry et al., 2008). We combined analyses of crayfish behaviour and physiology as indicated by locomotion and cardiac activity. We anticipated detectable reactions, as crayfish are confirmed sensitive to natural and artificial chemicals even at low concentrations (Kuklina et al., 2019, 2013, Stara et al., 2019a, 2019b). We used fluids of injured conspecifics to provide a scent associated with predation that has been shown to stimulate cardiac activity to a greater degree than other

Table 2
Mean heart rate (beats min^{-1}) of individual tramadol-exposed crayfish over 30 min (12 samples min^{-1}) pre- and post-exposure to stress stimulus. Dark grey indicates significant increase, light grey indicates significant decrease, and white indicates no significant difference in cardiac activity ($\alpha = 0.05$).

Crayfish	Stress	Day 0	Exposure, days				Depuration, days			
			1	7	14	21	1	2	7	14
1	Pre	82.0	88.0	95.4	94.0	71.0	69.8	74.8	97.6	74.8
	Post	99.1	102.7	100.8	93.0	82.6	87.0	91.6	107.3	91.6
2	Pre	46.6	44.7	46.1	52.9	46.1	45.1	42.6	62.1	42.6
	Post	68.6	57.4	56.3	62.5	51.1	48.6	47.3	41.9	47.3
3	Pre	45.6	35.6	38.4	69.7	36.7	41.3	39.4	43.7	39.4
	Post	44.0	39.1	39.7	59.6	35.0	37.7	41.2	46.1	41.2
4	Pre	53.0	48.6	54.9	60.9	41.2	64.3	47.3	55.8	47.3
	Post	63.4	59.4	61.2	55.4	60.6	54.2	77.8	53.0	77.8
5	Pre	125.0	83.2	107.8	94.8	70.3	71.9	60.0	81.7	60.0
	Post	121.3	114.9	101.7	87.1	86.3	83.6	67.4	88.8	67.4
6	Pre	61.2	49.8	66.9	64.2	60.2	47.9	48.4	58.8	61.7
	Post	82.3	65.2	75.1	64.3	60.5	71.4	65.2	56.0	77.9

Table 3
Mean heart rate (beat min^{-1}) of individual unexposed control crayfish 30 min (12 samples min^{-1}) pre- and post-exposure to the stress stimulus. Dark grey indicates significant increase, light grey indicates significant decrease (α = 0.05), and white indicates no significant difference of cardiac activity. M = Moulted.

Crayfish	Stress	Day 0	Exposure, days				Depuration, days			
			1	7	14	21	1	2	7	14
1	Pre	82.0	77.2	110.6	133.3	70.2	77.4	58.4	97.9	62.6
	Post	112.0	106.0	114.6	138.5	82.8	60.1	83.6	106.4	73.6
2	Pre	51.6	43.4	60.7	88.8	90.4	84.6	82.1	M	M
	Post	51.9	46.1	53.3	88.6	86.1	79.7	81.5		
3	Pre	74.9	45.9	59.8	92.0	78.6	49.1	56.8	58.5	71.4
	Post	62.7	91.1	67.4	93.4	71.6	55.1	64.0	63.7	57.6
4	Pre	88.4	86.6	81.6	80.1	84.2	82.6	78.9	73.2	75.4
	Post	90.3	91.4	82.6	79.5	81.6	77.2	74.0	76.0	78.1
5	Pre	67.1	53.1	53.1	113.8	98.6	70.2	58.1	69.9	53.8
	Post	58.7	59.1	52.8	115.7	107.5	87.1	74.6	61.8	50.6
6	Pre	67.6	66.4	60.8	105.4	52.7	86.0	46.9	50.8	61.3
	Post	94.8	76.2	67.2	106.9	52.6	56.0	47.1	55.5	56.6

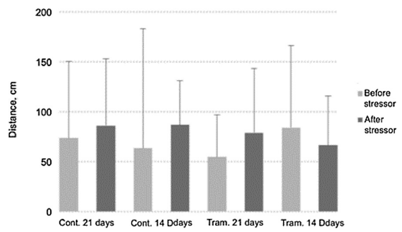

Fig. 2. Mean distance moved by control and tramadol-exposed crayfish during exposure and depuration before (light grey) and after (dark grey) stress initiation. Columns correspond to the mean results of trials conducted on days 0, 1, 7, 14, and 21 of the 21-day exposure period and on days 1, 2, 7, and 14 of the 14-day depuration period. Whiskers correspond to standard error; n = 6 in each trial.

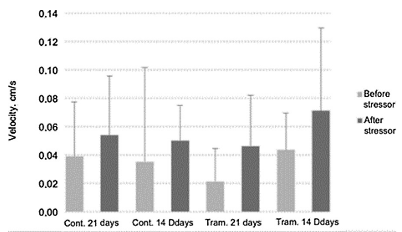

Fig. 3. Mean velocity of control and tramadol-exposed crayfish during exposure and depuration, before (light grey) and after (dark grey) stress initiation. Columns correspond to the means of trials conducted on days 0, 1, 7, 14, and 21 of the 21-day exposure period and on days 1, 2, 7, and 14 of the 14-day depuration period. Whiskers correspond to standard error; n = 6 in each trial.

natural stressors (Fossat et al., 2014; Kuklina et al., 2018). Tramadol-exposed crayfish showed greater HR change in response to stress odour than did controls (Fig. 1), showing possible evidence of tachycardia as an opioid side-effect (Sun-Edelstein et al., 2008)

Conversely, tramadol-treated crayfish spent less time in locomotion (Fig. 4). This behavioural difference may be related to the mode of action of the opioid painkiller on the central nervous system. Reduced locomotion of crayfish juveniles exposed to SSRI fluoxetine has been reported (Tierney et al., 2016). Side-effects of tramadol treatment in humans include decreased alertness, drowsiness, dizziness, and impaired sensitivity to environmental cues (Langley et al., 2010). However, unlike controls, which typically showed higher activity after stressor application, likely associated with escape and avoidance behaviour during whole trial, tramadol-exposed crayfish covered shorter distances prior to stress stimulus during exposure and greater distances during the depuration phase compared to controls. The tramadol-

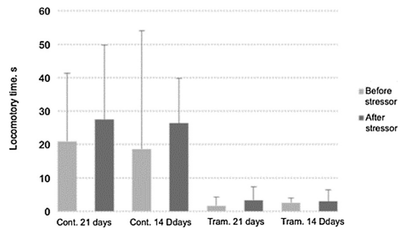

Fig. 4. Mean time spent in locomotion of control and tramadol-exposed crayfish during exposure and depuration, before (light grey) and after (dark grey) stress initiation. Columns correspond to the means of trials conducted on days 0, 1, 7, 14, and 21 of the 21-day exposure period and on days 1, 2, 7, and 14 of the 14-day depuration period. Whiskers correspond to standard error; n = 6 in each trial.

exposed crayfish demonstrated reduced locomotion after stressor administration, potentially impairing predator avoidance. This is in contrast to findings of Hedgespeth et al. (2018) who reported no effect of the SSRI sertraline on freshwater snail *Radix balthica* predation risk.

5. Conclusions

The odour of an injured conspecific is a stressful cue to aquatic organisms, including crayfish, in the present study leading to significant heart rate increase in both control and tramadol-exposed animals. Exposure to tramadol at an environmentally relevant level was associated with significant stress-related heart rate disturbance in signal crayfish.

A potential ecological impact of tramadol on crayfish was shown by lower distance moved, velocity, and time spent in locomotion prior to stressor application over the period of exposure. Thus, predator-prey relationships such as timely and adequate reaction to odour of an injured conspecific can potentially be disturbed by tramadol.

During acute stress, crayfish can often decrease locomotion or stay motionless, while heart rate can vary; therefore it is critical to observe both parameters in etho-physiological studies.

The recorded effects on crayfish cardiac physiology present a new area of research in relation to pharmaceutical opioid residue environmental impact.

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CHAPTER 5

CARDIAC AND LOCOMOTOR RESPONSES TO ACUTE STRESS IN SIGNAL CRAY-FISH *PACIFASTACUS LENIUSCULUS* EXPOSED TO METHAMPHETAMINE AT AN ENVIRONMENTALLY RELEVANT CONCENTRATION

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Cardiac and Locomotor Responses to Acute Stress in Signal Crayfish *Pacifastacus leniusculus* Exposed to Methamphetamine at an Environmentally Relevant Concentration

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Abstract: Methamphetamine (METH), a central nervous system stimulant used as a recreational drug, is frequently found in surface waters at potentially harmful concentrations. To determine effects of long-term exposure to environmentally relevant levels on nontarget organisms, we analysed cardiac and locomotor responses of signal crayfish *Pacifastacus leniusculus* to acute stress during a 21-day exposure to METH at 1 μg L^{-1} followed by 14 days depuration. Heart rate and locomotion were recorded over a period of 30 min before and 30 min after exposure to haemolymph of an injured conspecific four times during METH exposure and four times during the depuration phase. Methamphetamine-exposed crayfish showed a weaker cardiac response to stress than was observed in controls during both exposure and depuration phases. Similarly, methamphetamine-exposed crayfish, during METH exposure, showed lower locomotor reaction poststressor application in contrast to controls. Results indicate biological alterations in crayfish exposed to METH at low concentration level, potentially resulting in a shift in interactions among organisms in natural environment.

Keywords: aquatic environment; invertebrates; illicit drug; behaviour; predator–prey relationship; pollution

1. Introduction

Aquatic environments are contaminated by a wide variety of pharmaceutically active compounds (PhACs) including illicit drugs such as opium, peyote, coca leaf and its derivatives heroin and cocaine, as well as synthetic amphetamine-type stimulants [1] usually used as recreational drugs [2]. Amphetamine-type stimulants are the third most commonly used recreational illicit drug after cannabis and opioids worldwide [3]. Methamphetamine (METH) has historically been popular in Central Europe due to its low cost, and has more recently appeared in Cyprus, Germany, Spain, Northern Europe and, especially, in Asia and North America [3–5].

Similar to other pharmaceutically active compounds, residues of illicit drugs originating from human excretion, due to their chemical and physical properties, may leave wastewater treatment plants [6] unaltered, or slightly transformed, before reaching surface waters [7,8]. Concentrations of these drug residues are reported to reach levels from ng to μg L^{-1} in wastewater and surface waters [9–15]. At these concentrations, illicit drug residues are not considered likely to be acutely toxic to nontarget organisms, i.e., organisms which are exposed unintentionally.

Drug residues may be integrated into the tissue of aquatic invertebrates [16] and vertebrates [17], and can modify the physiology of invertebrates, i.e., zebra mussels [18–20]. Planarians [21,22] and crayfish [23–27] showed, similarly like target organism, reinforcing effect to higher concentrations of dopaminergic drugs. Impact of drugs to physiology and behaviour were reported by Liao et al. (2015) [28] on aquatic vertebrates such as fish *Oryzias latipes* and European eel *Anguilla anguilla* [29].

Effects of METH on mammals are well documented [30,31], whereas information of its impact on nontarget aquatic invertebrates at environmentally relevant concentrations is limited. Exceptions are Hossain et al. [32] who observed disrupted sheltering behaviour in METH-exposed crayfish and Guo et al. [33] who reported disrupted burrow excavation in METH-exposed crayfish. The crayfish is a promising model in investigating physiological and behavioural alterations associated with low concentrations of PhACs [34–36]. In this study, our aims were to quantify cardiac and locomotor responses to acute stress in signal crayfish *Pacifastacus leniusculus* during, and following, exposure to environmentally relevant concentrations of METH and to infer the potential impact of the drug residues on the wider aquatic ecosystem, particularly with respect to predator–prey relationships.

2. Material and Methods

2.1. Experimental Animals, Experimental Setup, Data Acquisition, Chemicals

Signal crayfish *P. leniusculus* were collected in spring 2018 from Křesanovský Brook (49°03′35.2″ N 13°45′33.8″ E) positioned outside the possible sources of pollution, flowing from Šumava National Park, Czech Republic. Crayfish were transferred to a recirculation system at the Faculty of Fisheries and Protection of Waters USB, Czech Republic. We separated 12 males and 12 females (carapace length 44.8 ± 0.82 mm) in intermoult, and divided into a METH exposure group and a METH-free control group (50:50 sex ratio). A noninvasive sensor for monitoring cardiac activity was attached to each crayfish as described by Kuklina et al. [37], and then the fish were placed individually into 10 L static tanks containing shelters made from half of a plastic plant pot with a narrow oblong opening cut in the upper part to ensure unimpeded entrance of the crayfish carrying sensors. Opaque walls of tanks prevented disturbance; the tanks were maintained at constant water temperature of 20 °C and a 12:12 dark: light regime, arranged in an arena system with a single video camera. Crayfish were fed on chironomid larvae every 48 h.

Cardiac activity, as heart rate (HR), and locomotor activity, as distance moved (cm), time spent in locomotion (%), and velocity (cm s^{-1}) in response to a natural stress odour were measured on Days 0, 1, 7, 14, and 21 of drug exposure and Days 1, 2, 7, and 14 of a depuration phase in both METH-exposed and METH-free control groups. Heart rate and locomotor responses were recorded for the 30 min before and 30 min after stress application. The stress stimulus, scent of an injured conspecific to simulated predation, consisted of haemolymph drawn from an anaesthetised crayfish cut sagittally in 2 L dechlorinated water. Haemolymph was distributed at 50 mL per tank by a system of peristaltic pumps over the course of 1 min [38]. Video recordings were processed using EthoVison XT software (Noldus Information Technology, Wageningen, The Netherlands).

A stock solution of METH (Sigma Aldrich, Darmstadt, Germany) was prepared at 10 mg L^{-1} in ultrapure water, and stored at 4 °C. The METH exposure bath was prepared by adding stock solution to aged tap water to obtain a concentration of 1 μg L^{-1}. The exposure solution was renewed every 48 h. Four times during exposure and four times during depuration, at time 0 after METH solution addition and at time 48 h before the water change, water was sampled by plastic syringe, filtered through 0.20 μm regenerated cellulose (Labicom, Prague, CR), and stored at −20 °C until analysis. The concentration of METH in water was determined by liquid chromatography with tandem mass spectrometry (TSQ Quantiva, Thermo Fisher Scientific, San Jose, CA, USA) using isotope dilution with D_5-METH from Cerilliant as isotopically labelled internal standard as described by Hossain et al. [32].

2.2. Statistical Analysis

Data were evaluated using Statistica 13 (StatSoft Inc., Tulsa, OK, USA). In cases of non-normality of data distribution and heterogeneity of variance, nonparametric tests were used. Mean crayfish HR pre- and poststimulus were compared using a paired t test for dependent samples with results expressed as percentage difference. Differences in HR of control and exposed crayfish were evaluated by t test for independent samples. Distance moved, velocity, and time spent in locomotion were compared by nonparametric Mann–Whitney and Kruskal–Wallis tests before and after stress exposure as well as between control and METH-treated groups during exposure and depuration. Results were considered significant when $p < 0.05$.

3. Results

3.1. Analysis of Water Samples

The concentrations of METH in analysed water samples (n = 4) was 1.5 ± 0.1 μg L^{-1} at time 0 (freshly prepared solution) and 1.3 ± 0.4 μg L^{-1} after 48 h. Water of the control group showed METH concentration lower than the limit of quantification (< 0.04 μg L^{-1}).

3.2. Cardiac Activity

During the exposure and depuration periods, mean HR of METH-exposed crayfish demonstrated weaker poststress cardiac reaction, except on Day 21 of the exposure when HR of groups did not differ ($p > 0.05$) and Day 2 of depuration when the mean HR poststress of the exposed group was significantly higher than that of controls (Figure 1).

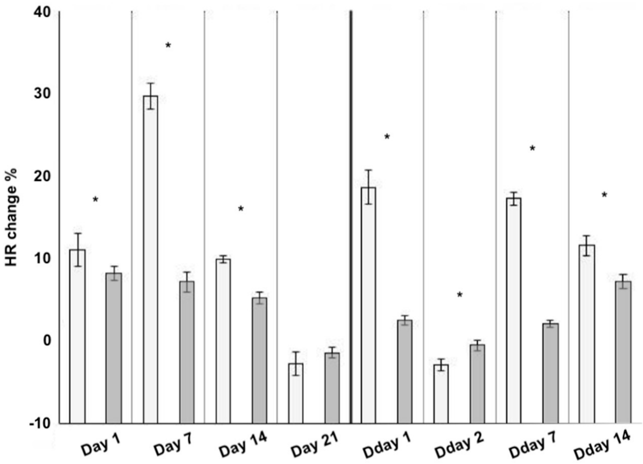

Figure 1. Mean heart rate change in control (light grey) and methamphetamine-exposed (dark grey) signal crayfish (n = 12) pre- and poststress initiation at each sampling day (Day 1–Day 21 days of exposure and Day 1–Day 14 days of depuration). * indicates significant difference ($p < 0.05$). Columns correspond to the mean value and whiskers correspond to standard error of mean.

3.2.1. Exposure Period

In 61% of recordings, the mean HR of individual specimens during the exposure period significantly increased ($p < 0.05$) after stressor application in METH-exposed (Figure 2A) compared to 73% in the control crayfish (Figure 2C). Following stress application, 33% of METH-exposed crayfish and 25% of control crayfish demonstrated significantly decreased HR, while 6% of exposed and 2% of controls showed no significant cardiac response to stress odour.

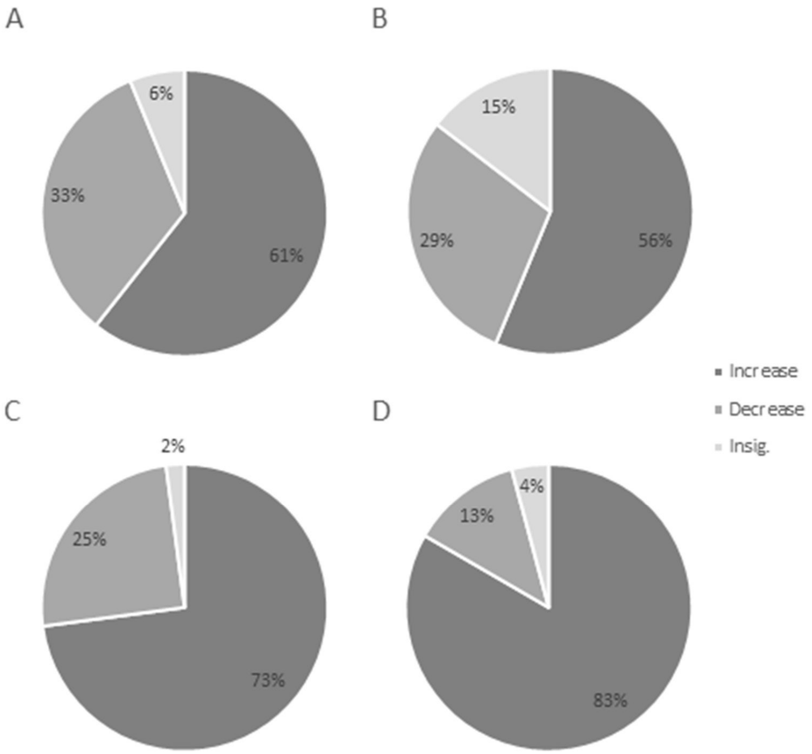

Figure 2. Cardiac response, as percent of recordings showing a significant change in mean heart rate of signal crayfish over 30 min after exposure to stress stimulus compared to the 30 min before stimulus. **A** = METH exposure, **B** = METH exposure depuration, **C** = control exposure, and **D** = control depuration. Four trials during exposure and four during the depuration period × 12 crayfish = 96 recordings.

3.2.2. Depuration Period

Significant increase ($p < 0.05$) of HR after stressor application was detected in 56% of recordings in previously METH-exposed crayfish (Figure 2B) compared to 83% in the control group (Figure 2D) during depuration period. In 29% of previously exposed and 13% of controls, crayfish demonstrated a significant decrease in HR after stress application. No significant cardiac response to stress stimulus was detected in 15% of recordings of previously exposed crayfish and in 4% of control recordings. A single moulted crayfish in the control group was not included in analysis of the two final sampling days.

Information of individual mean HR of exposed and control crayfish over 30 min pre- and poststress during exposure and depuration periods is shown in the Supplementary Materials (Tables S1 and S2).

3.3. Locomotion

Locomotor activity of METH-exposed crayfish did not significantly differ from that of controls ($p > 0.05$). However, crayfish within control group showed significant ($p < 0.05$) increase of distance moved (Figure 3) and velocity (Figure 4) in response to stress during exposure period in contrast to the METH-exposed group where distance and velocity before and after stress application did not differ.

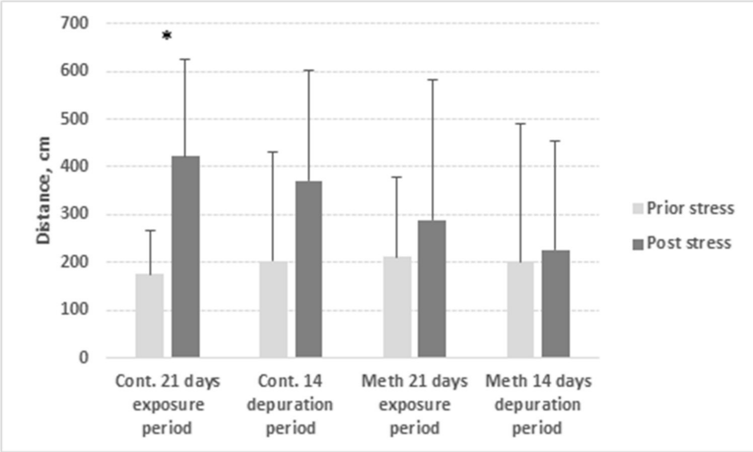

Figure 3. Mean distance moved by control (Ctr.) and METH-exposed (Meth) signal crayfish during exposure and depuration (D). Before (light grey) and after (dark grey) stress application. Columns correspond to the mean results of trials conducted on days 0, 1, 7, 14, and 21 of the 21-day exposure period and on days 1, 2, 7, and 14 of the 14-day depuration period. Whiskers correspond to standard deviation; n = 12 in each trial. * indicates significant difference ($p < 0.05$).

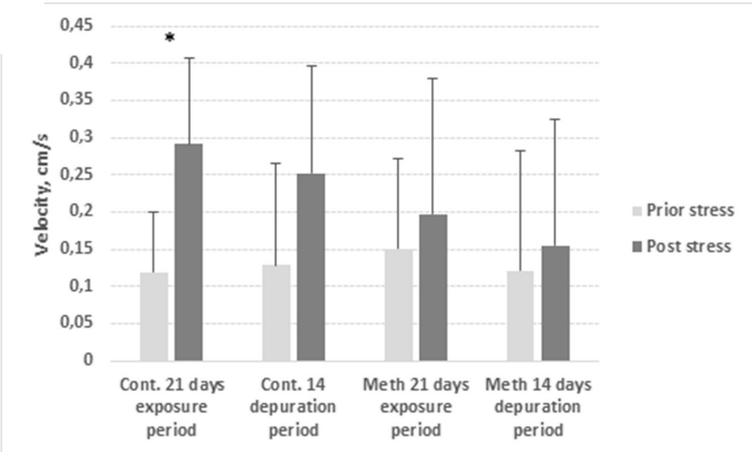

Figure 4. Mean velocity of control (Ctr.) and METH-exposed (Meth) signal crayfish during exposure and depuration (D). Before (light grey) and after (dark grey) stress application. Columns correspond to the means of trials conducted on days 0, 1, 7, 14, and 21 of the 21-day exposure period (21 days) and on days 1, 2, 7, and 14 of the 14-day depuration period. Whiskers correspond to standard deviation; n = 12 in each trial. * indicates significant difference ($p < 0.05$).

4. Discussion

Despite Hazlett (1994) [39] found that the crayfish *Orconectes propinquus* did not show an alarm response to crushed conspecifics, while *O. virilis* could show strong feeding response, or a mixture of alarm and feeding responses to crushed crayfish, depended on how the crayfish was prepared. We used fluids of injured conspecifics to provide a scent associated with predation based on our previous experiences that has been shown to stimulate locomotion and cardiac activity in greater degree than other natural stressors, i.e., food [35]. So, the stressor effect was confirmed for signal crayfish as well as for method of preparation of the stressor cue.

Cardiac activity expressed as percentage difference in mean heart rate pre- and poststimulus in METH-exposed group showed significantly lower stress reaction compared to controls (Figure 1), in contrast to the reported effect of the synthetic opioid tramadol on crayfish cardiac activity [38].

Despite altered physiological patterns, the locomotor activity of METH-exposed crayfish did not differ from that of the controls, which could be explained by high variation of individual locomotor reaction to stimuli (Figures 3 and 4). Hossain et al. [32] exposed marbled crayfish to the same concentration of methamphetamine as used in the present study, and reported similar results with respect to locomotion, but found reduced sheltering behaviour in the exposed group. However, we found the reaction to a natural stressor to be lower compared to that observed in unexposed crayfish, implying increased risk of predation, especially when combined with lower shelter use [32]. This may lead to disruption of predator–prey balance and indicate potential for food web and biodiversity alteration, as was observed by Bláha et al. [40] in a study of predatory insects. Guo et al. [33] reported female METH-exposed crayfish excavated burrows of lower depth and volume relative to individual weight than did controls, which had possible negative consequences especially during periods of drought. In general, disturbance in behaviour or a physiological process at individual-to-population level leads to the disruption of related functions/system and breakdown of ecosystem processes [41].

Planarians have been used as a simple neural system model to study reinforcement effects of licit and illicit drugs [21,22], and crayfish as a model organism to study sensitivity of their reward system [26]. Our findings were in contrast to those of Imeh-Nathaniel et al. [24,42], who observed METH injected into crayfish to increase locomotor activity. In these studies, a higher concentration of METH was used and was injected directly into neural tissue. Changes induced by METH are likely dose specific.

Alteration of cardiac activity during acute stress, and its previously reported neurotoxicity [43] and cardiotoxicity [44], suggests a possible link to METH disruption of catecholamine production/reception in aquatic organisms. Moreover, under natural conditions, organisms are often exposed to a cocktail of contaminants [16]. Ascertaining the impact of such mixtures on nontarget organisms is complex, requiring prediction of synergistic or antagonistic mechanisms of action of individual components.

Organism sensitivity to chemical compounds and natural stimuli (phenotypic plasticity) is considered an evolutionary mechanism to deal with dynamic changes in environmental conditions. In addition, species- and dose-specific effects, as reported by Brodin et al. [41] and Buřič et al. [45], of particular PhACs further complicate the situation. Many substances persist, and may bioaccumulate, in freshwater ecosystems that have diverse effects on organisms, communities, and entire ecosystems [16,41], potentially leading to substantial biodiversity loss and habitat changes.

5. Conclusions

Impact on crayfish biology of METH at environmentally relevant concentrations is apparent in the recorded cardiac and locomotor responses to acute stress and could influence predator–prey relationship with potential ecological consequences. Alteration in physiological processes lead to the disruption of related functions of an organism, which may modify larger scale ecosystem processes [41]. Further research is needed to provide information on the risks of micropollutants to freshwater ecosystems.

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-4601/17/6/2084/s1, Table S1: Mean heart rate (beats min-1) of individual METH-exposed signal crayfish over 30 min pre- and post-exposure to stress stimulus. Dark grey indicates significant increase, light grey indicates significant decrease, and white indicates no significant difference in cardiac activity (α = 0.05), Table S2: Mean heart rate (beats min-1) of individual unexposed control signal crayfish 30 min pre- and post-exposure to the stress stimulus. Dark grey indicates significant increase, light grey indicates significant decrease (α = 0.05), and white indicates no significant difference of cardiac activity. M, moulted.

Author Contributions: Conceptualization, F.L., I.K., T.R. and P.K.; methodology, T.R., I.K. and F.L.; software, P.C., J.K.; validation, K.G., P.K. and T.R.; formal analysis, F.L.; investigation, F.L., I.K.; resources, F.L., I.K, J.K. and K.G.; data curation, P.C.; writing—original draft preparation, F.L.; writing—review and editing, F.L., M.B., K.G. and P.K.; visualization, F.L., I.K., J.K.; supervision, P.K.; project administration, T.R.; funding acquisition, T.R. All authors have read and agreed to the published version of the manuscript.

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CHAPTER 6

General discussion

Suitability of the method

Freshwater crayfish is an important bio-indicator of water quality (Schilderman et al., 1999) and a model organism for behavioural and physiological studies of invertebrates (Imeh-Nathaniel et al., 2017; Koutnik et al., 2017; Stara et al., 2019; van Staaden and Huber, 2018; Velisek et al., 2019). Because behavioural responses to pollutants might not always occur immediately, we implemented the evaluation of physiological parameters in combination with behaviour as a more reliable approach for recording crayfish reactions. In our study, we present a simple, reliable and robust noninvasive method for continuous long-term recording cardiac activity expressed as a heart rate (Kuklina et al., 2019). In contrast to invasive methods (Bierbower and Cooper, 2009), the use of noninvasive biomonitoring omits stress from implantation of extraneous material (electrodes) into crayfish pericardum, theoretically providing more relevant ethophysiological data during long term investigations. Even if crayfish moult, sensors can be reattached to the crayfish carapace within a few days, when the exoskeleton has hardened. Among significant benefits of the current method is the ability to detect biological changes in motionless or freely moving animals during long-term investigation. According to the mentioned benefits, our method shows potential to be used in various approaches from laboratory testing to monitoring of water quality under practical water treatment conditions (Malinovska et al., 2019), and is potentially applicable to various species of decapods. In order to increase general awareness and to provide possible approaches in further studies detailed methodology has been published in the form of a video article (Kuklina et al., 2019).

Practical verification of the method

The practicability of our method under laboratory conditions was verified by investigation of cardiac and locomotor activity of signal crayfish exposed to selected natural and one anthropogenic chemicals. The heart rate differences measured before and after stimulation were arranged as follows: odour of conspecific crayfish (9.2 ± 7.1%) < predator (18.4 ± 13%) < food (33.5 ± 15.7%) < chloramine-T (41.1 ± 14.7%) < injured conspecific (51.8 ± 28.4%). The highest stress was recorded after detection of scent of an injured conspecific rather than after detecting scent of a predator. This is in agreement with observations of (Gherardi et al., 2011), where a stronger reduction in feeding of red swamp crayfish was reported after exposure to injured conspecific odour rather than exposure to odour of predatory fish. Based on our findings we consider the scent of an injured conspecific as predation rather than feeding stimuli, however crayfish are known to be omnivorous (Bondar et al., 2005) and their cannibalistic behaviour should not be underestimated (Hazlett, 1994).

Recently the aquatic environment has faced pollution by numerous micropollutants (e.g. PhACs, pesticides, personal care products, etc.) (Lindberg et al., 2014). Psychoactive compounds (especially antidepressants and illicit drugs) present in the aquatic environment, even at low concentration levels (below μg L^{-1}) can, due to their physicochemical properties, affect vital physiological functions of crayfish and other aquatic organisms. Crayfish reactions are complex chains of subresponses, thus an imbalance in one unit can cause some dysfunctions of the whole organism (Bergman and Moore, 2005). Outputs of standard toxicity tests, where the organisms are exposed to compounds for a short period of time, are mortality or immobilization observed for higher concentrations of selected PhACs on *Daphnia magna*:

sertraline (EC50 = 1.15 mg L^{-1}) > clomipramine (2.74 mg L^{-1}) > amitriptyline (4.82 mg L^{-1}) > fluoxetine (5.91 mg L^{-1}) > paroxetine (6.24 mg L^{-1}) > mianserine (7.81 mg L^{-1}) > citalopram (30.14 mg L^{-1}) and venlafaxine (141.28 mg L^{-1}) (Minguez et al., 2015). However, without the possibility to reveal the mode of action during long-term exposure in environmentally relevant concentrations to non-target organisms. Based on characterisation of ethophysiological patterns of crayfish to natural stimuli (Kuklina et al., 2018), we were able to record evidence of effects on biological parameters during long-term exposure of selected PhACs.

Effects of pharmaceutically active compounds on crayfish ethophysiology and ecology

We investigated the effects of long-term exposure to the synthetic opioid tramadol, used as analgesic, and an illicit drug from the amphetamine group, methamphetamine, in environmentally relevant concentration (~1 μg L^{-1}) on signal crayfish biological parameters expressed as cardiac and locomotor activity. Results indicate that both substances impacted heart rate, while effect of tramadol on locomotor activity was observed as time spent in locomotion, and methamphetamine altered distance moved. Exposure to methamphetamine, which is considered as a potent CNS stimulant, shows decreased excitation of cardiac activity faced with acute stress and locomotor activity expressed (Ložek et al., 2020a). Distance moved by exposed animals did not differ before and after induced stress as it did in the control. Similarly, reaction to stress caused by the lack of water, expressed as decreased burrowing activity was reported by (Guo et al., 2020) in red swamp crayfish exposed to the same concentration of methamphetamine. Hossain et al. (2019) reported that crayfish exposed to methamphetamine showed decreased hiding behaviour indicated by spending significantly more time outside the shelters. It could be concluded that the mentioned ethophysiological alterations caused by exposure to environmentally relevant concentration of methamphetamine may potentially decrease ability to avoid danger from predation to changing environmental conditions. However, in real environmental conditions not just one species but the whole food web is exposed, from which follows the importance of combining laboratory studies with long term observations of species composition in the environment to gain a more accurate image of PhACs impact on the environment.

Despite tramadol acting as an analgesic, excitation of crayfish cardiac activity was higher following the scent of an injured conspecific in comparison with the controls. Also, time spent in locomotion of exposed animals decreased, which is consistent with the report of (Buřič et al., 2018) where marbled crayfish exposed to the same concentration of tramadol showed decreased locomotor activity. Heart rate frequency is usually dependent on activity of the organism (Fedotov et al., 2006). Our findings of cardiac versus locomotor activity in the tramadol exposure highlight the importance of observation of both physiological and ethological parameters as the effective approach for assessing actual reactions of crayfish to stimuli. This approach makes it possible to observe the reason(s) for changes in the crayfish heart rate and whether or not they occur as a result of chemical alterations in the ambient or because of locomotion initiation. Generally, results from our experiments and those of other authors indicate the potential impact of PhACs even at concentrations commonly detected in aquatic environments to biological functions of non-target organisms, resulting in shifts in food webs (Bláha et al., 2019), with possible ecological risks including loss of diversity in the natural environment. In addition, as European native crayfish species are perceived as more sensitive to pollution, PhACs may also have a potential effect on the spread of non-native crayfish species.

Limitations and future approach

We present the effects of single compound on biological parameters of crayfish during acute stress (predation), from which we tried to conclude potential ecological consequences. However, in real conditions of the aquatic environment organisms face not only single compounds, but a cocktail of a variety of compounds with different modes of action and at various concentrations (Gašo-Sokač et al., 2017; Nannou et al., 2015). This fact makes it extremely difficult to simulate real conditions in laboratory studies. For simplification, predictive mathematical models were used for evaluation of possible toxicological interactions among these pharmaceuticals (Godoy et al., 2019), but limitations are similar to those of the standard toxicological studies (Christensen et al., 2007), or with use of water from a defined source (with known concentrations of PhACs in the effluent of WWTP, as done by (Bláha et al., 2019). Our results show the effects of a cocktail of pollutants on experimental animals just at the time of water sampling, without taking into account seasonal variability or water flow changes. Nevertheless, we consider information about the effect of a single compound as a basis for the study of multi-compound cocktails. With this intent we set up experiments where crayfish were exposed to a cocktail of antidepressants (sertraline, citalopram, and venlafaxine), anxiolytic (oxazepam), analgesic (tramadol) and illicit drugs (methamphetamine), each compound at a concentration level of 1 $\mu g\ L^{-1}$. Results suggested that exposed crayfish showed lesser locomotor activity when a shelter was absent compared with controls. Conversely, when shelter was available, exposed crayfish showed greater locomotor activity and spent a significantly higher proportion of time outside the shelter than did controls (Hossain et al., 2020). Also, seasonal variability in input and, consequently, water concentrations of psychoactive compounds, (during the year winter/summer season – antidepressants, on working days/weekends – recreational drugs after weekend) could play a role in affecting various biological processes from spawning season to embryonal development during early neural system ontogenesis (Archana et al., 2017; Golovko et al., 2014; Mackuľak et al., 2016). At this critical point of development we exposed crayfish eggs to methamphetamine, observing differences in cardiac activity, development stage and behaviour in young crayfish during stress (Ložek et al., 2020b).

Although we used a non-invasive method for detection of crayfish cardiac activity, which is suitable in long-term experiments such as exposure to environmentally relevant concentrations of PhACs, we noticed some limitations: the wires of sensors made it impossible to use more than one animal in one arena (for observation of social behaviour, etc.) and the size of the animal had to be sufficient to attach the sensor). In future experimental work we plan to eliminate this limitation by the use of a contactless crayfish cardiac activity monitoring system where the crayfish carapace is marked just by two tiny pieces of highly reflective tape, allowing software to automatically recognize the cardiac area (Císař et al., 2018). The elimination of sensors and their respective wires would provide wider possibilities in experimental designs, e.g. in the case of study of the effect of selected PhACs on a wider range of behavioural patterns within social or reproductive behaviour, in combination with cardiac activity which can vary greatly during these situations (Listerman et al., 2000) with the possibility to keep multiple crayfish in one experimental area. Among the other important benefits of a contactless system is the use of animals of any size for the monitoring procedure, making it possible to compare ethophysiological reactions between juveniles and adult animals; and also the use of smaller species.

Conclusions

Our findings contribute to exploiting the crayfish's ability to respond to various stimuli and their biological parameters, expressed as cardiac and locomotor activity, as a tool for studying effects of emerging aquatic pollutants. Decapods are higher invertebrates and, comparable to vertebrates, they demonstrate unique behaviours throughout their life. Crayfish generally show strong individualistic behaviour, not only in our experiments. This could be explained by phenotypic plasticity reflected by individualistic reactions to changes in conditions as a probable evolutionary ability to prevent distinction of species. In future research, which is already running, we would like to focus on this individualistic behaviour and observe individual effect of emergent pollutants.

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English summary

Crayfish cardiac and locomotor activity as a tool for study of pharmaceutically active compounds effect

Filip Ložek

Environmental pollution by pharmaceutically active compounds (PhACs) is increasingly recognized as a major threat to the aquatic environment. The presence of PhACs in the aquatic environment is not associated with acute toxicity, but can impact on physiology, resulting in behavioural alterations in non-target organisms followed by disturbance of predator-prey relationships with ecological impacts. Some species such as crayfish are sensitive bioindicators of water quality to which they react by ethophysiological changes. Previous studies of PhACs effects on aquatic organisms at environmentally relevant concentrations have been primarily focused on physiology or behaviour; our study combines both.

As a first objective of this study, we described and standardised a noninvasive method for recording cardiac activity of aquatic macroinvertebrates, for example crayfish or other decapods (chapter 2). In the presented article "Continuous noninvasive measuring of crayfish cardiac and behavioural activities" readers can find a link to detailed video-demonstration of animal preparation for experimental work, containing recording of ethophysiological parameters.

In the second objective, we characterised the intensity of signal crayfish *Pacifastacus leniusculus* cardiac and locomotor reactions to stimuli of natural and anthropogenic origin, as odour of conspecific crayfish < predator (predatory fish) < food (chironomids larvae) < chloramine-T < injured conspecific (chapter 3). Studies revealed that from selected stimuli the strongest ethophysiological reaction was recorded to scent of injured conspecific, which we considered that as a stress reaction to predation.

To detect the effect of psychoactive compounds on ethophysiological parameters of crayfish in the third objective, we investigated cardiac and locomotor activity of the signal crayfish in long-term exposure to selected PhACs, the synthetic opioid analgesic tramadol and the illicit drug stimulant methamphetamine, at environmentally relevant concentrations (1 $\mu g\ L^{-1}$), during acute stress – application of an injured conspecific scent (chapter 4, 5). Crayfish exposed to tramadol showed a higher increase of heart rate (beats min^{-1}) indicating a possible side effect of opioids which is tachycardia, however locomotor activity – time spent in locomotion (s) was slightly decreased after the stressor application (chapter 4). Crayfish in exposure to methamphetamine showed a weaker heart rate (beats min^{-1}) and insignificant difference in distance moved (cm), velocity (cm s^{-1}) before and after the stressor application (chapter 5). The opposite results between cardiac and locomotor activity indicates the suitability of observing both biological parameters.

From the results obtained we concluded that the crayfish organism with its biological functions is sensitive to detect selected psychoactive compounds in the relatively low concentrations that were found in the aquatic environment. In addition, ethophysiological alterations highlight possible impacts of these compounds on the ecology of aquatic invertebrates.

The recorded effects on crayfish cardiac physiology could present a new area of research in relation not only to residual PhACs, but to a wide range of contaminants with possible impacs on the aquatic environment.

Czech summary

Srdeční a pohybová aktivita raků jako nástroj ke studiu vlivu farmaceuticky aktivních látek

Filip Ložek

Znečištění životního prostředí farmaceuticky aktivními látkami (PhACs) je čím dál více považováno za jednu z hlavních hrozeb ve vodním prostředí. Přítomnost PhACs ve vodním prostředí nebývá spojena s akutní toxicitou, ale může mít dopad na fyziologii, s následnou změnou chování necílových organizmů, vyúsťující v narušení vztahu kořist-predátor s vyplývajícím ekologickým dopadem. Někteří živočichové, například raci, jsou citlivými indikátory kvality vody, na niž reagují ethofyziologickými změnami. Předešlé studie zabývající se efektem PhACs na vodní organizmy v environmentálně relevantních koncentracích byly primárně zaměřeny na fyziologii nebo chování, přičemž naše studie kombinuje oba tyto biologické parametry.

Jako první cíl této práce jsme popsali a standardizovali neinvazivní metodu zaznamenávání srdeční aktivity větších vodních bezobratlých, např. raků, nebo jiných dekapodů (kapitola 2). V prezentovaném článku „Continuous noninvasive measuring of crayfish cardiac and behavioural activity" čtenáři najdou odkaz na podrobnou video prezentaci přípravy zvířat pro experimentální práci, jejímž obsahem je záznamenávání etofyziologických parametrů.

Ve druhém objektivu jsme charakterizovali intenzitu srdeční a pohybové odezvy raka signálního (*Pacifastacus leniusculus*) na chemické podněty přírodního a umělého původu, jakými jsou: pach druhého raka < predátora (dravá ryba) < potrava (larvy pakomára) < chloramine-T (dezinfektant vody) < zraněného raka (kapitola 3). Studie odhalila nejsilnější etofyziologickou odezvu na pach zraněného raka, který považujeme za stresovou reakci na predaci.

K zachycení efektu psychoaktivních látek prostřednictvím etofyziologických parametrů raků jsme ve třetím objektivu sledovali srdeční a pohybovou aktivitu raka signálního během dlouhodobé expozice vybraných PhACs, syntetického opioidu analgetika tramadolu a ilegální drogy psychostimulantu methamphetaminu v environmentálně relevantních koncentracích (1 $\mu g\ L^{-1}$), během akutního stresu – aplikaci pachu zraněného raka (kapitola 4,5). Srdeční aktivita raků vystavených tramadolu vykázala po aplikaci stresoru vyšší tepovou frekvenci (tepy min^{-1}) poukazující na možný vedlejší efekt analgetika, jímž je tachykardie, nicméně pohybová aktivita – čas v pohybu (s) byla nižší oproti kontrole (kapitola 4). U raků vystavených methamphetaminu bylo zaznamenáno snížení srdeční aktivity (tepy min^{-1}) a statisticky nevýznamný rozdíl mezi pohybovou aktivitou – vzdálenost (cm), rychlost pohybu (cm s^{-1}) před a po aplikaci stresoru na rozdíl od kontrolní skupiny (kapitola 5). Rozdílné výsledky mezi srdeční a pohybovou aktivitou poukazují na hodnotu pozorování obou biologických parametrů.

Ze získaných výsledků jsme vyhodnotili biologické funkce raků jako citlivé k detekci vybraných psychoaktivních látek v relativně nízkých koncentracích, které se běžně vyskytují ve vodním prostředí. Navíc etofyziologické změny zvýrazňují dopad těchto látek na ekologii vodních bezobratlých.

Zachycený efekt na srdeční fyziologii raků může představovat novou oblast výzkumu nejen v souvislosti s PhACs znečištěním, ale rovněž se širokou škálou kontaminantů s možným dopadem na vodní prostředí.

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List of publications

Peer-reviewed journals with IF

Ložek, F., Kuklina, I., Grabicová, K., Kubec, J., Buřič, R., Randák, T., Císař, P., Kozák, P., 2020. Cardiac and locomotor responses to acute stress in signal crayfish *Pacifastacus leniusculus* exposed to methamphetamine at an environmentally relevant concentration. International Journal of Environmental Research and Public Health 17: 2084. (IF 2019 = 2.849)

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Ložek, F., Kuklina, I., Císař, P., Kubec, J., Grabicová, K., Randák, T., Buřič, M., Kozák, P., 2019. Locomotor and cardiac responses of crayfish exposed to simulated predation risk at environmental concentration of methamphetamine. In: 11th Symposium for European Freshwater Sciences (SEFS 11), June 30 – July 5, 2019, Zagreb, Croatia, p. 77

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Training and supervision plan during study

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Ichthyology and fish taxonomy	2018
English language	2019

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Seminar days of RIFCH and FFPW	2016 2017 2018 2019 2020

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Ložek, F., Kuklina, I., Randák, T., Kozák, P., Císař, P., Buřič, M., 2018. Effect of an analgesic at environmental concentration on crayfish locomotion and cardiac physiology. In: 22nd Symposium of International Association of Astacology (IAA 22), 9–13 July 2018, Pittsburgh, Pennsylvania, USA, p. 46.	2018

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Dr. Robert Huber, Ph.D., Life Sciences, Bowling Green State University, Bowling Green, Ohio (2 months, experimental designing, automatization – microcontrollers)	2017
Sandra Hudina, Ph. D., University of Zagreb, Zagreb, Croatia (2 months, fieldwork – collecting of material; laboratory – examination of immune response of collected signal crayfish from different sites of invasion)	2019

Pedagogical activities	**Year**
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