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Effects of psychoactive compounds on fish behaviour

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I hereby confirm that this PhD. thesis “Effects of psychoactive compounds on fish behaviour” was elaborated independently with the usage of quoted literature and based on consultations and the recommendations of my supervisor.

I agree with publishing this PhD. Thesis according to Czech law 111/1998 Sb. About the universities in its current valid wording. This agreement is independent from the results of defense.

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Effects of psychoactive compounds on fish behaviour

Summary

The aquatic environment has been found to be polluted by psychoactive compounds on a global scale. Four of these compounds - citalopram, methamphetamine, sertraline, and tramadol - have been selected in the present thesis to investigate their long-term effect on fish behaviour and condition. We specifically asked whether (i) there are significant effects on behaviour of fish under environmental concentration of the psychoactive compounds; (ii) the effects persist after a depuration period; (iii) their concentration in the brain influences changes in behaviour. Experimental and control fish were subjected to different behavioural tests under the effect of each substance. The behavioural tests included assays on activity, aggression, boldness, exploration, and sociability. In addition, we performed observation of food intake, withdrawal, and the condition of fish. We found that citalopram and methamphetamine increased aggression during exposure. Sertraline induced effects that resemble the behavioural inhibition performed by subordinate fish. Tramadol reduced boldness, exploration, activity and shoaling of fish. The depuration phase induced behaviours indicating withdrawal from methamphetamine and tramadol. Some of the compounds may, therefore, cause changes even after water purification. The pollutant concentration in the brain enabled finer evaluation of behavioural effects and should be performed in further studies in laboratory and in field. Significant changes were thus found in fish exposed to environmental concentrations of psychoactive compounds. These changes have potential to be manifested in the wild and should be investigated in field studies.

Keywords: citalopram, methamphetamine, sertraline, tramadol, aggression, condition, withdrawal

Contents

Introduction

1. Psychoactive pollutants

Pharmaceuticals are small molecules used for treatment of human or animal diseases (Boxall et al. 2012). Consumption of specific pharmaceuticals, such as antidepressants used to treat major depressive disorder, have increased dramatically over the last decades (Figure 1; OECD 2019). The consumption of pharmaceuticals increases probably due to their easier accessibility, ageing of the general population, higher standards of living, and Western lifestyle (Kümmerer 2010). They are synthesized to target specific molecules in a patient's body and cause a specific effect on the health of the patient. Some of these pharmaceuticals may be labelled as psychoactive. They may undergo metabolization (detoxication) in the patient's body. During the first phase of this process, the organism uses oxidative or reductive processes to make the parent compound more hydrophilic (van der Oost et al. 2003; Kümmerer 2010). The second phase is characterized by binding of conjugate (e.g. glucuronide) to the compound (van der Oost et al. 2003; Kümmerer 2010) resulting in production of metabolites that could be biologically active. Many of these compounds are excreted from the patient in form of a parent compound or as a metabolite (Kümmerer 2001, 2010).

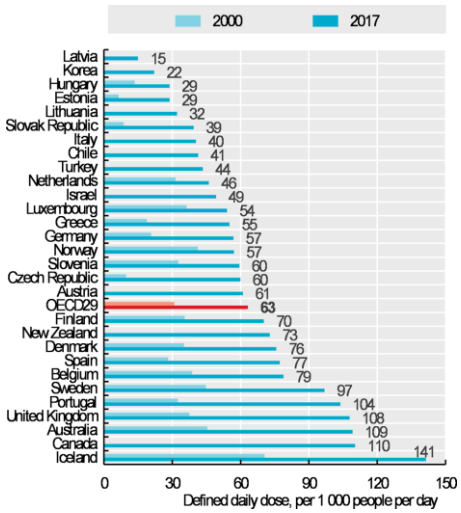

Fig. 1: Comparison of consumption of antidepressants around the world from year 2000 and 2017. Available from OECD ilibrary (<https://www.oecd-ilibrary.org/>).

The excreted pharmaceuticals and their metabolites reach the influents of wastewater treatment plants (WWTPs; Komesli et al. 2015). The polluted water is then subdued to the treatment processes. During primary treatment, the water particles are allowed to sediment to form sludge (Sonune & Ghate 2004). The sludge is also tainted with pollutants (Jelic et al. 2011; Komesli et al. 2015) and must be disposed of accordingly. The water, free of heavy particles, is then exposed to intense bacterial metabolism during secondary treatment of the wastewater treatment process

(Sonune & Ghate 2004). This process dramatically reduces the amount of organic matter. However, bacteria may also cleave the conjugate from the parent compound and reactivate it (Kümmerer 2010). This was observed in the cases of oxazepam and temazepam (Baker & Kasprzyk-Hordern 2013). The rest of the water can be exposed to the tertiary treatment including methods such as membrane treatment technology (Sonune & Ghate 2004; Kim et al. 2018) or exposure to adsorbents (Joseph et al. 2019; Quesada et al. 2019) or ozonation (Rizzo et al. 2019). Finally, the treated water is discharged into surface waters (Baker & Kasprzyk-Hordern 2013). The depuration of water from pharmaceuticals during the wastewater treatment process varies depending on the compound (Baker & Kasprzyk-Hordern 2013; Vymazal et al. 2017). For example, there was 100% clearance in the case of acetaminophen (Komesli et al. 2015), but 3,4-methylenedioxymethamphetamine (MDMA) was removed only from 13 to 19 % (Baker & Kasprzyk-Hordern 2013). The efficiency of removing pharmaceuticals, however, varies even among different WWTPs (Komesli et al. 2015). Furthermore, the final concentration in surface water depends also on how much the effluent is diluted by the receiving river or by precipitation (dilution factor; Mackuľak et al. 2016; Grabicová et al. 2020). The final concentration of pharmaceuticals in their parental form in surface waters is generally in the range of ng/L to μg/L (Baker & Kasprzyk-Hordern 2013). Pharmaceuticals are so-called pseudo-persistent in the environment, because of their continuous release.

Aquatic organisms in natural environments may be under the effect of pharmaceuticals from the WWTP effluent, and this issue is currently being thoroughly investigated. Fish uptake the pharmaceuticals into their bodies, which is demonstrated by finding their traces in fish tissue (Grabicová et al. 2020). The psychoactive pollutants possess a specific mode of action by acting on their target molecules. These molecules are often evolutionarily conserved. For example, it has been proved in the study of Gunnarsson et al. (2008) that zebrafish (*Danio rerio* (Hamilton, 1822)) had orthologs to 86 % of molecules targeted by pharmaceuticals. The interaction of drugs with the aquatic organism could cause changes in gene expression (Overturf et al. 2014; Gröner et al. 2017), physiology (Carney Almroth et al. 2015), histology (Galus et al. 2013; Sancho Santos et al. 2020) and consequently in behaviour of the organism (Brodin et al. 2013; da Silva Santos et al. 2018). The modified behaviour and physiology of individuals could then have an impact on the whole population (Figure 2; Ankley et al. 2010).

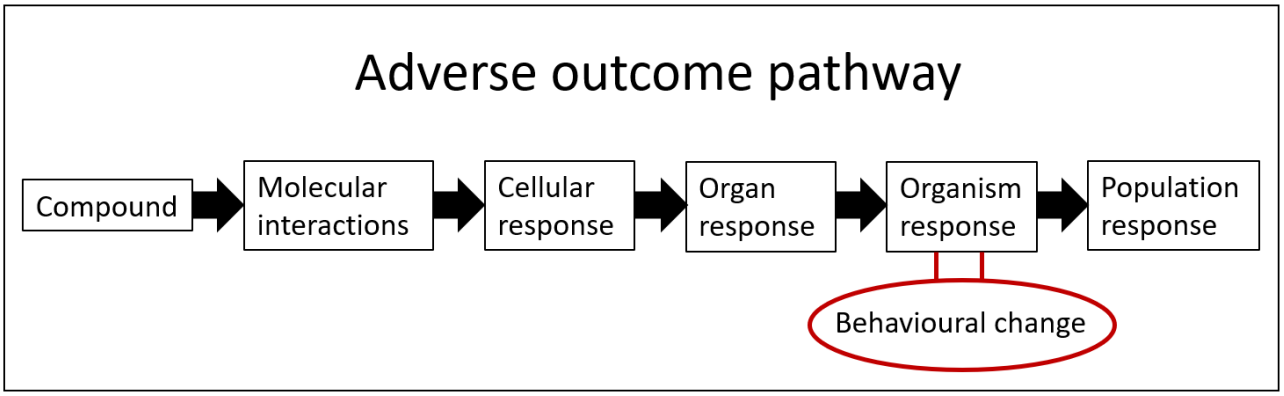

Figure 2: Diagram of key features of the adverse outcome pathway (AOP). This process begins with toxicant/pollutant binding to target molecules in organism, which leads to cascade of processes that produce adverse effects. The effects may impair organism's survival, development, and reproduction, which drive changes in population dynamics. Figure was adapted from Ankley et al. (2010).

2. Selected compounds

The following description of each selected psychoactive compound was composed of parts: (i) description of the compound, (ii) metabolization of the compound, (iii) uptake of contaminant by fishes, (iv) the known effects on behaviour of fish. Only results close to the environmental concentration were discussed.

2.1. Citalopram

Citalopram belongs to a group of compounds called the selective serotonin reuptake inhibitors (SSRIs), which share a similar mode of action. These compounds have been used in the treatment of depressive disorders, obsessive-compulsive disorder (OCD), and panic disorder. Its mode of action involves binding to the serotonergic transporter SERT and inhibiting its function of reuptake of serotonin from synaptic cleft (Milne & Goa 1991). This action leads to an increase of serotonin concentration in the synaptic cleft, which should alleviate symptoms of depression in humans (Milne & Goa 1991). This compound also shows weak affinity to other receptors such as dopamine$_1$, dopamine$_2$ or benzodiazepine receptors in human patients (Milne & Goa 1991; Noble & Benfield 1997). Fish express two gene forms of the SERT transporter (*serta*, *sertb*; Wang et al. 2006), dopamine$_1$ receptor (Messias et al. 2016), dopamine$_2$ receptors (Thörnqvist et al. 2019) and

benzodiazepine receptors (Wolkers et al. 2015). We may expect that these molecules will be targeted by citalopram in fishes.

Citalopram was detected in fish tissues such as liver, kidney, brain, gonads, and muscle (Grabicova et al. 2014; Arnnok et al. 2017; Grabicova et al. 2017; Ziegler et al. 2020b). The brain and gonads are highly fatty tissues that attract lipophilic compounds such as citalopram (LogP = 3.76; (NCBI 2022)). Its concentration in the brain signifies that citalopram reaches its target molecules and may exert its mode of action. The concentration of citalopram in the brain was compared to mammalian brain concentration causing therapeutic effects in the study of Grabicova et al. (2014). Those concentrations did not differ highly, which allowed authors to assume that citalopram's therapeutic effect could be manifested in fish (Grabicova et al. 2014). According to Milne & Goa (1991), the therapeutic action of this compound should manifest in humans during one to four weeks of exposure. Despite reaching its targeted molecules, citalopram did not increase the level of β-esterase (biomarker of neurotoxicity) at the concentration of 1 μg/L in fish (Ziegler et al. 2020a). The antidepressant concentration in the liver and the kidney of fish indicate their involvement in metabolization and excretion of the compound. Human trials proved that citalopram is converted by CYP isoenzymes in the liver (Noble & Benfield 1997) and its half-life elimination is approximately 33 hours in the human body (Milne & Goa 1991), but no such description was found in literature for fish. Instead, the ecotoxicological parameters focus on markers of damage of organs in fish. The environmental concentration of 1 μg/L did not cause any significant changes in liver histology, in concentration of superoxide dismutase (biomarker of oxidative stress) or in the level of heat shock protein 70 (hsp70; stress protein involved in protection of cells against damage) in fish (Ziegler et al. 2020a). The biochemical parameters used in ecotoxicological screening were thus generally unchanged at the environmental concentration, but the neurological targets in the brain could have been altered and cause further changes in behaviour.

Citalopram is one of the most frequently detected compounds in waters, as was the case of Portugal surface waters in study of Silva et al. (2014). Its aquatic concentration does usually not overcome 0.426 μg/L in global surface waters (Mole & Brooks 2019). However, the effluent concentration may reach up to 9.2 μg/L (Mole & Brooks 2019) and that poses a risk for effluent-dominated

streams. Fish may uptake the compound not only from water, but perhaps also from benthic organisms (Grabicova et al. 2015). That would require the citalopram to be present in benthic organisms, which was detected in Grabicova et al. (2015). Citalopram was also labelled as potentially bioaccumulative based on comparison of aquatic and tissue concentrations (Grabicova et al. 2017). This property of compounds is dangerous, as the pollutant could impair the fish's physiological processes after prolonged occupation in contaminated waters due to its increasing concentration in tissues. Additionally, citalopram was shown to inhibit enzyme cytochrome P450 2D6 involved in detoxication of different pollutants in humans (Noble & Benfield 1997). This indicates that there could be a risk of decreased metabolization of other substances during their co-administration. We could expect to detect such an effect in the environment, which is polluted by many different compounds.

The antidepressant had a significant effect on the behaviour of fish even at environmentally relevant concentrations. The food intake was reduced at concentrations of 1.5 μg/L during an exposure length of 15 days (Kellner & Olsén 2020), and at concentrations of 0.07 and 1.15 μg/L during 21 days of exposure in three-spined sticklebacks (*Gasterosteus aculeatus* L.) (Kellner et al. 2015). Citalopram had a significant effect on locomotion. Three-spined sticklebacks tended to occupy a higher position of the tank at the same concentrations as above (Kellner et al. 2016). However, no effect was found on locomotion at these concentrations in the case of brown trout (*Salmo trutta* L.) (Ziegler et al. 2020b). Fish exposed to citalopram also had a lower tendency to be afraid of a novel object (Kellner et al. 2016). The higher the applied dose was, the stronger effects of citalopram were (Ziegler et al. 2020b). The found effects on behaviour indicate anxiolytic properties of citalopram (Ziegler et al. 2020b). Although this antidepressant does not induce changes in biochemical biomarkers used in ecotoxicological evaluation at environmental concentrations, the changes in behaviour occur.

2.2. Methamphetamine

Methamphetamine can be used for treatment of attention deficit hyperactivity disorder (ADHD), but is generally abused as an illegal drug (Boles & Wells 2010). The compound has an affinity to transporters of various neurotransmitters (noradrenaline, dopamine, serotonin; Cruickshank & Dyer

2009; Carvalho et al. 2012). Additionally, methamphetamine also binds to vesicular monoamine transporter-2 (Sulzer et al. 2005; Elkashef et al. 2008). After binding, the compound reverses function of its target molecules (Carvalho et al. 2012). As was shown in case of citalopram, fish possess orthologues to a serotonergic transporter (Wang et al. 2006), transporters of noradrenaline and dopamine (Roubert et al. 2001) and vesicular monoamine transporter-2 (Wang et al. 2016). These molecules would be likely targeted by methamphetamine in fish brains.

The organs concentrating the highest levels of methamphetamine in brown trout were the kidney, liver, brain, muscle and plasma (Sancho Santos et al. 2020). The brain under methamphetamine exposure shows significant changes. Wang et al. (2020b) found reduced gliosis, neuronal loss, and necrosis in brain histology at environmentally relevant concentrations in medaka fish (*Oryzias latipes* (Temminck & Schlegel, 1846)). Also, brain gene expression differed at concentrations as low as 0.2 μg/L (Wang et al. 2020b). For example, brains of dosed fish had downregulated expression of genes *cacna1c* (calcium channels), upregulated expression of gene *oxtr* (oxytocin system), and *c-fos* (gene signifying early immediate gene expression) (Wang et al. 2020b). The changes show that the brains of fish could be damaged by methamphetamine administration at environmental concentrations. The liver is the main organ of methamphetamine metabolization in humans (Cruickshank & Dyer 2009). The liver of brown trout was found to be damaged by the 1 μg/L methamphetamine exposure lasting 35 days (Sancho Santos et al. 2020). Amphetamine is the result of methamphetamine metabolization and was detected in the same organs as methamphetamine in fish tissues (Sancho Santos et al. 2020). This indicates that fish can metabolize methamphetamine in their body. In the same study, non-apoptotic changes were observed also in the case of heart tissue (Sancho Santos et al. 2020). The plasma concentration allowed for comparison to human therapeutic plasma concentration in compliance with the "Read Across Hypothesis" (Huggett et al. 2003). According to this hypothesis, the concentration of 1 μg/L for 35 days did not reach the plasma concentration known to exert therapeutic effects in humans (Sancho Santos et al. 2020). Based on the evidence, it is possible to assume that methamphetamine may harm the organ tissue of the exposed organism in addition to its mode of action on target molecules in the brain.

Methamphetamine concentration in influents of WWTPs was reaching maximal values of 1.805 μg/L in Slovakia (Mackuľak et al. 2016). The maximal effluent concentrations were found to be up to 1 μg/L in the Czech Republic (Fedorova et al. 2014), 0.35 μg/L in Missouri, USA (Bartelt-Hunt et al. 2009), 0.095 μg/L in Canada (Metcalfe et al. 2010) and 0.0076 μg/L in Spain (Postigo et al. 2010). The drug enters the bodies of fish, which can be proven by its presence in fish tissues (Sancho Santos et al. 2020). However, the uptake of the compound from water could differ between benthic and pelagic fishes, as the intensity of the found effects differ depending on these microhabitats (Wang et al. 2020a). Unequal exertion of effects on fish species depending on their microhabitat preference could possibly lead to reduction of fish population in more affected microhabitats.

There is a scarcity of published literature on methamphetamine's effect on behaviour of fish. Medaka fish exposed to methamphetamine in the range of 0.05-25 μg/L for 90 days had significantly increased locomotion (Wang et al. 2020b). Specifically, the fish stayed longer in the central zone, travelled higher total distances with higher speed and performed spontaneous turns (Wang et al. 2020b). Authors explain that this behaviour may have happened due to methamphetamine's effect on expression of *c-fos* gene and, additionally, they assume the fish manifested anxiety behaviour (Wang et al. 2020b). Zebrafish were able to establish conditioned place preference (proxy for addiction) after being injected with methamphetamine (Jiang et al. 2016). Sailfin molly (*Poecilia latipinna* (Lesueur, 1821)) exposed to a single high concentration of methamphetamine (0.1, 0.5, 1 mg/L for 7 days) differed in their courtship (Ghazilou & Ghazilou 2011); however, these concentrations are much higher than those in the environment. Generally, methamphetamine at low concentrations alter locomotion and boldness of fish.

2.3. Sertraline

Sertraline is another compound constituting the SSRI group of compounds and is used for treatment of patients with major depressive disorder, panic disorder and OCD (Murdoch & McTavish 1992; De Vane et al. 2002). Sertraline's mode of action is constituted of inhibition of the serotonergic transporter SERT, similar to citalopram (Stahl 1998b). The inhibition itself is not enough to cause the antidepressant effect, as the adaptive changes in serotonergic neurons and receptors are more

likely to be involved in the formation of therapeutic effects (Stahl 1998a; Yang et al. 2021). Sertraline also binds to noradrenergic and dopaminergic transporters and inhibits uptake of these neurotransmitters as well (Stahl 1998b). The affinity, with which sertraline binds to the transporters other than SERT, is higher than in the case of citalopram (Stahl 1998b). The serotonergic system shows high conservation of pharmaceutical target molecules among humans and fishes (Gunnarsson et al. 2008), which could allow sertraline to bind and exert its mode of action in fishes. The dopaminergic and noradrenergic transporters can be also found in fishes (Roubert et al. 2001). We may expect that sertraline will have an effect on fish.

Sertraline presence was found in specific organs of the fish body such as liver, kidney, brain, and gonads (Grabicova et al. 2014, 2017; Xie et al. 2015; Arnnok et al. 2017). It is generally assumed that sertraline shows attraction towards fatty tissues because of its lipophilic properties (Xie et al. 2015). It was estimated that sertraline could cause therapeutic effects on fish, because the brain concentration of sertraline and mammalian brain concentrations exerting therapeutic effects was comparable (Grabicova et al. 2014). There was a relative increase in *5-ht2c* receptor transcripts in zebrafish larvae exposed to 1 μg/L of sertraline over 6 days (Yang et al. 2021), indicating activation of sertraline's mode of action. Xie et al. (2015) notices an effect on acethylcholinesterase (biomarker of neurotoxicity) at concentrations as low as 4.36 μg/L after 7 days of exposure. Acethylcholinesterase was, however, unaffected at lower concentration (1 μg/L for 6 days on zebrafish larvae (*Danio rerio*; Yang et al. 2021). Sertraline seems to exert its mode of action at concentrations that do not cause neurotoxicity based on the level of acethylcholinesterase. Liver and kidney concentration of pollutants usually indicate elimination pathway of the compound. In human trials, different forms of CYP enzymes are responsible for detoxication of sertraline to norsertraline (De Vane et al. 2002) and the elimination half-life is approximately 26 hours (Murdoch & McTavish 1992). In fishes, the elimination pathway is tested in ecotoxicological studies for their changes due to toxicant exposure. But the effects on superoxide dismutase, catalase (biomarkers of oxidative stress), and glutathione peroxidase (biomarker of second phase detoxication) were not significant at environmentally relevant concentrations in the study of Xie et al. (2015). From this information it seems that the liver does not show significant alterations at the environmental concentration, but the detoxication of sertraline may take place in fish bodies due to a presence of

norsertraline in their tissues. Additionally, sertraline was reported to be present in fish gonads (Arnnok et al. 2017). A trace dose of 5.2 ng/L of sertraline was shown to damage testis interstitial cell prominence after 21 days of exposure in fathead minnow (*Pimephales promelas* Rafinesque, 1820) (Schultz et al. 2011). Sertraline thus seems to exert its effects on the gonads as well as on targeted molecules in the brain.

Sertraline concentration in effluent was found to be as high as 1.93 μg/L globally, but its concentration in surface water was found to be very low (up to 0.075 μg/L; Mole & Brooks 2019). This antidepressant enters the fish body rapidly through inhalation or the food web (Arnnok et al. 2017; Grabicova et al. 2017). The uptake of sertraline is higher in waters with a higher pH (Alsop & Wilson 2019). Sertraline is often accompanied in waters by its metabolite norsertraline (Arnnok et al. 2017). Norsertraline was found to be a potent bioaccumulative compound (Arnnok et al. 2017), eliciting even stronger bioaccumulation than its parent compound, which was proved to be bioaccumulative as well (Grabicova et al. 2017). Based on comparisons between sertraline/norsertraline concentrations in tissues and water, it was found that the parent compound is easier to eliminate from the body than norsertraline (Arnnok et al. 2017). Arnnok et al. (2017) indicates that the position in the food web did not have a high impact on accumulation of sertraline (no biomagnification), but the exposure length and dosage mattered.

The behaviour of fish exposed to sertraline close to environmental concentration was significantly altered. The fathead minnow exposed to 3 μg/L of sertraline for 28 days stayed under shelter for a longer time (Valenti et al. 2012). Similarly, crucian carp (*Carassius auratus* (L.)) exposed to 4.36 μg/L had higher swimming activity and reduced shoaling (Xie et al. 2015). Zebrafish larvae performed higher dark avoidance at 1 μg/L after 6 days (Yang et al. 2021). These effects may indicate anxiolytic effects of sertraline. The aggression of Siamese fighting fish (*Betta splendens* Regan, 1910) decreased during a 14-day exposure to 0.4 μg/L of sertraline (Kania & Wrońska 2015). The perch (*Perca fluviatilis* L.) marginally reduced food intake at a concentration of 0.12 μg/L (Hedgespeth et al. 2014) and lowered feeding was also observed at a concentration of 4.36 μg/L in crucian carp (Xie et al. 2015). Sertraline caused increased mortality at a concentration as low as 5.2 ng/L (Schultz et al. 2011). Generally, the behavioural effect of sertraline seems to

comply with the regulation characteristic for the brain serotonergic system, which modulates anxiety responses, feeding behaviour, and aggression. In addition, there is concern over sertraline bioaccumulative properties, which might activate biochemical markers and other behavioural effects when tissue concentration overcome their threshold values.

2.4. Tramadol

Tramadol is an analgesic acting through its effect on the central nervous system (CNS; Dayer et al. 1994; Scott & Perry 2000; Grond & Sablotzki 2004). The pain relief is elicited by a dual mode of action binding to μ opioid receptors and inhibiting the reuptake of noradrenaline and serotonin (Dayer et al. 1994; Grond & Sablotzki 2004; Minami et al. 2015). Tramadol has a biologically active metabolite, O-desmethyl-tramadol, which was found to be even more effective in causing analgesia than its parent compound (Hennies et al. 1988). Regarding the target molecules of pain relief, fish brain contains μ opioid receptors (gene *oprm1*; Thörnqvist et al. 2019) and transporters of noradrenaline and serotonin (Roubert et al. 2001; Wang et al. 2006). Tramadol has thus a potential to affect fish physiology and behaviour through its mode of action.

Tramadol was found in the liver and the kidney of brown trout (Grabicova et al. 2017). The liver is the main organ of tramadol metabolization in humans (Grond & Sablotzki 2004). The metabolites of first phase detoxication were not elevated (Plhalova et al. 2020), but this process results in creation of the biologically active metabolite O-desmethyl-tramadol. Tramadol was found to elevate levels of glutathione-S-transferase (GST) in juvenile zebrafish exposed to 2 μg/L for 28 days (Plhalova et al. 2020). This enzyme is involved in phase II metabolization of the compound (van der Oost et al. 2003). The second phase metabolization is highly likely an important step in elimination of this compound and this process occurs in fish liver. Tanoue et al. (2017) proved that fish metabolize tramadol similarly to humans and could reach therapeutic effects if the concentration of O-desmethyl-tramadol is high enough. Dysfunction of the liver in humans was found to increase the time required for its metabolization (Grond & Sablotzki 2004) and we might expect a similar problem in fishes. Kidneys are responsible for excretion of the compound in fishes. In human trials, the elimination half-life of tramadol is only 5 to 6 hours (Grond & Sablotzki 2004), but this information was not determined in fishes. At higher concentrations, tramadol was observed to cause

changes in histology during early stages of fish life (Sehonova et al. 2016). Specifically, the skin of zebrafish embryos was darker and contained more mucous cells (Sehonova et al. 2016, 2017). There were evident changes on the gill histological structure as well (Sehonova et al. 2016, 2017).

Tramadol can reach high concentrations in influents to the WWTP, such as measured tramadol concentration of 4.6311 μg/L in WWTP in England (Baker & Kasprzyk-Hordern 2013). The effluent contains concentrations up to 1.6033 μg/L in the same study (Baker & Kasprzyk-Hordern 2013). Sometimes, the concentration of tramadol in effluent may be higher than in influents (Gunnarsson & Wennmalm 2008). This phenomenon is attributed to cleavage of conjugates from metabolization phase II during the wastewater treatment process leading to reactivation of the compound. Tramadol is found in surface waters with concentrations peaking at 1.4 μg/L as was measured in the Czech Republic (Grabicova et al. 2017). Tramadol metabolite O-desmethyl-tramadol can be found in the presence of its parent compound (Rúa-Gómez & Püttmann 2012). This metabolite is believed to be formed only as the metabolite in patients' bodies and is not formed during bacterial degradation in WWTPs (Rúa-Gómez & Püttmann 2012). Fish organs were positively detected for tramadol (Grabicova et al. 2017), which indicates the drugs uptake from water.

Behavioural effects of tramadol on fish received little attention. High concentration of tramadol (320 μg/L for 144 hours post-fertilization) on zebrafish embryos ameliorated locomotion under darkness (Bachour et al. 2020), but this concentration is not environmentally relevant. Tanoue et al.(2019) investigated the effect of 1, 10 and 100 μg/L of tramadol on fathead minnow for 23 or 24 days and found behavioural differences in novel tank diving tests in low and high doses. However, this study points to the challenge of data interpreting the data of results from these tests. Tramadol, however, generally does not increase mortality of fish even at higher concentrations (Sehonova et al. 2016, 2017). It seems that environmental concentrations do not produce many significant biochemical or behavioural effects on fish, but a lot of information is missing. The unavailability of information on behavioural effects highlights the importance of its investigation.

3. Animal behaviour

Animal behaviour has fascinated scientists for hundreds of years. In history, there were several prominent scientists, skilled observers of animal behaviour, who crafted well-known hypotheses in order to understand animal behaviour. Most of the early ethologists like Darwin (e.g., Darwin, 1872) and Lorenz (e.g., Lorenz 1972) compared behaviour of numerous species of divergent taxonomic groups to reveal universal patterns. Their theories were then used to explain several human features like the human tendency to wage wars, suffer with diseases relating to stress or body posturing in specific situations (Darwin, 1872; Lorenz 1972; Tinbergen et al. 1974).

Classification of behaviour has changed throughout the years. Originally, ethologists comparing divergent species classified behaviour into innate and learned (Tinbergen 1952). These scientists were interested in the so-called four big instincts – hunger, love, fight and flight (Lorenz 1972). These terms are not used in the literature anymore. Modern ethologists focus on behaviours that were previously termed as "tool activities" and they include fixed motor patterns, such as locomotion, pecking, and digging (Lorenz 1972). The expression of these fixed motor patterns was found to be easily stimulated (using behavioural tests), it provided quantitative data and – especially – was found to be expressed at different levels among conspecifics. Today the "instincts" are functions of the behaviour, while the "tool activities" seem to be the behavioural traits themselves (compared with Réale et al. 2007). Animal personalities or behaviours are consequently individual differences in behavioural traits that are relatively stable throughout different situations (Réale et al. 2007).

Behavioural traits are most frequently classified into five axes – activity, aggressiveness, boldness, exploration and sociability (Figure 3; Réale et al. 2007; Conrad et al. 2011). The detailed description of each trait is provided in subchapters below together with certain behavioural aspects of interest (feeding behaviour and substance-related addiction) that are investigated in this thesis. These behavioural traits were found to correlate with one another in specific situations (Réale et al. 2007). This correlation is termed "behavioural syndrome", "animal personality" or "coping style". For example, exploration indices were found to be positively related to boldness indices in zebrafish (Wisenden et al. 2011). However, finding a positive correlation between traits may not necessarily

mean that this relationship is genetically constrained (Bell 2005). On the contrary, the evidence points towards the fact that the expression of behavioural syndrome may be promoted by the character of the location/situation/experience (Bell 2005; Moretz et al. 2007). For example, three-spined sticklebacks from a high predation location manifested behavioural syndrome between boldness and aggression, which was not found or was weak in a location with low predation risk (Bell 2005). This assumption indicates that there is no optimal level of behaviour, but the major environmental factors matter. The manifested behaviour is then a significant factor involved in important decisions of animals such as the selection of a mate (Doutrelant & McGregor 2000), and its contribution to animal fitness is thus unquestionable.

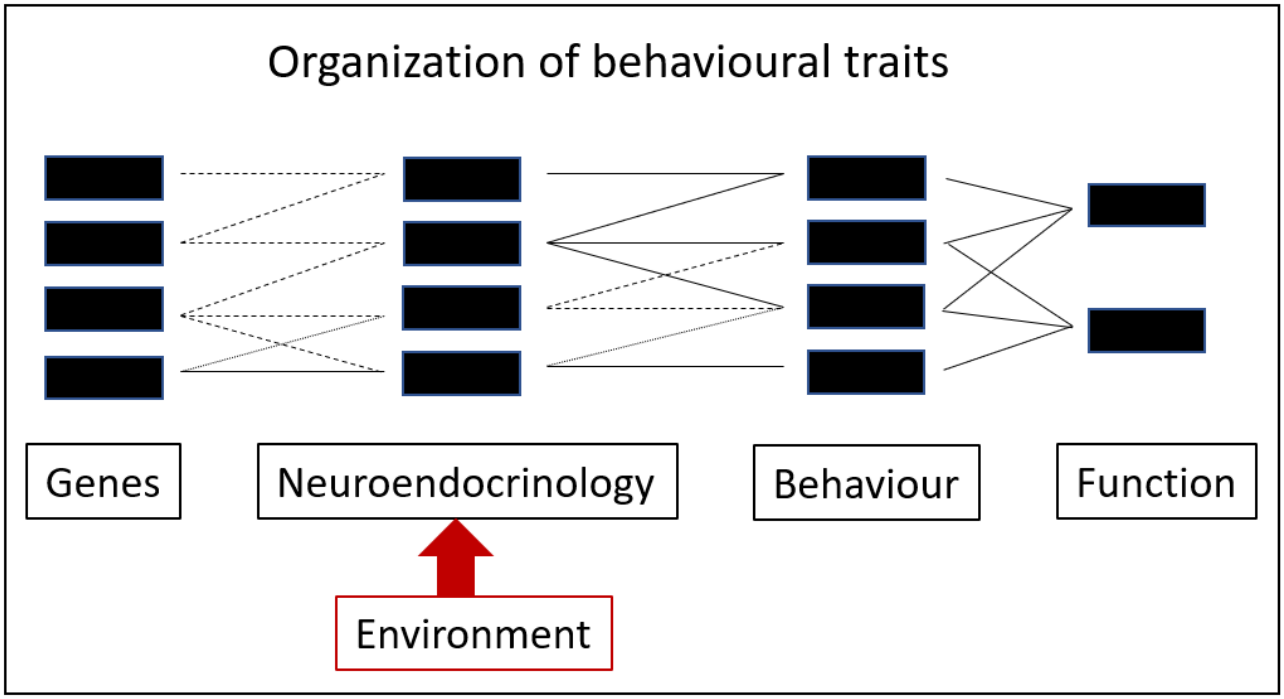

Figure 3: Model of organization of behavioural traits adapted from Réale et al. (2007). The black blocks represent individual components of each structure. The fullness of the line represents the strength of the link between components of different structures. The "Genes" structure is composed of single (major effect) or multiple genes (minor effect). The genes affect neuroendocrinological parameters ("Neuroendocrinology" structure) such as cortisol and steroid production. The "Behaviour" structure is made of behavioural traits, such as activity and sociability. The "Function" structure may be represented by reproduction. Environmental factors ("Environment" structure) contain anthropogenic pollutants in addition to other factors, which target neuroendocrinological parameters.

4. Behavioural traits

The following subchapters will be divided into subsections (i) definition and description of the behaviour; (ii) neuroendocrinological background of the behaviour; (iii) behaviour in context.

4.1. Activity

Activity is generally referred to as an amount of movement performed by a focal individual (Kotrschal & Essler 1995). However, distance of movement is not the only variable tested. Swimming speed (velocity), orientation of movement and swimming path may be other important variables when activity is to be evaluated. Activity can be used for evaluation of diurnal rhythms, to investigate spatiotemporal orientation, and during exposure to various stimuli (Kotrschal & Essler 1995). However, behavioural studies divide general activity in three different traits: activity, boldness and exploration (Réale et al. 2007). Activity is defined, according to behavioural studies, as the movement activity in familiar environment (Réale et al. 2007). Thus, the tests for activity in behavioural studies avoid any signs of novelty and risk-taking in the environment and observe spontaneous swimming of the focal individual. The swimming movements of fish vary depending on body shape and size. Specifically, there are two main types of movement described: undulatory and movement driven by pectoral fins (Lauder 2015). Undulatory movement is especially useful for acceleration rate, while swimming using pectoral fins is more useful for careful manoeuvring (Sfakiotakis et al. 1999; Lauder & Madden 2007). Shape of the body and fins change the effectivity of the desired movement (Sfakiotakis et al. 1999; Lauder & Madden 2007). However, the final shape of body and fins are usually a compromise of both desired activities. Fishes exhibit a wide variety of body shapes and sizes and thus differences in activity may be expected.

The spontaneous activity is motivated by specific stimuli. For example, rats have shown spontaneous activity every two hours throughout a day, and this was associated with their hunger level (gastric motility; Richter 1927). Hunger often elicits searching (appetitive) behaviour and thus swimming activity in fish (Priyadarshana et al. 2006). Motor activity could also change with the season due to fluctuation of steroid hormones (Zhao et al. 2018). Motivation and motor actions are regulated by the central nervous system, especially the dopaminergic pathways (Ikemoto et al. 2015). The dopaminergic pathway is divided into three parts in mammals: mesolimbic (motivation),

mesocortical and nigrostriatal (locomotion) pathways (Haber 2014). Certain nuclei in fish brains have been attributed the function of these dopaminergic pathways and today they form a part of the so-called social decision-making network (O'Connell & Hofmann 2012). Serotonergic (Winberg & Nilsson 1993) and orexinergic/anorexinergic (Nakamachi et al. 2006) substances may also affect the swimming activity of fish. Thus, pollutants affecting these pathways might have a potential to change the natural activity pattern of fishes.

Activity of fish in the wild is strongly affected by temperature (Bartolini et al. 2015), dissolved oxygen (Palstra & Planas 2013), light conditions (Sánchez-Vázquez & Tabata 1998), food presence (da Silva Souza et al. 2020), social rank (Winberg & Nilsson 1993), and water turbulence (Liao 2007). These factors deviate the fish activity level higher or lower depending on whether the performance increases or decreases chances of fish gaining energy from that action. Manifesting higher activity leads to higher energetic costs (Boisclair & Sirois 1993) and consequently to slower growth (Rennie et al. 2005). Increased metabolic cost could induce food intake to replenish the energy reserves of fish as was demonstrated under increased temperatures (Biro et al. 2007). Thus, activity levels have a strong link to fish energetics and consequently to their food intake and growth.

4.2. Aggression

Lorenz (1972) described aggression as fighting behaviour directed to another conspecific. The types of fighting behaviours expressed differ among species but can be generally separated into two categories: passive and active. Passive agonistic behaviour includes different displays that intend to show the size of body and fins to the opponent (Arnott & Elwood 2009; Arnott et al. 2011). Active agonistic behaviour is constituted of behaviours in escalated phases of the fight (e.g., biting, mouth-wrestling) and increases the chances of receiving/inflicting injury (Neat et al. 1998). The fight can be divided into three phases: evaluation, contest and post-conflict resolution (Silva et al. 2013; Jalabert et al. 2015). The contest ends when one individual decides to flee the encounter, which marks the loser, while the winner claims the resource. Win/lose experience induces the winner/loser effect (Hsu & Wolf 2001; Oliveira 2009; Lan & Hsu 2011). These effects include changes in initiation of another contest and changes in expression of escalated aggressive behaviours. Repeated experience from contests results in dominance and subordination relationships between individuals.

The contest is stressful for both contestants (Summers et al. 2005) and thus no immediate differences between winner and loser can be found after resolution of the contest (Chang et al. 2012; Earley et al. 2013). However, dominant individuals vary greatly from subordinate in physiology and behaviour once the social hierarchy is established (Winberg & Nilsson 1993). Low serotonin release is assumed to be continuous in subordinate individuals, while decrease in serotonin release heightens the aggression typical for dominants (Loveland et al. 2014). Winners may have regionally elevated release of dopamine (Schwartzer et al. 2013; Teles et al. 2013). As stated in the subchapter focusing on activity, motivation is usually attributed to certain dopaminergic pathways (Ikemoto et al. 2015). Social factors and motivation show a connection in fish brains as they both contribute to the social decision-making network (O'Connell & Hofmann 2012). Furthermore, subordination has other detrimental effects on fish. Subordinate fish are under social stress (Gilmour et al. 2005) and they show so-called behavioural inhibition including repression of food intake and locomotion (Winberg & Nilsson 1993). Other compounds affect the level of expressed aggression, such as steroids (Scaia et al. 2018), somatostatin, and histamine (Filby et al. 2010). The social rank induced by the contest resolution has therefore a significant impact on fish health. Pollutants targeting these pathways, such as the evolutionarily conserved serotonergic system (Gunnarsson & Wennmalm 2008), have a potential to alter the aggression of fish.

The purpose of aggression is to monopolize limited resources like food, mating, shelter or territory (Filby et al. 2010; Silva et al. 2013; Baran & Streelman 2020). However, the aggressive contest is energetically costly, the fish exposes oneself to possible predation and risks injury or death inflicted by the opposing individual (Neat et al. 1998). Fish may claim several advantages by acting aggressively. Firstly, aggression between individuals may contribute to differences in their growth based on the differences in food intake (Wong et al. 2008). Secondly, dominant individuals or more aggressive individuals may be more reproductively successful (Paull et al. 2010). Lastly, aggression tends to keep conspecifics in a safe distance from one another and contribute to the distribution of fish (Tinbergen 1957). Distribution of individuals is an important factor in anti-predator behaviour. For example, fish may remember locations with high abundance of prey and might tend to return to that location (Brown et al. 2011). The site-attached fishes would then profit if their distribution

would be more spread out, which promotes their territorial lifestyle (Tinbergen 1957). On the contrary, non-attached fish would profit from shoaling together and reducing aggressive interaction between them to avoid predation (Heathcote et al. 2017). Aggression levels therefore significantly matter in various occasions of fish life.

4.3. Boldness

Boldness in fish is usually interpreted as the willingness to undergo risky situations (White et al. 2013). On the contrary, shy individuals will avoid risks (Chapman et al. 2010; White et al. 2013). In laboratory settings, this behaviour may be observed when the individual occupies a familiar environment, but a certain risk factor is added (predator, predator cues, novel object, risk of being caught in a net or simulated predator attack) (Hebert et al. 2014; Forsatkar et al. 2016; Moran et al. 2016; Lagesson et al. 2019). Alternatively, boldness is also often tested as activity or depth of submerging in novel environment and other tests including shelter (Hebert et al. 2014; Dzieweczynski et al. 2016; Forsatkar et al. 2016; Moran et al. 2016; Mustafa et al. 2019). Boldness is therefore also measured as an activity, only in context inducing fear in the focal fish.

Boldness has been assumed to be linked to stress-coping styles, because the behavioural activity is measured in stressful/fearful situations. Proactive coping style is manifested in animals by high sympathetic activity and low hypothalamo-pituitary-interrenal (HPI) axis activity (Koolhaas et al. 1999). The HPI axis activity can be evaluated based on cortisol release after acute stress. This was proved as the shy individuals had significantly higher post-stress plasma cortisol concentration (Raoult et al. 2012; Fürtbauer et al. 2015), but in other studies the difference was not significant (Thomson et al. 2011; Alfonso et al. 2019). The non-significant effects were assumed to be caused by higher behavioural plasticity in bolder fish (Thomson et al. 2012; Bensky et al. 2017), even though proactive individuals are generally supposed to be less behaviourally flexible (Koolhaas et al. 1999). Nonetheless, the difference between bold and shy fish were demonstrated also with significant differences in norepinephrine levels, in expression of dopaminergic receptors and in serotonergic system activity (Alfonso et al. 2019; Thörnqvist et al. 2019). Finally, stress-coping style is also involved in a divergence of release of testosterone in animals (Koolhaas et al. 1999). This assumption was not successful in fish in the study of Fürtbauer et al. (2015), but estrogenic compounds in waters have been demonstrated to change the level of boldness in fish (Hebert et al.

2014; Dzieweczynski et al. 2018). The selected pharmaceuticals bind to neuroendocrine structures involved in regulation of this behaviour, which is a subject of further investigation.

Boldness carries several advantages and disadvantages for the individual. Bolder fish tend to have a faster metabolic rate (Binder et al. 2016). It is then possible to expect a higher need to replenish their energetic reserves. Indeed, bolder fish proved to eat more than shy fish (Ioannou et al. 2008; Jolles et al. 2016). They also demonstrated faster growth (Brown et al. 2007). However, larger fish were also more prone to angling (Klefoth et al. 2017; Keiling et al. 2020), which puts bolder/faster-growing fish at risk of being captured (Klefoth et al. 2017). Not only are the bolder fish prone to angling, but they are also prone to being captured by predators, which links boldness to survival success (White et al. 2013). This was proved when a population of shy prey was introduced into an environment and the biomass of the predator significantly decreased (Wang et al. 2021a). Additionally, boldness of individuals has a significant impact in social groups as well. The shoal is composed of more or less bold and social fish (Bevan et al. 2018). Fish that are both bolder and asocial tend to become leaders of the group, which means that they have a significant impact on the movement of the whole group (Bevan et al. 2018). In the study of Leblond & Reebs (2006) leadership was motivated more by hunger than boldness; however, bolder fish with their higher metabolic rate might be expected to be hungrier (Caudwell et al. 2013). Bolder fish are also valuable to shoals during predator inspection, as they are more likely to approach and investigate the level of the threat and provide this information to the shoal (Brown et al. 2011). The individual level of boldness is therefore an important factor affecting fitness of the focal individual, shoal, and predators.

4.4. Exploration

The tendency to explore novelty has been determined as another behavioural trait (Réale et al. 2007; Martins et al. 2012). This trait may be measured as the willingness to approach a novel object, to explore novel environments or to taste novel food items (Riedel 1998; Réale et al. 2007; Basic et al. 2012; Martins et al. 2012; De Serrano et al. 2016). A careful reader will notice a similarity between tests studying boldness and exploration. Investigation of novelty may hold a certain risk-factor as well. Thus, the tasks studying these traits may often be marked as boldness/exploration tasks. However, in the study of Budaev (1997), exploration was linked to activity and shoaling, unlike

boldness. Fish exploring a new environment will usually tend to follow the walls of the tank to investigate, but this reaction will disappear after the fish habituates to its environment (Riedel 1998). Then, fish will settle in a preferred site of the tank and establish a ‘home’ site (Stewart et al. 2010, 2012).

Exploration has been highly associated with stress-coping styles in fish such as boldness (Basic et al. 2012). The neuroendocrinological background of the stress-coping styles has been described in the subsection devoted to boldness. In addition to stress-coping styles, dopamine levels have been demonstrated to increase after exposure to exploration tasks (De Serrano et al. 2016). The neuroendocrinological background of exploration is therefore very similar to boldness, highly likely because of the similarity of testing methods. Dopamine and other compounds involved in regulation of this behaviour may be altered by selected psychoactive compounds.

Animals need to explore to find food, a mate or shelter (von Helversen et al. 2018). Neophobia allows exploitation of known resources, but prevents finding better options (von Helversen et al. 2018). Adriaenssens & Johnsson (2011) found that less explorative brown trout grew faster, were more behaviourally flexible and less aggressive. They were also more likely to avoid predator threats (Jones & Godin 2010). However, the profitability of low exploration depends on the environment. This lifestyle is better in stable conditions, where residents and specialists are favoured over generalists (Mettke-Hofmann 2014). On the other hand, fast exploration was shown to correlate with success in gathering social information (Nomakuchi et al. 2009). Exploratory tendencies and migrations are favoured in variable environmental conditions (Mettke-Hofmann 2014). But high exploration also heightens the chance to pass on the best option (von Helversen et al. 2018). The alteration of this behavioural trait by anthropogenic pollutants may consequently contradict the favoured level of exploration by environmental conditions.

4.5. Sociability

Sociability is usually referred to as the behaviour directed towards conspecifics excluding aggressiveness (Conrad et al. 2011). The level of this behaviour varies among species, as fish sociability may range from solitary, through living in pairs, shoaling, and schooling (Rubenstein & Abbot 2017). Within species, the tendency to join a shoal (social group size) is commonly evaluated

as a proxy for sociability (Conrad et al. 2011). Fish generally prefer larger shoals composed of more social individuals (Cote et al. 2012). However, social learning (Brown & Laland 2003), reciprocity (Raihani & Bshary 2011), and recognition of familiar conspecifics (Cattelan et al. 2019) may allow further investigation into this topic. Sociality may also be employed to assess fish "numerical" ability by letting fish choose among differently sized shoals (Bai et al. 2019).

Social behaviour is localized to discrete nuclei in the brain which constitute a social decision-making network (Teles et al. 2015, 2016). This network intertwines the social behaviour network with the dopaminergic mesolimbic network (Teles et al. 2015; O'Connell & Hofmann 2012) similar to aggression. The nuclei constituting the social decision-making network are highly conserved in vertebrate evolution (O'Connell & Hofmann 2012). Asocial fish were found to have a higher standard metabolic rate (Killen et al. 2016; Kim et al. 2022), although in another study there was no significant difference (Killen et al. 2021). This has a probable link to the hunger level of the fish, because a higher metabolic rate might deplete the energy reserves of the fish faster, as described earlier. It was demonstrated that hungrier fish will move further from conspecifics in search of food (Killen et al. 2016; Aimon et al. 2019). It is therefore plausible, that pollutants affecting the centres of satiation could also alter the individuals' proximity towards their conspecifics.

Social living has been observed to be an important strategy in various species that may allow them to survive various conditions. For example, eusocial lifestyle allowed ants to spread globally successfully (Rubenstein & Abbot 2017). In fish, a social lifestyle is a part of an anti-predator strategy. Some fish species may grow solitary and aggressive to keep their conspecifics away from their territory (Tinbergen 1957). In certain cases, high aggressiveness may then be linked to low sociability (Lacasse & Aubin-Horth 2014). Other fish favour a social lifestyle, which reduces the probability of being predated on (Herbert-Read et al. 2017), but increases the chance to transmit diseases (Petkova et al. 2018). On the other hand, the level of sociability within species was investigated due to its probable connection to dispersal. This was proved in invasive mosquitofish (*Gambusia affinis* (Baird & Girard, 1853)), where asocial individuals were more likely to spread further from the original location (Cote et al. 2010). Within shoal, bolder and less social individuals may become leaders and have thus a high influence on the direction of movement of the whole group (Jolles et al. 2017; Bevan et al. 2018). Social/asocial lifestyle may therefore be used as an

anti-predator strategy, is important in disease transmission among individuals, and may be a significant factor in species invasions.

4.6. Feeding behaviour

Feeding behaviour is not a part of the classical five behavioural axes defined by Réale et al. (2007). The behaviour may start as a searching (appetitive) behaviour when the individual starts to feel hunger, proceeds with perception of a probable food source and ends by its digestion (Kasumyan 1997). The feeling of hunger is suggested to be present only in conscious (sentient) animals (DeGrazia 2009). Fish belong to this category together with other vertebrates and specific invertebrates according to The Cambridge Declaration of Consciousness in Nonhuman Animals, which was signed by scientists from around the world in 2012 for undeniable evidence of this phenomenon. Sensory organs have a prominent role in food intake as they function to detect food (Hamdani & Døving 2007; Preuss et al. 2014). For example, the optic tectum can distinguish the size of an object and stimulate either approach or avoidance (Preuss et al. 2014). Olfactory signals are useful in detecting amino acids signalling a food source (Hamdani & Døving 2007) and the lateral line may provide cues about prey movement (Pohlmann et al. 2004). The development of sensory organs and consequently the sizes of their primary sensory areas in the brain varies greatly among fish species (Kotrschal et al. 1998). For example, clear shallow waters support higher development of the optic tectum (Van Staaden et al. 1995; Yopak & Lisney 2012; White & Brown 2015a, 2015b). Vision is also more important in predators hunting active prey (Huber et al. 1997). Sensory stimulus is therefore essential for food intake to be stimulated (cue salience).

Hunger in fish is processed in the orexigenic and anorexigenic centres of the hypothalamus and hypophysis (Lin et al. 2000; Schwartz et al. 2000; da Silva et al. 2016). These centres are intertwined in brain tissue and integrate information such as gut emptiness, levels of leptin in blood (and therefore the energetic reserve of the organism) and levels of glucose and insulin (da Silva et al. 2016). Various neurotransmitters are involved in integration of the information, such as neuropeptide Y, orexins, galanin, corticotropin-releasing hormone (CRH), urotensin I, serotonin and growth hormones (GH) (Lin et al. 2000). Social rank is accompanied by change in serotonin release, and those fish also manifest change in food intake (Winberg & Nilsson 1993). Feeling of hunger promotes expression of orexigenic factors in the brain (Narnaware & Peter 2001; Hoskins &

Volkoff 2012) and manifestation of hyperphagia (Schwartz et al. 2000; Abdel-Tawwab et al. 2006), but it can also act as a stressor and cause elevation of cortisol in blood (López-Patiño et al. 2021). Long-term food deprivation could exhaust the energetic reserves of the individual, and metabolism is then depressed (Zhang et al. 2009; Sinnett & Markham 2015). The longer the fish starves, the lower is its growth (Abdel-Tawwab et al. 2006); however, once the fish feeds, it may have a better conversion of feed and grow to its expected size (compensatory growth) (Tian & Qin 2003; Won & Borski 2013). This is true only if the starvation period is not too long (Tian & Qin 2003). Pollutants acting centrally on mentioned neurotransmitters and satiation centres have a potential to alter the food intake of fish.

Hungry fish were proven to increase movement activity and stray further from a shoal (Priyadarshana et al. 2006; Killen et al. 2016; Aimon et al. 2019; Wilson et al. 2019). This behavioural change may make fish vulnerable to predators, as hungry fish are less reactive to alarm cues (Giaquinto & Volpato 2001; McCormick & Larson 2008) and are more likely to perform solitary predator inspection (Godin & Crossman 1994). On the other hand, bolder individuals fed low doses of food were able to adjust their behaviour more flexibly to predator threats than shyer fish (Thomson et al. 2012). Bolder and asocial individuals may also be followed by shyer fish (Nakayama et al. 2012) and thus become leaders. This is leadership based on the temperament of the fish, which thus have intrinsic background (Nakayama et al. 2012). In case of an unwillingness of bold individuals to leave a shelter due to satiation, shy fish start to emerge from shelter by themselves and the leadership is then rooted from the need to find a food source (Nakayama et al. 2012). Metabolic rate phenotype might explain the seeming connection between feeding, sociability, and boldness. A high standard metabolic rate could be beneficial for fish in food-abundant conditions and the fish could grow faster (Metcalfe et al. 2016). Larger body size would allow them to have more offspring (Barneche et al. 2018), but they would also be susceptible to angling (Keiling et al. 2020). If food is scarce, they might take more risks to make up for the faster depletion of energy reserves (Metcalfe et al. 2016). Food intake and related behaviour are essential for the fitness and survival of the individual (Matsuda 2009).

4.7. Substance-related addiction

Addiction is sometimes referred to as to disturbed motivation (Di Chiara 2002). Motivation has been noted in the previous subsections on behaviour. In the original ethological studies motivation has been described as the state of activation of specific drives - hunger, love, fight, and flight – which could vary in intensity (Tinbergen 1952). Modern studies inspect motivation as the result of functionality of the dopaminergic system in relation to "rewards" and characterize it as "the ability to respond to stimuli in relation to their individual needs and with the ultimate goal of the survival of the own species" (Di Chiara 2002; Hyman 2007). The natural rewards – a food source, safety, the opportunity for mating, aggression – hold a great survival value to the individual and as such their seeking is reinforced (Di Chiara 2002; Hyman 2007; Golden et al. 2019). Motivation then consists of learning of specific associations between stimuli and biologically meaningful outcomes (Di Chiara 2002). Pavlovian learning can be used to inspect observed behaviour. Pavlovian unconditioned stimulus (food) caused dogs to show an unconditioned response (salivation, food craving). Then, Pavlov used a conditioned cue (specific illumination, loud sound) to create association with the reward and, after its learning by the experimental subject, the conditioned stimulus caused the release of the unconditioned response even without the presence of the unconditioned stimulus (food). The presence of an incentive cue (conditioned or unconditioned stimulus) to the afflicted individual may further release a series of instrumental behaviours (i.e., actions performed to reach a goal/reward), which is called the Pavlovian-to-instrumental transfer (Hyman 2007; Heinz et al. 2019). As a result, a Pavlovian cue may cause approach/avoidance behaviour to the stimulus depending on its hedonic value, and inhibit unrelated instrumental behaviours (Hyman 2007; Heinz et al. 2019). Thus, an addict may go to great lengths to obtain a drug (Hyman 2007). However, the dopamine release by natural rewards is dependent on the incentive salience of external cues as well as on habituation to them (reviewed in Di Chiara 2002). This is not true for drugs, whose mode of action reliably alters the firing of the dopaminergic system (Di Chiara 2002). The drug and its cues are reinforced with powerful incentive properties that affect goal-directed motor behaviours of the subject and are resistant to extinction (Di Chiara 2002; Hyman 2007).

Stimulus of natural reward, as well as the mode of action of drugs of abuse, may cause dopamine and opioid release (Heinz et al. 2019). Both neurotransmitters were investigated for their involvement in craving, drug-seeking, conditioned place preference, and tolerance (Di Chiara 2002; Heinz et al. 2019). Tolerance is determined when an addict administers increasing amounts of a drug to feel the same effects (*DSM-5* 2013). Craving has been characterized by an intense urge to take the drug (*DSM-5* 2013). The striatum is the main brain area in control of addiction-related processes and is heavily intertwined with dopaminergic neurons (Di Chiara 2002). The dopaminergic pathways in fish are also concentrated in areas believed to be homologous to the striatum (Rink & Wullimann 2001). Furthermore, the social decision-making network in fish brains was found to consist of the social behaviour network with mesolimbic dopaminergic pathways and thus also explain the connection between motivation and social signals (O'Connell & Hofmann 2012).

Substance use disorder has several criteria in the Diagnostic and Statistical Manual of Mental Disorders (*DSM-5* 2013), which are important in understanding the context of this behaviour. The first four criteria relate to impaired control and could be described as the development of tolerance (1), the inability to stop using the drug despite the desire to do so (2), the investment of significant time in actions related to obtaining the drug and its administration (3) and craving (4) (*DSM-5* 2013). Kuhn et al. (2019) review that testing "progressive ratio" can be used to assess how much the animal will work to receive another dose of a drug as each subsequent trial increases the cost of the reward. The "wanting", "drug-seeking" or "craving" was demonstrated in animal models by their preference for a place associated with drug administration (reviewed in Spanagel 2017; Kuhn et al. 2019). The other three criteria of substance abuse focus on impairment of social life: drug use and the actions related to it cause the individual to fail at social roles at work or home (5), the drug abuse continues even after immense negative consequences on social relations (6) and the individual reduces the time spent on social/occupational/recreational activities for time investment on drug use (7) (*DSM-5* 2013). Laboratory studies therefore tested whether drug self-administration will persist even though natural rewards will be present (Lenoir et al. 2007; Venniro et al. 2018). Surprisingly, animals may prefer natural rewards such as sweetened water over cocaine (Lenoir et al. 2007) or social interactions over methamphetamine reward (Venniro et al. 2018). The last two

criteria refer to risky use of the substance: an individual will administer the drug even in a situation that could result in his/her physical harm (8) and will not stop using the drug despite being aware of its negative physical or psychological effects caused by the drug of abuse (9) (*DSM-5* 2013). Spanagel (2017) reviews that if laboratory animals are provided unlimited access to drugs of abuse, they may overdose themselves to death. Thus, the negative consequences of drug administration on health may be ignored in animal models. In summary of the criteria from DSM-5, addiction to drugs seems to be similar to the reward experienced by natural rewards. But substance addiction does not allow its own extinction, does not hold any survival value to the individual, and keeps the subject occupied, which thus reduces time that would be spent on obtaining rewards holding survival values. The most dangerous aspect of substance-related addiction in humans as well as animals is the preoccupation with the drug-related activities, and the activities ensuring its survival are minimized.

Scientific Hypotheses and Objectives

The aim of the present thesis is to investigate the effect of environmentally relevant concentrations of selected compounds (citalopram, methamphetamine, sertraline, tramadol) on specific fish behaviours and body condition. We asked whether (i) the environmental concentrations of the selected psychoactive compounds affect behaviours and condition during exposure; (ii) the substances alter specific behaviours and condition during the depuration phase; (iii) the found effects have any relation to a drug (and its metabolite) concentration in fish brains.

Chronologically sorted articles investigating the topic of fish behaviour altered by psychoactive substances

5 **Hubená, P.**, Horký, P., Grabic, R., Grabicová, K., Slavík, O. and Randák, T., ***2020***. Environmentally relevant levels of four psychoactive compounds vary in their effects on freshwater fish condition: a brain concentration evidence approach. ***PeerJ***, 8, e9356. https://doi.org/10.7717/peerj.9356

6 Sancho Santos, M.E., Horký, P., Grabicová, K., **Hubená, P**., Slavík, O., Grabic, R., Douda, K. and Randák, T., ***2021***. Traces of tramadol in water impact behaviour in a native European fish. ***Ecotoxicology and Environmental Safety***, 212, 111999.

7 Horký, P., Grabic, R., Grabicová, K., Brooks, B.W., Douda, K., Slavík, O., **Hubená, P**., Sancho Santos, E.M. and Randák, T., ***2021***. Methamphetamine pollution elicits addiction in wild fish. ***Journal of Experimental Biology***, 224(13), jeb242145.

8 **Hubená, P**., Horký, P., Grabic, R., Grabicová, K., Douda, K., Slavík, O. and Randák, T., ***2021***. Prescribed aggression of fishes: Pharmaceuticals modify aggression in environmentally relevant concentrations. ***Ecotoxicology and Environmental Safety***, 227, 112944.

5. Environmentally relevant levels of four psychoactive compounds vary in their effects on freshwater fish condition: a brain concentration evidence approach

Hubená, P., Horký, P., Grabic, R., Grabicová, K., Slavík, O. and Randák, T., 2020. PeerJ, 8, e9356. https://doi.org/10.7717/peerj.9356

Environmentally relevant levels of four psychoactive compounds vary in their effects on freshwater fish condition: a brain concentration evidence approach

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ABSTRACT

Background. The aquatic environment has been contaminated with various anthropogenic pollutants, including psychoactive compounds that may alter the physiology and behavior of free-living organisms. The present study focused on the condition and related mortality of the juvenile chub (*Squalius cephalus*). The aim of the study was to test whether the adverse effects of the antidepressants sertraline and citalopram, the analgesic tramadol and the illicit drug methamphetamine, on fish condition exist under environmentally relevant concentrations and whether these effects persist after a depuration period. Innovative analyses of the fish brain concentrations of these compounds were performed with the aim to show relationship between compound brain tissue concentration and fish condition.

Methods. The laboratory experiment consisted of 42 days of exposure and a subsequent 14-day depuration period with regular monitoring of the condition and mortality of exposed and control fish. Identical methodology, including individual brain concentration analyses for the tested compounds, was applied for all substances. Additional study on feeding under sertraline exposure was also conducted. The feeding was measured from the 28th day of the exposure, three times in a week, by observation of food intake during 15 minutes in social environment.

Results. The effects of particular psychoactive compounds on chub condition varied. While sertraline induced a lower condition and increased mortality, the effects of methamphetamine were inverse, and tramadol and citalopram had no significant effect at all. Individual brain concentrations of the tested compounds showed that the effects of sertraline and methamphetamine on fish condition were increased with brain concentration increases. Additionally, the food intake was reduced in case of sertraline. In contrast, there was no relationship between tramadol and citalopram brain tissue concentration and fish condition, suggesting that the concentration-dependent effect is strongly compound-specific. Methamphetamine was the only compound with a persistent effect after the depuration period. Our results demonstrate the suitability of the brain concentration evidence approach and suggest that changes in fish condition and other related parameters can be expected in freshwater ecosystems polluted with specific psychoactive compounds.

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INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) have received significant scientific attention because of their potential environmental fates and effects (*Evgenidou, Konstantinou & Lambropoulou, 2015*). Generally, drugs for human and veterinary use have been invented to invoke drug-specific reactions in patients' bodies because of theirinteraction with drug targets (*Corcoran, Winter & Tyler, 2010*). The drugs are incompletelydegraded in users' bodies, which leads to their excretion and the subsequent release of various substances into the environment via wastewater (*Heberer, 2002*). The presence of various drugs in waters has been demonstrated to alter the physiology and behavior of aquatic fauna (*Brodin et al., 2013*; *Gunnarsson et al., 2009*; *Kümmerer, 2009*; *Randak et al., 2009*; *Simmons et al., 2017*).

In the present study, we focused on four psychoactive compounds, sertraline, citalopram,tramadol and methamphetamine, which are widely found in water bodies worldwide (*Evgenidou, Konstantinou & Lambropoulou, 2015*; *Fedorova et al., 2014*; *Kim et al., 2007*). Sertraline and citalopram are selective serotonin reuptake inhibitors (SSRIs), which are used as antidepressants in humans. During the initial action of SSRIs, antidepressants in thehuman brain bind to the serotonin transporter SERT and thus block its function (*Hiemke& Härtter, 2000*; *Rossi et al., 2008*). After two to three weeks of treatment, the therapeutic effects manifest as enhanced serotonergic transmission, resulting in alleviation of the symptoms of depression (*McDonald, 2017*; *Murdoch & McTavish, 1992*; *Noble & Benfield, 1997*; *Rossi et al., 2008*; *Stahl, 1998a*). Therefore, the acute and chronic treatment effects ofSSRIs may vary (*Winberg & Thörnqvist, 2016*). Sertraline is of a particular interest in this manner, because of its bioaccumulative properties (*Grabicova et al., 2017*). Specifically, a metabolite of sertraline, norsertraline, reached the highest bioaccumulation factor (it meansthe ratio of concentration of norsertraline in biota to its concentration in water) within the antidepressants found in fish from natural conditions (*Arnnok et al., 2017*). In case of*Grabicova et al. (2017)*, which tested the bioaccumulation factor in two different natural sites, the bioaccumulation factor for sertraline in brain was 1500 and 680. It is generallyassumed that if the bioaccumulation factor is higher than 500, the compound has tendencyto bioaccumulate (according to *Organization for Economic Co-operation and Development, 2001*). Therefore, some effects of sertraline may be revealed throughout the chronic exposure, during which the concentration of compound in the tissue increases. Tramadol
is a drug with a weak affinity for opioid μ-receptors and weak inhibition of norepinephrineand serotonin reuptake (*Grond & Sablotzki, 2004*; *Scott & Perry, 2000*). This dual action oftramadol causes pain relief but could also result in antidepressant effects (*Barber, 2011*; *Kalra, Tayal & Chawla, 2008*; *Markowitz & Patrick, 1998*). Methamphetamine (METH) is in low dosages prescribed to treat the attention deficit hyperactivity disorder (*Castle et al., 2007*); however, it is generally known as an illicit substance with complex action on the central nervous system, which consequently leads to neural damage (*Moszczynska & Callan,*

2017; Schep, Slaughter & Beasley, 2010). Amphetamines reverse the function of dopamine, noradrenaline, serotonin and vesicular membrane transporters and increase the release of neurotransmitters into the synaptic cleft (Riddle, Fleckenstein & Hanson, 2006; Sulzer et al., 2005). Therefore, neurotransmitters bind to available receptors and intensify transmission. The predicted values of sertraline concentrations in sewage treatment plant effluents range from 0.26 to 3.5 μg/L, and the predicted citalopram concentration ranges from 0.27 to 2.6 μg/L (Christensen et al., 2009). The estimated surface water concentration for tramadol in the Czech Republic ranged from 12 to 210 ng/L, and for methamphetamine, it ranged from 0.82 to 107 ng/L (Fedorova et al., 2014). Generally, the average detected concentrationsof the present compounds in surface waters are in range of 24–58 ng/L for citalopram and240–1400 ng/L for tramadol (Grabicova et al., 2017). The average detected concentrationof methamphetamine in surface waters ranged from 13–1800 ng/L (Mackul'ak et al., 2016).Several studies have already reported the effects of pharmaceuticals and illicit drugs on thebehavior of fish, such as shelter-seeking in fathead minnow (*Pimephales promelas*; (ValentiJr et al., 2012) or aggressiveness in Siamese fighting fish (*Betta splendens*; Kania & Wrónska,2015).

Studies on fish condition assume that fish with a higher weight of a given length are in a better condition (Bolger & Connolly, 1989; Jones, Petrell & Pauly, 1999). Therefore, the condition hints at the 'well-being' or 'fitness' of individuals. The length and weight of individuals, which are necessary for the calculation of condition, may change with the season, locality or fish age (Froese, 2006). Moreover, growth can also be affected by environmental stressors, such as the presence of pollutants in the environment (Nallani etal., 2016). The possibility of evaluating the well-being of fish has led to the calculation of condition factors in studies and fisheries even today (Brauer et al., 2020; Konoyima et al.,2020).

Pharmaceuticals have been shown to affect food intake in various studies (Hedgespeth,Nilsson & Berglund, 2014; Gröner et al., 2017). However, information about changes in condition in relation to the brain levels of pharmaceuticals of fish has been scarcely assessed, even though changes in length-weight relationships in fish are known to occurin polluted environments (Hubená, Horký & Slavík, 2018). The present study applied a chronic treatment for 42 days to estimate the long-lasting effects of these compounds in polluted natural habitats and focused on the condition (measured from length and weight), brain concentration of the compound of interest and mortality of the juvenile chub (*Squalius cephalus*). The chub is a common omnivorous cyprinid of Europeanrivers (Balestrieri et al., 2006; Krywult, Klich & Szarek-Gwiazda, 2008), that reaches even highly polluted streams, presumably in search of a source of food (Silkina, Mikryakov & Mikryakov, 2016).

The aim of the study was to test: (A) whether the adverse effects of the various psychoactive compounds tested (i.e., sertraline, citalopram, tramadol and methamphetamine) exist under long-term exposure at an environmentally relevantconcentration; (B) whether these effects persist after the depuration period (i.e., a pharmaceutical-free period); and (C) whether there is a direct link between the individual brain concentration of the tested compounds and fish condition.

MATERIALS & METHODS

The experiments with particular psychoactive compounds were conducted separately inan identical manner as described in the sections below.

Experimental animals

The fish used in the experiment were hatchery-reared juvenile chubs obtained from a localfish supplier (Czech Fishery Ltd.; Czech Republic). A total of 320 similarly sized 0+ juvenilefish (five or ten months old for autumn and spring tests, respectively) were transported from the hatchery to the laboratory before testing each particular compound (i.e., A totalof 1280 individuals were used for all compounds; the overall average standard length of the fish was 101 ± 13 mm (mean ± S.D.), and the overall average weight of the fish was
7.56 ± 3.46 g (mean ± S.D.). Two weeks prior to the start of the experiment, the fish were divided into four separate holding tanks (200 l with 80 randomly selected individualseach). Half of the identical tanks were intended for the exposed groups, and the second half were intended for the control groups. The fish were fed *ad libitum* on food pellets once a day and kept under a photoperiod of 12 h of light/12 h of darkness, maintaining the same regime to which they were accustomed in the hatchery. Two-thirds of the watervolume was renewed with aged dechlorinated municipal tap water every day to habituate the fish to the experimental regime. The water temperature was controlled automatically
using air conditioning throughout the whole experiment and held at an average of 20.8 ± 0.4 °C (mean ± S.D.). Fish health, defined as normal appearance and behavior, including normal body position, movements and food intake (*FAO, 1983*), was monitored daily. Mortality during the experiment was also noted on a daily basis, but it was not primarilyanticipated in the study design because environmentally relevant concentrations of the compounds were used in the present study, aiming to simulate the conditions in real-lifepolluted waters.

Pharmaceutical exposure

After acclimatization, fish in the exposed groups were separately exposed to the pollutants atan environmentally relevant concentration of 1 μg/L for 42 days, followed by a depurationperiod with tested compound-free water until the 56th day (two weeks of depuration). Individual psychoactive compounds (citalopram and sertraline from AK Scientific, Inc., USA, METH and tramadol from Sigma-Aldrich) were dissolved in ultra-pure water (AquaMax Basic 360 Series and Ultra 370 Series instrument, Younglin, purchased from Labicom, CR) to prepare a stock solution at a concentration of 10 mg/L. For the exposurebath, one mL of stock solution was added to every 10 L of aged dechlorinated municipal tapwater in the holding tank. All environmental variables (i.e., the temperature, photoperiodand water renewal) were kept the same as those during the acclimatization period (see theExperimental Animals section). The compound was added during the water renewal to keep its concentration in the tanks at the required level. The control fish in the laboratory were kept under the same regime but without the psychoactive compound treatment. Thetested compound concentration in the holding tanks was checked every other week duringthe exposure as well as during the depuration (32 water samples altogether) to verify the real

concentrations in the exposed fish and exclude the possibility of cross-contamination in thecontrol groups. The chemical analysis was performed according to *Hossain et al. (2019)*, where isotopically labelled internal standards (citalopram-D_6, Toronto Research Chemicals;METH-D_5, Chiron Chemicals; sertraline-D_3 and tramadol-D_3, both from Lipomed) wereadded to filtered water (regenerated cellulose filter, 0.20 μm), and the samples were analyzedby liquid chromatography with tandem mass spectrometry (TSQ Quantiva, Thermo FisherScientific) for 10 min on a Hypersil Gold aQ column (50 × 2.1 mm, 5 μm particles, Thermo Fisher Scientific) with heated electrospray ionization in positive mode (HESI+). The limits of quantification (LOQs) ranged from 0.011 to 0.021 μg/L for citalopram, from 0.010 to 0.012 μg/L for sertraline, from 0.0077 to 0.017 μg/L for methamphetamine,and 0.030 to 0.060 μg/L for tramadol. During the exposure, the average concentration of individual psychoactive compounds in the treated tanks was as follows: citalopram 1.3 ±
0.2 μg/L (mean ± S.D.); sertraline 0.23 ± 0.07 μg/L (mean ± S.D.); methamphetamine 1.1 ± 0.1 μg/L (mean ± S.D.); tramadol 0.99 ± 0.18 μg/L (mean ± S.D.). The concentrations in the tanks of the control fish as well as in the tanks of the exposed fish during depurationwere below the limits of quantification.

Experimental design

The length and weight of eight randomly selected exposed and eight randomly selected control fish (four specimens per tank) were tested after 1, 7, 21 and 42 days of exposure andon the 56th day of the experiment (i.e., the day of exposure and two weeks after depuration).Thus, five trials with 80 specimens were conducted altogether. These specimens were killedaccording to valid law 246/1992, § 17 and the above-cited permit to analyze the psychoactivecompound concentrations in their brains. The brains of freshly killed fish were dissected,weighed and stored frozen at −20 °C for later pharmaceutical content analyses. Before the analyses, the brain samples were defrosted and extracted according to the procedure described in the work of *Grabicova et al. (2018)*. Briefly, isotopically labelled psychoactive compound and extraction solvent were added to approximately 0.1 g of brain tissue. The samples were homogenized, centrifuged, filtered and frozen for 24 h. Then, the extracts were centrifuged again, and the aliquots were analyzed using liquid chromatography witha high-resolution mass spectrometer (Q-Exactive; Thermo Fisher Scientific) on a HypersilGold aQ column for seven minutes in HESI+. The LOQs ranged from 0.12 to 0.34 ng/g wet weight (ww) for citalopram, from 0.10 to 0.44 ng/g ww for sertraline, from 0.16 to 0.43ng/g ww for methamphetamine and from 0.19 to 0.69 ng/g ww for tramadol.

The analyzed concentration of psychoactive compounds was standardized by brainweight (ng/g). Please see Table 1 for details regarding the conditions of the chemical analyses.

Additional experiment (sertraline)

Data on food intake under sertraline exposure were collected regularly from the fourth to eighth week of the experiment from Friday to Sunday (three consecutive days every week, resulting in 15 trials). Food intake was not measured in the first three weeks becauseof the delayed therapeutic effects of SSRIs, which usually take two to three weeks (*Rossi*

Table 1 Information about chemical analysis of water and brain samples by liquidchromatography with mass spectrometry.

TSQ Quantiva

Elution gradient for liquid chromatography

Time [min]	A [%]	B [%]	Flow [μL/min]
0	100	0	300
1	100	0	300
5	60	40	350
7	20	80	400
8	0	100	400
8.01	100	0	300
10	100	0	300

Q-Exactive

Elution gradient for liquid chromatography

Time [min]	A [%]	B [%]	Flow [μL/min]
0	100	0	350
1	100	0	350
3	50	50	350
4	20	80	450
5	0	100	450
6	0	100	450
6.05	100	0	350
7	100	0	350

Mass transitions for mass spectrometry

Analyte	Parent ion	Quan	Qual	RT [min]
citalopram	325.20	109.06	262.07	6.10
citalopram-D_6	331.27	108.825	–	6.10
methamphetamine	150.11	91.11	119.11	4.14
methamphetamine-D_5	155.22	92.11	–	4.14
sertraline	306.06	159.00	274.93	6.75
sertraline-D_3	309.18	159.00	–	6.75
tramadol	264.23	58.17	246.11	4.80
tramadol-D_3	267.17	58.11	–	4.80

Mass transitions for mass spectrometry

Analyte	Parent ion	Quan	Qual	RT [min]
citalopram	325.17	109.0449	262.1019	4.10
citalopram-D_6	331.21	109.0450	–	4.10
methamphetamine	150.13	91.0545	119.0854	3.45
methamphetamine-D_5	155.16	92.0611	–	3.45
sertraline	306.08	275.0389	158.9764	4.34
sertraline-D_3	309.10	158.9764	–	4.38
tramadol	264.20	58.0658	264.1953	3.68
tramadol-D_3	267.21	58.0658	–	3.68

Notes.

A–mobile phase ultra-pure water + 0.1% formic acid; B –mobile phase acetonitrile + 0.1% formic acid.

et al., 2008). Feeding experiments were conducted directly in the holding tanks to avoid causing distress to the fish and to prevent the results from being affected by the animal manipulation. Five grams of food pellets (i.e., 1% of the average weight of fish in the particular holding tank; food supply was adjusted according to mortality) were proffered to fish. The presence of non-ingested food in particular tanks was monitored for 15 min inone-minute intervals and noted every minute as a binomial value (0 –not all food pelletswere ingested; 1 –all food pellets were ingested). A limit of 15 min was established based on the preliminary testing of food intake during regular feeding, which lasted from sevento eight minutes (i.e., the limit was double the regular feeding time).

Statistical analyses

'Treatment' was defined as the class variable distinguishing whether fish were exposed to sertraline. 'Experimental phase' distinguished between the exposure and depuration phases.'Compound in brain tissue' expressed the relative concentration of particular psychoactivecompound in the individual fish brain tissue. Fulton's condition factor 'K' was evaluated as$K = M/LS3$, where M is mass and LS is the standard length. The compound concentrationin brain tissue and Fulton's condition factor were assigned for every sampled individual fish.

'Food ingestion' was assigned only for sertraline treatment as a count variable expressingnumber of one-minute intervals per feeding trial until all the food was eaten. Thus, it could

acquire values from 1 to 15 (tanks where the all food was not eaten got a maximum score of 15 out of the 15 intervals). 'Mortality' was assigned as a count variable expressing a number of dead individuals in a particular holding tank per day. Food ingestion and mortality were assigned for every tank.

The statistical analyses were performed using the SAS software package (SAS InstituteInc., version 9.4, http://www.sas.com). The effects of all four psychoactive compounds (i.e., sertraline, citalopram, tramadol and methamphetamine) were tested separately.

Differences between Fulton's condition factor 'K' of control and exposed fish and progress of 'brain compound concentration' of exposed fish during particular time samples(i.e., 1, 7, 21, 42, 56th day of the experiment) were determined with a separate *t*-tests andpresented as boxplots of raw data. Generalized linear mixed models (GLMM; SAS functionPROC GLIMMIX) with a Poisson distribution were used to analyze the variables 'food ingestion' and 'mortality'. Particular holding tank and date of sampling were used as class random factors for both dependent variables. As feeding could be hypothetically influenced by fish weight, we included mean weight and its standard deviation obtained from individual condition measures as continuous random factors for analyses of 'food ingestion' as well. A separate model for each dependent variable was fitted, using interactionof 'treatment', and 'experimental phase' as the class explanatory variable. Generalized linearmixed model (GLMM; SAS function PROC MIXED) with a normal distribution was usedto analyze Fulton's condition factor 'K'. Individual fish and date of sampling were used as a class random factors. Two models for dependent variable 'K' were fitted due to the 'treatment' and 'compound in brain tissue' variables overlap. Thus, first model contained'treatment' fixed factor variable, while second model included 'compound in brain tissue'fixed factor variable. The significance of the exploratory variables was assessed using
an *F*-test. Least-squares means (henceforth referred to and in bar charts presented as 'adjusted means' of model predictions) were subsequently computed for the particular classes of class variables. Differences between the classes were determined with a *t*-test, and a Tukey–Kramer adjustment was used for multiple comparisons. Association betweenthe dependent variables 'K' and other continuous variable 'compound in brain tissue' wasestimated by fitting a random factor model using PROC MIXED as described by *Tao et al. (2002)*. With this random coefficient model, we calculated the predicted values for thedependent variable 'K' and plotted them against the continuous variable 'compound in brain tissue' by using the predicted regression lines in particular scatter charts. The degreeof freedom was calculated using the Kenward–Roger method (*Kenward & Roger, 1997*).

Ethical note

All laboratory experimental procedures complied with valid legislative regulations (lawno. 246/1992, § 19, art. 1, letter c) and were carried out with the relevant permission from the Ministry of Education, Youth and Sports of the Czech Republic (permit no. MSMT-1972/2016-5, registered by the Ministry of Education, Youth and Sports of the Czech Republic). The Departmental Expert Committee supervised the animal welfare inthe present study. The research staff was trained according to valid legislative regulationson laboratory animal care, handling and welfare. The number of fish used corresponded

to the reduction in laboratory animals used, and the use of animals could not be replacedby any other known method to obtain the relevant data.

RESULTS

Condition factor progression

There was no difference between the chub condition under the control and exposure treatments at the beginning of all experiments (1st day: citalopram, $t = 0.71$, $P > 0.48$,$n = 16$; Fig. 1A; methamphetamine $t = 1.33$, $P > 0.2$, $n = 16$; Fig. 1B; sertraline, $t = 1.09$,$P > 0.29$, $n = 16$; Fig. 1C; tramadol, $t = 1.59$, $P > 0.14$, $n = 16$; Fig. 1D). Citalopram ($F_{1, 78} = 0.01$, $P > 0.93$) and tramadol ($F_{1, 78} = 0.96$, $P > 0.33$) treatment did not causeany overall changes in chub condition when compared to the control. Additionally,comparison of condition during particular days did not show any significant differences (Fig. 1A, citalopram: 7th day, $t = 0.57$, $P > 0.58$, $n = 16$; 21st day, $t = 1.79$, $P > 0.09$, $n = 16$;42nd day, $t = 1.15$, $P > 0.27$, $n = 16$; 56th day, $t = 0.66$, $P > 0.52$, $n = 16$; Fig. 1C, tramadol:7th day, $t = 0.21$, $P > 0.84$, $n = 16$; 21st day, $t = 0.96$, $P > 0.35$, $n = 16$; 42nd day, $t = 2.09$, $P > 0.06$, $n = 16$; 56th day, $t = 1.24$, $P > 0.23$, $n = 16$). In contrast, methamphetamine ($F_{1, 78} = 24.58$, $P < 0.0001$; Fig. 2A) and sertraline ($F_{1, 80} = 11.15$, $P < 0.0013$; Fig. 2B) treatment induced significant overall condition alterations. Methamphetamine caused acondition increase (Adj. $P < 0.0001$; Fig. 2A), which was detectable from the 21st day of exposure (7th day, $t = 0.64$, $P > 0.54$, $n = 16$; 21st day, $t = 2.52$, $P < 0.03$, $n = 16$; 42nd day, $t = 3.26$, $P < 0.01$, $n = 16$) and persisted after the depuration phase (56th day, $t = 9.51$, $P < 0.01$, $n = 16$). In contrast, sertraline caused a decrease in condition (Adj. $P < 0.0013$; Fig. 2B), which was detectable from the 21st day of exposure (7th day, $t = 0.54$, $P > 0.6$, $n = 16$; 21st day, $t = 2.31$, $P < 0.04$, $n = 16$; 42nd day, $t = 2.2$, $P < 0.04$, $n = 16$) and disappeared during the depuration phase (56th day, $t = 1.38$, $P > 0.19$, $n = 16$).

Psychoactive substances in the fish brain

In all the control fish, particular psychoactive substances in the brain tissue were below theLOQ throughout the whole experiment, confirming that the control fish were unaffected in this respect. In contrast, fish exposed to environmentally relevant concentrations of psychoactive substances showed positive brain concentrations from the 1st day of exposure(Fig. 3A, citalopram: 1.7 ± 0.79 ng/g (mean ± S.D.); Fig. 3B, methamphetamine: 1.4 ± 0.62ng/g (mean ± S.D.), Fig. 3C, sertraline: 590 ± 47 ng/g (mean ± S.D.); Fig. 3D, tramadol: 1.8 ± 0.78 ng/g (mean ± S.D.)). Citalopram, methamphetamine and tramadol brain tissueconcentrations did not show any clear exposure-related progression, and they did not differ between the 1st and 42nd days of exposure (citalopram:, $t = 0.53$, $P > 0.6$, $n = 16$; methamphetamine: $t = 0.45$, $P > 0.46$, $n = 16$; tramadol: $t = 0.34$, $P > 0.74$, $n = 16$). After the depuration period (56th day), the brain concentration of tramadol was completely below the limit of quantification, and a positive response of citalopram was observed inonly one of the eight individuals tested (mean 0.025 ± 0.070 ng/g (mean ± S.D.)), but methamphetamine was still detectable in all sampled fish (mean 0.82 ± 0.25 ng/g (mean ± S.D.)). In contrast to other compounds tested, the sertraline mean concentration in the exposed fish brain tissue subsequently increased during exposure (1st day—mean 590 ±

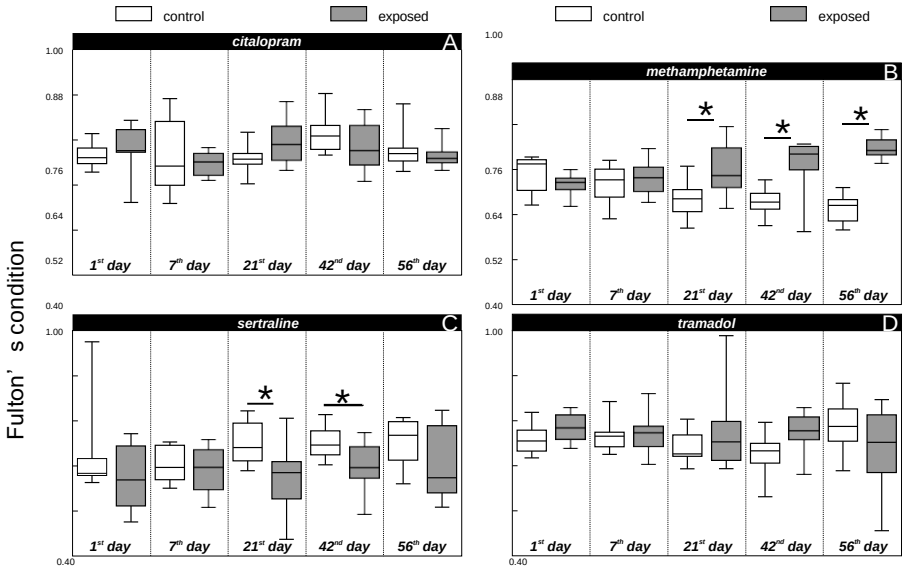

Figure 1 Fulton's condition factor 'K' of control and exposed fish during particular time samples (i.e., 1, 7, 21, 42, 56th day of the experiment) across citalopram (A), methamphetamine (B), sertraline (C) and tramadol (D) experiment. Boxplot with whiskers from minimum to maximum originate from raw data. Asterisks indicate significant differences.

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47 ng/g (mean ± S.D.); 7th day—mean 850 ± 120 ng/g (mean ± S.D.); 21st day—mean 1400 ± 660 ng/g (mean ± S.D.); 42nd day—mean 1800 ± 1000 ng/g (mean ± S.D.)) with significant differences between the 1st and 42nd days of exposure ($t = 3.25$, $P < 0.01$, $n = 16$). Despite this fact, the drug uptake was strongly individually driven as there were specimens reaching concentrations around 700 ng/g throughout the experiment. Lowbut still detectable levels of sertraline were also observed in all fish after depuration (56th day—mean 32 ± 17 ng/g (mean ± S.D.).

The direct link between methamphetamine ($F_{1,77} = 7.71$, $P < 0.0069$; Fig. 4A) and sertraline ($F_{1,80} = 5.86$, $P < 0.0178$; Fig. 4B) brain tissue concentrations and individual chub condition indicated that there is a strong influence of the individual amount of the specific drug received from the environmentally relevant water concentrations. The effect of methamphetamine and sertraline was opposite: increased methamphetamine concentrations were related to higher chub conditions (Fig. 4A) and vice versa for sertraline(Fig. 4B). In contrast, there was no significant relationship between tramadol ($F_{1, 78} = 3.47$,$P > 0.07$) and citalopram ($F_{1, 78} = 0.00$, $P > 0.96$) brain tissue concentration and fish condition, suggesting that the concentration-dependent effect was strongly compound- specific.

Mortality

Mortality varied across all control and most treatment groups (i.e., tramadol, citalopramand methamphetamine) from 0 to 8 individuals (i.e., 0–5%) throughout the whole

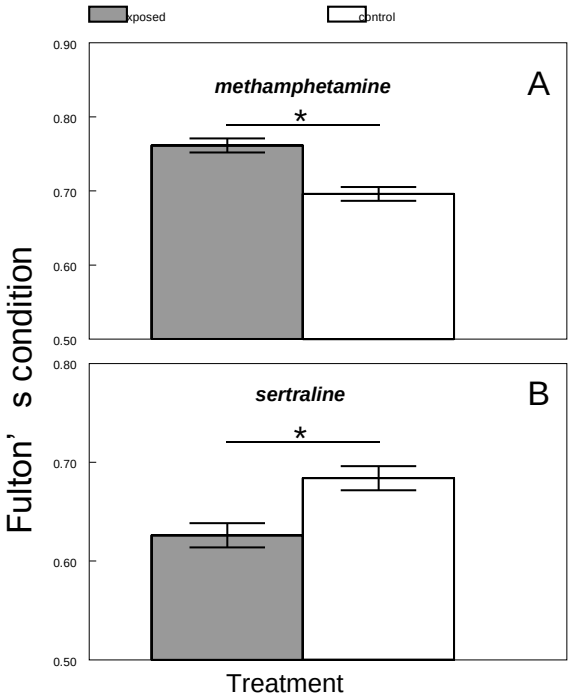

Figure 2 Differences between predicted Fulton's condition factor 'K' of control and exposed fish across methamphetamine (A) and sertraline (B) experiment. Values are adjusted means ± SE predicted from particular mixed models. Asterisks indicate significant differences. Figure covers the time span of whole experiment (i.e., exposure and depuration together).

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experiment lasting 56 days. There was never found more than 1 dead individual during the daily controls. Dead fish showed no obvious signs of disease- or condition-related problems. In contrast, sertraline treatment caused mortality in 42 individuals (i.e., 26%).Up to 4 dead individuals were found during daily controls, and dead fish showed signs of lowered condition. The increased mortality of sertraline-treated fish was significantly higher than that of the control fish during exposure ($F_{3,\ 230} = 6.22$, $P < 0.0004$; Adj. $P < 0.0008$ Fig. 5A). This adverse effect disappeared during the depuration phase (Adj. $P > 0.44$).

Feeding under sertraline exposure (additional experiment)

Sertraline affected the feeding behavior of chub during the exposure phase. The food ingestion was lower in the exposed fish than in the control fish ($F_{3,\ 8.571} = 5.49$, $P < 0.0218$; Adj. $P < 0.0206$; Fig. 5B). In other words, sertraline caused a decrease in food consumption. The adverse effects disappeared during the depuration phase (Adj. $P > 0.99$).

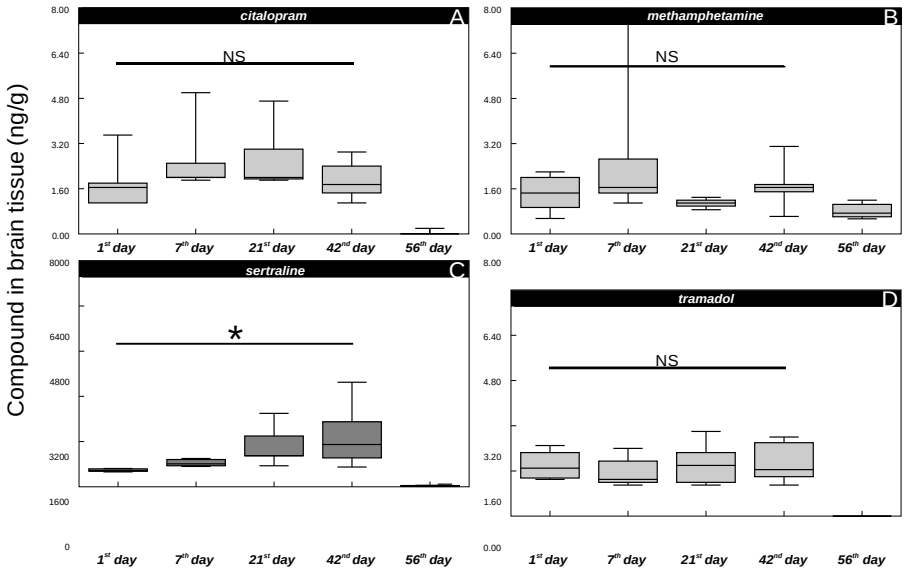

Figure 3 Citalopram (A), methamphetamine (B), sertraline (C) and tramadol (D) in brain tissue during particular time samples (i.e., 1, 7, 21, 42, 56th day of the experiment). Boxplot with whiskers from minimum to maximum originate from raw data. Asterisks indicate significant differences.
Full-size DOI: 10.7717/peerj.9356/fig-3

DISCUSSION

In the present study, we aimed to simulate the long-term exposure of fish to various psychoactive compounds. The effects of environmentally relevant levels of these compounds varied from a decrease in condition and related adverse effects in the case of sertraline to an increase in condition in the case of methamphetamine to non-detectable effects in case of citalopram and tramadol. Various impacts of particular compounds may reflect their different modes of action (*Stahl, 1998b*) as well as species-specific effects. For instance, chub in the present study did not show a significant change in condition under the environmental concentration of citalopram, while the opposite was shown in the three-spine stickleback (*Gasterosteus aculeatus*; *Kellner et al., 2015*). The condition effects, however, were also individually dependent, as was shown by our progressive brain concentration analyses. We found a direct link between individual brain concentrations of sertraline and methamphetamine and the degree of its influence on chub condition. Variations in individual compound consumption at the same aquatic concentration are presumably the result of personality (*Manning & Dawkins, 2012*) and related individual metabolic rates (*Metcalfe, Van Leeuwen & Killen, 2016*). If we adopt this concept, then particular individuals from the same polluted aquatic environment face different pressures based on their personality, which could influence the makeup of populations (*Brown et al., 2009*) based on which types of individuals are likely to be preferred if psychoactive substances alter their performance.

The sertraline concentration in the brain was negatively correlated with chub condition in the present study. The sertraline mechanism of action causes prolonged activation

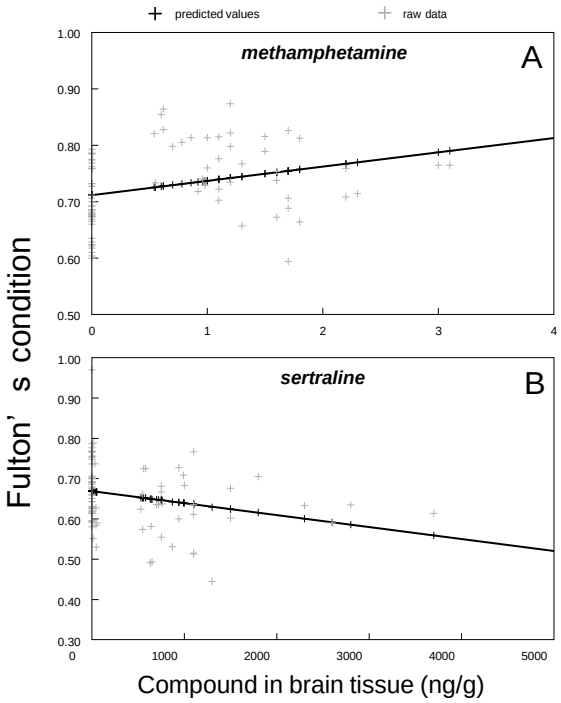

Figure 4 **Fulton's condition factor 'K' in relation to methamphetamine (A; predicted regression line is fitted by $y = 0.7116 + 0.0253x; r^2 = 0.9$) and sertraline (B; predicted regression line is fitted by $y = 0.6688 - 0.0001x; r^2 = 0.9$) in brain tissue.** Predicted values from particular mixed model are shown as dark markers, raw data are shown as shaded markers.

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of the serotonergic system (*Murdoch & McTavish, 1992*). Similar heightened activity of the serotonergic system has been found in subordinate individuals in species suchas rainbow trout (*Oncorhynchus mykiss*), Arctic charr (*Salvelinus alpinus*) and Atlantic
salmon (*Salmo salar*; *Alanärä et al., 1998*; *Cubitt et al., 2008*; *Øverli et al., 1998*; *Vindas et al., 2016*; *Winberg et al., 1993*; *Winberg & Nilsson, 1993*). These studies have demonstratedthat subordinates also reduce food intake and have lower growth rates than dominant fishcharacterized by lower serotonergic activity (*Alanärä et al., 1998*; *Cubitt et al., 2008*; *Øverliet al., 1998*; *Vindas et al., 2016*; *Winberg et al., 1993*; *Winberg & Nilsson, 1993*). Sertraline has been demonstrated to decrease food intake, but only at concentrations higher than theenvironmental levels (*Hedgespeth, Nilsson & Berglund, 2014*; *Chen, Gong & Kelly, 2017*; *Xieet al., 2015*). The environmental concentration of sertraline has been tested previously for six to eight days only (*Hedgespeth, Nilsson & Berglund, 2014*; *Chen, Gong & Kelly, 2017*). Weobserved a significant decrease in food intake in chub, which was measured from the 28thday of the supplementary experiment, following the change in the condition that appeared from the 21st day. Thus, we suggest that future experiments should consider the first threeweeks of sertraline exposure separately, as the effects during this time might not represent

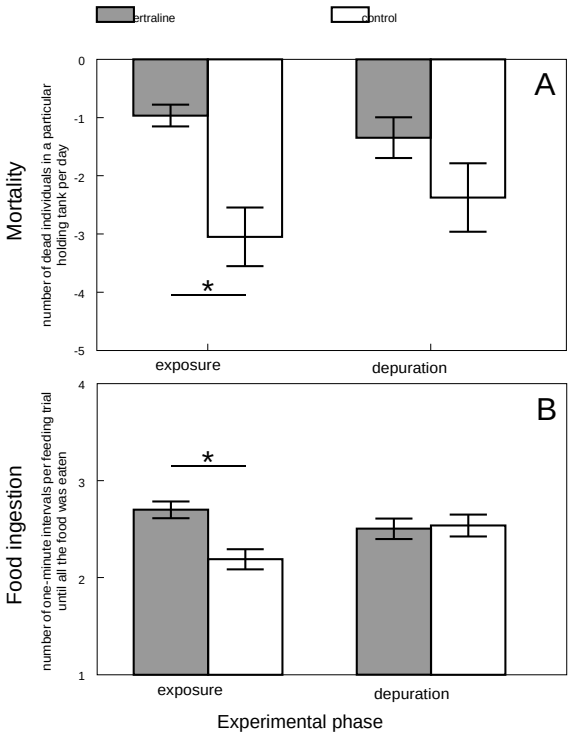

Figure 5 Predicted values of mortality (A) and food ingestion (B) in relation to experimental phase and treatment of sertraline experiment. Values are adjusted means ± SE predicted from particular mixed models. Asterisks indicate significant differences.

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the chronic effects of this compound because of the adaptive change in the serotonergic system after two to three weeks of treatment (*Cowen & Sargent, 1997*). Therefore, fish chronically exposed to sertraline could possibly have impaired energy intake through lowerfood ingestion. In the present study, we assume from the calculation of Fulton's conditionfactor that the exposed fish gained less weight at a given length than control individuals.Fulton's condition factor is also thought to be a good indicator of fish resistance to chemicalpollution or stress (*Owen et al., 2009*). Simultaneously, with lower resistance and energy storage, the fish might struggle with survival, as was proven in the present study, with a mortality of 26% in the exposure groups under environmental concentrations. We note that mortality following exposure to environmental concentrations of 5.2 ng/L of sertralinewas observed even in fathead minnow (*Schultz et al., 2011*). *Schultz et al. (2011)* warned ofthe chronic exposure effects of this compound because of its bioaccumulative properties. A plethora of studies have found a significant bioaccumulation of sertraline in tissues (*Chen,Gong & Kelly, 2017*; *Grabicova et al., 2017*; *Koba et al., 2018*; *Xie et al., 2015*; *Grabicova et al., 2014*). Specifically, sertraline was found in the liver and other tissues, including the

brain, kidneys, gills and muscles (*Xie et al., 2015*; *Grabicova et al., 2017*). The increasinglevels of sertraline in the brain in the present study support these findings.

The sertraline-free period resulted in compensatory growth of chub because no conditionchanges were detectable, even though a low sertraline brain concentration was still detected.Compensatory growth has also been demonstrated in other fish species, such as hybrid striped bass (*Skalski et al., 2005*), three-spined stickleback (*G. aculeatus*; *Inness & Metcalfe,2008*) and gibel carp (*Carassius auratus gibelio*; *Xie et al., 2001*). The growth after food deprivation in barramundi (*Lates calcarifer*) was dependent on the duration of food deprivation; thus, it may have resulted in partial compensation only (*Tian & Qin, 2003*). However, complete compensation was detected after six weeks of sertraline exposure followed by two weeks of depuration. Despite the fact that different situations could ariseover a lifetime of exposure in polluted natural habitats, these results are promising for remediation measures aimed at decreasing pollution in freshwaters.

Chronic methamphetamine exposure resulted in increased chub conditions in the present study. In contrast, goldfish (*Carassius auratus*) injected with amphetamine showeda decrease in food intake and feeding behavior at high concentrations of 25 to 75 μg/g (*Volkoff, 2013*). Decreases in weight and food intake were also observed in rats injectedwith methamphetamine, but only during the first days of exposure (*Hsieh et al., 2006*; *Kuo, 2003*). The contradictory evidence of methamphetamine may be explained by the concentration and length of the exposure dependency. For instance, *Irons et al. (2010)* described an increase in locomotion of zebrafish exposed to 0.2 to 6.6 μM amphetaminebut a decrease in locomotion at 20 μM. Additionally, most of the studies on fish and rats focus on exposure for only seven days (*Crowley et al., 2005*; *Hsieh et al., 2006*; *Kuo, 2003*; *Liao et al., 2015*; *Zheng et al., 2014*). Therefore, after demonstrating a significant change in the condition of fish after 21 days, we promote consideration of the length of the exposure in experiments with environmental concentrations of methamphetamine. Methamphetamine has a complex mode of action in organisms, acting on various substances and neurotransmitters. *Crowley et al. (2005)* found that rats fed *ad libitum* under methamphetamine exposure showed increased levels of neuropeptide Y in their brains. Neuropeptide Y has been found to stimulate the growth of abdominal fat in rats (*Kuo et al., 2007*). If we adopt this explanation, the stimulated growth of abdominal fat could explain the higher condition of chub in the present study. Furthermore, we provedthat the methamphetamine brain concentration and altered condition persisted after the depuration period. Despite the fact that a withdrawal period in rats has resulted in a significant restoration of metabolic processes, the levels of specific compounds have not returned to normal (*Zheng et al., 2014*). Specifically, there were altered levels of isoleucine,palmitic acid or compounds involved in the tricarboxylic acid cycle, which has a connectionto energy stores and lipid metabolism (*Zheng et al., 2014*).

Neither citalopram nor tramadol changed the condition of chub in the present study. Despite this fact, we should be cautious in this respect, as all pollutants in the environmentshould be assumed to be stressors (*Nallani et al., 2016*), and the effect of psychoactive compounds could be species (*Kellner et al., 2015*) as well as context specific (*Conners et al., 2009*; *Weinberger II & Klaper, 2014*; *Dorelle et al., 2020*). Nonetheless, in the present

study, we demonstrate that not all chemical compounds at environmental concentrations influence the condition factors of fish.

CONCLUSIONS

The present study demonstrates that particular psychoactive compounds in environmental doses may have a variable effect on fish condition during long-term exposure. Most adverse effects were detected for sertraline, as its effect on condition accompanied by disrupted food intake resulted in elevated mortality. Therefore, ecosystems polluted with this bioaccumulative SSRI should be managed with special attention. Our data from innovative brain concentration analyses indicate that particular individuals from the same polluted aquatic environment face different pressures, presumably as a result of their various personalities. Further research should be conducted in this respect to reveal the harmful potential of this phenomenon for the makeup of wild populations. We also highlight the need to consider the time-dependent modes of action and metabolic rates of compounds during establishing the lengths of experimental exposures because some effects may be detectable only after a certain amount of time (e.g., 21 days for sertraline and methamphetamine in our case).

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Pavla Hubená performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Pavel Horký conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Roman Grabic analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Kateřina Grabicová performed the experiments, prepared figures and/or tables, and approved the final draft.
- Ondřej Slavík and Tomas Randak conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

All laboratory experimental procedures complied with valid legislative regulations (law no. 246/1992, $19, art. 1, letter c) and were carried out with the relevant permission from the Ministry of Education, Youth and Sports of the Czech Republic (permit no. MSMT-1972/2016-5, registered by the Ministry of Education, Youth and Sports of the Czech Republic).

Data Availability

The following information was supplied regarding data availability:

The raw measurements are available in the Supplementary Files.

Supplemental Information

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6. Traces of tramadol in water impact behaviour in a native European fish

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Traces of tramadol in water impact behaviour in a native European fish

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ABSTRACT

Tramadol is a widely used analgesic with additional antidepressant and anxiolytic effects. This compound has been reported in continental waters reaching concentrations of μg/L as a consequence of its inefficient removal insewage treatment plants and increasing use over time. In this study, European chubs (*Squalius cephalus*) were exposed to 1 μg/L of tramadol in water for 42 days with a subsequent 14 days of depuration. Our results revealed that chubs exposed to this analgesic underwent changes in their behaviour as compared to the control group. The behavioural outcome was also influenced by the individual concentration of tramadol in brain tissue. In particular, experimental fish presented anxiolytic-like effects, characterized by less bold and less social individuals. Exposed animals were less frequently out of the shelter and moved a shorter distance, indicating that they explored the new environment less during the boldness test. In the novel object recognition experiment, although they distinguished the new item, they examined it less and displayed a reduced activity. Shoal cohesion was disrupted as observed in an increased distance between individuals. After the depuration phase, this alteration remained whereas the boldness effect disappeared. Moreover, the degree of behavioural changes was correlated with the concentration of the substance in brain. According to our findings, chronic presence oftramadol in the environment can impact the fitness of exposed aquatic fauna by altering evolutionary crucial behaviours.

1. Introduction

Pharmaceuticals are emerging contaminants within freshwaters given their potential impacts over wildlife. Psychopharmaceuticals and their active metabolites are increasingly detected in waters ranging in concentrations from nanograms (ng) to micrograms (μg) per litre on account of their insufficient removal from the sewage treatment plants (STPs) (Asimakopoulos and Kannan, 2016; Ebele et al., 2017; Golovko et al., 2014).

Tramadol is a synthetic opioid that exerts its analgesic action over the central nervous system. It possesses a double mode of action, being a μ-opioid receptor agonist and as an inhibitor of norepinephrine and serotonin reuptake in the synaptic terminals (Miotto et al., 2017). For this reason, it is used as a painkiller for acute and chronic pain, as well as an anaesthetic, anxiolytic and antidepressant (Vazzana et al., 2015). Chronic pain is a frequent issue in daily medicine Its management is constrained to limited treatment options that results in an over prescription of tramadol. For instance, prescriptions of tramadol in the United States rose 22.8% from 2012 to 2015 (Bigal et al., 2019). The use of tramadol in combination with non-opioid analgesics increased from 1.8 to 5.3 DDD (defined daily dose per 1000 inhabitants per day) in the period from 2008 to 2015 in Spain (AEMPS and Ministerio de Sanidad, 2017). In the Czech Republic, the rate increased from 5.3 to 9.5 DDD during the years 2008–2019 (State Institute for Drug Control, n.d). In addition, an illegal trade of this pharmaceutical has been reported due to its potential for abuse (Miotto et al., 2017). In 2010, 2013 and 2017, global seizures of tramadol were 10 kg, 9 tons and 125 tons, respectively (United Nations Office on Drugs and Crime, 2019).

Tramadol s both extensive use and low rate of removal in STPs contribute to the observed high concentrations, up to 1 μg/L, in effluents and surface waters (Campos-Mañas et al., 2019; Coelho et al., 2019; Fedorova et al., 2014; Golovko et al., 2014; Grabicova et al., 2017; Loos et al., 2013). This substance was even detected at low levels in seawater (Alygizakis et al., 2016). Furthermore, tramadol was present in several

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tissues of fish living in ponds or in STPs effluent impacted streams (Grabicova et al., 2017; Grabicová et al., 2020).

The neuroendocrine pathways, such as monoaminergic and opioid systems, are evolutionary well-conserved in teleosts (Dreborg et al., 2008; Soares et al., 2018). Consequently, even low levels of chronic exposure to psychoactive compounds can impact neurotransmitter systems and produce similar effects as human and experimental animals in non-targeted organisms (Fent et al., 2006). Behavioural responses correlate to these complex physiological processes (Saaristo et al., 2019). Thus, they can be analysed to better understand the complex effects psychoactive compounds can exert at individual and ecosystem levels (Brodin et al., 2014).

Several studies have elucidated behavioural effects of tramadol in aquatic invertebrates. Impairments in feeding behaviour of dragonfly larvae (*A. cyanea*) were observed from 1 μg/L of tramadol for 7 days (Bláha et al., 2019). Marbled crayfish (*P. virginalis*) spent more time in shelters and diminished locomotion from 1 μg/L of the analgesic for 7 days (Buřič et al., 2018). Red swamp crayfish (*P. clarkii*) exposed to the aforementioned conditions showed a sex-dependent change in burrowing behaviour (Guo et al., 2020). Ložek et al. (2019) detected changes in locomotion and heart rate in signal crayfish (*P. leniusculus*) exposed to 1 μg/L of the analgesic for 21 days. Douda et al. (2019) recorded a slightly different parasitic interaction between chub and glochidia (larvae of *A. anatina*), both previously exposed to tramadol at 1 μg/L for 42 days and 24 h, respectively.

In the case of fish, early stage tests were performed in common carp (*C. carpio*) using concentrations of 10–200 μg/L tramadol in a mixture with naproxen for 32 days, reporting several disruptions in their development (Sehonova et al., 2017). Similarly, a delay of hatching in zebrafish (*D. rerio*) and impairments in development of common carp were observed in Sehonova et al. (2016). Bachour et al. (2020) recorded an anxiolytic effect in the early-stages of zebrafish at 320 μg/L. Zebrafish larvae exposed to 1–100 μg/L exhibited slightly decreased activity, although not significant (Huang et al., 2019). Tanoue et al. (2017) reported very similar metabolism of this pharmaceutical in fish compared to humans. Then, Tanoue et al. (2019) aimed to explain behavioural changes in fathead minnows (*P. promelas*), using concentrations from 1 μ to 100 μg/L during 23–24 days however, the results were ambiguous and difficult to interpret.

Despite the high levels and frequency reported in environmental tramadol, there is still a lack of research about its potential consequences to aquatic wildlife. European chub (*Squalius cephalus*) is a small cyprinid chosen as an experimental fish for being widely distributed in European freshwaters. It has been successfully used as a bioindicator (Douda et al., 2019; Hájková et al., 2007) and as a model for behavioural research (Hubená and Horký, 2020a, 2020b). European chub is easily reared and maintained under laboratory conditions. This study aimed to reveal currently unidentified effects on European chubs that are exposed to an environmentally relevant concentration of1 μg/L of tramadol. For this purpose, we (1) monitored the progression of the behavioural changes over time and (2) related the concentration of tramadol in brain tissue with the observed variations in the basic behavioural traits (boldness, activity, exploration, and sociability). A set of three behavioural experiments were conducted after 1, 7, 21, 42 days of exposure, and after 14 days of depuration, i.e. boldness, novel object exploration and shoal cohesion tests. We specifically addressed potential anti-anxiety effects in fish associated with the time-linked concentration of the substance in brain.

1. Material and methods

1.1. Chemicals

The tramadol hydrochloride (purity ≥98%) used for exposure was obtained from Sigma-Aldrich Corporation (USA) and the isotopically labelled tramadol (D_3), as analytical standard, from Lipomed (USA). The stock solution of pure tramadol (10 mg/L) was prepared with ultra-pure water (AquaMax Basic 360 Series and Ultra 370 Series instrument, Younglin, purchased from Labicom, CR) and stored at 4 °C. From this solution, the exposure treatment of 1 μg/L was added to aged dechlorinated municipal tap water and provided to the respective aquaria. Ultra-pure water and acetonitrile (Merck) and isopropanol (Merck), all acidified with formic acid (Labicom, CR), were utilized as mobile phases for liquid chromatography and as extraction solvents for the biota samples.

1.2. Experimental animals

This study uses the same experimental population as Hubená and Horký (2020a) (2020b) who studied tramadol effect on chub condition. This approach was adopted according to the general need for the reduction of animals utilized in research (e.g. de Boo and Hendriksen, 2005). The experimental fish were hatchery-reared chubs (*Squalius cephalus*, mean weight 5.58 ± 1.24 g, mean standard length 92 ± 7 mm), obtained from a local fish supplier (Czech Fishery Ltd.; Czech Republic). 320 0 juvenile fish (ten months old) were acclimatized under laboratory conditions for two weeks prior to the start of the experiment. The population was allocated at random in four separate holding tanks, two for control (0 μ/L) and the other half for exposed (1 μ/L), with 80 randomly selected individuals in each one. Fish were maintained in 200 L of aged tap water with constant aeration, fed daily ad libitum on food pellets, and kept on a photoperiod of 12 h of light/12 h of darkness (OECD, 2012). The water temperature was controlled automatically using air conditioning throughout the whole experiment and held at an average of 20.8 ± 0.4 °C (mean S.D.). Because tramadol is stable in water, every second day, two-thirds of the water volume were renewed with aged dechlorinated municipal tap water, and fresh stock solution of tramadol was added to reach the final concentration. Fish health, defined as normal appearance and behaviour, including normal body position, movements, and food intake (FAO, 1983) was monitored daily.

1.3. Tramadol exposure

After acclimatization, fish in the experimental groups were separately exposed to the tramadol at an environmentally relevant concentration of 1 μg/L. The treatment lasted 42 days, with a subsequent 14 days of depuration in tramadol-free water (56 days of experiment intotal). The tramadol concentration in the holding tanks was checked analytically during the whole experiment to verify the real concentrations in the exposed fish and exclude the possibility of cross-contamination in the control group. Testing days for behavioural analyses and collection of brain samples were set after 1, 7, 21, 42 days of exposure and 14 day-depuration. Four fish per aquaria (a total of eight fish per group) were randomly taken in each sampling day for behavioural and chemical analyses. This study was performed in agreement with the EU-harmonized Animal Welfare Act of the Czech Republic. All laboratory experimental procedures complied with valid legislativeregulations (law no. 246/1992, 19, art. 1, letter c) and were carried out with the relevant permission from the Ministry of Education, Youth and Sports of the Czech Republic, Ethical approval committee MSMT-1972/ 2016-5.

1.4. Behavioural testing

1.4.1. General information

Five trials with 80 focal specimens were conducted altogether. Every specimen was subsequently subjected to a series of behavioural testswith a minimum one hour long resting period between the particular tests. Fish were placed separately into individual aerated tanks between tests (every specimen had its own tank). Digital cameras (GoPro Hero, USA) were used to record fish behaviour from above. Then, obtained data were analysed using the automated video tracking system

(LoliTrack v. 4, Loligo Systems; please see data analyses section for details).

1.1.1. Boldness test

Individual fish were tested for boldness by recording latency to enter into a novel environment (e.g. Brodin et al., 2013). Fish were inserted into an opaque, white plastic arena (footprint 30 × 15 cm, water depth 10 cm) in an initial shelter. After five min, we remotely opened the doorof the refuge. The latency to emerge from the shelter into the novel habitat and the distance moved outside the refuge were recorded for 15 min

1.1.2. Novel object recognition test

Novel object recognition is a highly validated test for identification memory, used predominantly in rodents (Ennaceur and Delacour, 1988), but also in fish (Burns, 2008). It was performed in a square arena (footprint 30 × 30 cm, water depth 10 cm) with opaque, white plastic walls. During trial 1, two identical objects (yellow balls, 4 cm diameter) were located in opposite corners. Each fish was placed individually inthe third corner, facing the wall, and allowed to explore for five min.After an inter-trial interval of 15 min, the fish were reintroduced for trial 2 with one familiar object (identical shape and colour but different piece to avoid influence of olfactory cues) and one novel object (black cube of identical size, i.e. side 4 cm) as compared to trial 1. Once again, the fish were allowed to explore, and their behaviour was recorded for five min.

1.1.3. Shoal cohesion

A group of four individuals (1 3) was used to score the shoal cohesion. Two identical experimental aquaria (footprint 30 × 15 cm, water depth 20 cm) were placed side by side with the walls covered with a grey paper. The focal individual used in the previous tests was placed in one aquarium, and three familiar conspecifics to the other aquarium, separated by a transparent wall. Conspecifics were matched for the size of the focal individual (the size of the fish was determined visually to prevent actual handling stress, according to Lahti et al., 2002), and all fish were left to acclimate for three minutes. Shoal cohesion was quantified as the distance between the focal individual and the othermembers of the shoal (Miller and Gerlai, 2007) during a six min interval.

1.2. Chemical analyses

The concentration of tramadol in water was determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS; TSQ Quantiva, heated electrospray in positive mode (HESI+), Accela 1250 LC pump, Hypersil Gold aQ column (50 × 2.1 mm; 5 μm particles), PAL autosampler, Thermo Fisher Scientific, USA) in all aquaria four times during the experiment. Water samples were taken before and after the water was changed, and stored at − 20 °C. After defrosting, the water obtained from the aquaria was filtered (0.20 μm regenerated cellulose filter) and internal standard (D_3-tramadol, Lipomed) was added. Then, the sample was analysed in gradient elution of acidified water and acetonitrile (with formic acid). The complete procedure is specified in Douda et al. (2019). The mean concentration of tramadol in water in the treated tanks was 0.99 ± 0.18 μg/L (mean ± S.D.) during exposure. The depuration phase and during the whole experiment in control remained below the limit of quantification (LOQ; Hubená and Horký, 2020a, 2020b). LOQs ranged from 0.030 to 0.060 μg/L for tramadol (Hubená and Horký, 2020a, 2020b).

Following behavioural testing, eight fish per group and time were beheaded according to valid law 246/1992, § 17. Their brains were dissected, weighted, and stored at −80 °C until further analyses. The complete method of sample preparation and analysis is described in Grabicova et al. (2018). After defrosting, internal standard, extraction solvent (acidified acetonitrile and isopropanol), and a stainless-steel ball were added to 0.25 g of tissue. The samples were extracted (TissueLyser II, Quiagen, Germany; 30 Hz), centrifuged (Mini spin, Eppendorf, Germany; 6708 g), filtered (Labicom; regenerated cellulose, 0.45 μm pores) and frozen at − 20 °C for 24 h. After defrosting were centrifuged again. Then, the resulting aliquots were analysed by liquid chromatography with high resolution mass spectrometry (LC-HRMS, QExactive, Accela 1250 LC pump, Hypersil Gold aQ column (50 × 2.1 mm; 5 μm particles) PAL autosampler, Thermo Fisher Scientific). TraceFinder 3.3 software (Thermo Fisher Scientific) was used for the processing of the data. The concentration of tramadol in brain tissue of control individuals was below LOQ (range 0.19–0.69 ng/g; Hubená and Horký, 2020a, 2020b). In the treated group, the compound was detected in all the fish brains at an average of 1.6 ng/g wet weight during exposure, while no residues were found in the brain after depuration (Hubená and Horký, 2020a, 2020b). The Bioconcentration factor was determined by dividing the mean concentration of tramadol in brain tissue by the average concentration in water (OECD, 2012). The calculated bioconcentration factor (BCF) was 1.8 in the present study.

1.3. Statistics

The statistical analyses for behaviour was performed with the SAS software package (SAS Institute Inc., version 9.4, www.sas.com).

1.3.1. Data analyses

'Treatment' was defined as the class variable distinguishing whether fish were exposed to tramadol. 'Experimental phase' was also assigned as a class variable distinguishing sample groups for the course of the experiment. It acquired five values, 1, 7, 21, 42 and 56 days, from the beginning of the experiment. 'Tramadol in brain tissue' expressed the relative concentration of this particular psychoactive compound in the individual fish's brain tissue.

We used a regular time grid approach to address a kind of M-dependence structure hidden within the data (e.g. Moon and Velasco, 2013). The M-dependence refers to dependent structured variables which can be transformed into independent ones by removing some consecutive observations. In our case, M-dependence was caused by several records per second on a video file. Preliminary data analyses comprised a grid for particular variables assigned as follows: 'Boldness' was assigned as a binary class variable (0 – fish in shelter, 1 – fish in an open field) at a sampling rate of once every 5 s 'Distance' was designated as a continuous variable, expressed as a distance moved (in cm) in the open field during the whole boldness test (15 min). 'Social distance' was assigned as a class variable at a sampling rate of once every 10 s and used as an indicator of shoal cohesion. Social distance could acquire values from 0 to 3 (0 – both individuals close to each other, mostly side by side; 1 - individuals within one body length of each other; 2 – individuals separated by at least one body length; 3 – individuals separated on the maximum possible distance – i.e. in opposite corners of the aquarium). 'Exploration' during the novel object test was designated as a count variable expressing number of framerates spent closer than 5 cm near a particular object (novel or familiar) within the 30 s grid. 'Object' was assigned as a class variable acquiring two values, 'novel' or 'familiar'. Every individual was continuously determined as being active (i.e. fish moved between two framerates) or not (i.e. fish did not move between two framerates). 'Activity' during the novel object 'exploration' was assigned as a count variable, expressing the number of active statuses within the 30 s grid.

1.3.2. Statistical analyses

Separate generalized linear mixed models with random factors were fitted to analyse the variables 'boldness'; 'distance'; 'social distance'; 'exploration' of both objects (novel and familiar); 'exploration' of novel object only in order to analyse influence of tramadol in brain tissue on novel object exploration; and 'activity' during the novel object exploration. Mixed models are a generalization of standard models, e.g., GLM, with the generalization indicating that the data are permitted to exhibit correlation and nonconstant variability. This method is used to cope

with repeated measures experiments in which people or animals are the subjects, and the subjects are declared random because they are selected from a larger population in which generalizations are required (SAS Institute Inc, 2004). Therefore, in the present study, factors were applied to account for the repeated measures introduced by using the same experimental units (i.e., individual fish). More detailed information about mixed models can be found elsewhere (e.g. Breslow and Clayton, 1993; SAS Institute Inc, 2004; Searle et al., 1992). Generalized linear mixed models (GLMM; SAS function PROC GLIMMIX) with a Poisson distribution were applied to analyse the variables 'activity' and 'exploration'; GLMM with a multinomial distribution was used to analyse 'social distance', and GLMM with a binary distribution to analyse 'boldness'. GLMM with a normal distribution (SAS function PROC MIXED) was applied to analyse 'distance', squared root transformed to meet normality requirements prior to the analyses. The significance of the explanatory variables (treatment, experimental phase, tramadol in brain tissue, focal individual weight), and their interactions, were assessed using F-tests. Separate models containing 'treatment' or 'tramadol in brain tissue' along with other explanatory variables were fitted due to the 'treatment' - 'tramadol in brain tissue' overlap (intercorrelation), in order to avoid misinterpretation of the results. Least-squares means (henceforth referred to and in bar charts presented as 'adjusted mean' of model predictions) were subsequently computed for the particular classes of class variables. Differences between the classes were determined with *t*-test, and Tukey-Kramer adjustment was utilized for multiple comparisons. The association between the dependent variables and other continuous variables, 'tramadol in brain tissue' and 'weight', was estimated by fitting a random factor model as described by Tao et al. (2002). With this random coefficient model, we calculated the predicted values for the dependent variable and plotted them against the continuous variables. The degree of freedom was calculated using the Kenward-Roger method (Kenward and Roger, 1997).

For testing cross-trait correlations we used Spearman correlations according to Brodin et al. (2013), and adjusted for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Every individual tested during the exposition period (i.e. 1-42 days from the start of the experiment) was assigned one mean value per particular trait (i.e. 'boldness', 'distance', 'exploration', 'activity' and 'social distance'). Cross-trait correlations for various treatments (control and tramadol exposure) were tested separately. Significant relationship indicate existence of a behavioural syndrome between the two individual traits tested.

1. Results

1.1. Behavioural effects of tramadol exposure

Chub exposed to environmental concentrations of tramadol showed significant behavioural differences compared to control. Exposed chubs were generally less bold ($F_{1,\ 1009} = 5.51$, $p < 0.0191$; Fig. 1A). Both treatments showed a similar temporal pattern in leaving the shelter ($F_{4,\ 1004}$ 22.02, $p < 0.0001$; Fig. 1B), with nonsignificant differences during particular time intervals. Exposed fish also moved on a shorter distance when they were out of the shelter ($F_{1,\ 77}$ 3.98, $p < 0.0495$; Fig. 1C), indicating that they did not explore the new environment as thoroughly as in the control treatment.

The novel object recognition test was performed in order to evaluate the possible alterations in the novel item identification memory. Both treatments (tramadol and control) explored the novel object more than the familiar one ($F_{3,\ 1582}$ =8433.79, $p < 0.0001$; Fig. 2A). Nevertheless, tramadol exposed chubs showed significantly less exploration towards the new item than control fish (Fig. 2A). Exposed individuals were also less active during the novel object exploration ($F_{1,\ 601.5}$ =4.48, $p < 0.0346$; Fig. 2B). Both the lower novel object exploration and lower activity during exploration indicate an impairment of novel object recognition in tramadol exposed chubs.

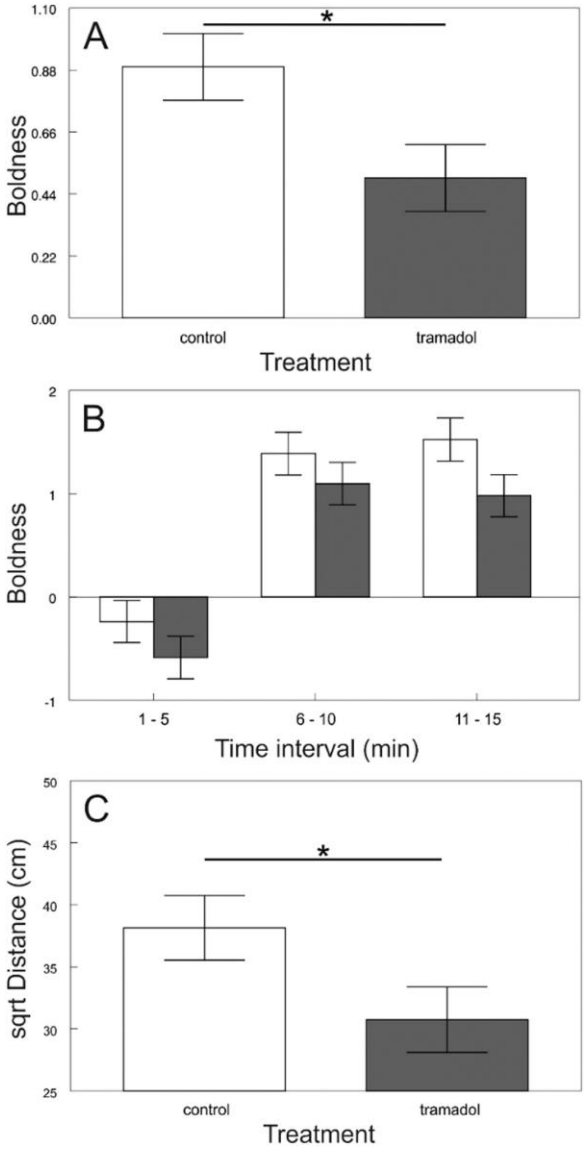

Fig. 1. Boldness across the treatment (A), time interval (B), and distance moved during boldness test across the treatment (C). Values (+/- S.E.) are predicted from the particular mixed model. Asterisks indicate significant dif- ferences (Adj. $p < 0.05$).

The shoal cohesion test was performed in order to evaluate the possible effects on social behaviour in exposed fish. Chub exposed to tramadol maintained larger inter-individual social distances within the experimental shoal than controls ($F_{1,\ 230.1}$=41.77, $p < 0.0001$; Fig. 3), indicating that their shoal cohesion was disrupted.

1.2. Tramadol exposure and behavioural syndromes

The complex results of cross-trait correlations can be found in Table 1. Boldness was positively related to distance moved in the open field for both treatments. Exploration of the novel object and activity during its exploration were positively correlated under the tramadol treatment only, while exploration and boldness were positively corre- lated exclusively in control.

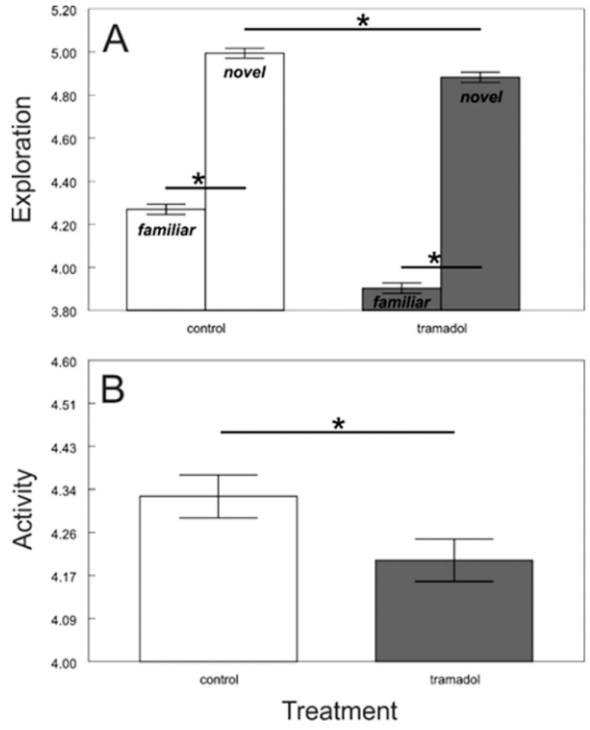

Fig. 2. Exploration of familiar and novel object (A) and activity during exploration of novel object (B) across the treatment. Values (+/- S.E.) are predicted from the particular mixed model. Asterisks indicate significant differences (Adj. $p < 0.05$).

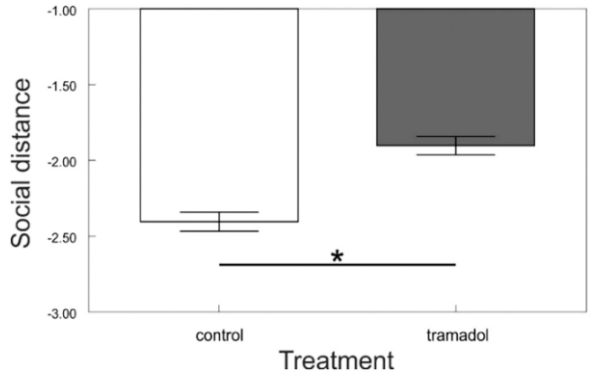

Fig. 3. Social distance across the treatment. Values (+/− S.E.) are predicted from the particular mixed model. Asterisks indicate significant differences (Adj. $p < 0.05$).

1.1. Progression of behavioural effects

The effect of tramadol on particular behavioural traits varied throughout the course of the experiment. Boldness was only significantly affected after 42 days of exposure ($F_{8,\ 1007} = 7.46$, $p < 0.0001$; Fig. 4A). In contrast, the effect on social distance ($F_{8,\ 228.6} = 8.41$, $p < 0.0001$; Fig. 4B) was consistent from the first to last day of exposure and remained significant even after two weeks of depuration (Fig. 4B). The consequence of the temporal effect on novel object recognition was not observed.

1.2. Tramadol brain concentration vs. behavioural variability

Increased tramadol concentrations in brain were linked to a decrease of boldness ($F_{1,\ 1068} = 53.00$, $p < 0.0001$; Fig. 5A), lower exploration of a novel object ($F_{1,\ 558.3} = 39.95$, $p < 0.0001$; Fig. 5B) and a larger inter-individual distances, indicating a disruption of the shoal cohesion ($F_{1,\ 8510} = 27.19$, $p < 0.0001$; Fig. 5C). The behavioural outcome was also influenced by the individuals weight, as larger fish were bolder ($F_{1,\ 1068} = 53.00$, $p < 0.0001$; Fig. 5A), spent more time during exploration of the novel object ($F_{1,\ 558.3} = 39.95$, $p < 0.0001$; Fig. 5B) and maintained larger inter-individual distances ($F_{1,\ 8510} = 27.19$, $p < 0.0001$; Fig. 5C).

2. Discussion

Our results indicated that exposure to environmentally relevant concentrations of tramadol impaired the behaviour of a native European fish. The direct link between the concentration of tramadol in brain tissue and the individual behaviour highlighted the strong influence of this drug received from the water.

2.1. Behavioural effects

Behaviour is the external response that results from the complex combination of several biological processes, e.g. biochemical or physiological (Hellou, 2011; Legradi et al., 2018). It has been extensively reported as a major change related with psychoactive compounds exposure (Buřič et al., 2018; Cunha et al., 2019). Our previous study has also shown that tramadol in water at 1 μg/L displayed impairments in the normal behaviour of invertebrates, compared to unexposed in- dividuals (Bláha et al., 2019; Buřič et al., 2018; Ložek et al., 2019), underscoring the potential ecological effects of tramadol even at low concentrations. Specifically, the changes reported by Buřič and Ložek et al., (2018, 2019) also denoted a decrease in activity and locomotion. Furthermore, their results indicated that exposed crayfish showed a tendency to spend more time inside shelters than in the control treatment, which is similar to the decreased boldness observed in our experiment.

The few studies available in the literature do not provide solid results about behavioural disruption in fish induced by tramadol at environmental levels. In Tanoue et al. (2019), even though tramadol was detected in the brain, the behavioural testing did not provide satisfactory results, and the committee of experts questioned in this respect could not find the reason why. Several similar studies including carp larvae, tilapia, and zebrafish showed impairments in behaviour and other parameters -e.g. histopathology, development or oxidative stress - caused by tramadol exposure (Bachour et al., 2020; Huang et al., 2019; Sehonova et al., 2017, 2016; Soliman and Sayed, 2020). However, the levels of the substance used in these toxicology tests were generally much higher than in realistic conditions, so it is difficult to compare to the current subject.

In contrast, our findings clearly demonstrated changes in some of the major behavioural axes - i.e. boldness, activity, exploration, and sociability (Réale et al., 2007) - underlying an apparent anxiolytic-like effect. This assumption can be justified by the concurrent impairments in all of the previously cited patterns, since the alterations over behavioural traits should be taken into consideration as a whole (Conrad et al., 2011). Our results indicated that, in general, exposed fish were less bold, less active, less social, and did not explore the new environment nor the new object as much as the controls.

Personality traits can be correlated in order to disclose a behavioural syndrome (Conrad et al., 2011). As an example, previous research regarding invasive species - i.e. mosquitofish (*Gambussia affinis*) - indicated a syndrome in which activity, exploration, and boldness were positively associated, while sociability was just scarcely correlated with the others (Cote et al., 2010). Additionally, bolder individuals are generally more exploratory and more active- providing an advantage

Table 1
The cross-trait correlation table showing the Spearman correlation coefficients for all observed behavioural traits. Significant relationships ($p < 0.05$) are highlighted in bold and indicate the existence of a behavioural syndrome between two individual traits under a particular treatment (tramadol or control).

	Boldness		**Distance**		**Exploration**		**Activity**		**Social distance**	
Boldness	Control	Tramadol								
	ρ = 1.00	*ρ = 1.00*								
	n = 32	*n = 32*								
	N.A.	*N. A.*								
Distance	**Control**	**Tramadol**	Control	Tramadol						
	ρ = 0.51	***ρ = 0.64***	*ρ = 1.00*	*ρ = 1.00*						
	n = 32	***n = 32***	*n = 32*	*n = 32*						
	p < 0.01	***p < 0.01***	*N.A.*	*N. A.*						
Exploration	**Control**	Tramadol	Control	Tramadol	Control	Tramadol				
	ρ = 0.35	*ρ = 0.08*	*ρ = −0.07*	*ρ = −0.18*	*ρ = 1.00*	*ρ = 1.00*				
	n = 32	*n = 32*	*n = 32*	*n = 32*	*n = 32*	*n = 32*				
	p < 0.05	*p > 0.67*	*p > 0.69*	*p > 0.31*	*N.A.*	*N. A.*				
Activity	Control	Tramadol	Control	Tramadol	Control	**Tramadol**	Control	Tramadol		
	ρ = −0.23	*ρ = 0.22*	*ρ = −0.20*	*ρ = −0.04*	*ρ = 0.18*	***ρ = 0.67***	*ρ = 1.00*	*ρ = 1.00*		
	n = 32	*n = 32*	*n = 32*	*n = 32*	*n = 32*	***n = 32***	*n = 32*	*n = 32*		
	p > 0.20	*p > 0.21*	*p > 0.28*	*p > 0.82*	*p > 0.32*	***p < 0.01***	*N.A.*	*N. A.*		
Social distance	Control	Tramadol	Control	Tramadol	Control	Tramadol	Control	Tramadol	Control	Tramadol
	ρ = 0.16	*ρ = −0.02*	*ρ = 0.17*	*ρ = 0.05*	*ρ = 0.11*	*ρ = −0.17*	*ρ = −0.31*	*ρ = −0.20*	*ρ = 1.00*	*ρ = 1.00*
	n = 32	*n = 32*	*n = 32*	*n = 32*	*n = 32*	*n = 32*	*n = 32*	*n = =32*	*n = 32*	*n = 32*
	p > 0.37	*p > 0.93*	*p > 0.36*	*p > 0.79*	*p > 0.55*	*p > 0.34*	*p > 0.09*	*p > 0.28*	*N.A.*	*N. A.*

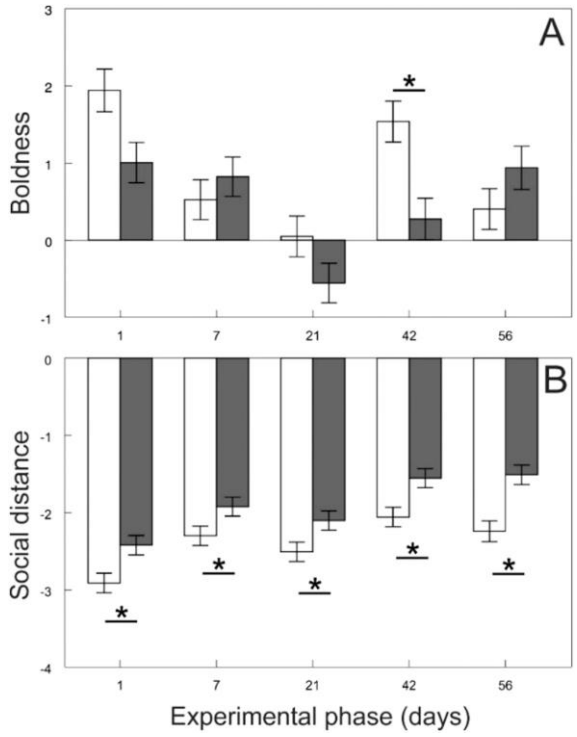

Fig. 4. Boldness (A) and social distance (B) across the experimental phase. Shaded bars indicate tramadol treatment. Values (+/- S.E.) are predicted from the particular mixed model. Asterisks indicate significant differences (Adj. $p < 0.05$).

over other congeners in dispersing and/or foraging (Conrad et al., 2011). In line with this concept, we observed a relation between bold- ness and distance for both treatments, suggesting that bolder individuals from both groups explored the new environment more by covering an increased distance. Boldness and exploration of the new object were only correlated in control, and activity and exploration of the novel item only in exposed fish. These differences in trait relations suggest that the tramadol treatment influenced the expression of the behavioural syndromes among the personality traits. The data also indicated that, although there were differences between the treatments, boldness seemed to be more related with activity, exploration, and distance; while sociability remained aside. Accordingly, previous studies could not relate boldness and sociability (Bevan et al., 2018; Cote et al., 2011; Jolles et al., 2016).

Among exposed fish, the individual weight of exposed chubs was related with the degree of behavioural changes. Bigger fish were bolder, less social, and explored the new object more. Overall, bolder in- dividuals are expected to be bigger naturally because they can deal with opponents better and subsequently obtain more sustenance (Conrad et al., 2011; Jolles et al., 2016). As they were bolder, they would likely explore the new object more in comparison to shyer individuals. Moreover, bolder individuals were reported to be less social (Conradet al., 2011; Jolles et al., 2015; Tang and Fu, 2020).

Several studies comprising other psychopharmaceuticals at lowlevels indicated similar impairments concerning those subjects. For example, *Perca fluviatilis* exposed to 1.8 μg/L of oxazepam for 7 days became less active and less social, although boldness remained unchanged (Brodin et al., 2013). *Rutilus rutilus* exposed to 0.84 μg/L of oxazepam for 7 days were bolder and more active than unexposed individuals (Brodin et al., 2017). Escitalopram increased boldness and induced alterations in swimming behaviour in zebrafish exposed to 1.5 μg/L of the substance for 21 days (Nielsen et al., 2018). *Gasterosteus aculeatus* exposed to citalopram at 1.5 μg/L for 21 days showed higher swimming activity and stayed closer to the novel object (Kellner et al., 2016). *Carassius auratus* exposed downstream to a STP for 21 days, contained six serotonin reuptake inhibitors in plasma and appeared bolder and more exploratory, compared to individuals from a less polluted upstream area (Simmons et al., 2017). In accordance with our findings, Brodin et al. (2014) suggested that the precise alterations in behaviour from exposure to this group of substances could be equiva- lent, although in different directions.

Our results suggest that the treated fish downplay the stimulus of the surrounding environment resulting from a calming effect. The end result of these changes has been mostly related to an increase in their vulnerability to predators (De Abreu et al., 2014; Dugatkin, 1992; Saaristo et al., 2019; Soares et al., 2018). Alterations in the fish social network have been suggested to produce direct effects over feeding behaviour, dispersion and/or reproduction (Brodin et al., 2014).

Behavioural analyses are more sensitive than other ecotoxicity as- says because they provide information about possible effects without

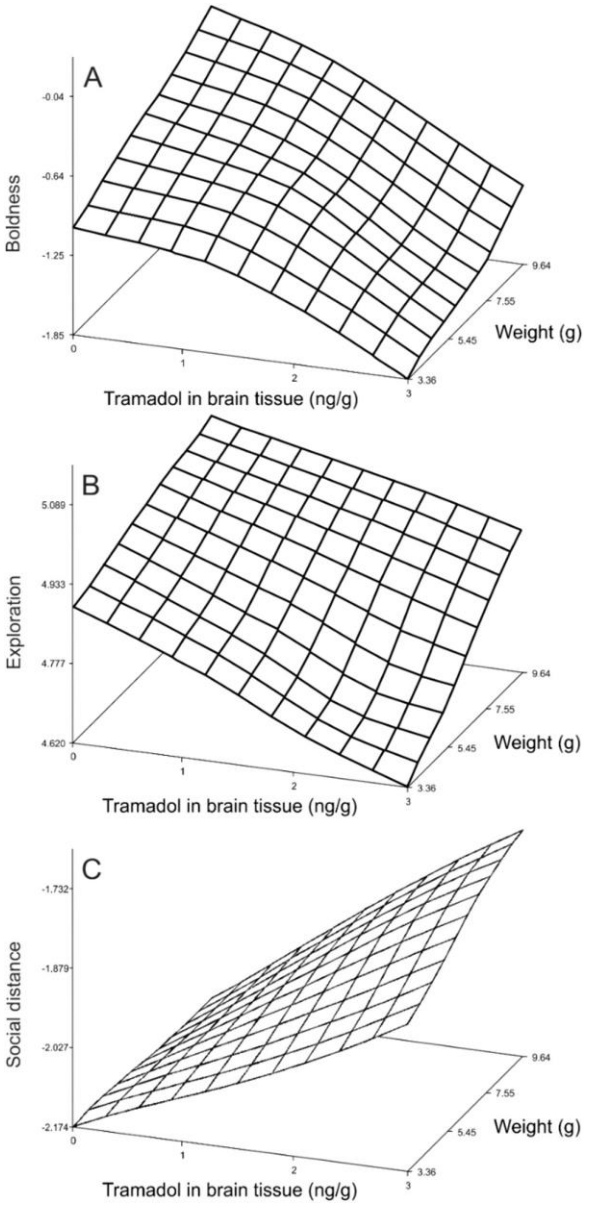

Fig. 5. Boldness (A), exploration (B) and social distance (C) in relation to tramadol in brain tissue and weight. Values are predicted from the particular mixed model.

reaching lethal concentrations (Hellou, 2011; Peterson et al., 2017). Changes of individual behaviour outcomes can induce alterations in the whole population, and thus, the fitness of aquatic ecosystems as a result of the existing interrelationships between species (Bisesi et al., 2014; Brodin et al., 2013). Therefore, the impacts over evolutionary behavioural patterns should be considered as a threatening signal and as a model to predict what could be happening in the aquatic ecosystems due to pharmaceutical contamination (Cunha et al., 2019; Klaminder et al., 2016).

1.1. Chemistry vs. behaviour

Tramadol, a weak base with n-octanol-water partition coefficient (log K_{ow}) of 1.35 at pH 7 (World Health Organisation, 2014), is not suspected to bioconcentrate in tissues due to its chemical properties. Accordingly, we observed a low BCF (< 500; OECD, 2001) in brain. Despite the low capacity of bioconcentration, the pseudo-persistence in the environment for the unceasing discharge into the water could pro- vide a continuous presence in fish tissues (Rosi-Marshall et al., 2015; Schultz et al., 2011).

The constant concentration of tramadol in brain in this experiment suggested a steady state reached after one day of exposure. In agreement with this, the changes in boldness and shoal cohesion were alsocontinuous from the first day of exposure. The BCF was very close to the values in plasma reported in Tanoue et al. (2017), which ranged from 1.4 to 1.8, depending on the pH of the water. In the same study, a relation between the concentration of tramadol in plasma and brain was observed, although higher amounts of the drug was detected in the brain compared to plasma (Tanoue et al., 2017). This phenomenon has been described in previous studies, where the concentration of the psycho- active substance in the brain was the highest (Huerta et al., 2016), or higher (Grabicova et al., 2018; Sancho Santos et al., 2020; Xie et al., 2015), compared to other organs. Among the 74 pharmaceuticals found, Grabicova et al. (2018) only detected psychoactive compounds in thebrains of common carp in ponds where a STP effluent and precipitation were the only source of water. Furthermore, Grabicová et al. (2020) detected psychoactive compounds such as sertraline and citalopram in the brains of common carp. In pikeperch living in the same conditions, the psychoactives carbamazepine, sertraline, and venlafaxine as well as the antibiotic azithromycin were observed (Grabicová et al., 2020).

The read-across hypothesis is a model approach where the effects of some specific compound are estimated due to the comparison of the fish's drug concentration in plasma compared to the human therapeutic plasma concentration (Huggett et al., 2003). Even though this is commonly accepted for toxicological studies (Malev et al., 2020), the importance of measuring the chronic presence of the psychopharmaceutical in the target organ over the plasma should be reinforced, especially in the case of psychoactive substances. Several authors emphasized the same idea considering the chemical characteristics and pharmacokinetics of each compound specifically (Grabicova et al., 2014; Schultz et al., 2011). Thus, direct impairments of the neuro- transmitter pathways can be expected even at minimal concentrations of these substances in brain (Grabicova et al., 2014). In addition, Hubená and Horký (2020a) (2020b) observed a correlation between brain concentration of sertraline and methamphetamine in fish and their condition, substantiating the brain concentration evidence approach, but the authors did not observe this link in the case of tramadol. However, behavioural endpoints are generally considered more sensitive than other parameters (Cunha et al., 2019). Therefore, alterations due to chronic exposure of tramadol should not be discarded, as we have confirmed in this study.

The particular acting of tramadol provides a combination of effects over several neurotransmission pathways. As previously described, tramadol has a dual mode of action. For one side, the parent compound, and its main metabolite, O-desmethyltramadol (M1), act on μ-receptors providing anti-nociception properties. On the other hand, it behaves as a non-opioid, inhibiting the reuptake of noradrenaline and serotonin over synaptic terminals, producing the anxiolytic and antidepressant-like effects. The metabolite M1 has 200 times higher affinity for the opioid receptor than the parent compound, consequently the analgesic efficiency depends on the metabolism of the pharmaceutical (Zhuo et al., 2016). However, Tanoue et al. (2017) reported a lower metabolism of M1 in fish compared to humans and other mammals. Regardless, in the same study, low amounts of M1 were detected in brain, supporting an analogous metabolism in fish compared to mammals (Tanoue et al., 2017).

Social distance was the only behavioural trait disrupted even after 14 days of depuration, when no traces of the parent compound were detected in the brain. Shoal cohesion has been related to rewarding

support neurocircuitries in zebrafish, as well as it is likely to be enhanced by stress (Soares et al., 2018). Moreover, it has been reported that active metabolites of drugs can remain in the tissues over more time than the parent compound (Sancho Santos et al., 2020). Hence, although metabolites of tramadol were not quantified in our study, they can be suspected to produce this finding. Further studies should address this uncertainty along with the specific effects of tramadol metabolites on fish.

In this experiment, concentrations of tramadol measured in fish brain were directly related with behavioral effects in terms of boldness, exploration of a new object, and social interindividual distance. These results indicated that the presence of the parent compound in the brain generates direct impairments in the neurocircuitry pathways, resultingin a concurrent gradation of changes in behaviour. Therefore, we suggest the application of this approach in future studies to confirm whether this correlation also occurs after the exposure to other psychoactive substances, along with a lowest-observed-adverse-effect level (LOAEL) at this context.

1. Conclusions

Our findings clearly demonstrated that tramadol at 1 μg/L in water – here considered as environmental relevant concentration - can impair the individual behaviour of European native fish. In particular, the exposure to the pharmaceutical for 42 days triggered a reduction in activity, boldness, exploratory and social behaviour of chubs. Even after 14 days of depuration, the shoal cohesion was still disrupted, indicating the possible action of metabolites apart from the parent compound. The apparent anxiolytic-like effect was correlated with the concentration of the pharmaceutical in the brain, underlining the importance of the presence of the compound in the main target organ. These results indi- cate the potential environmental effects of tramadol and emphasize the need for further ecotoxicological studies focused on this pharmaceutical.

CRediT authorship contribution statement

Sancho Santos Maria Eugenia: Writing - original draft, Conceptualization. **Horký Pavel:** Writing - Review & Editing, Investigation, Methodology. **Grabicová Kateřina:** Writing - Review & Editing, Investigation. **Hubená Pavla:** Writing - Review & Editing, Investigation. **Slavík Ondřej:** Writing - Review & Editing, Funding Acquisition. **Grabic Roman:** Writing – Review & Editing, Investigation, Methodology. **Douda Karel:** Writing - Review & Editing, Methodology. **Randák Tomáš:** Experimental design, Writing - Review & Editing, Funding Acquisition, Supervision.

Declaration of Competing Interest

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

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7. Methamphetamine pollution elicits addiction in wild fish

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SHORT COMMUNICATION

Methamphetamine pollution elicits addiction in wild fish

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ABSTRACT

Illicit drug abuse presents pervasive adverse consequences for human societies around the world. Illicit drug consumption also plays an unexpected role in contamination of aquatic ecosystems that receive wastewater discharges. Here, we show that methamphetamine, considered as one of the most important global health threats, causes addiction and behavior alteration of brown trout Salmo trutta at environmentally relevant concentrations (1 µg l^{-1}). Altered movement behavior and preference for methamphetamine during withdrawal were linked to drug residues in fish brain tissues and accompanied by brain metabolome changes. Our results suggest that emission of illicit drugs into freshwater ecosystems causes addiction in fish and modifies habitat preferences with unexpected adverse consequences of relevance at the individual and population levels. As such, our study identifies transmission of human societal problems to aquatic ecosystems.

KEY WORDS: Behavior, Brain metabolome, Drug residues in brain, Withdrawal

INTRODUCTION

Illicit drug abuse is widely acknowledged as a global public health challenge that elicits profound societal costs, including financial burdens of hundreds of billions of dollars each year in the USA alone (NIDA, 2010). Users of illicit drugs indirectly introduce these drugs into surface waters following excretion to sewage collection systems and discharge from wastewater treatment plants, because these systems were not designed to treat such such contamination (Ort et al., 2014). Other contaminants of emerging concern, including prescription medicines and other consumer chemicals, are similarly introduced into surface waters with the potential to alter the physiology and behavior of aquatic organisms at relatively low levels (Brodin et al., 2013). Unfortunately, whether illicit drugs alter fish behavior at levels increasingly observed in surface water bodies is not known.

Though amphetamines and methamphetamines could be used to treat various diseases including bipolar disorder, abuse and addiction potential limit their usage during psychiatric drug therapies

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(Perugi et al., 2017). In fact, amphetamine-type drug consumption is dramaticallyincreasing,somuchsothat methamphetamine addiction is now considered one of the most important global health threats (UNODC, 2017). Such global abuse of methamphetamine translates to surface water contamination worldwide (Xu et al., 2017). In some parts of Europe, methamphetamine use is elevated; for example, sewage- (or wastewater-) based epidemiology studies of illicit drugs in raw sewage identified relatively high consumption in regions of the Czech and Slovak Republics (Ort et al., 2014). Consequently, methamphetamine was previously observed in surface waters of the Czech Republic at levels of hundreds of nanograms per liter (Koba et al., 2018).

Fish are sensitive to adverse effects of many neurologically active drugs from alcohol to cocaine and are employed as model organisms to study nervous system disorders (Collier et al., 2014). Though behavioral perturbations by neurologically active contaminants may have fundamentally important consequences to individual-, population- and community-level dynamics (Saaristo et al., 2018), behavioral response variables are rarely employed during environmental assessments (Ågerstrand et al., 2020). However, Ford et al. (2021) recently provided consensus perspectives and recommendations to advance behavioral ecotoxicology interfaces between the basic and translational sciences. These recommendations are particularly relevant for illicit drugs because information on their potential behavioral impacts in aquatic ecosystems are poorly understood.

Because fish can develop drug addiction such as behavioral dependencies related to the dopamine reward pathway in a similar manner to humans (Bossé and Peterson, 2017), we tested whether fish exposed to environmentally relevant methamphetamine concentrations show signs of addiction during withdrawal. We then examined potential

is a globally important species that is native primarily in Europe with a range extending to western Asia and North Africa (MacCrimmon et al., 1970) but with naturalized populations on all continents except for Antarctica (Elliott, 1994). Furthermore, brown trout has been employed as a model species in toxicology (e.g. Luckenbach et al., 2001). Thus, the results obtained in the present study are broadly relevant to numerous ecosystems.

MATERIALS AND METHODS

Experimental animals

All laboratory experimental procedures complied with appropriate animal welfare regulations (Law no. 246/1992, § 19, article 1, letter c), which were derived from Directive 2010/63/EU; the permit was awarded to O. Slavík, qualified according to Law no. 246/1992, § 17, article 1: permit no. CZ02233. All laboratory procedures were performed with relevant permission from the Departmental Expert Committee for authorization of experimental projects of the

Ministry of Education, Youth and Sports of the Czech Republic (permit no. MSMT – 1972/2016-5). Fish used for experimentation were hatchery-reared juvenile brown trout, Salmo trutta Linnaeus 1758, obtained from a local fish supplier that was verified as uncontaminated (Czech Fishery Ltd). A total of 120 similar-sized fish of the same age (1 year; mean standard length 117 mm) were transported from the hatchery to the laboratory and were kept in two separate holding tanks (350 l, each with 60 randomly distributed organisms) with aeration for 2 weeks prior to the start of the experiment. Fish were fed adlibitum food pellets (Biomar Ltd) once a day (except on days when behavioral assays were performed) and were kept under the natural photoperiod (i.e. daylight varied from 13 to 14 h), thus maintaining the same regime to which they were accustomed in the hatchery. Three-quarters of the water

volume was renewed with aged tap water filtered through activated charcoal every other day. Mean water quality parameters were as follows: pH 7.2, NH_4^+ <0.05 mg l^{-1}, NO_3^- 7.08 mg l^{-1}, NO_2^- <0.04 mg l^{-1}, PO_4^{3-} <0.05 mg l^{-1}, chemical oxygen demand by manganese ($CHSK_{Mn}$ 1.1 mg l^{-1}, Cl^- 8.9 mg l^{-1}, $\Sigma Ca^{2+}+Mg^{2+}$ 1.00 mmol l^{-1}, Ca^{2+} 34.1 mg l^{-1}. Water temperature was controlled automatically and held at a mean(±s.d.) of 17.6 ± 0.2 °C throughout the entire experiment.

Methamphetamine study

Following a 2 week acclimation period, 60 fish in one holding tank were nominally exposed to methamphetamine (Sigma-Aldrich, Steinheim, Germany) at the environmentally relevant concentration of 1 µg l^{-1} for 8 weeks. Because methamphetamine affects fish condition after at least 3 weeks of exposure (Hubená et al., 2020), we employed a period of 8 weeks to examine longer-term chronic conditions that may be expected in lotic systems continuously receiving municipal effluent discharge. A nominal concentration of 1 µg l^{-1} was selected as an intermediate methamphetamine treatment level between lower (tens or hundreds of nanograms per liter; e.g. Lin et al., 2010) and higher (25 µg l^{-1}; Paciuszkiewicz et al., 2019) levels reported in surface waters around the world. All environmental variables (i.e. temperature, photoperiod, food) were consistent with the acclimatization period. Two-thirds of the water volume was renewed with aged tap water filtered through activated charcoal every other day. No significant pH differences were detected between the treatment and control (mean pH 7.2 versus 7.17; P>0.11, n=56). Methamphetamine was added during every water renewal in order to maintain its concentration in the tank at the required level. Sixty negative control fish were kept under the same regime in a separate 350 l tank without methamphetamine. Methamphetamine concentrations in these two holding tanks were analytically determined 10 times during the 8 week study to verify exposure conditions. The mean (±s.d.) level of methamphetamine in aquaria with exposed fish was 1.2±0.4 µg l^{-1} (n=10) and the concentration in the control tank was below our limit of quantification (<0.023 µg l^{-1}).

Behavioral experimental design

Behavioral observations were conducted in a two-current choice flume for examining preference of aquatic animals (Fig. 1) that was designed according to Jutfelt et al. (2017). Two separate tanks of 100 l volume were used to feed the system by gravity. Baffle plates, fine mesh and honeycomb collimators were designed to create two separate currents of laminar flow in the following choice arena (40×40 cm; volume of water ca. 30 l). The choice arena was free of any obstacles, allowing fish to choose freely between control and methamphetamine-contaminated areas. Methamphetamine levels in this choice arena were maintained at the same environmental concentration (1.2±0.4 µg l^{-1}) as during the 8 week exposure period. The methamphetamine-dosed part of the observation arena (left or right) and fish treatment (of either control or previously exposed individuals) were regularly rotated in order to randomize experimental observations.

After completing the 8 week experiment, fish were transferred to clean water to initiate depuration for 10 days. We then investigated the behavior of 8 randomly selected fish from the control and methamphetamine-treated tanks beginning 48 h after completion of the 8 week study (Cachat et al., 2010) and then every 48 h thereafter during the 10 day depuration period. Behavioral observations over this time period were intended to simulate 'withdrawal' following methamphetamine exposure. Thus, 5 separate trials (on the 2nd, 4th, 6th, 8th and 10th day of withdrawal) with 80 total specimens (40 control, 40 treated) were conducted. Every specimen was placed in the choice arena separately and its behavior was subsequently recorded for 10 min using a GoPro HERO digital camera (GoPro Inc.) placed above the arena. The flume was emptied and rinsed thoroughly between observations with each fish, and the order of control and treated specimens was changed regularly (i.e. first control, first treated, second control, second treated, etc.). Immediately after each behavioral observation, individual fish were measured (standard length mean 117 mm, range 88–146 mm), weighed (mean 23 g, range 9–41 g) and killed by cervical dislocation, followed by exsanguination (method approved by the valid legislative regulations; law no. 246/1992, § 17). No significant size differences were detected between the two groups (control, treated) of fish (standard length P>0.3, n=80; mass P>0.59, n=80). Brain tissues were dissected from freshly killed fish, weighed and stored frozen at −20°C for subsequent methamphetamine and amphetamine analyses. Before the analysis, brain samples were defrosted and extracted according to procedures described in Grabicová et al. (2018). Tissue aliquots were analyzed using liquid chromatography with high resolution mass spectrometry (QExactive, Thermo Fisher Scientific). High resolution product scan (HRPS) was used for quantitative analysis

of methamphetamine and amphetamine (i.e. targeted analyses), while full scan data (100–800 m/z range) in both positive and negative electrospray ionization modes were acquired for consequent metabolomics in another LC-HRMS run for non-targeted analyses.

Data analyses

To address an obvious M-dependence structure hidden within the data, mainly caused by multiple records per fish within a short time period, we employed a regular 15 s grid approach to reduce the number of observations to 40 records per fish (i.e. 4 records per minute). These reduced data formed a regular and balanced longitudinal profile while distinguishing for trial repetitions. The 'fish position' values within the choice arena were binomial and varied from 0 (i.e. preference for the control part) to 1 (i.e. preference for the methamphetamine-dosed part). The fish position values that were used to define a binomial variable 'probability of methamphetamine source preference' included just the data from the last minute of the observation (i.e. 4 records per fish). Thus, we used data based on the final individual decision, without accounting for the prior potential exploration and habituation effects. Fish movement values within the choice arena varied from 0 (i.e. fish was stationary) to 1 (i.e. fish was moving) and were used to define a binomial 'probability of movement' variable. Fish movement values from thewhole 10 min observation period were entered in the analyses, accounting for the exploration and habituation effects in this variable. 'Amphetamine in brain tissue' and 'methamphetamine in brain tissue' variables were defined as binomial variables with 1

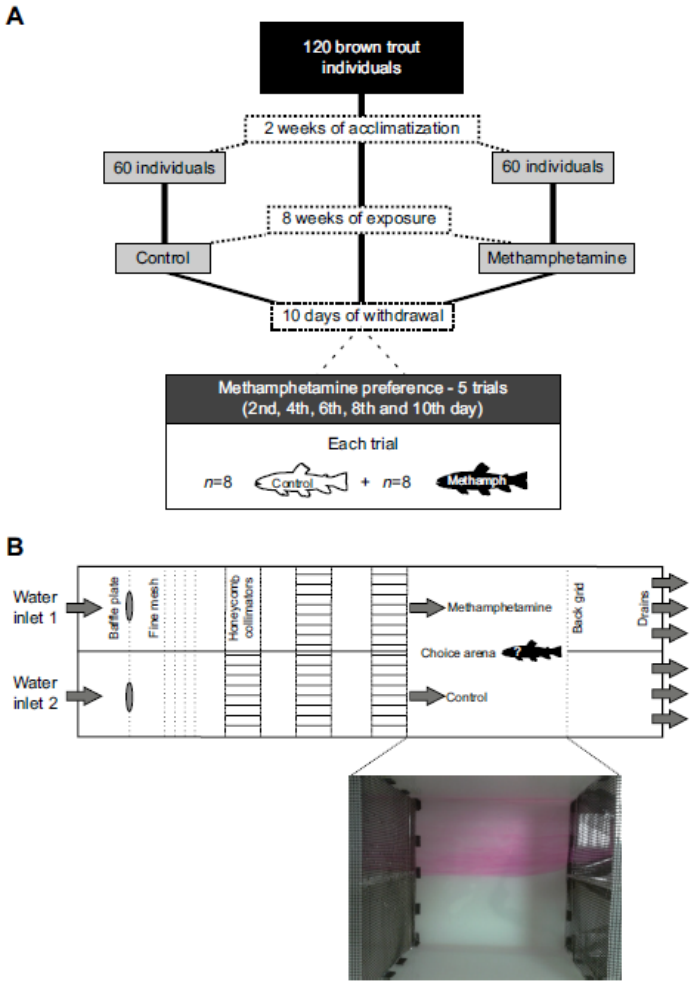

Fig. 1. Experimental testing. (A) Outline of the experiment. (B) Illustration of the two-current choice flume. Water from two different tanks fed the choice flume by gravity. Water flow through the baffle plates, fine mesh and honeycomb collimators creates two laminar, non-mixing currents in the choice arena as can be seen in the illustrative photo during the purple dye test.

indicating presence of particular substance. ‘Treatment’ was defined as the class variable distinguishing whether fish were previously exposed to methamphetamine or not. Class variable ‘trial’ defined the order of the experiment based on the day of the simulated withdrawal period (2nd, 4th, 6th, 8th and 10th day).

Statistical analyses

Statistical analyses were performed using the SAS software package (SAS Institute Inc., version 9.4, www.sas.com). The binomial dependent variables ‘probability of methamphetamine source preference’, ‘probability of movement’ and ‘methamphetamine in brain tissue’ were analyzed using mixed models with random factors (PROC GLIMMIX with binomial distribution and logit link). Two models for dependent variables ‘probability of methamphetamine source preference’ and ‘probabilityof movement’ were fitted because of the ‘treatment’ and ‘amphetamine in brain tissue’ variables overlap (intercorrelation). Thus, the first model contained ‘treatment’ and the interaction between ‘treatment’ and ‘trial’ fixed factor variables, while the second model included ‘amphetamine in brain tissue’ and ‘methamphetamine in brain tissue’ fixed factor variables. An additional model for dependent variable ‘methamphetamine in brain tissue’ was fitted in order to determine whether the presence of methamphetamine in brain tissue was dependent on ‘fish

position’ or not. Random factors were used to account for the repeated measures collected for the same experimental units (individual fish) across the duration of the experiment. Whether exploratory variables were significant was assessed using an F-test. Least-squares means (henceforth referred to as ‘adjusted means’) were subsequently computed for particularclasses. Differences between the classes were tested with a t-test, and a Tukey–Kramer adjustment was used for multiple comparisons. Degrees of freedom were calculated using the Kenward–Roger method.

LC-HRMS data evaluation

LC-ESIpos HRMS full scan data were processed using Compound Discoverer 2.0 software (Thermo Fisher Scientific). Blank samples were used to filter out all possible interference from the solvents we used and the chromatographic system. All chromatograms were aligned with a retention time tolerance of 0.2 min. We used 5 ppm mass tolerance through the entire workflow. We attributed data files with categorical variables that were used later for filtering groups from the dataset. Controls were set as control while exposed fish were set as sample. The categorical variable depuration time was used to separate corresponding sample and control groups. We set diagnostic ratios for differential analysis as follows: sample to corresponding control for each day of experiment, all controls to control 2nd day and all samples to sample 2nd day of depuration. Differential analysis resulted in plots showing statistically significant relationships as P-values and differences as log foldchange. We set criterions of significant difference as $P<0.05$ and log fold-change >1. Consequently, only significantly different signals from exposed fish on day 2 were selected and assumed as markers of methamphetamine effect. All other signals were

filtered out from all studied groups and then principle components analysis was performed to reveal whether selected markers can be used as a tracers of persisting methamphetamine effects during withdrawal.

RESULTS AND DISCUSSION

We observed control fish to cross from one side of the choice arena to the other during 21.6% (347 of 1600) of all observations. These controls spent 41.5% (665 of 1600) of all observations in the methamphetamine-dosed part of the arena. Similarly, we observed previously exposed fish to cross from one side of the choice arena to the other during 21.1% (339 of 1600) of observations, but these animals were in the methamphetamine-dosed part of the choice arena in a higher number of observations (50.5%, or 809 of 1600). Following 56 days of exposure (1 $\mu g\, l^{-1}$), preference for methamphetamine during a simulated withdrawal period was considered an indicator of addiction. These brown trout showed higher probability of methamphetamine source preference compared with controls ($F_{1, 306}$=28.02, P<0.0001; Fig. 2A), and this difference was apparent for the first 4 days of depuration following treatment ($F_{8, 306}$=2.18, P=0.029; Fig. 2B). Such methamphetamine preference was positively correlated with levels of amphetamine residues in fish brains ($F_{1, 314}$=18.56, P<0.0001; Fig. 2C), suggesting that addiction is linked to the presence of this drug metabolite in nervous system tissue. Amphetamine was only identified in brain tissue of exposed trout and its presence decreased from 100% to 12.5% of individuals throughout the 10 day depuration period (Fig. S1A).

Exposed brown trout displayed a lower probability of movement than controls during the withdrawal period ($F_{1,3190}$=4.94, P=0.0263; Fig. 3A). This behavioral modification was also observed until the 4th day of depuration ($F_{8,3190}$=7.18, P<0.0001; Fig. 3B) and significantly correlated with amphetamine in brain tissue ($F_{1,3157}$=6.47, P=0.011; Fig. 3C). However, the opposite effect of movement increase was observed when methamphetamine was found in fish brain ($F_{1,3075}$=42.96, P<0.0001; Fig. 3D). Methamphetamine was observed in brains of individuals that occurred more frequently in the dosed part of the observation arena ($F_{1, 3158}$=51.00, P<0.0001; Fig. S1B), suggesting that methamphetamine presence in the brain resulted from acute drug intake.

Biochemical changes in fish brains were revealed using differential non-target analysis of LC-HRMS data. Significant differences (both up- and down-regulated signal intensities) between control fish and those experiencing the withdrawal period during depuration gradually decreased from 210 signals

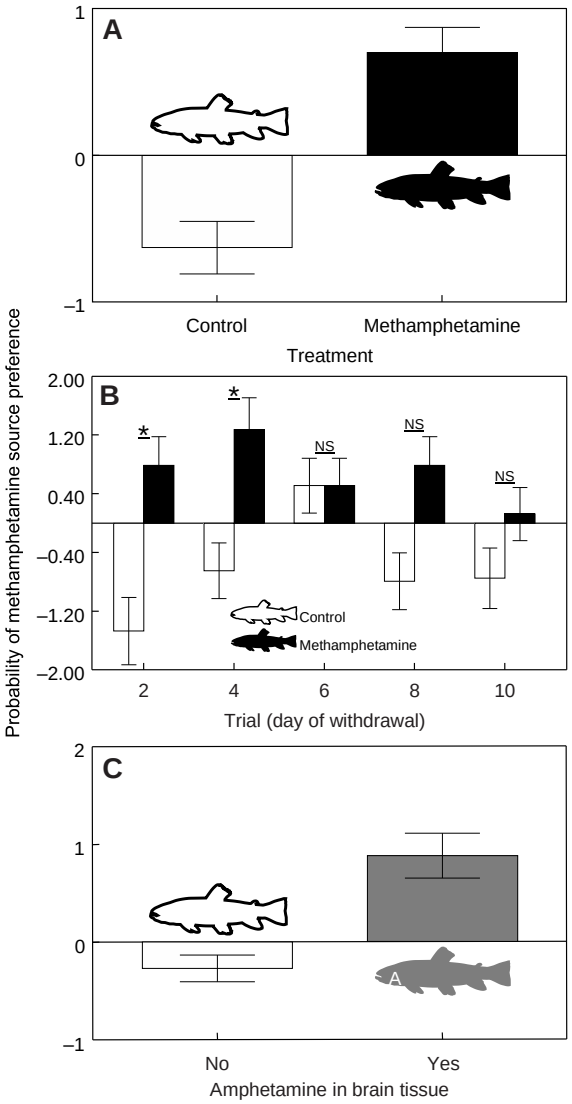

Fig. 2. Brown trout methamphetamine source preference. Probability of methamphetamine source preference (A) in relation to previous treatment, (B) across trials, i.e. days of withdrawal and (C) for amphetamine in brain tissue of focal individuals. Values are adjusted means±s.e. based on a mixed model with random factor analyses (PROC GLIMMIX; significance was assessed using an F-test; P<0.05). Five separate trials (on the 2nd, 4th, 6th, 8th and 10th day of withdrawal) with 80 total specimens (40 control, 40 treated) were conducted. Estimates could be negative because of use of the logit link, where probabilities between 0 and 1 are specified as logits, ln[p/(1−p)], which can be less than 0 when the estimated probability is less than 0.50. Differences among classes in particular figure parts are significant (*adjusted P<0.0001).

(substances) during the 2nd day of depuration (Fig. S2A) to 36 substances during the 10th day (Fig. S2B). These novel markers of methamphetamine exposure were consequently applied for description (principle component analysis where variables were revealed markers only) of changes in brain metabolomes across all experimental groups. Similar to our observations of brain tissue residues, differences in overall brain metabolites were significant

are further suggested to stimulate drug-seeking behavior in humans (Piper, 2015). We found that brown trout withdrawn from waterborne (or inhalational) exposure to an environmentally relevant level of methamphetamine displayed similar drug-seeking behavior. Fish preference for a drug is often dependent on the dopamine pathway (Bretaud et al., 2007), confirming that fish can display signs of addiction and withdrawal symptoms (Tran et al., 2015).

Withdrawal symptoms include increases in anxiety and stress (Piper, 2015). Exposed trout in the present study similarly displayed lower probability of movement, suggesting that their ability to explore a novel environment was reduced as a response to stress caused by methamphetamine withdrawal (Cachat et al., 2010). For example, Bossé and Peterson (2017) found that reduction of fish exploration rate indicated suffering from withdrawal symptoms without drug access.

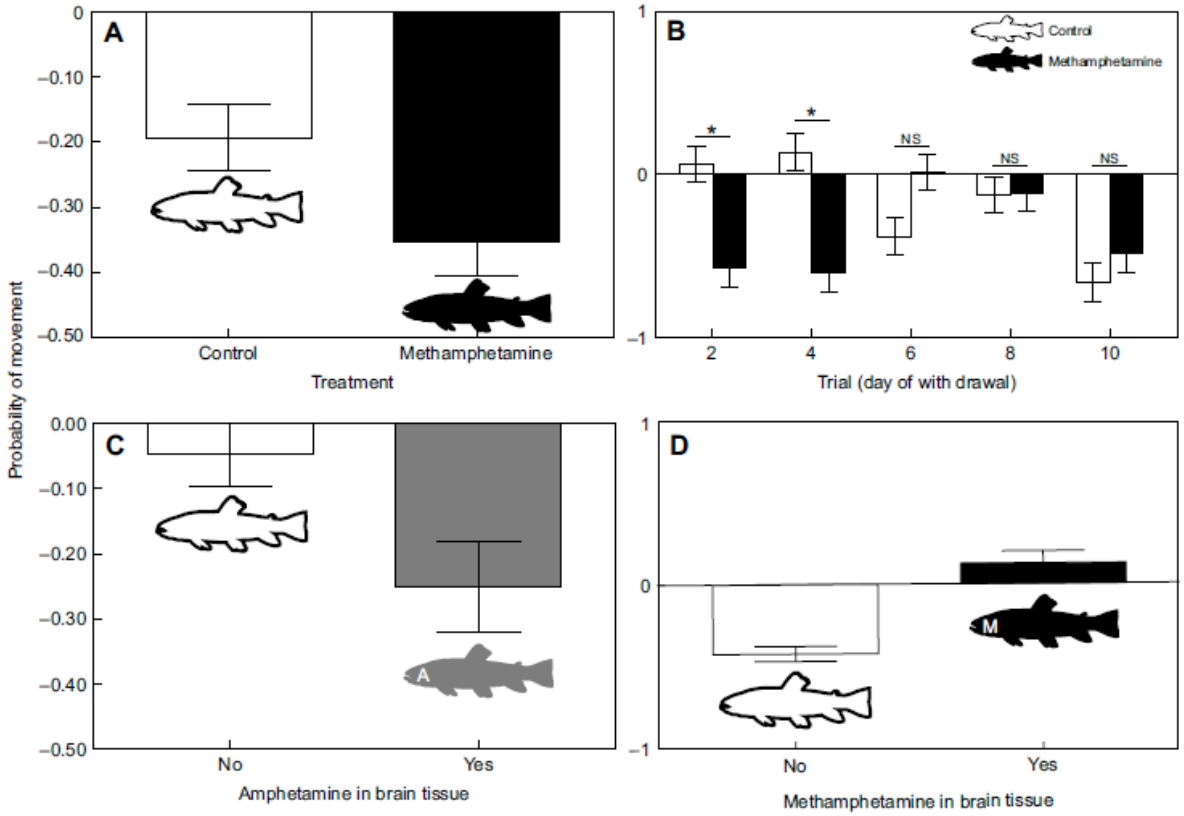

Fig. 3. Brown trout movement. Probability of movement (A) in relation to previous treatment, (B) across trials, i.e. days of withdrawal (B), and for
(C) amphetamine and (D) methamphetamine in brain tissue of focal individuals. Values are adjusted means±s.e. based on a mixed model with random factor analyses (PROC GLIMMIX; significance was assessed using an F-test; $P<0.05$). Five separate trials (on the 2nd, 4th, 6th, 8th and 10th day of withdrawal) with 80 total specimens (40 control, 40 treated) were conducted. Estimates could be negative because of use of the logit link, where probabilities between 0 and 1 are specified as logits, $\ln[p/(1-p)]$, which can be less than 0 when the estimated probability is less than 0.50. Differences among classes within a
panel are significant (*adjusted $P<0.01$).

until the 4th day of depuration and then leveled out thereafter (Fig. S3).

Intense physical and psychological manifestations triggered by withdrawal from a drug are considered major signs of addiction and are further suggested to stimulate drug-seeking behavior in humans (Piper, 2015). We found that brown trout withdrawn from waterborne (or inhalational) exposure to an environmentally relevant level of methamphetamine displayed similar drug-seeking behavior. Fish preference for a drug is often dependent on the dopamine pathway (Bretaud et al., 2007), confirming that fish can display signs of addiction and withdrawal symptoms (Tran et al., 2015).

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Our observations of withdrawal symptoms in brown trout were significantly related to the presence of the methamphetamine metabolite amphetamine in fish brain. These withdrawal behaviors were prevalent for 96 h and then disappeared as the rate of amphetamine-positive individuals decreased. Similar observations have been made in time course studies of methamphetamine

withdrawal in humans, with an initial peak within the first 24 h and subsequent decline to near-control levels by the end of the first week of abstinence (McGregor et al., 2005). Despite this fact, amphetamine was found in the brain of one trout specimen after the 10 days, suggesting differential metabolism among individuals (Metcalfe et al., 2016) can influence the internal dose with related behavioral consequences. Voluntary methamphetamine intake during the preference test led to a detection of methamphetamine in the brains of individual fish. The voluntary drug intake is highly individual in animals, depending on factors such as dominance or previous social disturbance, as shown by Wolffgramm and Heyne (1995). We found that methamphetamine in fish brain significantly increased its activity. Such performance-enhancing stimulant effects are not unexpected; for example, methamphetamine was strategically used to elicit such effects under the brand name Pervitin by the German army during World War II (Defalque and Wright, 2011).

Our results also indicate that environmental concentrations of methamphetamine alter fish brain metabolomes. Effects of drugs of abuse on brain metabolic activity have previously been observed in humans, with methamphetamine-specific decreases in dopamine transporters within the striatum (Volkow et al., 2001). Using nontarget analyses, we found that 36 endogenous molecules in the brain metabolome of exposed fish differed after the 10th day of withdrawal, while Volkow et al. (2001) found that the dopamine transporter was reduced in the striatum even after 11 months, suggesting that adverse effects of methamphetamine exposure could be long lasting in fish.

In conclusion, observations in the present study suggest that fish exposed to environmental concentrations of methamphetamine in surface waters will develop addiction and be attracted to reside near wastewater treatment effluent discharges. The wastewater effluents are often nutrient rich, offering additional bioenergetic incentives for

for fish attraction to outfall mixing zones. Such unnatural attraction to one area together with documented changes in behavior could result in unexpected ecological consequences influencing whole ecosystems (Boulêtreau et al., 2011). Furthermore, drug reward cravings by fish could overshadow natural rewards such as foraging or mating that provision homeostatic and reproductive success (Hyman et al., 2006) and further reinforce adverse ecological consequences of pollutants in aquatic environments (Prokkola and Nikinmaa, 2018). The elicitation of drug addiction in wild fish could represent another example of unexpected evolutionary selection pressure for species living in urban environments (Johnson and Munshi-South, 2017) along with ecological side effects of human societal problems within aquatic ecosystems. Further field research is needed to examine the withdrawal effects of methamphetamine observed in this experimental study under mesocosm conditions and natural ecosystems.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Methodology: P. Horký, R.G., K.D., T.R.; Investigation: P. Horký, R.G., K.G.; Writing original draft: P. Horký; Writing - review & editing: R.G., K.G., B.W.B., K.D., O.S., P. Hubená, M.E.S.S., T.R.; Funding acquisition: O.S., T.R.

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8. Prescribed aggression of fishes: Pharmaceuticals modify aggression in environmentally relevant concentrations

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Prescribed aggression of fishes: Pharmaceuticals modify aggression in environmentally relevant concentrations

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ABSTRACT

Traces of psychoactive substances have been found in freshwaters globally. Fish are chronically exposed to pollution at low concentrations. The changes of aggressive behaviour of chub (*Squalius cephalus*) were determined under the exposure to four psychoactive compounds (sertraline, citalopram, tramadol, methamphetamine) at environmentally relevant concentrations of 1 μg/L for 42 days. We tested whether (A) the behavioural effect of compounds varies within a single species; (B) there is a correlation between the individual brain concentration of the tested pollutants and fish aggression using the novel analysis of pollutants in brain; and (C) there is detectable threshold to effective pollutant concentration in brain. Behaviour and pollutant concentrations in brain were determined repeatedly (1st, 7th, 21st, 42nd and 56th days), including a two-week-long depuration period. The effect of particular compounds varied. Citalopram and methamphetamine generally increased the fish aggression, while no such effect was found after exposure to tramadol or sertraline. The longitudinal analysis showed an aggression increase after depuration, indicating the presence of withdrawal effects in methamphetamine- and tramadol-exposed fish. The analysis of pollutant concentration in brain revealed a positive linear relationship of citalopram concentration and aggression, while no such effect was detected for other compounds and/or their metabolites. Structural break analyses detected concentration thresholds of citalopram (1 and 3 ng/g) and sertraline (1000 ng/g) in brain tissue, from which a significant effect on behaviour was manifested. While the effect of sertraline was not detected using traditional approaches, there was a reduction in aggression after considering its threshold concentration in the brain. Our results suggest that pursuing the concentration threshold of psychoactive compounds can help to reduce false negative results and provide more realistic predictions on behavioural outcomes in freshwater environments, especially in the case of compounds with bioaccumulation potential such as sertraline.

1. Introduction

The presence of biologically active compounds in waters has been confirmed on a global scale (aus der Beek et al., 2016). Hospitals, households and industry have been identified as the main sources of pharmaceutical pollution in waters (Quesada et al., 2019). The biologically active compounds found in waters include antibiotics, lipid regulators, antiepileptics, antidepressants, anti-inflammatories, hormones, X-ray contrast agents and others (Fent et al., 2006; Corcoran et al., 2010; Aubertheau et al., 2017; Pereira et al., 2017; Bojarski et al., 2020).

Presence of these substances in waters has been a subject of public concern for example due to growing resistance of bacteria against antibiotics (Bojarski et al., 2020). The adverse effects of pharmaceuticals may demand time to develop (Hubena et al., 2020a´), but in other cases fish may weaken their response to compounds with time such as under exposure to enrofloxacin (Sehonova et al., 2019). Chronic exposure to low concentrations of pharmaceuticals among aquatic organisms may induce significant changes to histology and physiology of aquatic vertebrates (Galus et al., 2013; Sehonova et al., 2017b, 2017a; Burgos-Aceves et al., 2018; Perez et al., 2018; Turani et al., 2019; ´ Nowakowska et al., 2020; Kondera et al., 2020). This leads to changes in behaviours such as boldness, nest cleaning, startle and freezing reaction to predator and locomotor activity (Weinberger and Klaper, 2014; Martin et al., 2017; Nielsen et al., 2018; Sehonova et al., 2018; Ziegler et al., 2020). The response of fish to certain pharmaceuticals has been even compared to human idiopathic autism (Thomas and Klaper, 2012). We focused on four psychoactive compounds in the present study. The selected compounds included selective serotonin reuptake inhibitors (SSRIs) citalopram, and sertraline, which are prescribed as a treatment for depression (Arroll et al., 2005) but vary in their mode of action (Stahl, 1998). Citalopram binds to the serotonergic transporter SERT only, while sertraline has an affinity for noradrenergic and dopaminergic transporters (Stahl, 1998). Both SSRIs represent two very different uptake patterns in fish and have bioaccumulative

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potential (Grabicova et al., 2017). The next selected compound was the analgesic tramadol. Tramadol binds to μ– , δ– and κ-opioid receptors (Dayer et al., 1997; Grond and Sablotzki, 2004) but also inhibits the reuptake of serotonin and noradrenaline (Dayer et al., 1997; Grond and Sablotzki, 2004; Caspani et al., 2014). The last selected compound was the illicit drug methamphetamine, which causes addiction in users (Petit et al., 2012). Methamphetamine reverses or blocks the transport of neurotransmitters through interaction with dopamine, noradrenaline and serotonin transporters and vesicular monoamine transporter-2 (Sulzer et al., 2005; Elkashef et al., 2008). All selected compounds (i.e., citalopram, sertraline, tramadol, and methamphetamine) have been repeatedly found in surface waters (Kasprzyk-Hordern et al., 2008; Fedorova et al., 2014; Grabicova et al., 2017). The fish are known to react to traces of these substances, shown by changes in locomotion, boldness or food intake (Xie et al., 2015; Kellner et al., 2016; Wang et al., 2020). Nevertheless, the effect of individual drugs on the aggressiveness of fish has not been well documented in methamphetamine and tramadol. The effect of SSRIs on aggressiveness has received more attention, but fluoxetine has mainly been focused (Dzieweczynski and Hebert, 2012; Eisenreich et al., 2017; Aliko et al., 2019). We investigated aggression under sertraline and citalopram exposure, which differ significantly in their uptake pattern (Grabicova et al., 2017).

Aggressive behaviour is a way of social rank maintenance (Øverli et al., 2004; Grosenick et al., 2007; Ang and Manica, 2010) and a way to monopolise the limited resources across species (Ryer and Olla, 1991; Basquill and Grant, 1998; Silva et al., 2013). The individual level of aggressiveness is stable across different situations (Roy and Bhat, 2018) but can be modulated in a specific range. Aggressiveness has a genetic basis (R´eale et al., 2007), but the neuroendocrinology enabling the range of behaviour alters aggression via many pathways. Specifically, dopamine, serotonin, histamine, nitric oxide, somatostatin, hypothalamo-neurohypophysial, hypothalamo-pituitary-interrenal and hypothalamo-pituitary-gonadal pathways play an essential role in the behavioural outcome of aggression (Winberg and Nilsson, 1993; Chiavegatto and Nelson, 2003; Filby et al., 2010; Reichmann et al., 2020). Additionally, the neural pathways controlling aggression show a high degree of comparability between fish and mammals (Filby et al., 2010). Pharmaceuticals binding to receptors in any of the pathways may have the potential to change the aggressiveness of fish.

Organ-specific uptake of psychoactive compounds has been found in fish, with examples such as the liver, kidney, and brain (Grabicova et al., 2017; Grabicova et al., 2020a´). Specifically, brain sample analysis is preferred in postmortem forensic toxicology due to its delayed postmortem redistribution of drugs (Rohrig and Hicks, 2015). The concentration of pollutants in brain correlates with changes in condition (Hubena et al., 2020a´), making it a suitable tool for evaluating behaviour under compound exposure. Dose-response modelling underlies modern risk assessment (Ritz, 2010). The dose has usually been accounted for as the aquatic concentration in fish ecotoxicological studies. However, the uptake of compounds differs based on their physico-chemical parameters (Chen et al., 2017), and the real concentration affecting the behaviour may therefore differ as well. In the present study, we focused on concentration of pollutants in brain, which represents the result of the specific compound uptake altering the behaviour. We aimed to detect changes in the aggressive behaviour of fish exposed to four psychoactive compounds (i.e., sertraline, citalopram, tramadol, and methamphetamine) at an environmentally relevant concentration of 1 μg/L. The selected compounds vary in targeted receptors in living organisms (Stahl, 1998; Grond and Sablotzki, 2004; Elkashef et al., 2008), and thus, we tested whether (A) their behavioural outcome would vary within a single species; (B) there is a direct link between the individual psychoactive compound and/or its metabolite brain concentration and fish aggression; and (C) there is detectable an effective psychoactive compound and/or its metabolite brain concentration. Behaviour and concentrations of pollutants in brain were determined repeatedly (1st, 7th, 21st, 42nd and 56th days from the start of the experiment), enabling longitudinal analysis of the studied effects, including a 14-day depuration period.

The chub (*Squalius cephalus* L.) was used as an experimental species due to its known aggressive ethogram under laboratory conditions (Hubena et al., 2020b´) and wide distribution in European rivers (Kottelat and Freyhof, 2007), which enables a more realistic view of the effect of psychoactive compounds in freshwaters.

2. Materials and methods

Design of the experiment was approved by Ministry of Education, Youth and Sports of the Czech Republic (permit no. MSMT-1972/2016- 5) and was in accordance with the valid legislation of the Czech Republic (law no. 246/1992, §19, art. 1, letter c). Animal welfare during experiments was superintended by the Departmental Expert Committee. Due to the increasing ethical standards for experiments, such as the reduction of animals in animal research (Flecknell, 2002), the experimental trials in the present study were conducted using the same experimental population as in the study of Hubena et al. (2020a)´ . The combination of multiple test guidelines into one protocol is a preferred reduction strategy in animal research (De Boo and Hendriksen, 2005; Terry et al., 2014).

Juvenile chubs (*Squalius cephalus*) were purchased from the local fish supplier (Czech Fishery Ltd.; Czech Republic) and transported from the hatchery to the laboratory before each trial. The sex of juvenile chub was not determined as both sexes are visually indifferent, and their gonads are immature. Each experiment required 320 size-matched fish (i.e., a total of 1280 fish were used; the average length was 101 ± 13 mm, and the average weight was 7.56 ± 3.46 g). The fish were acclimated to the experimental holding tanks, and the water renewal procedures started two weeks prior to the beginning of the experiment (four tanks of 200 L, each containing 80 random individuals per trial). Two holding tanks represented the experimental groups for each trial, and two tanks represented the control groups. The fish were fed daily ad libitum on food pellets under a photoperiod of 12 h of light and 12 h of darkness. The water temperature was controlled throughout the whole experiment using air conditioning (the average temperature was 20.8 ± 0.4 °C). The water (150 L) was renewed with dechlorinated municipal tap water. The frequency of water renewal depended on the stability of the tested compound in water and ranged from daily water renewal (sertraline) to once in two days (citalopram, methamphetamine, tramadol). Relatively low mean initial stocking density of ca. 3.2 kg/m^3 (e.g., Adams et al., 2007) and frequent water renewal resulted in the nitrogen compounds concentrations that were below the following limits (NO_2^- < 0.04 mg/L; NH_4^+< 0.05 mg/L; NH_3 < 0.0005 mg/L) in all aquaria. For sensitive cold-water species like juvenile cod (*Gadus morhua*) or Atlantic salmon (*Salmo salar*) for example is the safe threshold limit of total ammonia nitrogen (TAN; NH_3 + NH_4^+) considered to be 1 mg/L. (Fivelstad et al., 1995; Bjornsson and ¨ Olafsd´ ottir, 2006´). With respect to the fact that the warm-water fish are more tolerant of ammonia toxicity than cold-water fish and freshwater fish are more tolerant than saltwater fish (Timmons et al., 2002), for freshwater chub staying in temperatures around 20 °C can be TAN values < 0.06 mg/L considered safe. The normal body position, locomotion, food intake and fish mortality were monitored daily by visual examination. A detailed description of the conditions is included in the study of Hubena et al. (2020a)´ .

The water of the experimental and control groups was sampled every second week to maintain exposure at the selected nominal level of 1 μg/ L. The water sample concentrations were compared to isotopically labelled internal standards (citalopram-D_6, methamphetamine-D_5, sertraline-D_3, tramadol-D_3; see the chapter 'Chemicals'), which were added to filtered water using a regenerated cellulose filter (0.20 μm, acrylic copolymer housing, cellulose acetate/surfactant-free membrane; Sartorius Lab Instruments GmbH & Co. KG). The analysis of the samples and standards in liquid chromatography with tandem mass spectrometry (TSQ Quantiva, Thermo-Fisher Scientific) followed. The analysis was conducted for 10 min on a Hypersil Gold aQ column (50 × 2.1 mm, 5 mm particles, Thermo Fisher Scientific) with heated electrospray ionisation in positive mode (HESI+). The limits of quantification (LOQs) differed among compounds (0.011 – 0.021 μg/L for citalopram; 0.010 –

0.012 µg/L for sertraline; 0.0077 – 0.017 µg/L for methamphetamine; 0.030 – 0.060 µg/L for tramadol). The determined sampled water concentration was 1.3 ± 0.2 µg/L (mean ± S.D.) for citalopram; 0.23 ± 0.07 µg/L (mean ± S.D.) for sertraline; 1.1 ± 0.1 µg/L (mean ± S.D.) for methamphetamine; 0.99 ± 0.18 µg/L (mean ± S.D.) for tramadol. The water concentration of the control groups was, in all cases, below the limits of quantification. *2.1. The experimental trials*

Fish were exposed to each of the selected pharmaceuticals for 42 days, followed by a 14-day depuration period (until the 56th day). The behavioural tests were conducted on the 1st, 7th, 21st, 42nd and 56th days from the start of the experiment. Four random fish were used from each holding tank (i.e., eight individuals from the control group and eight individuals from the experimental groups) during every test day, resulting in the overall use of 80 individuals (40 exposed, 40 control) per compound. The behavioural test followed the same design as the real opponent test in Hubena et al. (2020b)´ . Two nine-litre testing aquaria (30 × 15 × 20 cm) were placed next to each other facing the longer side. All sides of the tanks were covered in removable grey cover (RGB: 238, 238, 238) except for the distant longer side, which was used to record the experiment using a digital camera (GoPro Hero, GoPro Inc.). The focal individuals were placed in one of the aquariums and left to habituate for one minute. Consequently, the removable grey cover between the aquaria was lifted, and the focal fish faced opponent for six minutes recorded by the camera.

After the termination of the aggression trial, the fish were killed according to valid legislative regulations (law no. 246/1992, § 17) and permitted by the Ministry of Education, Youth and Sports of the Czech Republic (permit no. MSMT-1972/2016-5) to analyse the compound concentration in their brain. The brain was dissected, weighed and stored in Eppendorf tubes at − 20 °C for further analyses. Brain samples were extracted by the procedure described in Grabicova et al. (2018). Briefly, ~ 0.1 g of brain tissue was homogenised with isotopically labelled internal standards of the tested compounds and extraction solvent (acidified mixture of acetonitrile and isopropanol, 3/1 vol/vol). The samples were extracted (TissueLyser II, Quiagen, Germany; 30 Hz), centrifuged (Mini spin, Eppendorf, Germany; 6708 g), filtered (Labicom; regenerated cellulose, 0.45 µm pores) and frozen at − 20 °C for 24 h followed by second centrifugation. Then, the resulting aliquots were analysed by liquid chromatography with high resolution mass spectrometry (LC-HRMS, QExactive, Accela 1250 LC pump, Hypersil Gold aQ column (50 × 2.1 mm; 5 µm particles) PAL autosampler, Thermo Fisher Scientific). TraceFinder 3.3 software (Thermo Fisher Scientific) was used for the processing of the data. The LOQs were 0.12 – 0.34 ng/g wet weight (ww) for citalopram; 0.13 – 0.37 ng/g ww for N-desmethylcitalopram; 0.10 – 0.44 ng/g ww for sertraline; 0.21 – 0.92 ng/g ww for norsertraline; 0.16 – 0.43 ng/g ww for methamphetamine; 0.11 – 0.32 ng/g ww for amphetamine; 0.19 – 0.69 ng/g ww for tramadol; 0.15 – 0.47 ng/g ww for O-desmethyltramadol; and 0.14 – 0.48 ng/g ww for N-desmethyltramadol.

2.2. Chemicals

The selected compounds were purchased from AK Scientific, Inc. (USA; citalopram hydrobromide, sertraline HCl) and Sigma–Aldrich Corporation (USA; methamphetamine hydrochloride, tramadol hydrochloride). Individual stock solutions were prepared in 10 mg/L ultrapure water (AquaMax Basic 360 Series and Ultra 370 Series, Young Lin Instruments, Korea) and stored at 4 °C. The nominal environmentally relevant concentration of 1 µg/L was prepared as a dilution of the individual stock solution with dechlorinated tap water added to the experimental groups.

Isotopically labelled standards were purchased from Lipomed (USA; tramadol-D_3, methamphetamine-D_5), Cerilliant Corporation (Round Rock TX, USA; sertraline-D_3) and Toronto Research Chemicals (Canada; citalopram-D_3). Ultra-pure water and acetonitrile (Merck), both acidified with formic acid (Sigma–Aldrich, Germany), were used as mobile phases in liquid chromatography. Acetonitrile and isopropanol (Merck) were used as the extraction solvents for brain samples.

2.3. Data analysis

The ethological software Boris (Friard and Gamba, 2016) was used to evaluate the recorded video files. The aggressive behaviours were distinguished based on the chub ethogram provided in Hubena et al. ´ (2020b) and defined as lateral display, frontal display, circling, and up and down swimming. The sum of all individual aggressive behaviours represented a count variable 'aggressiveness'. 'Treatment' was defined as the class variable distinguishing the control and exposed groups. The 'experimental phase' was also assigned as a class variable distinguishing sample groups for the course of the experiment. Five values, 1, 7, 21, 42 and 56 days, were acquired from the beginning of the experiment. 'Concentration in brain tissue' expressed the relative concentration of the corresponding psychoactive compound or its metabolite in ng/g in the individual fish's brain tissue.

2.4. Statistical analysis

The statistical analyses were performed using the SAS software package (SAS Institute Inc., version 9.4, www.sas.com). The effects of all four psychoactive compounds (i.e., sertraline, citalopram, tramadol, and methamphetamine) were tested separately (Table 1). Generalized linear mixed models (GLMM; SAS function PROC GLIMMIX) with a Poisson distribution were fitted to analyse the dependent variable 'aggressiveness'. Mixed models are a generalisation of standard models, e.g., GLM, with the generalisation indicating that the data are permitted to exhibit correlation and nonconstant variability. This method is used to cope with repeated measures experiments in which people or animals are the subjects, and the subjects are declared random because they are selected from a larger population in which generalisations are required (SAS Institute Inc, 2004). Therefore, in the present study, factors were applied to account for the repeated measures introduced using the same experimental units (i.e., individual fish). More detailed information about mixed models can be found elsewhere (e.g., Searle et al., 1992; Breslow and Clayton, 1993; SAS Institute Inc, 2004). The significance of the explanatory variables and their interactions were assessed using F-tests. Separate models containing 'treatment' or 'concentration in brain tissue' and other explanatory variables were fitted due to the 'treatment' - 'concentration in brain tissue' overlap (intercorrelation) to avoid misinterpretation of the results. The first set of models was fitted using the explanatory variable 'treatment' and the interaction between

Table 1
The summary table showing the significance of specific effects on chub aggressiveness under the influence of individual compounds and their metabolites (< 0.05 – significant; NS – nonsignificant; NA – not available).

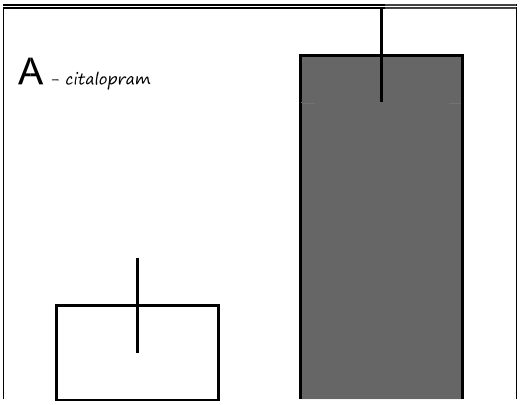
Effect on aggressiveness	Psychoactive compound			
	Citalopram/N- desmethylcitalopram	Sertraline/ norsertraline	Tramadol/O- and /N- desmethyltramadol	Methamphetamine/ amphetamine
Treatment	***< 0.05*** */ NA*	*NS / NA*	*NS / NA / NA*	***< 0.05*** */ NA*
Treatment (experimental phase)	*NS / NA*	*NS / NA*	***< 0.05*** */ NA / NA*	***< 0.05*** */ NA*
Brain concentration (continuous variable)	***< 0.05*** */ NS*	*NS/ NS*	*NS / NA / NA*	*NS / NS*
Structural breaks (brain concentration)	***< 0.05*** */ NS*	***< 0.05*** */ NS*	*NS / NA / NA*	*NS / NS*
Brain concentration (class variable)	***< 0.05*** */ NA*	***< 0.05*** */ NA*	*NA / NA / NA*	*NA / NA*

variable 'concentration in brain tissue'. Furthermore, the Chow test (Chow, 1960) was applied to search for significant structural breaks in the relationship between 'aggressiveness' and 'concentration in brain tissue'. Significant structural breaks were used to restructure 'concentration in brain tissue' as a class variable with two levels – above and below the significant structural break. The third set of models was subsequently fitted using the class explanatory variable 'threshold concentration in brain tissue'. Least-squares means (henceforth referred to and in bar charts presented as 'adjusted mean' of model predictions) were subsequently computed for the particular classes of class variables. Differences between the classes were determined with a *t*-test, and Tukey–Kramer adjustment was utilised for multiple comparisons. The association between the dependent variables and a continuous explanatory variable 'concentration in brain tissue' was estimated by fitting a random factor model as described by Tao et al. (2002). With this random coefficient model, we calculated the predicted values for the dependent variable and plotted them against the continuous variables. The degree of freedom was calculated using the Kenward–Roger method (Kenward and Roger, 1997).

3. Results

Particular compounds differed in their influence on chub aggressiveness (Table 1). The treatment itself increased aggressiveness in citalopram ($F_{1, 66.05}$ = 22.6, P < 0.0001; Fig. 1A) and methamphetamine ($F_{1, 73.8}$ = 4.57, P < 0.0359; Fig. 1B), while no such general influence was observed in sertraline and tramadol. The progression of effects on aggressiveness was found to be significant only in the case of methamphetamine ($F_{8, 45.88}$ = 4.1, P < 0.0009; Fig. 2A) and tramadol ($F_{9, 78.37}$ = 8.41, P < 0.0001; Fig. 2B). Both compounds significantly increased aggressiveness after 14 days of depuration only (56th day).

Diverse effects of concentrations of individual compounds in brain and their metabolites on aggressive behavioural outcomes were detected. When accounting concentrations as continuous variables, the effect was significant only in the case of citalopram ($F_{8, 75.57}$ = 12.88, P < 0.0006; Fig. 3), as its increased concentrations in brain were related to an increase in aggressiveness. Further effects were detected when accounting for the structural breaks. Significant structural breaks in the relationship between aggressiveness and compound brain concentration were observed in the case of citalopram (1 ng/g of concentration in brain; $F_{2, 316}$ = 4.76, P < 0.0092 and 3 ng/g of concentration in brain; $F_{2, 316}$ = 4.73, P < 0.0094) and sertraline (1 000 ng/g of concentration in brain; $F_{2, 316}$ = 4.3, P < 0.0143) only. These breaks were then applied to test for the effect of pollutant brain concentrations as class variables dividing concentration on two levels (below and above the defined threshold). The analyses confirmed that citalopram brain concentrations above 1 ng/g ($F_{1, 76.85}$ = 17.98, P < 0.0001; Fig. 4A) and above 3 ng/g ($F_{1, 73.43}$ = 4.44, P < 0.0384; Fig. 4B) induced aggressiveness increases, while sertraline brain concentrations above 1 000 ng/g induced aggressiveness decreases ($F_{1, 82.51}$ = 7.66, P < 0.0070; Fig. 4C). There were no detectable effects of brain concentrations of metabolites on fish aggression. Our results highlight the importance of the pollutant concentration in brain approach in behavioural outcome analyses, as there



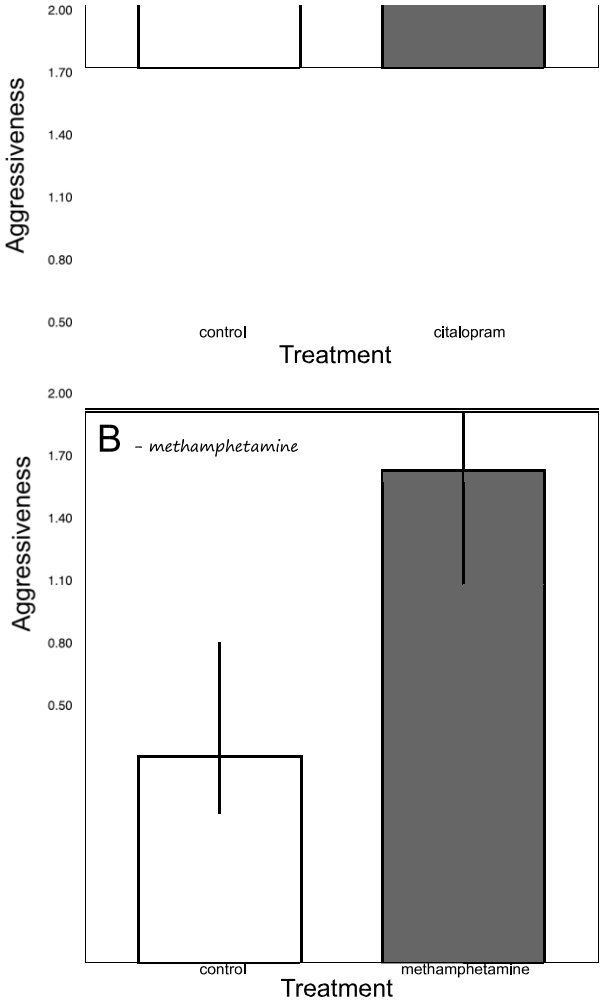


Fig. 1. Aggressiveness across the citalopram (A) and methamphetamine (B) treatment. Values (adjusted means +/- S.E.) are predicted from particular mixed model.

was no significant effect of sertraline treatment until accounting for the effective level of its brain concentration.

4. Discussion

All selected psychoactive compounds influenced the aggressive behaviour of chub at environmentally relevant concentrations; however, their effects varied and were detectable under different statistical methods.

4.1. Citalopram

In general, exposure to citalopram resulted in an increase in aggression. In previous studies, the influence of citalopram on fish aggression varied from a nonsignificant effect in three-spined stickleback (*Gasterosteus aculeatus*; 1.5 µg/L for 31 days; Kellner et al., 2016) and rainbow trout fry (*Oncorhynchus mykiss*; 1 µg/L for 6–7 days;

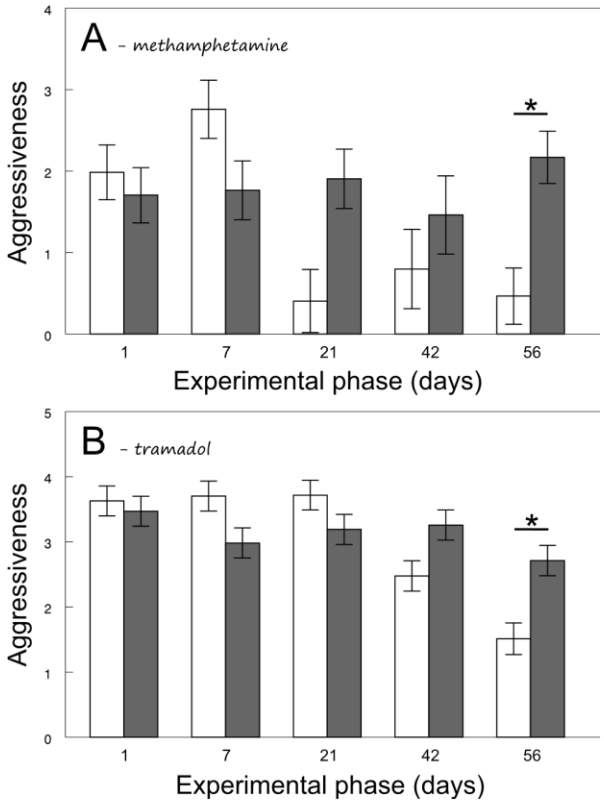


Fig. 2. Aggressiveness across the experimental phase. Shaded bars indicate methamphetamine (A) and tramadol (B) treatment. Values (adjusted means +/- S.E.) are predicted from particular mixed model. Asterisks indicate significant differences.

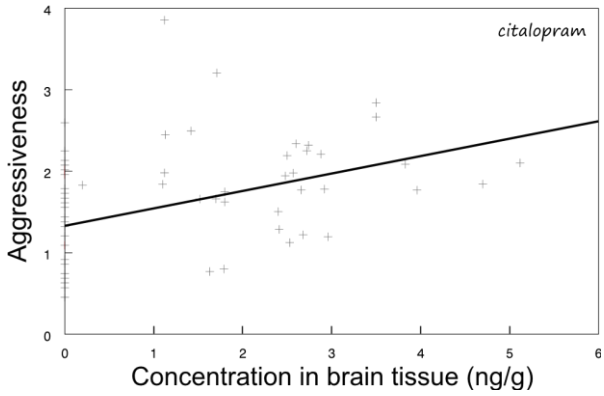


Fig. 3. Aggressiveness in relation to citalopram in brain tissue. Values are predicted from particular mixed model.

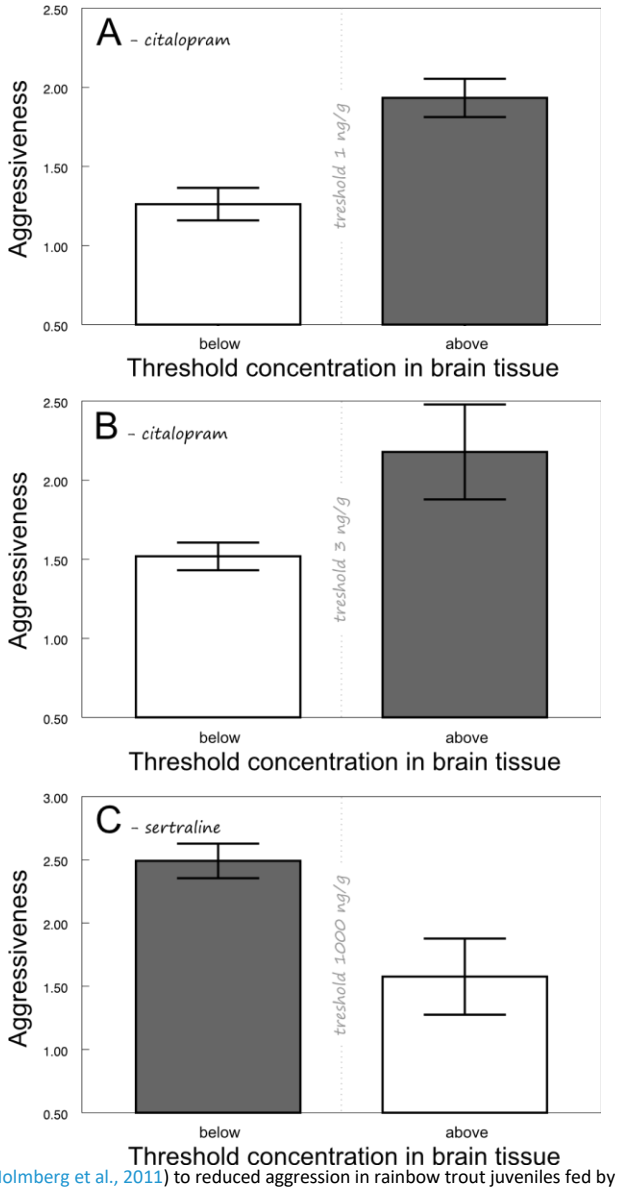


Fig. 4. Aggressiveness across citalopram (A –1 ng/g; B –3 ng/g) and sertraline (C –1000 ng/g) threshold concentration in brain tissue. Values (adjusted means +/- S.E.) are predicted from particular mixed model.

Holmberg et al., 2011) to reduced aggression in rainbow trout juveniles fed by contaminated pellets with an approximate intake of 100 µg of citalopram per 1 kg of fish body mass (Lepage et al., 2005) and three-spined stickleback (maximal dose 380 ng/L for 21 days; Kellner and Ols´en, 2020).

In humans, citalopram was noted to decrease anger and hostility compared to placebo at a dose of 40 mg (Kamarck et al., 2009). In rodents, citalopram at a low dose (maximal dosage to 0.00025 µmol/kg) caused anxiogenic responses, at an intermediate dose (maximal dosage 0.25 µmol/kg) induced anxiolytic responses and at the highest concentrations (2.5 µmol/kg) had no effect (Sanchez and Meier, 1997´). The anxiety of rodents is usually accompanied by changed aggression (Kuniishi et al., 2017; Walker et al., 2017).

The study of Sanchez and Meier (1997) ´ on rodents may explain the behavioural change in fish. The citalopram intake by chub in the present study could have been very small and thus could have caused the anxiogenic responses noted in the case of rats in Sanchez and Meier (1997)´ . On the

"high dose" from the study of Sanchez and Meier ´ (1997) for three-spined stickleback as no effect was found. Therefore, this could be the reason why the smaller dose of 0.38 µg/L caused a decrease in aggression (Kellner and Ols´en, 2020), as it could represent the "intermediate dose". This would indicate different sensitivity towards citalopram among species. Additionally, a specific concentration of citalopram in a single location could evoke divergent responses in different species and disturb the balance among them.

4.2. Sertraline

There was no detectable effect of sertraline on chub aggression in the present study when accounting for the traditional treatment/control comparison. However, after accounting for the innovative pollutant threshold concentration method, it was found that a concentration of 1000 ng/g sertraline in brain tissue was required to decrease the aggression of fish. This level was breached between the 7th and 21st days of exposure (Hubena et al., 2020a´). Sertraline decreased aggression in Siamese fighting fish (*Betta splendens*) after 7 and 14 days of exposure to waterborne concentrations ranging from 0.4 to 4 µg/L (Kania and Wronska, 2015´). Thus, a similar effect on aggression was found after the threshold of brain concentration was breached.

We highlight the importance of the exposure length. The threshold was breached between the 7th and the 21st day of exposure in the present study, while the Siamese fighting fish lowered its aggression already after 7 days (Kania and Wronska, 2015´). Therefore, certain species could react faster to compounds. One of the factors contributing to the early effects could be the relatively higher temperature (22–24 °C) in the Siamese fighting fish tanks (Kania and Wronska, 2015; ´ Freitas et al., 2020).

In comparison to other species, aggressiveness decreased under exposure to sertraline in *Anolis carolinensis* (Larson and Summers, 2001) and increased in virile crayfish (*Orconectes virilis*; Woodman et al., 2016). At a concentration of 100 µg/L, aggression increased in Siamese fighting fish in the same experimental setting (Kania and Wronska, ´ 2015). Thus, sertraline could induce different effects depending on the concentration. The maximal concentration found in the literature detected in effluent from sewage treatment plants was 1.93 µg/L (Mole and Brooks, 2019). From an ecological perspective, the concentration of 100 µg/L is very high and is not found in open waters. Consequently, the fish in polluted streams would be more likely to express decreased aggression. Furthermore, we recommend using the analysis of pollutant concentration in brain, which allows for further calculation of the pollutant threshold level, which enables finding more intricate effects. *4.3. Methamphetamine*

The environmental level of methamphetamine induced aggressiveness in chub in the present study. Information on the aggressiveness of fish under methamphetamine was not previously available to our knowledge. However, the increase in aggressiveness under chronic methamphetamine exposure is relatively well established among rodents (Sokolov et al., 2004; Wang et al., 2019) and humans (Mcketin et al., 2006; Sommers and Baskin, 2006; Payer et al., 2011; Lederer et al., 2016; Leslie et al., 2018; Uhlmann et al., 2018; McKetin et al., 2020). The concentrations of methamphetamine used in rodents and humans are aimed at therapeutic concentrations. Using a concentration of 1 µg/L, the fish plasma concentration should reach the therapeutic dose in the case of methamphetamine (Sancho Santos et al., 2020). Therefore, there is clear evidence that methamphetamine can be used to induce aggression even in fish at environmental concentrations.

In rodents, changes in the striatum and prefrontostriatal circuit under chronic treatment with methamphetamine have been suggested to be involved in manifestations of aggression (Sokolov et al., 2004). Additionally, in mammals, impulsivity in methamphetamine-dependent groups is linked to both dopaminergic and serotonergic systems (Lee et al., 2009; Wang et al., 2019). Curiously, the fish showed the same behavioural response to methamphetamine, even though the typical mammalian neocortex is not developed. In conclusion, the increase in aggressiveness under methamphetamine exposure is well known in mammals; however, such information was missing for fish. Therefore, fish could be substitutes for rodents in related studies, as they show comparable results, and their usage in laboratories has an increasing trend due to 3R strategies in animal research.

Recently, fish were demonstrated to suffer from withdrawal to methamphetamine (Horký et al., 2021). The withdrawal of methamphetamine can increase aggression in humans (Sekine et al., 2006; Hamilton and Goeders, 2010); however, it does not belong to the most prominent withdrawal symptoms (McGregor et al., 2005; Zorick et al., 2010). Aggression during methamphetamine withdrawal was associated with the density of serotonin transporters in cortical and cingulate areas in humans (Sekine et al., 2006). Chub increased its aggression after the depuration period in the present study despite the fact that the neocortical structures are not reliably proven in the fish brain.

The withdrawal symptoms of methamphetamine in humans can last from three to five weeks; however, the symptoms are most prominent in the acute phase, usually lasting a week (McGregor et al., 2005; Zorick et al., 2010). We found a significant increase in aggression after two weeks of depuration; therefore, the aggression would already be declining from the peak. This is in agreement with the study of Horký et al. (2021), who demonstrated a decline in amphetamine (a metabolite of methamphetamine) in the brain during the depuration period related to withdrawal symptoms. In contrast to the study of Horký et al. (2021), amphetamine was not responsible for the change in aggression in the present study. Instead, the persisting methamphetamine concentration found in Hubena et al. (2020a) ´ caused the effects. In addition to Horký et al. (2021), who noted a change in locomotion activity as a withdrawal symptom, the present study also includes heightened aggression found in fish exposed to methamphetamine.

4.4. Tramadol

Addiction to tramadol has received less attention. Tramadol abuse itself may be associated with violent acts in humans (Fawzi, 2011). Chronic exposure to 20 mg/kg tramadol increased aggression in rats (Balogun and Osuh, 2020). Such an effect of chronic exposure was not proven in the present study.

Tramadol caused increase in aggression of fish during depuration period as a possible withdrawal symptom in the present study. Humans after prolonged intake of tramadol may experience withdrawal symptoms, including increased aggression (El-Hadidy and Helaly, 2015; Sidana et al., 2019). Tramadol acts on the serotonergic system, indicating the source of the change in behaviour (Hopwood et al., 2001; Fawzi, 2011). Information about its effects on the aggression of fish is scarce, even though tramadol is a highly detected compound in aquatic environments (Fedorova et al., 2014; Grabicova et al., 2020b; Sancho ´ Santos et al., 2020). To conclude, our results indicate that abstinence from tramadol caused a withdrawal symptom manifested as an aggression increase in chub.

4.5. Threshold level of pollutants in brain

The lowest observable effect concentration (LOEC) was usually calculated in ecotoxicology to find the lowest aquatic concentration inducing behavioural changes (Ferrari et al., 2003; Triebskorn et al., 2007). However, LOEC was questioned in subsequent studies (Laskowski, 1995; Warne and van Dam, 2008). Recently, an innovative analysis of pollutant concentration in brain has emerged for a more precise estimation of the effect of psychoactive compounds (Borik et al., 2020; Hubena et al., 2020a; Santos et al., 2021´).

Our results indicate that the pollutant concentration in brain and its threshold level approach can be an important tool in this respect. The two SSRIs (citalopram and sertraline) possessed a different uptake pattern (Grabicova et al., 2017), resulting in specific behavioural outcomes, which would be unrevealed without determination of threshold concentration of compounds. There are also dissimilarities in the bioaccumulation of the compounds in the brain. Citalopram brain concentration did not show any progression during the 42 days of exposure, while sertraline concentration increased significantly (Hubena et al., ´ 2020a). The brain uptake of sertraline could have been magnified by its high lipophilicity, overcoming that of citalopram (Grabicova et al., 2014, 2017; Grabicova et al., 2020a´). The high lipophilicity parameter indicates better cellular penetration to assure its

biological action (Angelov et al., 2008). Sertraline consequently shows a higher bioaccumulation factor than citalopram, calculated from the concentration in tissue and the concentration in water (Grabicova et al., 2014, 2017; Arnnok et al., 2017). Therefore, the difference in brain uptake due to inequal lipophilicity may be one of the explanations for the varying results of the behavioural outcome. The concentration thresholds of methamphetamine and tramadol in brain were not found in the present study. We assume that the threshold of these compounds was out of the range of the concentrations found in the fish brain in the present study.

4.6. Limits of the study

The calculation of pollutant in brain has a potential for comparison of behaviour found in laboratory and field studies. We assume that pollutant concentration in brain could differ among species because, for instance, amounts of certain compounds could increase with trophic level (Rojo et al., 2021). The calculation of threshold level triggering the behavioural differences could be useful in this sense for finding affected species in a polluted area. It is, however, necessary to compare this method for other compounds and find out what the thresholds are in different compounds and if they are similar among divergent species. Secondly, the concentration of psychoactive compounds in brain could indicate how strong the effects on fish behaviour will be; however, some pollutants does not have to reach brain to cause an effect. In this case, the calculation of its concentration in brain would probably not provide the necessary support for the findings. Lastly, fish are exposed to wide array of compounds of different concentrations in their natural environments (Fedorova et al., 2014; Hossain et al., 2018). In addition, the compounds could interact between one another, compete in an organism for target molecules or work synergistically (Mason and Blackburn, 1997; Di Nica et al., 2017; Jijie et al., 2020, 2021). This limits the results of the present study, as the compounds were tested separately. However, we provide well-defined building stones for further studies testing these compounds in mixtures.

5. Conclusions

To conclude the findings, we demonstrated that all selected psychoactive compounds induced changes in the aggression of fish at environmentally relevant concentrations. However, there was a need to use different methodological approaches to prove the effect of individual compounds. The traditional treatment/control comparison successfully proved the effect of citalopram and methamphetamine, whereas the effect of tramadol was found only when accounting for such a difference after 14 days of withdrawal. Thus, tramadol affected the aggression of fish more during the depuration period indicating withdrawal symptoms. The effect of sertraline was highly specific and proven only after accounting for the innovative calculation of pollutant brain concentration threshold. Therefore, we recommend being cautious when reporting negative results of psychoactive substances on fish behaviour with one methodological approach only. We propose applying the analysis of pollutant concentration in brain and its threshold level to determine of behavioural changes in further studies to reduce false negative results.

CRediT authorship contribution statement

Pavla Hubena´: Investigation, Writing – original draft. **Pavel Horký**: Formal analysis, Writing – review & editing. **Roman Grabic**: Methodology, Investigation. **Kate˘rina Grabicova´**: Methodology, Investigation. **Karel Douda**: Resources, Writing – review & editing. **Ond˘rej Slavík**: Resources, Project administration. **Toma´˘s Randak´** : Conceptualization, Supervision, Funding acquisition.

Declaration of Competing Interest

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

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Summary Discussion

Generally, we found significant differences between the control and drug-exposed groups of fish. Each compound caused a different set of behavioural changes depending on their physico-chemical properties, toxicokinetics and toxicodynamics. The results from the published articles will thus be discussed in subchapters dedicated to each compound. The structure of the text of subchapters reflects the scientific hypotheses in the following order: (i) the effect of the compound on fish condition and behaviour during exposure; (ii) the effect of the compound on fish behaviour and condition during a depuration period; (iii) the relationships between compound concentration in the brain and behavioural indices.

9. Citalopram

Citalopram has not caused any overall change in the condition of chub throughout the trial (Chapter 5). No significant differences in condition were found even among individual days of exposure. The Fulton's condition factor is calculated from length and weight measurements (Rätz & Lloret 2003) and it is thus possible to compare the results of the study in Chapter 5 with literature. The weight of three-spined sticklebacks exposed to citalopram (0.15 μg/L and 1.5 μg/L) did not change throughout a three-week exposure (Kellner et al. 2015). This would comply with the results we found in our study (Chapter 5). Although, it is necessary to mention that the food intake of fish in the study of Kellner et al. (2015) was lower, which could potentially lead to lower condition after longer exposure. The aggressiveness of exposed chub was significantly higher than the aggressiveness of control fish in our study (Chapter 8). Generally, the effect of citalopram on aggressiveness varies in literature. There was no significant effect detected after 31 days of exposure to 1.5 μg/L in three-spined sticklebacks (Kellner et al. 2016) and after 6 and 7 days of exposure to 1 μg/L in rainbow trout fry (*Oncorhynchus mykiss*) (Holmberg et al. 2011). However, the dose of 380 ng/L for 21 days resulted in reduced aggression in three-spined sticklebacks (Kellner & Olsén 2020) and in rainbow trout juveniles fed by citalopram-contaminated pellets at a dose of 100 μg/kg (Lepage et al. 2005). Furthermore, there were diverging results of citalopram on anxiety of rodents (Sánchez & Meier 1997). The authors found that a low dose caused anxiogenic responses, an intermediate dose caused anxiolytic responses and a high dose induced no effect

(Sánchez & Meier 1997). These effects could be attributed to the dosage in water, the uptake of the compound caused by its physico-chemical properties and by the observed species as there are significant differences between metabolic rates of different species (Hill et al. 2017). All of these mentioned effects alter the internal dose that affects the neuroendocrinological system. The serotonergic system has been found to be very plastic in its activity, as low continuous activity is known to reduce aggression and inhibition increases aggression (Winberg & Nilsson 1993). Citalopram, an SSRI compound, prolongs the duration of released serotonin in synaptic cleft, and the neurotransmitter binds to its receptors (Stahl 1998a). However, serotonin also binds to its auto-receptors on the presynaptic terminal and inhibits another release of serotonin (Stahl 1998a). At this phase, the animal could perform heightened aggression based on an inhibited serotonergic system from the study of Winberg & Nilsson (1993). But this is not certain, as the activity pattern in specific brain areas should be measured. After two to three weeks of exposure (Rossi et al. 2008) therapeutic effects occur, which are probably caused by disinhibition of the serotonergic system or its adaptive change (Stahl 1998a). It is uncertain whether the citalopram dose could reach the therapeutic effects in Chapter 8, and it is thus possible that the change of aggression could be caused by the serotonergic system. But citalopram also shows low affinity for benzodiazepine receptors, where the $GABA_A$ receptor belongs. Miczek et al. (2003) reviewed that stimulation of this receptor may suppress aggression, but some compound such as alcohol may act on this receptor and heighten the aggression performed by the focal animal. Citalopram also shows affinity for D_2 receptors, which could induce aggression if activated in specific brain regions (Schwartzer et al. 2013). The underlying processes of the change should be investigated, and we suspect either the serotonergic system, D_2 receptors, or $GABA_A$ receptors are responsible for the change.

There were no significant differences in observed behaviours during the depuration period (Chapter 5 and 8). Thus, purification of waters from citalopram, using the modern methods of tertial treatment, could result in amelioration of the adverse effects caused by this compound.

The citalopram concentration in the brain was detectable from the first day of exposure and there was no trend of increase throughout the exposure (Chapter 5). The fish, in which concentration in the brain reached the level of 1 or 3 ng/g, started to manifest changes in aggressiveness in the present study (Chapter 8). This structural-breaks analysis is not applied in the literature, which does

not allow us to properly discuss the findings. However, ecotoxicological studies were trying to find a so-called “lowest observable effect concentration”, but the concentration they were evaluating was the aquatic concentration (Ferrari et al. 2003; Triebskorn et al. 2007). The physico-chemical properties of chemicals, such as the partition coefficient, may cause differences in uptake among compounds as lipophilic compounds penetrate cells with higher efficiency (Angelov et al. 2008). The pharmacological studies are pursuing “the minimal effective concentration”. That refers to the minimal concentration of compound in plasma that assures its therapeutic effects (Loftsson 2015). Thus, this variable is already purified by its uptake to organisms and devoid of significant effects such as the first-pass effect. Receiving blood samples from small laboratory fish may be challenging and there was also a study from the field showing no presence of compounds in fish plasma, even though the compounds were detected in the brain (Grabicova et al. 2017). Additionally, the brain is also the important integrative centre crucial in manifestation of behaviour. Therefore, we promote analysing the brain for the compound concentration and subsequent comparison with behavioural indices.

10. Methamphetamine

Methamphetamine caused significant increase in condition and aggression of fish throughout the exposure (Chapter 5 and 8). The increased condition was unexpected (Chapter 5). This compound is known to cause weight loss in humans (Cho & Melega 2002) as well as in rats (Berquist et al. 2020). The reduction of body weight in mammals is often associated with induced hyperthermia (Fukumura et al. 1998; Rusyniak et al. 2012; Halpin et al. 2013). This cannot happen to fish as they thermally conform to their environment. If weight loss would be caused only by hyperthermia, then there would be no effect detected in the case of fish. Significant decrease in body weight, however, happens in rats also in doses that should not induce hyperthermia (Berquist et al. 2020). Another explanation was offered by Kobeissy et al. (2008), who associated hypophagia with changes in the serotonergic system under methamphetamine exposure. Recently, methamphetamine was demonstrated to cause significant degeneration of adipose tissue in rats (Jaafari-Sheybani et al. 2021), which could be offered as an explanation for weight loss in rats. To our knowledge, this information was not demonstrated on fish in the literature. However, certain fish species were proved to feed more under methamphetamine exposure (Wang et al. 2021b), which could

potentially lead to an increase of condition found in the present thesis (Chapter 5). Methamphetamine also increased general aggression of fish (Chapter 8). There is a scarcity of information about this effect on fish in the literature. But the increase of aggression under exposure to methamphetamine is well-known in rodents (Sokolov et al. 2004; Wang et al. 2019) and well-established in humans as well (Sommers & Baskin 2006; Payer et al. 2011; Lederer et al. 2016; Leslie et al. 2018; Uhlmann et al. 2018). This effect could be associated with methamphetamine's impact on the serotonergic system, but this interaction was found to be minimal on aggression (Payer et al. 2012). The change could have been attributed to methamphetamine binding to the dopamine transporter and releasing the neurotransmitter, which activates dopaminergic receptors (Schwartzer et al. 2013). However, regional monoamine turnover should be measured to investigate the underlying changes induced by this drug. Generally, the found effects were not well described in scientific literature on fish and the underlying mechanisms of these effects should be investigated in further studies.

The increased condition of fish persisted throughout the depuration period in the present thesis (Chapter 5). Also, aggression was significantly higher in exposed fish compared to control even after 14 days of depuration (Chapter 8). The effect on condition is driven by the methamphetamine concentration in the brain, which is discussed in the paragraph below. However, the aggressiveness increase during the depuration phase is interesting. Sudden cessation of administration of many drugs may result in withdrawal symptoms, such as the case with benzodiazepines (Reidenberg 2011; Brett & Murnion 2015) and methamphetamine in our study (Chapter 7). The withdrawal effects of some compounds may even lead to life-threatening events (Reidenberg 2011). Withdrawal from methamphetamine was characterized on humans for the first three weeks of abstinence in the study of McGregor et al. (2005). The first week is governed by the acute effects of withdrawal, which include sleepiness, hyperphagia, anhedonia, motor retardation, agitation, anxiety, and craving (McGregor et al. 2005; Mancino et al. 2011). These symptoms persisted at low levels for another week (McGregor et al. 2005; Mancino et al. 2011). The acute phase of withdrawal is investigated in Chapter 7 of this thesis and shows that exposed fish tended to stay in methamphetamine-contaminated area and could indicate craving for the drug. The fish withdrawal symptoms detected after 14-day detoxification should be much lower than in the acute withdrawal

phase. One of the less mentioned symptoms of withdrawal in humans is the increase in aggression (Payer et al. 2012), which is an object of study in the present thesis (Chapter 8). This effect was explained by a reduced number of serotonergic transporters in the human brain persisting even throughout abstinence (Sekine et al. 2006). Fish aggression could follow a similar pattern in the brain; however, this should be investigated further. Withdrawal from methamphetamine in rodents was found to also alter multiple genes involved in neuroendocrinological transmission, explaining the found changes (Cadet & Brannock 2015). The preference for contaminated water and the increase in aggression is interpreted as a sign of methamphetamine withdrawal. The most severe effect of methamphetamine administration is thus the addiction potential in wild fish.

The concentration of methamphetamine in the brain was positively detected in fish brains throughout exposure and persisted there even after depuration in lower amounts (Chapter 5). The condition positively scaled with methamphetamine concentration in the brain during exposure (Chapter 5). Detoxication was not enough to cleanse fish of the compound, even though elimination half-life for this compound in humans is approximately 10 hours (Cruickshank & Dyer 2009). Considering that there was no bioaccumulation of the compound observed and yet the compound lasted in the brain throughout depuration (Chapter 5) could indicate slower metabolization, or the excreted methamphetamine could have re-entered their body. The excretion of methamphetamine can reach up to 50 % (Cruickshank & Dyer 2009), which could make the compound available for repeated uptake to fish bodies. The persistent level of methamphetamine in the brain was, however, responsible for heightened aggression (Chapter 8), and this information should thus not be dismissed. Nonetheless, the acute phase withdrawal (first 4 days) resulted in fish with amphetamine present in their brain to prefer methamphetamine-contaminated water in our study (Chapter 7). Also, amphetamine was associated with less movement activity (Chapter 7) and that was therefore characteristic for fish that were in the process of methamphetamine detoxification. As mentioned above, one of the prominent symptoms of human withdrawal includes motor retardation (McGregor et al. 2005; Mancino et al. 2011). This effect could have been reduced in rats that were exposed to environmental enrichment in addition to methamphetamine (Hajheidari et al. 2015). The opposite effect (increased locomotion) was true for methamphetamine concentration in fish brains, which was found in fish inhaling methamphetamine from the contaminated source in the preference test

(Chapter 7). The locomotory stimulation by methamphetamine was attributed to serotonergic receptors in rats (Steed et al. 2011; Ortman et al. 2021). Interestingly, acute administration of amphetamine and methamphetamine to rodents was found to increase locomotion in both cases (Hall et al. 2008, 2009). This effect was inhibited by silencing the D1 receptor (Hall et al. 2009). Thus, both amphetamine and methamphetamine must be evaluated for explanation of behavioural effects found in animals.

11. Sertraline

Sertraline generally decreased the condition of fish throughout the exposure (Chapter 5). This effect was significant from the 21st day of exposure in our study (Chapter 5). In addition, we observed decreased food intake, heightened mortality, and no general effect on aggression (Chapter 5 and 8). The effect of sertraline on food intake has already been the subject of interest in previous studies (Hedgespeth et al. 2014; Xie et al. 2015; Chen et al. 2017). But a significant decrease was observed only in concentrations higher than environmental during exposure lasting only 6 to 8 days (Hedgespeth et al. 2014; Xie et al. 2015; Chen et al. 2017). We propose in studies in Chapter 5 and 8 that the first three weeks of exposure to sertraline should be evaluated separately if the chronic effect of the compounds are focused, as we could not find a significant effect on behaviour and condition during this interval. This complies with data published in human medicine, where the compound is believed to manifest its therapeutic effects after two to three weeks of exposure (Rossi et al. 2008) when the serotonergic system plastically adapts to SSRI effects (Stahl 1998a). The change in food intake could have resulted in a lower weight of individuals at a given length, thus in lower condition (Chapter 5). The food intake is well-known to be influenced by the serotonergic system in fish (Lin et al. 2000). The condition of fish might consequently lower the probability of survival (Chapter 5). The effect of sertraline on fish mortality was also observed previously in the study of Schultz (Schultz et al. 2011). The increase of mortality was significant even at a much lower dose of 5.2 ng/L in the case of fathead minnow (Schultz et al. 2011). The effect on mortality is a severe adverse effect suggesting a concern over the presence of this compound in waters. Sertraline mode of action is the inhibition of the reuptake transporter SERT (Murdoch & McTavish 1992). This mode of action should assure chronically increased levels of serotonin in the synaptic cleft (Murdoch & McTavish 1992). However, even fish descending in social rank (subordinates)

manifest low continuous release of serotonin (Winberg & Nilsson 1993; Alanara et al. 1998; Cubitt et al. 2008; Vindas et al. 2016). Thus, we could compare our results (Chapter 5 and 8) with a manifestation of subordination. The subordination results in dramatic changes in fish behaviour. The most prominent signals of social subordination are lower aggressiveness, inhibition of food intake, reduced growth (Winberg et al. 1992; Olsen & Ringø 1999), and general behavioural inhibition (Winberg & Nilsson 1993). This information fits the results found in our studies in Chapters 5 and 8, and we suggest that sertraline could have induced behavioural inhibition in fish.

A depuration period of two weeks successfully eliminated the adverse effects of sertraline (Chapters 5 and 8). In other words, no effect on condition, food intake or mortality was found. Purification of waters of sertraline could be used to eliminate the negative consequences of its intake observed in our studies (Chapter 5 and 8).

Sertraline was found in brain tissue from the first day of exposure and its concentration in the brain grew with the length of exposure (Chapter 5). This indicates bioaccumulative properties of this compound, which was already proved in the study of Grabicova et al. (2017). This property is significantly influenced by the physico-chemical parameters of the chemical (Grabicova et al. 2017). Sertraline has a high partition coefficient (LogP = 5.51; NCBI 2021), which could facilitate substance uptake. The knowledge of the amount of sertraline in the brain allowed its comparison with condition and behavioural indices (Chapters 5 and 8). The higher the level of sertraline in the brain, the lower the condition, which proved a direct link between these parameters (Chapter 5). Additionally, there was no overall effect of sertraline on aggression (Chapter 8). But the structural-breaks analysis uncovered a threshold of 1000 ng/g of sertraline in the brain, from which a decrease in aggression was significant (Chapter 8). The application of the structural-breaks analysis allowed a discovery of deeper effects of compounds on behaviour, which could not be determined otherwise at this aquatic concentration (Chapter 8).

12. Tramadol

Tramadol did not cause any significant changes in overall condition of fish throughout exposure (Chapter 5). However, the behavioural indices differed significantly (Chapter 6). Exposed fish were generally less bold, less explorative, less active, and less social in our study (Chapter 6). There is a

scarcity of studies on tramadol causing differences in boldness. Only a recent study by Thörnqvist (Thörnqvist et al. 2019) associated boldness with the expression of opioid and dopamine receptors in fish. Perhaps the activation of the opioid receptors could have been affected by tramadol, but this assumption will require further investigation. Tramadol has also been observed to decrease exploration of a novel object in rats, which was assumed to signify impaired memory (Hosseini-Sharifabad et al. 2016). This is in accordance with the results of our study (Chapter 6), because exposed fish also explored the novel object less. However, the underlying mechanisms proving that memory was impaired were not investigated in our study (Chapter 6). We assume that the differences in novel object recognition are simply based on differences in exploratory behaviours (Chapter 6). The impact of tramadol on locomotion has been investigated in crayfish (Buřič et al. 2018; Ložek et al. 2019). Specifically, crayfish *Procambarus virginalis* (Lyko, 2017) and *Pacifastacus leniusculus* (Dana, 1852) moved shorter distances and with lower velocity in open field tests (Buřič et al. 2018; Ložek et al. 2019). This behavioural effect could have been diminished by the implementation of shelter in the tank (Buřič et al. 2018). Also, zebrafish embryos were less active when exposed to tramadol and this effect was translated as anxiolytic (Bachour et al. 2020). This could be supported by the fact that tramadol may be used as an anxiolytic drug because of its inhibitory role on serotonergic and noradrenergic reuptake in addition to the opioid mode of action (Dayer et al. 1994). On the contrary, tramadol-exposed crayfish were more reactive to acute stress (Ložek et al. 2019) and that would suggest that this compound could be anxiogenic. The contradictory conclusions may be caused by missing tests (cortisol assays, catecholamine reactivity) or species-specific effects, as tramadol shows differences in metabolization efficiency even among rats and humans (Epstein et al. 2006). We also could not determine whether anxiolytic or anxiogenic effects were in effect in this thesis as we did not measure the level of stress (Chapter 6). Shoaling has been suggested to depend on opioid stimulation as inhibition of opioid receptors reduces this behaviour in fish (Kavaliers 1981). The effect of tramadol on shoaling could be attributed to its effect on opioid receptors in our study (Chapter 6), but the neuroendocrinological parameters should be investigated to clarify the processes. Generally, the behavioural effects of tramadol on fish promote locomotory inhibition and asociality. It is unclear whether the found effects imply apathy or anxiety as cortisol/catecholamine reactivity have not been measured.

The abrupt cessation of tramadol administration resulted in a significant increase of aggression of wild fish (Chapter 8). As mentioned previously, the cessation of drug intake may lead to withdrawal symptoms, which may include heightened irritability in humans. Thus, we assume that the high aggression found after 14 days of depuration could have been an induced withdrawal symptom in fish (Chapter 8). Tramadol has previously been investigated for its abuse potential in humans and rodents (Epstein et al. 2006). Generally, the abuse potential for this drug in humans is not high (Epstein et al. 2006), but the data on rodents prove robust addiction potential for this compound. Rodents have been subdued to the conditioned place preference test, their tendency for self-administration and development of tolerance showing the drug's addiction potential (Szkutnik-Fiedler et al. 2012; Cha et al. 2014). The difference between humans and rodents in their addiction probability is the metabolization efficiency (Epstein et al. 2006). We could not determine whether the data on fish metabolization efficiency is rather similar to human or rodent reaction in our thesis (Chapter 8). However, they seem to develop at least one symptom of drug-withdrawal, aggression (Chapter 8). We hope that the present results could help in the investigation of this issue.

Tramadol was detected in fish brains from the first day of exposure but did not show any significant increase in its concentration (Chapter 5). The compound did not persist in fish brains throughout the depuration period (Chapter 5). However, the higher the tramadol concentration in the brain was, the lower the locomotion-related behaviours and shoaling was (Chapter 6). Tramadol can be found in open waters quite commonly; however, its concentration in the brain was not detected in fish from the field (Grabicova et al. 2017). This information should not imply safety from effects of compounds in the wild. Instead, it should be meant as a concern, due to increasing consumption of pharmaceuticals. The discussion would require more studies from laboratory and field detecting this compound in fish brains, which would allow for thorough comparison.

Conclusions

Each selected psychoactive compound induced behavioural effects characteristic to its unique mode of action and physico-chemical properties. For example, sertraline and methamphetamine had opposite effects on condition due to their mode of action. Even the compounds with a similar mode of action (sertraline, citalopram) differed in their effects based on their physico-chemical properties. It cannot be stated that the compounds behave as general stressors. Also, it cannot be expected that compounds with opposite behavioural effects in a single mixture would maintain the behaviour in balance, as they could affect one another by compound-compound interactions, differences in affinities, synergism, and antagonism (Mason & Blackburn 1997; Di Nica et al. 2017; Jijie et al. 2020, 2021).

Citalopram increased aggression of fish, sertraline induced behaviour typical for behavioural inhibition, methamphetamine increased the aggression condition and tramadol-exposed fish were less locomotory active and less social. Each of these effects would have a different impact on fish behaviour in the wild. Heightened aggression could result in injury (Neat et al. 1998), but aggressive individuals could also monopolize resources (Silva et al. 2013) and keep conspecifics away (Tinbergen 1957). The impact of citalopram and methamphetamine in the wild could therefore vary strongly depending on food availability, fish density and predation risk. Behavioural inhibition is a specific condition caused by neurotransmitters in the brains of fish, which reduces the food intake and aggression performed by subordinate individual (Winberg & Nilsson 1993). This mechanism could keep subordinate fish alive inside territory dominated by hostile dominant fish, but this behaviour is not optimal for individuals' fitness. Sertraline is thus highlighted as a compound of concern, as such its concentration in water and in brain should be monitored in the field continuously. The effect of tramadol is now still uncertain due to the discrepancy in behavioural testing as the cortisol level should have been measured. It is unclear whether this compound produces apathy or anxiety in fish.

The effectivity of depuration varied among compounds. Methamphetamine persisted in fish brains even after 14 days of depuration, although its concentration was decreasing. The depuration phase elucidated withdrawal effects from methamphetamine and tramadol in fish. This could indicate

abuse potential in fish. The behaviour of fish in the field could then be affected by their drug-seeking.

The concentration of compounds in the brain allowed the discovery of intricate effects on behaviour that could not be determined otherwise. For instance, there was no significant effect of sertraline on aggression until the structure-breaks analysis was performed, which enabled finding a threshold concentration causing decrease in aggression. These analyses have the potential to be employed in field studies for their comparison with laboratory results.

The results in the present study show that evaluation of behaviour under the exposure to a targeted compound requires several different behavioural tests, as a single test could result in falsely negative conclusions stating that the compound does not have any significant effect. The length of exposure to envision the chronic effects might require conducting experiments with prolonged exposure time. For example, seven-day exposure was not long enough to produce chronic effects in case of sertraline in this thesis. Certain behavioural effects thus require a much longer time to manifest. We recommend exposing fish for a prolonged duration of time at first and performing repeated testing at selected intervals, as was performed in our studies. This design enabled finding the shortest duration of time at which chronic effects may be observed.

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