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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 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 (Perugi et al., 2017). In fact, amphetamine-type drug consumption is dramatically increasing, so much so that 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 mechanisms of addiction by identifying the extent of methamphetamine and its metabolite amphetamine presence in brain tissues of brown trout, *Salmo trutta*. Brown trout 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

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(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 *ad libitum* 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 the whole 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

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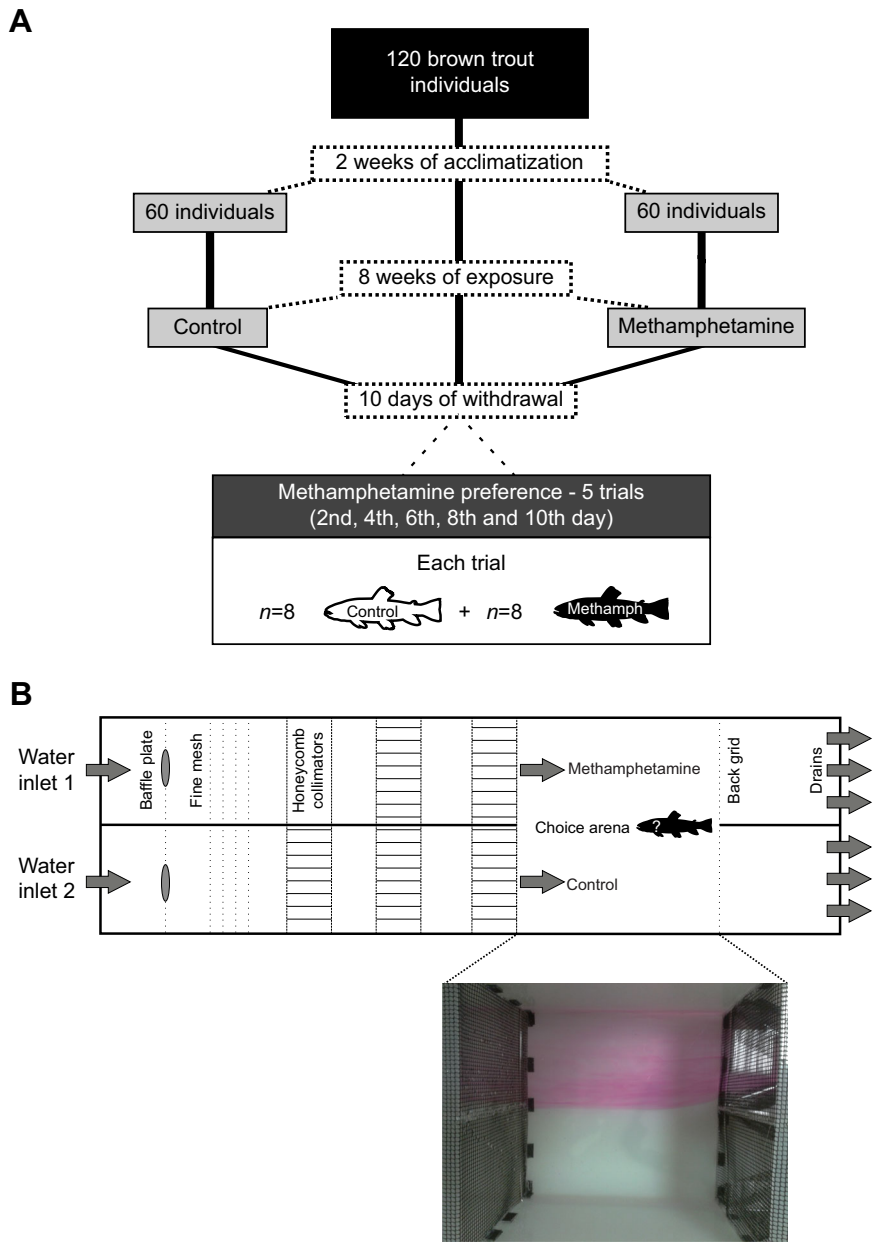

Fig. 1. Experimental testing. (A) Outline of the experiment. (B) Illustration of the two-current choice flume. Water from two different tanks fed the choice flume by gravity. Water flow through the baffle plates, fine mesh and honeycomb collimators creates two laminar, non-mixing currents in the choice 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 'probability of 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 particular classes. 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

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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 fold-change. 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 µg 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

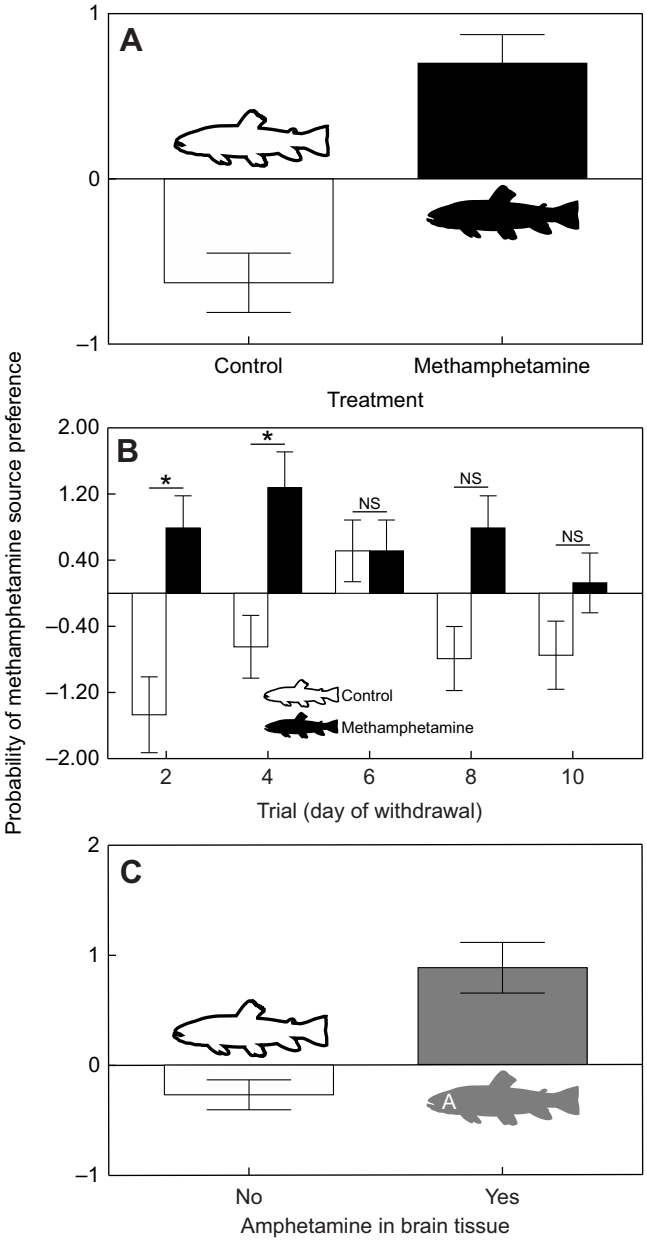

Fig. 2. Brown trout methamphetamine source preference. Probability of methamphetamine source preference (A) in relation to previous treatment, (B) across trials, i.e. days of withdrawal and (C) for amphetamine in brain tissue of focal individuals. Values are adjusted means±s.e. based on a mixed model 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

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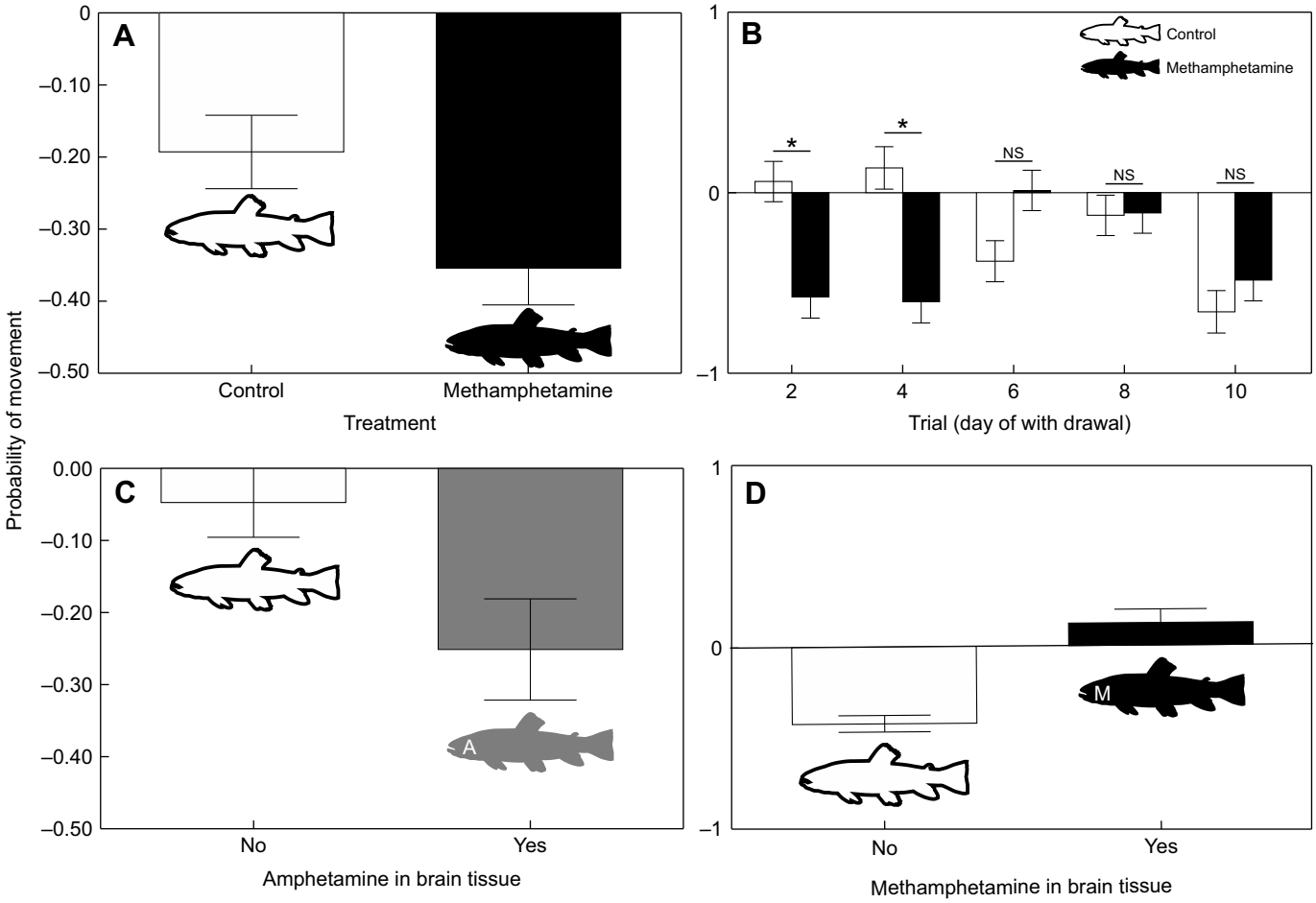

Fig. 3. Brown trout movement. Probability of movement (A) in relation to previous treatment, (B) across trials, i.e. days of withdrawal (B), and for (C) amphetamine and (D) methamphetamine in brain tissue of focal individuals. Values are adjusted means±s.e. based on a mixed model with random factor analyses (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).

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.

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 non-target 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 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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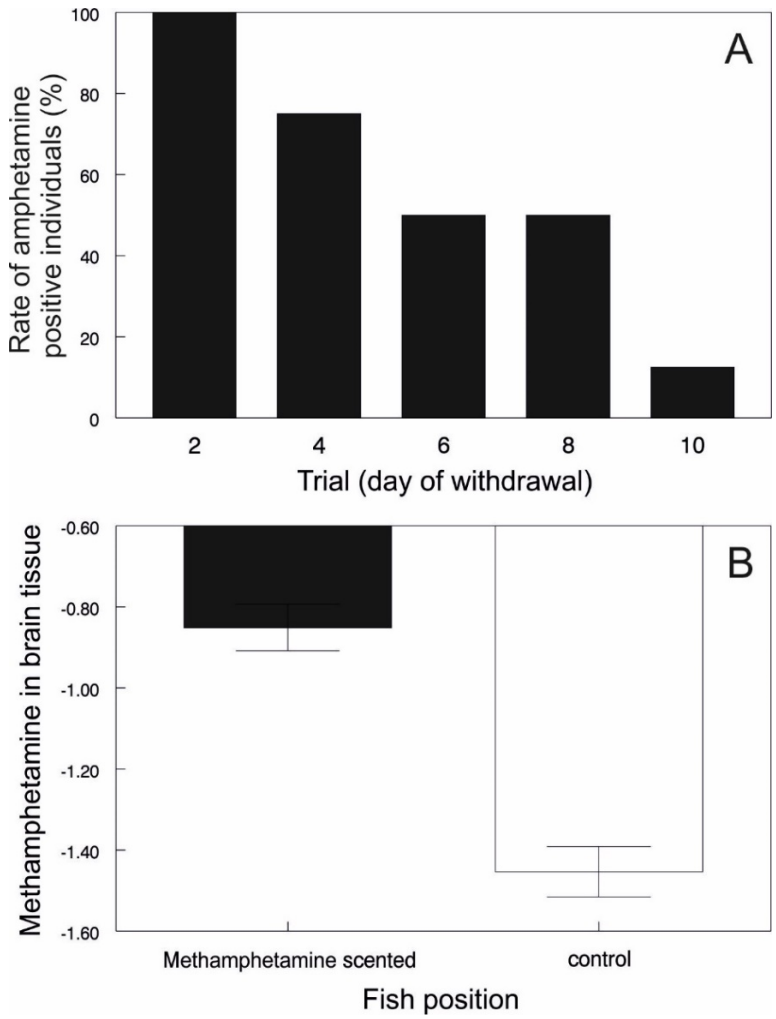

Fig. S1. Amphetamine and methamphetamine in brain tissue.

Rate of amphetamine positive brown trout specimen across the days of withdrawal (A) and probability of methamphetamine in brain tissue occurrence in relation to fish position during the preference test (B). The raw data in the every single trial in part A originates from the 8 previously exposed fish (i.e. rate 100% means 8 amphetamine positive fish). Values in part B are adjusted means ± SE based on 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 zero when the estimated probability is less than 0.50. Differences among classes in part B are significant (Adj. $P < 0.0001$).

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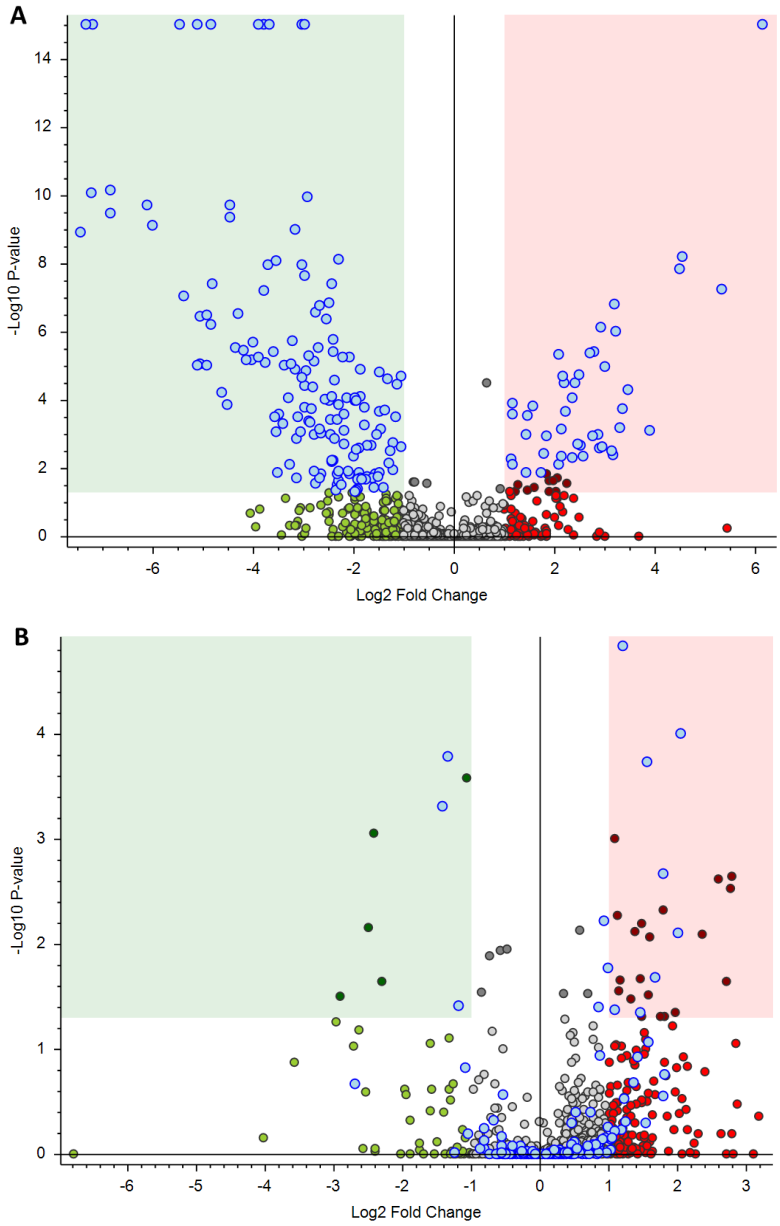

Fig. S2. Differential analysis of brain extracts.

Results of differential analysis of brain extracts between control and withdrawn fish groups in the 2nd day (A) and 10th day (B) of depuration. Colored squares (green down regulated and red up regulated) represent differences between sample and control groups at statistical significance of $P<0.05$ and at least one fold of magnitude difference in signal intensity. Light blue dots represent up and down regulated signal in exposed compare to control on 2nd observation day in both figures.

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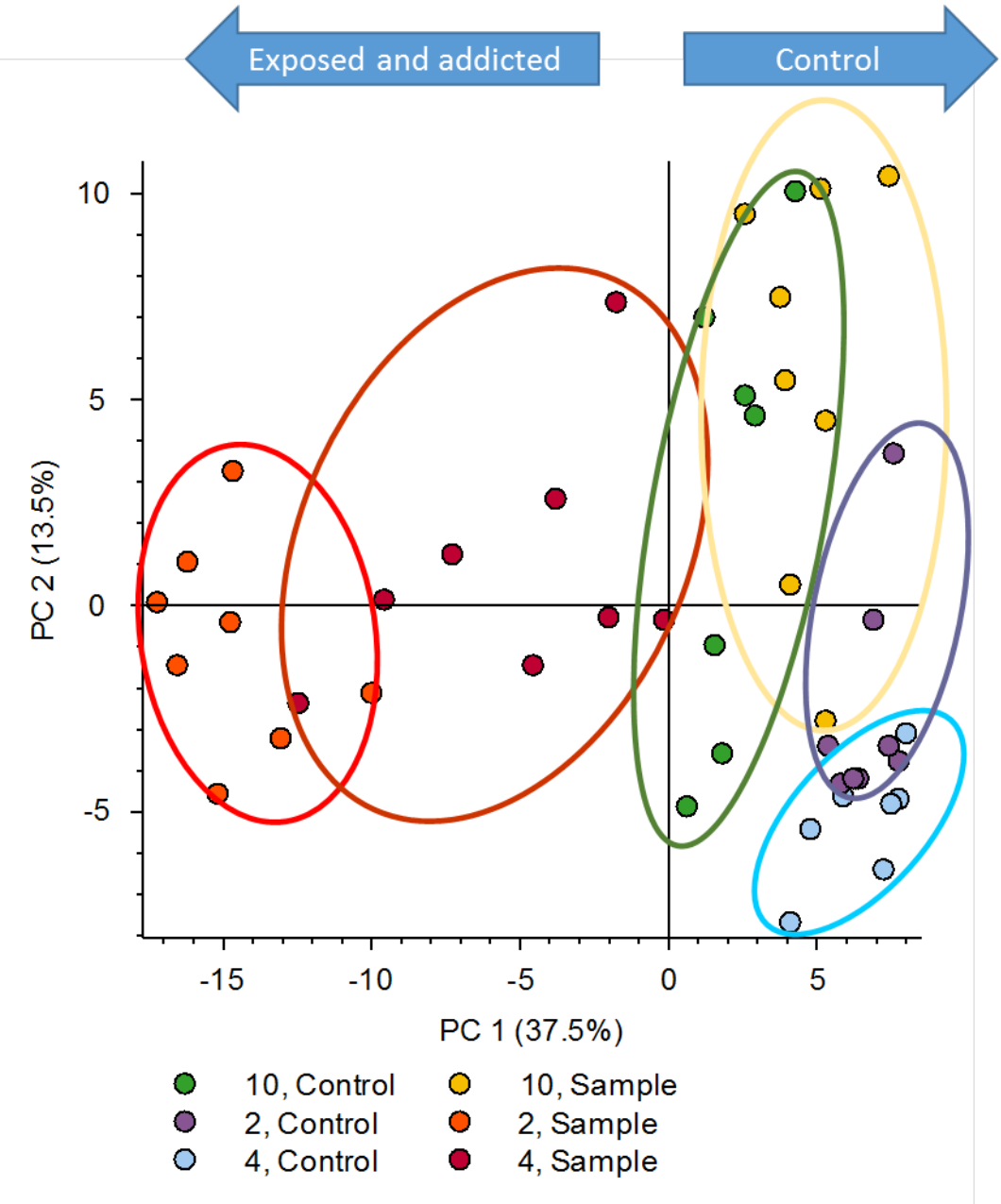

Fig. S3. Brain metabolites.

Principal component analyses of fish individuals from both control and exposed groups from 2nd, 4th and 10th day of depuration. Only significantly different signals (both up and down regulated with intensity difference at least one fold of magnitude and statistical significance at $p < 0.05$) from 2nd day were used as effect markers.

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