Science of the Total Environment 605–606 (2017) 578–588

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Exposure to wastewater effluent affects fish behaviour and tissue-specific uptake of pharmaceuticals

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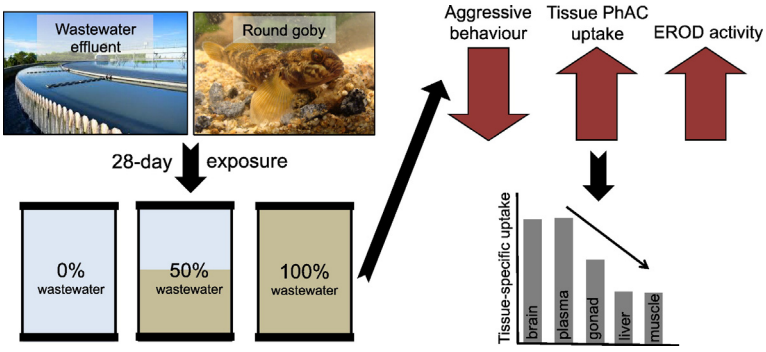
HIGHLIGHTS

- Round goby exposed for 28-days to 0, 50 or 100% wastewater effluent
- Exposure to 100% effluent reduced aggressive acts towards a mirror
- Exposure to 50% and 100% increased tissue uptake of pharmaceuticals
- Pharmaceutical uptake was greatest in brain ≥ plasma > gonads > liver ≥ muscle
- Increased hepatic EROD activity in relation to effluent exposure

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 March 2017
Received in revised form 9 June 2017
Accepted 9 June 2017
Available online xxxx

Editor: D. Barcelo

Keywords:
round goby
PhACs
PPCPs
Hamilton Harbour
EROD
aggression

ABSTRACT

Pharmaceutical active compounds (PhACs) are increasingly being reported in wastewater effluents and surface waters around the world. The presence of these products, designed to modulate human physiology and behaviour, has created concern over whether PhACs similarly affect aquatic organisms. Though laboratory studies are beginning to address the effects of individual PhACs on fish behaviour, few studies have assessed the effects of exposure to complex, realistic wastewater effluents on fish behaviour. In this study, we exposed a wild, invasive fish species—the round goby (*Neogobius melanostomus*)—to treated wastewater effluent (0%, 50% or 100% effluent dilutions) for 28 days. We then determined the impact of exposure on fish aggression, an important behaviour for territory acquisition and defense. We found that exposure to 100% wastewater effluent reduced the number of aggressive acts that round goby performed. We complimented our behavioural assay with measures of pharmaceutical uptake in fish tissues. We detected 11 of 93 pharmaceutical compounds that we tested for in round goby tissues, and we found that concentration was greatest in the brain followed by plasma, then gonads, then liver, and muscle. Fish exposed to 50% and 100% effluent had higher tissue concentrations of pharmaceuticals and concentrated a greater number of pharmaceutical compounds compare to control fish exposed to no (0%) effluent. Exposed fish also showed increased ethoxyresorufin-*O*-deethylase (EROD) activity in liver tissue, suggesting that fish were exposed to planar halogenated/polycyclic aromatic hydrocarbons (PHHs/PAHs) in the wastewater effluent. Our findings suggest that fish in effluent-dominated systems may have altered behaviours and greater tissue concentration of PhACs. Moreover, our results underscore the importance of

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http://dx.doi.org/10.1016/j.scitotenv.2017.06.073

characterizing exposure to multiple pollutants, and support using behaviour as a sensitive tool for assessing animal responses to complex contaminant mixtures, like wastewater effluent.

1. Introduction

There has been rising concern over the effects that wastewater treatment plant (WWTP) effluents have on wild aquatic animals (Strayer and Dudgeon, 2010; Sumpter, 2009). Part of this concern stems from the fact that WWTP effluents contain anthropogenic, endocrine-active contaminants like steroid hormones, bisphenol-A (BPA), personal care products, and pharmaceutical active compounds (PhACs; Klecka et al., 2010; Pal et al., 2010). Human consumption of PhACs has been increasing in developed countries (Bernhardt et al., 2017; Hemels et al., 2005; OECD, 2013), and traditional WWTPs remain ill-equipped to remove these compounds before wastewater effluent is discharged into the environment (Jelić et al., 2012). Accordingly, reports of PhACs in WWTP effluents and in surface waters have been increasing (Blair et al., 2013; Kolpin et al., 2002; Verlicchi et al., 2012). Although PhACs are specifically designed to modulate human physiology and behaviour, many other vertebrates have well conserved biological targets (e.g., receptors, enzymes) on which PhACs can act (Brown et al., 2014; Gunnarsson et al., 2008). When present in the environment, PhACs are usually found at low concentrations (ng l^{-1} – μg l^{-1}; Blair et al., 2013; Klecka et al., 2010; Kolpin et al., 2002; Pal et al., 2010). However, after chronic and continuous exposure, PhACs can concentrate in fish tissues (Brooks et al., 2005; Ramirez et al., 2009; Schultz et al., 2010). There is therefore a growing awareness that even low concentrations of PhACs may cause sub-lethal changes to animal physiology and behaviours important for survival and reproduction (Brodin et al., 2014; Hellström et al., 2016; Söffker and Tyler, 2012). But, how exposure to the complex PhAC mixtures found in WWTP effluent affects wildlife remains poorly understood (Backhaus, 2014).

Many classes of PhACs are present in wastewater effluent, but psychiatric pharmaceuticals are particularly concerning because of their potential effects on animal behaviour and physiology in the wild (Brodin et al., 2014; Calisto and Esteves, 2009; Corcoran et al., 2010). Compounds such as anxiolytics or antidepressants that are prescribed for treatment of human behavioural disorders are commonly measured in wastewater effluents and in surface waters (typically in low ng l^{-1}; Calisto and Esteves, 2009; Fick et al., 2017; Klaminder et al., 2015; Metcalfe et al., 2010; Verlicchi et al., 2012). Psychiatric pharmaceuticals are known to bioconcentrate in fish tissues, especially in the brain (Brooks et al., 2005; Grabicova et al., 2014), but also in fish plasma, liver, and muscle tissue (Heynen et al., 2016; Schultz et al., 2010). In the laboratory, fish behaviours have been altered following exposures to environmentally relevant concentrations of psychiatric pharmaceuticals. For example, the anxiolytic oxazepam increased boldness in European perch (*Perca fluviatilis*), making exposed fish more active and exploratory (Brodin et al., 2013). Likewise, the antidepressant fluoxetine reduced predator response behaviours in guppy (*Poecilia reticulata*), making them slower to respond to a threat (Pelli and Connaughton, 2015). While the number of studies connecting pharmaceutical exposures to changes in fish behaviour has grown (e.g., Dzieweczynski and Hebert, 2012; Greaney et al., 2015; Hedgespeth et al., 2014; Olsén et al., 2014; Painter et al., 2009; Weinberger and Klaper, 2014), there are also studies that have reported few changes to behaviour following exposure to environmentally relevant concentrations (Holmberg et al., 2011; Margiotta-Casaluci et al., 2014; McCallum et al., 2017a). The behavioural effects documented in the laboratory may not generalize to the wild because wastewater effluent contains a mixture of PhACs and other compounds.

To better address how wastewater effluent might affect fish behaviour, several studies have now measured behavioural endpoints following exposure to wastewater effluent in the laboratory or in the field. In the laboratory, Sebire et al. (2011) found that male three-spine stickleback (*Gasterosteus aculeatus*) exposed to 50% and 100% wastewater built fewer nests and spent less time courting female mates. Garcia-Reyero et al. (2011) and Martinović et al. (2007) found that male fathead minnow (*Pimphales promelas*) exposed to 100% wastewater effluent were less aggressive and less successful at securing a nesting site against unexposed competitors. In one of the only studies on fish collected directly from a wastewater-exposed field site, Saaristo et al. (2014) found that exposed male mosquitofish (*Gambusia holbrooki*) courted females more than males from a reference location. The few studies reviewed here have focused primarily on reproductive behaviours, and they were partnered with measures of the estrogenic or anti-androgenic activity of the wastewater effluent as a potential cause of behavioural disruption. However, many other behaviours impact animal fitness (e.g., foraging, territory defense; Brodin et al., 2014; Zala and Penn, 2004). For instance, Melvin (2016) showed that short-term exposure to wastewater effluent reduced activity and swimming performance in empire gudgeons (*Hypseleotris compressa*). It is also worth noting that two studies have reported no change to fish behaviour following chronic exposures to WWTP effluent (McCallum et al., 2017b; Schoenfuss et al., 2002).

Of the handful of studies that have addressed the impacts of WWTP effluents on behaviour to date, none have measured PhAC uptake into fish tissues to characterize the extent of exposure. In this study, we used an invasive fish species, the round goby (*Neogobius melanostomus*), to test how chronic (28-day) exposure to wastewater effluent (at 0%, 50%, or 100% dilutions) affected fish behaviour alongside tissue-specific uptake of pharmaceuticals. We measured aggression as an ecologically relevant behavioural endpoint because round goby use aggression to acquire and defend sheltered territories from conspecifics and heterospecifics (Balshine et al., 2005; Bergstrom and Mensinger, 2009; Dubs and Corkum, 1996; Janssen and Jude, 2001). They use these shelters to reproduce and care for offspring during the breeding season (Corkum et al., 1998; MacInnis and Corkum, 2000), and without shelter they are susceptible to avian and aquatic predators (Belanger and Corkum, 2003; King et al., 2006; Reyjol et al., 2010; Somers et al., 2003). Round goby aggressiveness is also thought to have contributed to their invasion success in the Laurentian Great Lakes, Western Europe, and the Baltic Sea (Corkum et al., 2004; Kornis et al., 2012). We then investigated in which bodily tissues the pharmaceuticals concentrated the most, and predicted that fish exposed to 50% and 100% effluent would concentrate a greater number of PhACs and higher concentrations of PhACs in their tissues. We further hypothesized that round goby exposed to 50% and 100% effluent would exhibit reduced aggressive responses when compared to controls (0%) if we also detected psychiatric medications like antidepressants or anxiolytics in the exposed fish tissues. Such medications alter monoamine signaling to ameliorate depressive or anxious behaviours in humans (e.g., antidepressants improve mood via increased serotonergic signaling, anxiolytics sedate via increased GABA signaling: Argyropoulos and Nutt, 1999; Stahl, 1998), and prior laboratory exposures have accordingly found that psychiatric pharmaceuticals reduced fish aggression and altered social interactions (Brodin et al., 2013; McCallum et al., 2017a; Paula et al., 2015; Perreault et al., 2003).

Finally, because wastewater effluent is a complex mixture of PhACs and other pollutants, we also assessed 7-ethoxyresorufin-*O*-deethylase (EROD) activity in fish liver tissue. Cytochrome P450 enzymes are involved in Phase 1 metabolism of planar halogenated/polycyclic aromatic hydrocarbons (PHHs/PAHs) and other similarly structured xenobiotic

chemicals, and EROD activity is an indicator of CYP1A activity (Whyte et al., 2000). Such pollutants are common in WWTP effluent from combined sewer systems (like the one in our present study) as a by-product of petroleum combustion (Gasperi et al., 2010; Jones et al., 2012). We aimed to establish if fish that were exposed to PhACs were also exposed to other types of pollutants from WWTP effluents.

2. Methods

2.1. Fish collection and housing

We collected 72 (36 male and 36 female) round goby between July 10, 2013 and October 10, 2013 from LaSalle Park, Burlington, Ontario, Canada (43°17′59″N; 79°50′45″W). We collected fish using baited minnow traps (for detailed collection methods see McCallum et al., 2014; Young et al., 2010) and transported the fish live to McMaster University. We housed fish in same-sex groups of six and held them on a 14 L:10D light schedule in 75 l aquaria equipped with ~1 cm of natural gravel substrate and a static renewal filter (Aquaclear). We fed fish until satiation with Nutrafin Basix Staple Food once daily (~0.2 g per tank). After 24 h acclimation to laboratory conditions, we uniquely tagged fish using non-toxic acrylic paint along their dorsal fin to facilitate later identification in behavioural trials (Capelle et al., 2015; Wolfe and Marsden, 1998). We held fish for 72 h before transferring them to exposure tanks.

2.2. Wastewater effluent exposure

We exposed fish to treated wastewater effluent collected from the Woodward Avenue Wastewater Treatment Facility in Ontario, Canada. The wastewater effluent from this facility discharges into Hamilton Harbour, an International Joint Commission Area of Concern currently being remediated to improve water quality, aquatic habitats, and fish and wildlife health (International Joint Commission, 1999; Hall et al., 2006). The Harbour has been degraded from historical industrial activities, urban run-off, wastewater effluent inputs, and combined sewer overflows (Hamilton Harbour RAP, 1992). Woodward is a secondary conventional activated sludge treatment plant serving the populations of Hamilton, Stoney Creek, and Ancaster, Ontario, Canada (~400,000 population). Woodward treats wastewater from residences, businesses, industry, and storm sewers throughout the city, and handles wastewater from both a combined (40%) and separated (60%) sewer system (City of Hamilton, 2011). Wastewater effluent is discharged at an average flow of 3750 l s^{-1}, while the receiving stream has a considerably lower flow of 600 l s^{-1}, creating an effluent-dominated environment (Bowlby et al., 2009; City of Hamilton, 2011; Gudimov et al., 2011). We conducted point source collections of wastewater effluent after the final stage of treatment (secondary clarifiers) and immediately before it would be returned to the watershed. We collected new effluent every three days throughout the exposure period (details below), transported it in opaque plastic containers to McMaster University, and stored it at 4 °C in dark conditions to prevent degradation.

We exposed round goby to the wastewater effluent in the laboratory for 28 days. We conducted the exposures between September 25, and October 31, 2013, in 20 l aquaria equipped with 1 cm of natural gravel substrate, black PVC tubes for shelter, an airstone, and a static renewal filter (Aquaclear) with the carbon insert removed. We exposed fish in same-sex groups of six fish and groups were placed in one of three exposure treatments: 0% wastewater effluent (control), 50% wastewater effluent (low), or 100% wastewater effluent (high). Four replicate tanks were used for each wastewater exposure treatment. Prior to adding the effluent, we allowed it to equilibrate to room temperature (maintained at 19–21 °C) overnight under dark conditions. When necessary, we diluted the effluent with the appropriate volume of dechlorinated tap water to create the treatment concentrations before adding it to the exposure tanks. Every 48 h, we performed a 50% water change on the exposure tanks and re-dosed them with the appropriate treatment concentration of effluent and dechlorinated tap water. Throughout the exposure duration, we monitored fish survival, health and noted all mortalities.

We summarized water quality parameters of the final treated effluent across our collection period (Table 1). These measures included: total suspended solids, total phosphorus, total nitrogen, ammonia, nitrate, nitrite, carbaceous oxygen demand (cBOD), *E. coli*, and conductivity. This data was measured by Hamilton Water (Hamilton Water, 2013, *unpublished data*). All parameters were measured five days per week, except cBOD, *E. coli* counts and conductivity, which were measured only once per week. All measures were taken from composite samples of the final treated effluent over a 24-h period. In the laboratory, each day we monitored water quality from a randomly selected exposure tank representing each of the wastewater exposure groups (Table 1). We monitored: dissolved oxygen, temperature (La Motte Pocket Tracer), pH, conductivity, total dissolved solids and salinity (Oakton Multiparameter PCS Testr 35).

2.3. Behavioural assay

We assessed the effect of exposure to wastewater effluent on round goby aggressive behaviour using a mirror aggression assay. Mirror assays are increasingly being used to investigate aggression because they reduce the number of fish needed (no stimuli fish needed), remove any risk of inflicted harm (potentially elicited in a dyadic contest), and provide more experimental control and standardization (the variable motivation of stimulus fish is not a factor; Balzarini et al., 2014; Elwood et al., 2014; Wilson et al., 2011). Moreover, mirror assays are a repeatable measure of aggression in round goby (McCallum et al., 2017a). For each fish, we conducted behavioural trials on the final (28th day) of exposure. To conduct the behavioural assays, we used a 20 l experimental tank of identical dimensions to the exposure tanks, each also containing 1 cm natural gravel substrate, a static renewal filter with the carbon insert removed, and an airstone. Experimental tanks contained a mirror at one end, and this mirror was initially obscured from the focal fish by an opaque barrier. We tested fish in the same effluent concentrations as their wastewater treatment exposure condition to ensure that water quality parameters would not change between exposure and testing conditions. We filled the experimental testing tanks with the appropriate concentration of wastewater effluent (50% or 100%) or dechlorinated tap water (0%). We thoroughly cleaned experimental tanks and filters in between testing days. The behavioural trial would begin when we transferred a focal fish from its exposure tank to the experimental tank, with the opaque barrier in place to hide the mirror. We gave each fish 30 min to recover from handling and to habituate to the experimental tank. Then, we began the mirror assay by remotely removing the black opaque barrier and revealing the mirror to the focal fish (Fig. 1a). We video-recorded the subsequent 15-min trial (Canon HD Vixia HFS100 8.0 Megapixel) from behind an opaque blind to limit experimenter influence.

All videos were scored for aggressive behaviours towards the mirror. The researcher scoring the behavioural videos was blind to wastewater exposure treatment, and they based their behavioural scores on an ethogram constructed specifically for the round goby (see McCallum et al., 2017a, 2017b). For the mirror aggression assay, we recorded: 1) ***time taken*** *for the focal fish to move towards the mirror*, measured as the time elapsed from barrier removal to the first movement towards the mirror. This was used to indicate motivation to begin an aggressive interaction; 2) *the number of* ***aggressive acts*** *towards the mirror*; 3) *the number of* ***aggressive bouts*** *with the mirror*. In round goby, aggressive interactions are comprised of discrete bouts of multiple aggressive acts punctuated by periods of resting or non-aggressive activity (e.g., tank exploration, substrate manipulation; Sopinka et al., 2010). In the absence of a live competitor, we used bouts with the mirror as a measure of motivation to re-engage in aggression; and 4) *all* ***non-aggressive***

Table 1
Average (±standard error) water quality measures of the final treated effluent and laboratory exposure tanks during the exposure period. Final treated effluent data was provided by Hamilton Water and is taken daily from 24 h composite samples, tested by the Hamilton Water Environmental Laboratory following Wastewater Systems Effluent Regulations (Fisheries Act, SOR/2012-139), except for cBOD, *E. coli*, and conductivity, that were only measured once per week. Laboratory exposure tank measures were conducted daily on one randomly selected tank per treatment. cBOD = carbaceous oxygen demand, CFU = colony-forming units (of *E.coli*). Means with different letter superscripts are significantly different, ANOVA with Dunnet's post-hoc.

Final effluent	24 h composite samples $N = 28$		24 h composite samples $N = 5$
Total suspended solids mg l^{-1}	10.28 (±1.13)	cBOD mg l^{-1}	4.33 (±0.27)
Total phosphorus mg l^{-1}	0.51 (±0.024)	*E. coli*, CFU 100 ml^{-1}	10.00 (±0.00)
Total nitrogen mg l^{-1}	2.11 (±0.16)	Conductivity, μS cm^{-1}	1032.4 (±54.99)
Ammonia mg l^{-1}	0.88 (±0.13)		
Nitrate mg l^{-1}	13.15 (±0.37)		
Nitrite mg l^{-1}	0.23 (±0.014)		
Exposure tanks	Control (0%) $N = 31$	Low (50%) $N = 33$	High (100%) $N = 32$
Temperature °C	21.38^{a} (±0.12)	21.14^{a} (±0.17)	20.89^{a} (±0.15)
Dissolved oxygen mg l^{-1}	9.93^{a} (±0.093)	9.54^{a} (±0.14)	9.63^{a} (±0.12)
pH	8.33^{a} (±0.024)	8.20^{b} (±0.040)	8.13^{b} (±0.046)
Total dissolved solids ppm	275.74^{a} (±2.20)	610.27^{b} (±5.07)	891.16^{c} (±6.57)
Conductivity μS cm^{-1}	387.09^{a} (±2.89)	857.06^{b} (±7.01)	1253.56^{c} (±9.03)

behaviours (other activity: e.g., hops, swims, digging, substrate scraping) when not interacting with the mirror. We used this measure as a proxy for fish activity to measure whether wastewater exposure dampened all behavioural output more generally. Fish were considered to be interacting with their mirror image when they were oriented towards the mirror and within one body length of the mirror, or in direct contact with the mirror (e.g., attacking).

2.4. Morphological measures and tissue collection

Following the mirror aggression assay, we euthanized all fish with a cerebral concussion and spinal severance, measured body condition and reproductive status, and collected a number of tissues. We measured standard length (snout to caudal peduncle) using calipers accurate to 0.01 cm and body mass using a digital balance accurate to 0.001 g

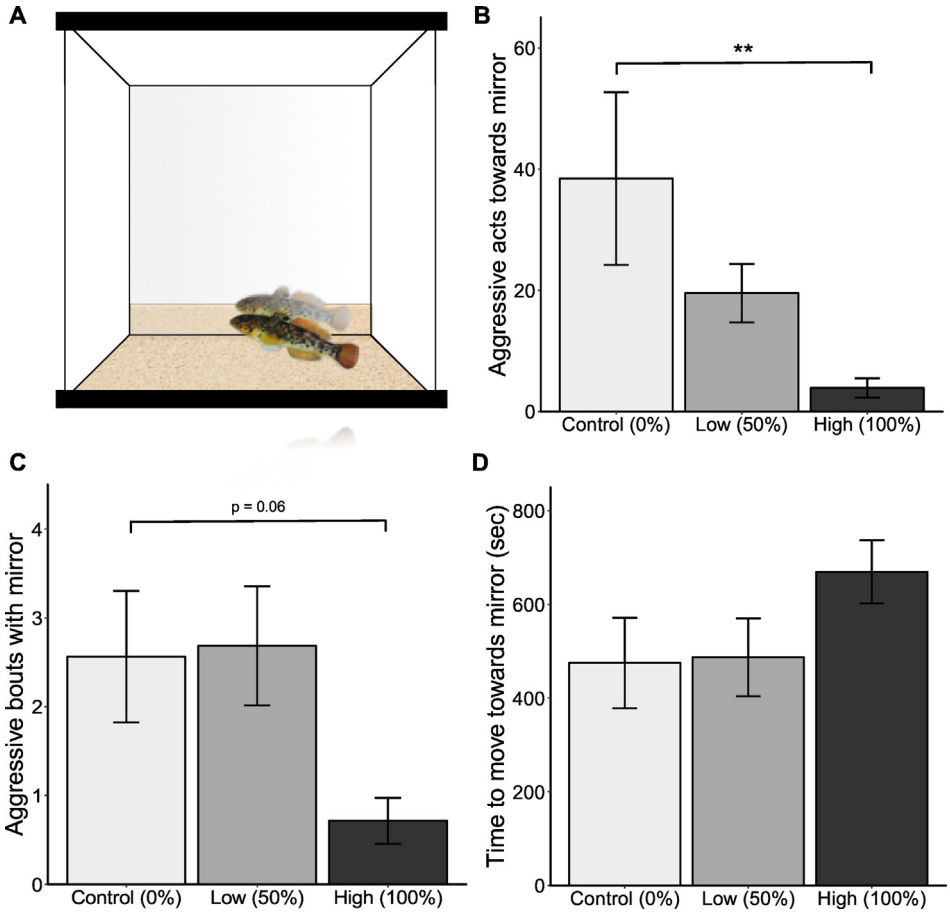

Fig. 1. Diagram of mirror aggression assay and behavioural results. **A**) Testing tank for the mirror aggression, showing a fish interacting with its mirror image (reprinted with permission from McCallum et al., 2017b). **B**) Number of aggressive acts performed towards the mirror. **C**) Number of discrete aggressive bouts with the mirror. **D**) Time taken for round goby to move towards the mirror. Error bars represent ±1 standard error. ** $p < 0.01$.

(Ohaus Adventurer Pro). Fish body size did not differ between treatment groups (mass, ANOVA $F_{(2, 54)} = 1.87$, $p = 0.16$; standard length, ANOVA $F_{(2, 54)} = 1.53$, $p = 0.23$); fish were (mean ± standard error) 8.95 ± 0.42 g in mass and 7.15 ± 0.11 cm in length. We used body length and body mass measurements to calculate body condition using Fulton's Index ([body mass / standard length]^3). We removed and weighed both the liver and the gonads and calculated a hepatosomatic index (HSI) using the liver weight as: [liver mass / (body mass – liver mass)]*100 and a gonadosomatic index (GSI) using the gonad mass as: [gonad mass / (body mass – gonad mass)]*100. We used GSI to assign reproductive status: male round goby were considered reproductive if they had a GSI > 1% and females were considered reproductive if they had a GSI > 8% (Marentette and Corkum, 2008; Zeyl et al., 2014). Note that given these established criteria for reproductive status, no females in our study were in reproductive condition and only one male fish was classified to be in reproductive condition.

Blood was collected via caudal vein puncture using a heparinized syringe, and was spun at 8000 *g* for 10 min (Eppendorf MiniSpin Plus 5453). The plasma supernatant was drawn off and frozen in liquid nitrogen. We removed, weighed, and froze the liver, brain, gonad, and muscle (taking a section from the dorsal-axial muscle) in liquid nitrogen. All tissues were stored at −80 °C for later EROD analyses (liver) and further quantification of pharmaceutical compounds.

2.5. Tissue pharmaceutical analysis

We determined tissue-specific uptake of 93 pharmaceuticals in the plasma, brain, liver, muscle and gonad tissues from our exposed fish. These 93 pharmaceuticals were selected based on their potencies and predicted ability to bioconcentrate in fish (Fick et al., 2010; see Supplementary Table 1 for full list of compounds). Liver, brain, gonad, and muscle tissue samples (0.1 g) were extracted sequentially after the addition of 50 ng of internal and surrogate standards, all internal and surrogate standards used have been presented in a previous publication (Grabic et al., 2012). Only female gonad tissue was extracted as male gonad tissue was of insufficient mass for analysis (note, all fish were in non-reproductive condition). Extraction of tissue samples were done with 1.5 ml acetonitrile, repeated twice. Samples were homogenized for four minutes at 42000 oscillations per minute, using a Mini Beadbeater (Biospec. Bartlesville, USA) with zirconium beads and then centrifuged at 17500*g* for 10 min (Beckman Coulter Microfuge 22R Centrifuge). This protocol was followed for both eluent mixtures individually and the supernatants were combined, evaporated to 20 μl and reconstituted in 100 μl methanol.

Plasma samples (20 μl) were pretreated by adding 50 ng of each internal surrogate standard, 50 μl methanol and 20 μl of water (with 0.1% formic acid), samples were then frozen at −18 °C for 1 h. Samples were thawed and centrifuged at 17500*g* for 10 min.

Samples were analyzed using a system with a triple-stage quadrupole mass spectrometer (Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, CA)) coupled with a liquid chromatographic pump (Accela, Thermo Fisher Scientific) and an autosampler (PAL HTC, CTC Analytics AG, Zwingen, Switzerland). Heated electrospray (HESI), krypton 10.6 eV, in positive ion mode were used for ionization of the pharmaceuticals. Specific details related to the determination of the pharmaceuticals including HESI ionizations, polarities, precursor/product ions, collision energies, tube lens values, etc. have been described in detail elsewhere (Grabic et al., 2012; Lindberg et al., 2014), an overview of the analytical method is presented in the supplementary information.

To ensure proper quality assurance and quality control, two MS/MS transitions were used for positive identifications of analytes with the criterion that the ratio between the transitions was not allowed to deviate more than ±30% from the ratio in the corresponding calibration standard. Retention times for all analytes also had to be within ±2.5% of the retention time in the corresponding calibration standard. Limit of quantification (LOQ) was determined from standard curves based on repeated measurements of low level spiked plasma and tissue samples, and the lowest point in the standard curve that had a signal/noise ratio of 10 was considered to be equal to the LOQ. A seven-point matrix adjusted calibration curve over the range of 0.05–100 ng ml^{-1} was used for linearity evaluation and quantification. Carry-over effects were evaluated by injecting standards at 100 ng ml^{-1} followed by two mobile phase blanks. Several instrumental and field blanks were included in each analytical run. LOQs and recoveries for tissues and plasma are presented in Supplementary Table 2 and Supplementary Table 3.

2.6. EROD assay

We assessed 7-ethoxyresorufin-*O*-deethylase (EROD) activity in round goby liver tissue as a biomarker of exposure to PHH/PAH pollutants in the wastewater effluent. EROD indicates CYP1A activity, and CYP1A proteins have been successfully identified in round goby and other gobioid species (Moore et al., 2003; Vincelli, 2016). We used a subset of $N = 22$ livers from our exposed males ($N_{Control} = 7$, $N_{Low} = 6$; $N_{High} = 9$), and followed a protocol specific to round goby (adapted from Marentette et al., 2010). Larger exposed males were selected for this assay because of the large volume of liver tissue available for analyses (female round goby are generally smaller; Kornis et al., 2012; McCallum et al., 2014). Briefly, each liver was first thawed and homogenized in buffer (50 mM Tris HCl, 0.15 M KCl, pH 7.4, 4 ml g^{-1} tissue). Liver homogenates were then centrifuged for 10 min at 750*g* and 10 min at 12,000 *g*, at 4 °C (Eppendorf 5904 R). S9 fractions were immediately frozen and stored at −80 °C until analysis. The protein levels with 5 μl of the homogenized sample were estimated spectrophotometrically according to Bradford method (Bradford, 1976; SpectraMax Plus 384, Molecular Devices). EROD activity was measured as the amount of resorufin produced from the addition of 1.33 mM NADPH and 2 μM 7-ethoxyresorufin in buffer (50 mM Tris, 0.1 M NaCl, pH 7.8) at 25 °C using a black 96-well microplate fluorometer (excitation 530 nm and emission 590 nm; SpectraMax Gemini XPS, Molecular Devices). Final reaction volume was 200 uL and the LOQ for resorufin in our assay was 0.0061 pmol. Stock protein content added to each reaction was (mean ± standard error) 7.98 ± 0.23 mg ml^{-1} and the final diluted protein content was 0.08 ± 0.23 mg ml^{-1}. EROD activity was expressed as pmol of resorufin formed min^{-1} mg^{-1} of S9 fraction protein in hepatic tissue.

Table 2
Summary of sample sizes (*N*) used in exposures, behavioural analyses, morphology measures, EROD assay, and tissue pharmaceutical quantification. Sample sizes vary based on what tissues were available for collection and the amount of tissue needed for analysis. *Gonad tissues were only collected from female fish, as male gonads were of insufficient mass.

Survival	Total	Control (0%)	Low (50%)	High (100%)
Exposed	72	24	24	24
Survived	57	17	19	21
Behavioural assay				
Mirror aggression	57	17	19	21
Morphology and EROD				
HSI, GSI, & body condition	57	17	19	21
EROD	22	7	6	9
Tissue-specific uptake				
Brain	54	17	19	18
Plasma	36	8	15	13
Gonad*	31	10	10	11
Liver	53	17	19	17
Muscle	53	14	19	20

2.7. Statistical analyses

All statistical analyses were conducted in R (version 3.3.2: R Core Team, 2016). Unless otherwise stated, we tested for the impact of wastewater exposure treatment on our response variables using linear mixed effects models (LMMs; nlme package; Pinheiro et al., 2016) or generalized linear mixed effects models (GLMMs, when data were non-parametric; lme4 package; Bates et al., 2015), including a random effect of exposure tank. We assessed the effect of wastewater exposure on fish survival using a binomial GLMM. We used a LMM to assess the latency for fish to move towards the mirror. We used negative binomial GLMMs appropriate for fitting over-dispersed count data to analyze the number of aggressive acts performed towards the mirror, the number of aggressive bouts with the mirror, and the number of non-aggressive acts (as a proxy of activity). We used an LMM to analyze tissue uptake of pharmaceuticals. We additionally included fish ID as a random effect in this analysis to account for non-independence of the tissue samples, and we weighted our variance by wastewater treatment to account for heteroscedasticity between the exposure groups. We used a GLMM with a poisson distribution to analyze the number of pharmaceuticals detected in the tissues, including a random effect of fish ID. We removed all non-significant interactions from the models. We analyzed the effect of wastewater exposure on EROD activity in liver tissue using an ANOVA. We followed our primary analyses with Dunnett's post-hoc tests to investigate any main effects of wastewater exposure treatment. In our tissue analyses, we further investigated tissue-specific pharmaceutical uptake within each wastewater exposure group using Tukey's post-hoc test to compare tissues. See Table 2 for a summary of sample sizes used in the above analyses. Because sex effects were not the focus of our study and because our sample size for each sex was small, data were collapsed across sex before analysis. A significant difference was recognized when $p < 0.05$.

3. Results

3.1. Survival and morphological measures

Across treatment groups, 79% of the fish survived the 28-day wastewater exposure, and fish survived similarly across exposure groups (Binomial GLMM: $N_{tanks} = 12$; LRT_{Dose} $\chi^2 = 0.24$, $p = 0.89$). We did not detect any impact of the wastewater exposure on fish liver investment (hepatosomatic index, LMM: $N = 57$, $F = 0.43$, $p = 0.66$), body condition (Fulton's Index, LMM: $N = 57$, $F = 0.004$, $p = 0.99$), or gonad investment (gonadosomatic index, LMM: $N = 57$, $F = 0.41$, $p = 0.68$). Across treatments, liver investment was (mean ± standard error) 6.81 ± 0.28%, Fulton's body condition was 2.24 ± 0.03 g/cm^3, and gonad investment was 0.56 ± 0.06%.

3.2. Behavioural assay

Fish exposed to the high dose of effluent were less aggressive towards the mirror and preformed fewer aggressive acts towards the mirror (Fig. 1b; Negative binomial GLMM: $N = 57$; LRT_{Dose} $\chi^2 = 7.75$, $p = 0.021$; Dunnett's post-hoc: high vs control, $Z = -3.09$, $p = 0.002$, low vs control, $Z = -0.90$, $p = 0.37$). Fish exposed to the 100% wastewater effluent also tended to have fewer aggressive bouts with the mirror (Fig. 1c; Negative binomial GLMM: $N = 57$; LRT_{Dose} $\chi^2 = 7.15$, $p = 0.09$; Dunnett's post-hoc: high vs control, $Z = -2.12$, $p = 0.061$, low vs control, $Z = 0.04$, $p = 0.99$), though this only approached statistical significance. In contrast to aggression towards the mirror, wastewater exposure did not affect the non-aggressive activity observed during the trials (Negative binomial GLMM $N = 57$; LRT_{Dose} $\chi^2 = 3.23$, $p = 0.20$), or the latency to begin moving towards the mirror in the aggression trials (Fig. 1d; LMM: $N = 57$; $F = 0.81$ $p = 0.47$).

3.3. Tissue-specific pharmaceutical uptake

Pharmaceuticals were successfully determined in tissue and plasma samples, with stable and reproducible results. No carry-over effects were observed, no pharmaceuticals were detected in the instrumental or field blanks, and R^2 values were above 0.99 for all calibration curves in given concentration range. We detected 11 of the 93 tested target compounds in the tissues and in the plasma of round goby in our exposure study (Table 3, and also see Supplementary Materials for full list of all the tested compounds). We found that with increasing wastewater exposure, fish had a higher concentration of pharmaceuticals in their tissues, and generally, this was most pronounced in the brain and plasma compared with the gonad, liver or muscle tissues (Fig. 2a; LMM: $N = 232$; $F_{Dose*Tissue} = 15.00$, $p < 0.0001$). Post-hoc tests revealed that within exposure treatment, the brain and plasma indeed had the highest tissue pharmaceutical concentrations, while the female gonads were intermediate, and the liver and muscle concentrated the lowest concentrations of pharmaceuticals (see Fig. 2a for post-hoc results). We also detected a greater number of pharmaceutical compounds in the tissues of fish exposed to 100% wastewater and 50% wastewater, when compared to fish exposed to 0% wastewater (Fig. 2b; Negative binomial GLMM: $N = 232$; LRT_{Dose} $\chi^2 = 42.9$, $p < 0.0001$; Dunnett's post-hoc high vs control, $Z = 9.99$, $p < 0.0001$, low vs control, $Z = 7.94$, $p < 0.0001$). Generally, a greater number of pharmaceutical compounds were detected in the brain, plasma and gonads, than in the liver and muscle tissues (LRT_{Tissue} $\chi^2 = 87$, $p < 0.0001$; see Fig. 2b for results of post-hoc analyses).

3.4. EROD activity

Exposure to wastewater effluent increased hepatic EROD activity in fish exposed to the 100% wastewater compared to fish exposed to 0% wastewater effluent (Fig. 3; ANOVA: $N = 22$, $F_{Dose(2, 19)} = 4.36$, $p = 0.027$; Dunnett's post-doc: high vs control, $t = 2.77$, $p = 0.024$, low vs control, $t = 0.49$, $p = 0.84$).

4. Discussion

We exposed round goby, a non-model fish species, to 0%, 50% or 100% dilutions of treated wastewater effluent for 28 days, and assessed behavioural responses, tissue-specific uptake of pharmaceuticals, and hepatic EROD activity. We found that fish exposed to 100% effluent showed reduced aggression in our study: fish performed fewer aggressive acts at the mirror and had fewer bouts of aggressive interaction with the mirror. Our findings are similar to those of Martinović et al. (2007), and Garcia-Reyero et al. (2011) who both found that male fathead minnow exposed to 100% wastewater effluent for 21 days performed fewer aggressive behaviours and were more likely to lose their nesting site to a competitor fish. The reduced aggression that we observed was not a result of an overall suppression of fish behaviour following wastewater exposure, as we found no evidence that non-aggressive activity differed across the wastewater exposure groups.

Aggression is an important behaviour for round goby fitness, as they compete for access to sheltered territories in the littoral zone (Balshine et al., 2005; Bergstrom and Mensinger, 2009; Dubs and Corkum, 1996; Janssen and Jude, 2001). When in contests against native logperch (*Percina caprodes*), round goby were more aggressive than logperch, took over the shelter resources from the logperch, and then spent more time in the shelter (Balshine et al., 2005). In the field, round goby without access to shelter are more likely to be predated (Belanger and Corkum, 2003) and they are prevalent in the diets of waterbirds, water snakes, and piscivorous fish (King et al., 2006; Somers et al., 2003; Reyjol et al., 2010). Our findings suggest that round goby exposed to 100% wastewater effluent, as would be found in effluent-dominated environment, may be less adept at competing for resources or

Table 3
Mean ± 1 standard error of samples in which a pharmaceutical compound was detected. Brackets show number of samples in which this compound was detected over the total number of samples tested (sample size). Tissue concentrations were measured in ng g^{-1}, plasma was measured in ng ml^{-1}. [a]Gonad tissues were tested only from female fish, as male gonad tissue was of insufficient mass. – shows < limit of quantification (LOQ).

		Compound										
	Tissue	Bupropion	Carbamazepine	Citalopram	Clotrimazol	Diltiazem	Diphenhydramine	Donepezil	Flecainide	Oxazepam	Risperidone	Venlafaxine
Control (0%)	Brain	2.09 (1/17)	–	–	–	–	2.23 (1/17)	–	–	15.54 (1/17)	0.8 ± 0.06 (13/17)	–
	Plasma	–	–	–	–	–	0.41 ± 0.11 (2/8)	0.80 ± 0.10 (3/8)	–	–	0.76 ± 0.14 (8/8)	–
	Gonad[a]	–	–	–	–	–	–	–	–	–	0.42 ± 0.04 (10/10)	–
	Liver	–	–	–	–	–	0.08 (1/17)	–	–	–	0.19 ± 0.02 (12/17)	1.30 (1/17)
	Muscle	–	–	–	–	–	–	–	–	–	0.15 (1/14)	–
Low (50%)	Brain	11.01 ± 2.30 (19/19)	–	–	4.81 ± 3.38 (3/19)	–	8.59 ± 5.63 (19/19)	–	–	–	0.94 ± 0.94 (19/19)	2.74 (1/19)
	Plasma	2.47 ± 0.38 (15/15)	–	6.35 ± 1.00 (3/15)	–	0.71 ± 0.02 (3/15)	6.72 ± 0.90 (15/15)	2.29 ± 0.59 (11/15)	0.16 (1/15)	–	0.66 ± 0.07 (15/15)	–
	Gonad[a]	0.62 ± 0.07 (9/10)	–	–	–	1.08 ± 0.15 (7/10)	3.85 ± 0.50 (10/10)	–	0.16 ± 0.02 (4/10)	–	0.37 ± 0.08 (10/10)	–
	Liver	0.19 (1/19)	–	–	–	0.51 (1/19)	0.79 ± 0.13 (19/19)	–	–	–	0.18 ± 0.01 (11/19)	–
	Muscle	0.14 ± 0.02 (11/19)	–	–	–	0.56 (1/19)	0.38 ± 0.05 (19/19)	–	–	–	–	–
High (100%)	Brain	22.03 ± 4.19 (18/18)	1.36 ± 0.08 (7/18)	–	7.17 ± 1.4 (15/18)	1.48 ± 0.27 (9/18)	25.66 ± 5.63 (18/18)	–	–	–	0.82 ± 0.08 (18/18)	10.79 ± 8.52 (2/18)
	Plasma	3.82 ± 0.58 (13/13)	–	8.04 ± 1.10 (7/13)	–	0.94 ± 0.18 (6.13)	14.87 ± 1.95 (13/13)	4.26 ± 0.59 (11/13)	0.29 ± 0.03 (4/13)	–	0.79 ± 0.07 (13/13)	3.27 (1/13)
	Gonad[a]	0.81 ± 0.09 (11/11)	1.26 (1/11)	–	–	1.37 ± 0.23 (11/11)	8.22 ± 0.82 (11/11)	0.55 ± 0.05 (2/11)	0.21 ± 0.02 (8/11)	–	0.41 ± 0.04 (11/11)	1.94 ± 0.17 (4/11)
	Liver	0.20 ± 0.03 (2/17)	–	–	1.07 (1/17)	1.03 ± 0.35 (4/17)	1.63 ± 0.29 (17/17)	–	–	–	0.15 ± 0.01 (10/17)	0.92 (1/17)
	Muscle	0.22 ± 0.01 (20/20)	–	–	–	0.74 ± 0.21 (2/20)	0.98 ± 0.15 (20/20)	–	0.23 (1/20)	–	–	0.90 (1/20)

defending themselves from predators. However, all other aquatic organisms (including many of the predators of round goby) could be similarly exposed to wastewater effluet, potentially negating any overall effect on competitive or defensive behaviour. Different species might be differentialy affected by the same pharmaceutical concentration (i.e. asymmetrical effects, Brodin et al., 2014) it is therefore difficult to say if exposure to wastewater would benefit interspecific interactions of the round goby or not. Here, we used a mirror assay to measure round goby aggression, which provides a standardized measure of individual aggressive tendencies by omitting a live competitor that introduces variablity in a contest situation (Balzarini et al., 2014; Elwood et al., 2014; Wilson et al., 2011). Future work should expand our findings to aggression in dyadic interactions. For instance, conducting resource contests against live opponents (intra- and inter-specific contests) to better understand resource acquisition and complex interactions over territory, or investigating how wastewater effluent affects predator-prey interactions when predators may have a different degree of exposure given their mobility in the environment (e.g., larger piscivorous fish, avian predators).

To compliment our behavioural measures, we characterized fish exposure to PhACs by measuring tissue-specific uptake of pharmaceuticals. Generally, we found that bupropion, diphenhydramine, diltiazem, risperidone, and venlafaxine were detected most frequently. Bupropion and diphenhydramine showed a distinctive exposure response because they were detected in all fish tissues in the 50% and 100% exposure groups, and they often doubled in tissue concentration between the 50% and 100% treatment groups. When we assessed the total pharmaceutical concentration in fish tissues, we found that fish exposed to 50% and 100% effluent had higher tissue concentrations of pharmaceuticals compared to 0% effluent control fish. We detected the antidepressant medications bupropion, venlafaxine, and citalopram, and these compounds may be linked to the behavioural changes we observed. Individually, all compounds were below plasma therapeutic levels in humans (Horst and Preskorn, 1998), but human therapeutic plasma concentrations do not always clearly predict behavioural changes in fish (Huerta et al., 2016). The effect of simultaneous exposure to multiple psychiatric PhACs is largely unexplored in the laboratory to date (but see Schoenfuss et al., 2016). Likewise, changes to water quality parameters characteristic of wastewater effluent, such as increased ammonia, may also alter fish behaviour. Ammonia itself was unlikely to cause the behavioural change we observed in our study, while ammonia has been shown to both increase and decrease swimming activity and/or performance in fish

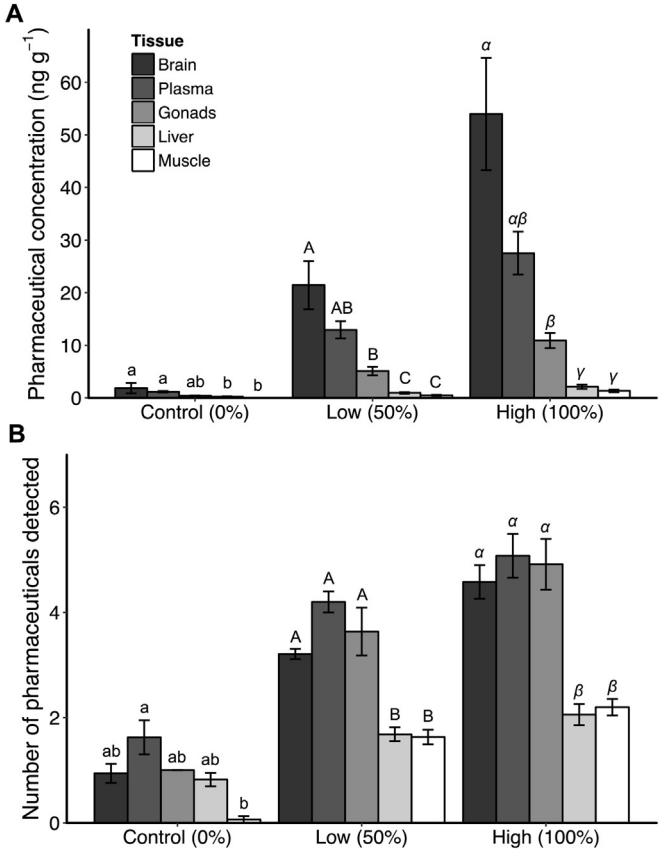

Fig. 2. Results of pharmaceutical quantification in round goby tissues and plasma following exposure to wastewater effluent. **A**) Mean pharmaceutical concentration in tissues and plasma, plotted by exposure group (note: plasma concentrations were measured in ng mL^{-1}). **B**) Mean number of pharmaceutical compounds detected in tissues and plasma, plotted by exposure group. Lowercase, uppercase, and Greek letters show the results of post-hoc analyses comparing the tissue-specific uptake of pharmaceuticals within each exposure group. Error bars represent ±1 standard error.

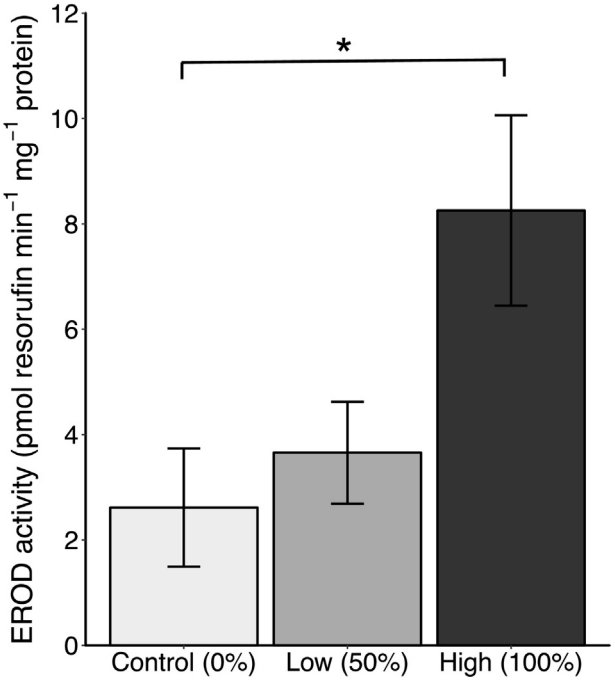

Fig. 3. 7-ethoxyresorufin-*O*-deethylase (EROD) activity in round goby liver tissue, plotted by exposure group. Error bars represent ±1 standard error. * $p < 0.05$.

(Israeli-Weinstein and Kimmel, 1998; Jian-yu et al., 2005; Shingles et al., 2001; Wicks et al., 2002), our findings were specific to aggression.

Pharmaceutical concentrations were highest in the brain and plasma, intermediate in female gonads (male gonads were not analyzed due to insufficient tissue size), and the lowest in the liver and muscle tissues. Previously, fish exposed to wastewater effluents (*Ictalurus punctatus, Pomoxis nigromaculatus, Lepomis macrochirus*, Brooks et al., 2005; *Oncorhynchus mykiss* Grabicova et al., 2014) had the highest concentrations of pharmaceuticals in the brain and liver. However, patterns in PhAC uptake are determined by many interacting factors, including environmental characteristics of the exposure regime, chemical properties of the PhAC, and species-specific factors (Zenker et al., 2014). Our current results suggest that plasma concentrations reflect some of the higher concentrations in the body, and this pattern of uptake was similarly noted in European perch exposed to oxazepam (*Perca fluviatilis*, Heynen et al., 2016). Plasma sampling would provide a useful non-lethal way to investigate PhAC concentration in the future alongside other plasma biomarkers (e.g., 'omics' approaches: Martyniuk and Simmons, 2016; Simmons et al., 2015). To better understand how pharmaceuticals present in wastewater effluent bioconcentrate in fish tissues, future work will measure PhACs in the collected effluent and fish tissues. Previous studies had quantified PhACs in the effluent from the WWTP used in our study (Csiszar et al., 2011; Galus et al., 2013; Metcalfe et al., 2003); however, these studies screened for very few of the compounds that we actually detected in the tissues and plasma of fish from our study.

To our knowledge, our study provides the first assessment of pharmaceutical uptake in fish gonad tissues following an exposure to wastewater effluent. Our findings extend only to females, but we showed that female fish concentrated pharmaceuticals in their gonads, even when in non-reproductive condition. A number of studies have begun evaluating the transgenerational effects of pharmaceuticals in aquatic organisms (Galus et al., 2014; Minguez et al., 2015; Schwindt et al., 2014), and further investigation into the transfer of pharmaceutical compounds to developing offspring would be intriguing. For instance, laboratory studies of maternal transfer of bisphenol-A (BPA, a monomer that leaches from plastic products) have indicated that BPA is transferred to developing eggs and can impact offspring development (Birceanu et al., 2015; Takao et al., 2008).

Fish in our study were collected from a wild population, and we did detect low amounts of some pharmaceuticals in the tissues of a small number of our control fish. For example, the anxiolytic oxazepam was detected in one fish from the control group. We also detected the antipsychotic medication, risperidone, in almost all tissues from fish in all exposure groups (0%, 50%, and 100% wastewater effluent). Because tissue concentrations of risperidone were uniform across treatment groups, this indicates that fish are likely to have been exposed to risperidone in the field and not directly from the wastewater exposure in the laboratory. Unfortunately, little is known about risperidone in fish tissues, though it has been measured in treated drinking water at 0.34 ng l^{-1} (Calisto and Esteves, 2009). The half-life of risperidone in human plasma is 24 to 48 h, however prolonged release formulas can manipulate the measured half-lives to be longer in humans (Taylor, 2009). As discussed above, other compounds that we detected did follow an exposure profile consistent with the wastewater treatments in our study. Moreover, the increased EROD activity in the liver tissue also followed an exposure profile consistent with the wastewater treatments in our study.

In conclusion, we have shown that exposure to a high dose (100%) of wastewater effluent reduced round goby aggression, an important behaviour linked to their success as an invasive species (Kornis et al., 2012). We showed that fish exposed to 50% or 100% wastewater effluent concentrated pharmaceuticals in their tissues and plasma, and that this concentration was greatest in the brain and plasma. We therefore underscore the importance of using brain tissue and plasma for quantifying PhAC exposure in wild, wastewater-exposed fish. Our results

describe the impacts of a "worst-case" scenario exposure to wastewater effluent, such as those found in effluent-dominated systems (Brooks et al., 2006), and caution should be applied when extrapolating these findings to systems with higher flow and/or greater dilution. Importantly, the behavioural changes we measured cannot *only* be a result of the pharmaceuticals we measured in fish tissues, as wastewater is a complex mixture of many pollutants. Comparing the effects of wastewater exposure between studies is inherently challenging because researchers use different endpoints, different study species, and wastewater effluent from different treatment facilities. However, such studies are important for evaluating current impacts of wastewater effluents on receiving environments. Our work has relevance for the remediation of Hamilton Harbour (Ontario, Canada), an International Joint Commission Area of Concern that is currently undergoing remediation to improve water quality, fish populations, and aquatic habitats after degredation by wastewater inputs, industrial activities, and urban run-off (International Joint Commission, 1999; Hamilton Harbour RAP, 1992; Hall et al., 2006). Our findings are also broadly relevant because understanding how wastewater effluents impact wild animals is of current international concern as human use of synthetic products continues to rise (Bernhardt et al., 2017; Boxall et al., 2012; Sumpter, 2009). Studies investigating the impacts of wastewater effluent on aquatic organisms are a natural next-step to connect controlled laboratory exposures to realistic field exposures. We anticipate that our study will further stimulate and develop the use of behaviour as a practical tool for assessing the sub-lethal effects of anthropogenic contaminants on fishes and other organisms in the wild.

Acknowledgements

The authors would like to thank P. Capelle and R. Charney for help with field collections; Hamilton Water, M. Bainbridge, B. Posedowski, and A. Bazzard for facilitating wastewater effluent collections; S. Du, G. Scott and G. McClelland for laboratory assistance; J. Wilson and M. Galus for advice on experimental design; A. Bose for comments on this manuscript. This project was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant (RGPIN-2016-05772), the Canada Research Chair Program, and a Canada Foundation for Innovation (CFI) Grant to S. Balshine, and an NSERC post-graduate scholarship (PGS-D) to E. McCallum.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.06.073.

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