Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com

In situ exposure to wastewater effluent reduces survival but has little effect on the behaviour or physiology of an invasive Great Lakes fish

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ARTICLE INFO

Article history:
Received 31 October 2016
Received in revised form 16 December 2016
Accepted 17 December 2016
Available online xxx

Keywords:
Caging
PPCPs
Respirometry
Activity
Cootes paradise marsh
Round goby

ABSTRACT

Treated effluents from wastewater treatment plants (WWTP) are a significant source of anthropogenic contaminants, such as pharmaceuticals, in the aquatic environment. Although our understanding of how wastewater effluent impacts fish reproduction is growing, we know very little about how effluent affects non-reproductive physiology and behaviours associated with fitness (such as aggression and activity). To better understand how fish cope with chronic exposure to wastewater effluent in the wild, we caged round goby (*Neogobius melanostomus*) for three weeks at different distances from a wastewater outflow. We evaluated the effects of this exposure on fish survival, behaviour, metabolism, and respiratory traits. Fish caged inside the WWTP and close to the outfall experienced higher mortality than fish from the reference site. Interestingly, those fish that survived the exposure performed similarly to fish caged at the reference site in tests of aggressive behaviour, startle-responses, and dispersal. Moreover, the fish near WWTP outflow displayed similar resting metabolism (O_2 consumption rates), hypoxia tolerance, haemoglobin concentration, haematocrit, and blood-oxygen binding affinities as the fish from the more distant reference site. We discuss our findings in relation to exposure site water quality, concentrations of pharmaceutical and personal care product pollutants, and our test species tolerance.

1. Introduction

Wastewater treatment plant (WWTP) effluents are a large and ubiquitous source of aquatic pollution (Johnson and Sumpter, 2014; Schwarzenbach et al., 2010; Strayer and Dudgeon, 2010). WWTP effluents reduce dissolved oxygen, contribute to eutrophication *via* nutrient inputs, and increase anthropogenic contaminants like endocrine active substances in receiving waters (Brooks et al., 2006; Carey and Migliaccio 2009; Kolpin et al., 2002). Such contaminants can include a mix of natural and synthetic compounds like pharmaceuticals and personal care products (PPCPs), manufacturing by-products, pesticides and herbicides, and steroid hormones (Klecka et al., 2010; Pal et al., 2010). Since many conventional WWTPs are still ill-equipped to remove these contaminants from the water they treat, many are regularly measured in low but consistent quantities in the environment (*i.e.* ng/l to µg/l; Jelic et al., 2012). The presence of anthropogenic pollutants in the environment has led to growing concern about the effects that wastewater effluent exposure might have on the survival and fitness of aquatic organisms (Boxall et al., 2012; Sumpter, 2009). Many endocrine systems have conserved functions across vertebrates, so these systems are likely to be disrupted in animals that are exposed to the endocrine-active substances found in wastewater effluent (Brown et al., 2014; Gunnarsson et al., 2008). While the effects of wastewater effluent on the reproductive physiology of fish has been examined (Fuzzen et al., 2015; Harris et al., 2011; Liney et al., 2006; Tetreault et al., 2012; Vajda et al., 2008), there has been comparatively little research on effects of effluent on fish behaviour or non-reproductive physiology (*e.g.*, metabolism). Behaviour and metabolic physiology can both contribute significantly to fitness (Brodin et al., 2014; Scott and Sloman, 2004), so understanding the impact of wastewater effluents have on these processes is crucial for informing wastewater remediation efforts.

Behaviour is strongly linked to fitness because it plays a critical role in successful reproduction, territory defense, predator evasion, and foraging abilities (Brodin et al., 2014; Söffker and Tyler, 2012). A growing number of studies have assessed how single contaminants found in wastewater effluent (often endocrine active) affect fish behaviour in the laboratory (*e.g.* Brandão et al., 2013; Brodin et al., 2013; Galus et al., 2014; Hedgespeth et al., 2013), but few have addressed the impacts of complex wastewater effluent mixtures. Notable exceptions do exist, and these studies indicate that effluent exposure can alter fish behaviour. For example, Garcia-Reyero et al. (2011) and Martinović et al. (2007) showed that fathead minnows (*Pimephales promelas*) were less able to compete and hold a nesting site against unexposed rival males following a three-week exposure to 100% wastewater effluent in the laboratory. Similarly, male three-spine sticklebacks (*Gasterosteus aculeatus*) exposed to 50% or to 100% effluent for three weeks built fewer nests and reduced female courtship (Sebire et al., 2011). In contrast, in one of the only studies conducted on fish exposed in the wild, Saaristo et al. (2014) showed

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http://dx.doi.org/10.1016/j.aquatox.2016.12.017
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that male mosquitofish (*Gambusia affinis*) collected downstream from a WWTP outfall actually courted females *more* than fish collected from a pristine site.

Metabolic physiology (energy utilization, respiration) is another major contributor to fitness, and provides the cellular energy needed to support behaviour (Biro and Stamps, 2010; Brown et al., 2004; Scott and Sloman, 2004). Not surprisingly, many previous studies have demonstrated a tight link between behaviour and metabolism (Biro and Stamps, 2010). For example, Ros et al. (2006) found that more active and more aggressive Mozambique tilapia (*Oreochromis mossambicus*) also had higher resting metabolic rates (O_2 consumption). When fish are faced with complex contaminant stressors like wastewater effluent, there may be a metabolic trade-off between contaminant detoxification and routine bodily and behavioural processes (Scott and Sloman, 2004). Handy et al. (1999) and Campbell et al. (2002) noted such a trade-off in rainbow trout (*Oncorhynchus mykiss*) exposed to copper; exposed fish had similar resting metabolic rates to control fish, but were much less active in their tanks. Contaminant-induced oxidative stress may increase the metabolic demands for tissue maintenance and repair, as well as reduce liver glycogen stores, each of which has been documented in fish exposed to WWTP effluents (Carney Almroth et al., 2008; Cazenave et al., 2014; Melvin, 2016). An increased metabolic cost of contaminant detoxification could also constrain tolerance of hypoxia, high temperature, and other environmental stressors (Kelly et al., 2014; Mandic et al., 2009). Consequently, wastewater exposure may increase routine metabolic costs, which could in turn limit environmental stress tolerance and the metabolic scope available to support normal activity and behaviour. Assessing the impacts of wastewater effluent on metabolism and respiratory physiology alongside behaviour is a useful way to assess such trade-offs (Handy et al., 1999; Killen et al., 2013; Scott and Sloman 2004).

The aims of our study were two-fold: (1) to establish the effects of an environmental exposure to wastewater effluent on fish behaviours important for fitness, and (2) to assess the impact of exposure to wastewater effluent on fish metabolism and respiratory physiology. We caged fish for three weeks at varying distances from a WWTP outfall to address these aims. A caging exposure provides certain advantages over collecting exposed fish from the wild or exposing fish in the laboratory. For example, with caging we can control for exposure duration and fish mobility, allowing us to better-connect measured effects to the wastewater exposure. Moreover, field exposures allow us to integrate ambient environmental conditions into the exposure regime, something laboratory wastewater exposures are unable to replicate (Oikari, 2006; Palace et al., 2005). In this study, we caged round goby (*Neogobius melanostomus*)—an invasive fish species that has become widespread throughout the Laurentian Great Lakes and Europe—at varying distances from a tertiary WWTP in Dundas (Ontario, Canada). This facility's effluent discharges into a canal that receives no significant flow from any other sources (Hamilton Water, *unpublished data*; T. Theÿsmeÿer, Head of Natural Lands, Royal Botanical Gardens, *personal communication*). The impact of wastewater effluent on aquatic organisms is especially important in effluent-dominated streams, such as our study, as there is little dilution of contaminants or effluent water quality (Brooks et al., 2006).

After a three-week caging exposure, we assessed behavioural and physiological endpoints important for round goby fitness. We evaluated aggressive, startle response, and dispersal behaviours, as they reflect a range of contexts important for fish survival such as locating and defending a territory and reacting to predators (Dell'Omo, 2002; Smith and Blumstein, 2008). To evaluate how fish cope metabolically with wastewater effluents, we conducted physiological assays measuring resting O_2 consumption rate, critical O_2 tension (a level of hypoxia tolerance that reflects the minimum level of O_2 needed to maintain routine metabolic processes), and haematology. If a trade-off existed between metabolism and routine behaviour and environmental stress tolerance (Handy et al., 1999; Scott and Sloman, 2004), then we expected that fish exposed closer to the wastewater effluent source would have had higher resting metabolic rates, reduced hypoxia tolerance, and reduced performance in our behavioural measures (*i.e.* dampened aggression, reactivity to startle stimuli, and dispersal). This would be in line with previous research showing that fish exposed to wastewater effluent have a general decrease in behaviours following exposure (Garcia-Reyero et al., 2011; Martinović et al., 2007; Sebire et al., 2011).

2. Methods

2.1. Fish collection & housing

We collected male round goby ($N = 239$) using baited minnow traps from Fifty Point Conservation Area, Lake Ontario, Canada (43°13′33″N; 79°37′27″W), a site 26 km from our exposure locations (for detailed collection procedures see McCallum et al., 2014; Young et al., 2010). We used only male round goby to reduce behavioural and physiological variability and because they are easier to capture in large numbers (McCallum et al., 2014; Young et al., 2010). We transported the fish to McMaster University and housed them in groups of 10–20 fish in 150 l housing tanks (H44 cm × W80 cm × D38 cm) equipped with coarse gravel substrate, an airstone, and a static renewal filter. We maintained fish on a 14L: 10D light cycle, and fed them a mix of fish pellets (Northfin) and flake food (Nutrafin Basix) once daily. We housed all fish for a minimum of 72 h under laboratory conditions to ensure health and regular feeding before we deployed them in cages for field exposures.

2.2. Caging exposure

We caged fish in four locations at varying distances from the Dundas Wastewater Treatment Plant (43°16′2″N; 79°56′37″W, Fig. 1). This facility serves a population of 41,000, and treats on average 18.2 million litres of wastewater daily from residences, businesses, and storm drains. The facility is a conventional activated sludge plant with tertiary sand filtration (City of Hamilton, 2011). The facility's effluent is released into the western-most end of the Desjardins Canal (Fig. 1), and is the main source of flow to the canal aside from a small run-off ditch (Hamilton Water, *unpublished data*; T. Theÿsmeÿer, *personal communication*). Characterizing the effluent from this canal is of special interest because it flows into Cootes Paradise Marsh, a nature reserve and the largest coastal wetland on Lake Ontario that serves as an important spawning habitat for fish species and bird migration stopover (Hamilton Harbour Remedial Action Plan, 1992). This wetland has been undergoing remediation after anthropogenic shoreline modifications, invasive species, combined sewer overflows, and wastewater effluents drastically reduced water quality and aquatic biodiversity in the early 1900's (Mayer et al., 2008; Thomasen and Chow-Fraser, 2012). We caged fish inside the secondary clarifiers of the Dundas Wastewater Treatment Facility (our highest exposure site, Fig. 1). Next, we caged fish close (50 m) to the effluent outfall, in the Desjardins Canal (43°16′0″N; 79°56′31″W), as well as 830 m downstream where the canal meets West Pond (43°16′9″N; 79°55′59″W). Our reference site was located in Beverly Swamp in Flamborough, ON (43°21′57″N; 80° 6′27″W), 17.4 km upstream from the outfall and the marsh. This reference site is on pro-

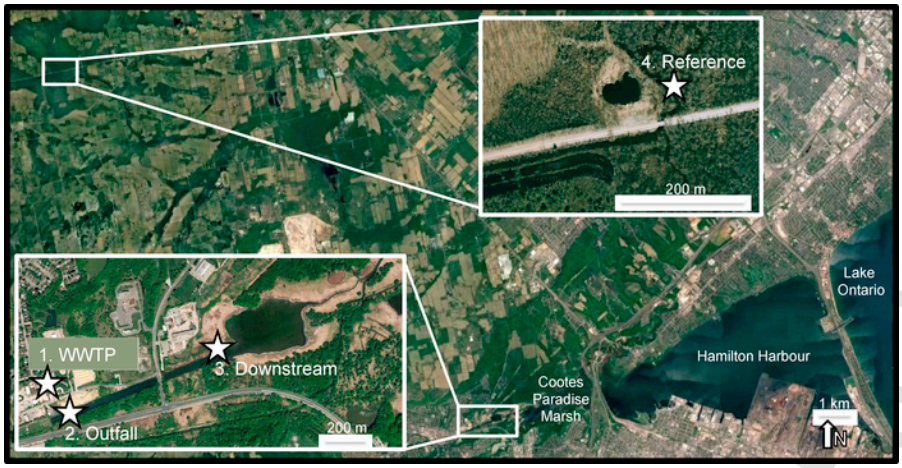

Fig. 1. A diagram of our caging locations near Cootes Paradise Marsh, which is connected to Hamilton Harbour, Lake Ontario, and our reference site in Flamborough, ON. The outfall site was located 50 m downstream from the wastewater treatment plant (WWTP) effluent discharge. The downstream site was located 830 m downstream from the effluent discharge. The reference site was located 17.4 km northwest in the headwaters to the Spencer Creek watershed, which empties into the marsh. Map data: Google, DigitalGlobe (2016).

tected lands and is part of the same watershed; specifically, it forms the headwaters for Spencer Creek that flows into the marsh. It does not receive wastewater effluent discharge from any wastewater treatment facilities (Hamilton Conservation Authority, 2009).

We caged fish in groups of 14–16 fish for 21 days at each location. The cages were 114 l plastic totes (Rubbermaid: H51 cm × W81 cm × D44.5 cm), each with approximately 200, 0.5 cm holes for water exchange. We tethered cages to concrete blocks using stainless steel chain, and submerged them so that 0.5 cm of the lid remained above the waterline. Although fish were always caged for a total of 21 days at each site, we staggered the deployment dates to facilitate behavioural and physiological processing. Each week we deployed one cage per site, and we repeated this for five weeks, creating five replicate cages per site. To measure contaminant exposure from pharmaceutical and personal care products (PPCPs) and other endocrine active compounds, we deployed passive polar organic chemical integrative samplers (POCIS) in triplicate at each site for two weeks during the caging experiment (POCIS-HLB, Environmental Sampling Technologies, Alvarez, 2010). We anchored samplers in an empty cage, identical to those in which the fish were held. During POCIS deployment and collection we used field blanks to detect background contamination during handling. Once each week, we conducted health checks on all cages and supplemented fish diet with fish pellets (Northfin). We also recorded water quality measures, including: temperature, pH, conductivity, total dissolved solids, salinity (Oakton Multi-Parameter Pocket Testr), dissolved oxygen (WTW Oxi 3310 SET 2), and flow (Hontzsch vane wheel flow sensor and interface RS232) at this time. Following the exposure, we transported fish live to McMaster University: two fish from each cage underwent resting metabolism and hypoxia tolerance assays, and six fish from each cage underwent behavioural assays. Fish held at the different sites did not differ in body mass (ANOVA on log body mass: $F_{(3,147)} = 1.93$, $p = 0.13$) or standard length (ANOVA on log standard length: $F_{(3,147)} = 1.89$, $p = 0.13$).

2.3. Behavioural assays

In the laboratory, we housed caged fish in site-matched groups of three in 40 l tanks (H33 cm × W51 cm × D28 cm) for 24 h before behavioural testing. We conducted three behavioural assays: (1) a mirror aggression assay, (2) a startle response assay, and (3) an activity and dispersal assay (Fig. 2). We conducted our first two assays in the same 40 l experimental tank between 08:00 and 12:00. A mirror was positioned at one end of the tank, with a removable opaque black barrier positioned over the mirror at the start of each trial. We transferred an exposed focal fish from their housing tank to the experimental tank and allowed them to habituate for 40 min. We then remotely lifted the removable opaque barrier to reveal the mirror, and a 20-min mirror aggression trial was video recorded (Fig. 2a, Canon Vixia HFS100 8.0 Megapixel). Following this trial, an opaque marble (1.25 cm diameter) was dropped from a fixed height (30 cm) into the testing aquaria (Fig. 2b) to assess the fish's startle response. The fish's response and any movement after the drop was recorded for an additional 20 min. An observer blind to exposure site later scored the behaviour from the video recordings. The observer recorded the latency for fish to move towards the mirror, the number of aggressive acts towards the mirror (following Supplementary Table 1), the startle response of each fish (categorical: freeze, dart/startle, or continued activity), the number of seconds elapsed for fish to move again after being startled, and the number of seconds elapsed for fish to resume interacting with the mirror.

We returned fish to their housing tank until 16:00, when we conducted the activity and dispersal assay. This assay occurred in a maze tank under simulated dusk conditions with red lights (dusk is when round goby are most active, Marentette et al., 2011). The dispersal tank (15 cm high × 175 cm wide × 75 cm deep) was separated into five compartments (Fig. 2c, adapted from Marentette et al., 2011). A removable barrier was placed over the exit from the first compartment to allow us to first assess activity in one compartment and then dispersal throughout the remaining compartments after we removed the barrier. We tested fish in site-matched groups of three fish, as previous work has shown that round goby are most active when tested in groups (Marentette et al., 2011). The group was transferred to the first compartment in a start-box where they remained for 10 min. The start box was then removed and the fish were allowed to freely explore the first compartment for 5 min. We live-scored total activity (all individual behaviours exhibited, see Supplementary Table 1) for each fish for 5 min in a pre-determined and random order. We then removed the barrier and fish were able to disperse through all compartments of the dispersal tank for a 20-min trial. We live-scored the time and direction of each compartment switch (Fig. 2c).

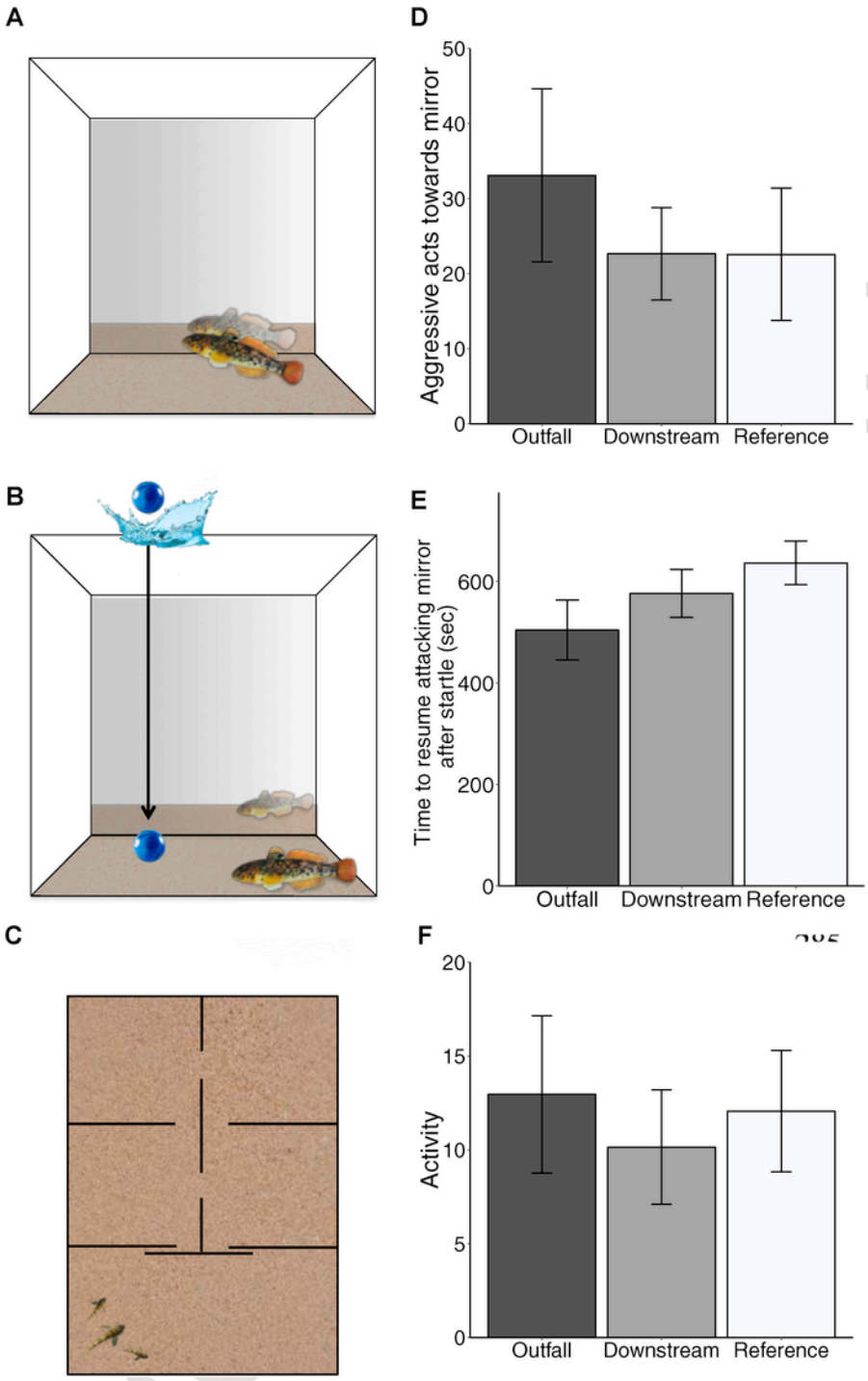

Fig. 2. Behavioural assays and results. Error bars represent ± 1 standard error. All findings were not significant (ns). (A) Mirror aggression task showing a fish interacting with its mirror image. (B) Startle response task showing a marble drop used to startle fish. (C) Dispersal task showing segmented maze, as seen from above. (D) Average number of aggressive acts towards the mirror plotted by exposure site. (E) Average time taken to resume aggressing at the mirror after being startled with the marble drop plotted against exposure site. (F) Average activity during the dispersal trials plotted against exposure site.

2.4. Physiological assays and fish sampling

2.4.1. Resting metabolism and hypoxia tolerance

We measured resting metabolism and hypoxia tolerance using stop-flow respirometry as previously described in detail (Borowiec et al., 2015; Crans et al., 2015). Briefly, we held fish in 425 ml respirometry chambers for that received a continuous supply of normoxic water (100% air saturation) for 10 h to allow fish to habituate to the respirometry chamber. First under normoxic conditions, we measured resting O_2 consumption rate (M_{O2}) as the change in water O_2 content over time using fibre-optic oxygen sensors (PreSens, Re-

gensburg, Germany). We then used a step-wise hypoxia protocol to determine each fish's critical oxygen tension [P_{crit}, the O_2 tension below which fish do not maintain resting M_{O2}; see Borowiec et al., 2015]. We did so by reducing air saturation from 100% to 10% air saturation in 10% increments every 20 min. At 10% air saturation, we closed the chamber and fish were allowed to consume the remaining oxygen until 0.5% air saturation was reached. We then flushed the chamber with normoxic water to recover the fish. The water O_2 content was recorded every second using a DAQ-M instrument and AutoResp software (Loligo Systems), and we measured M_{O2} twice at each O_2 level over 5 min measurement periods. We then used regress software (Yeager and Ultsch, 1989) to determine P_{crit} from the M_{O2} data.

2.4.2. Fish sampling & tissue collection

Fish were euthanized by cerebral concussion and spinal severance and sampled after behavioural and metabolism assays. We measured the standard length (snout to caudal peduncle) using calipers accurate to 0.01 cm. We measured total body mass using a digital scale accurate to 0.001 g. We collected blood from the caudal vein, either by puncturing with a chilled needle and syringe (pre-rinsed with ethylenediaminetetraacetic acid, EDTA; Sigma Aldrich) or by cutting off the tail and collecting the blood into a capillary tube, and a small volume (6 µl) was used to measure haemoglobin concentration using Drabkin's reagent (following instructions from the manufacturer, Sigma Aldrich). The remaining volume from samples collected *via* caudal vein puncture were centrifuged at 10,000 rpm at 4 °C for 4 min. Samples collected *via* capillary tubes were centrifuged at 14,000 rpm for 5 min at room temperature to measure haematocrit (%; volume of red blood cells/volume of total blood). From both collection techniques, packed red blood cells were frozen in liquid nitrogen and stored at −80 °C for later haemoglobin analyses. We removed and weighed the liver and gonads. We used gonad mass to calculate gonadosomatic index (GSI: gonad mass/(total mass − gonad mass) × 100). Males with a GSI over 1% were considered to be in reproductive condition (Marentette and Corkum, 2008; Zeyl et al., 2014). Overall, 31% of fish were reproductive, 67% were non-reproductive, and the percentage of reproductive fish was similar across caging sites (WWTP: 28%, Outfall: 29%, Downstream: 29%, Reference: 39%). Reproductive status did not impact behaviour or physiology in all statistical analyses (all analyses, $p > 0.1$).

2.4.3. Haemoglobin-oxygen binding

We used the lysate from frozen red blood cells to evaluate haemoglobin-O2 binding, in order to represent *in vivo* conditions of blood containing the natural levels of allosteric modifiers at the time of sampling. Haemoglobin-oxygen dissociation curves were generated at 25 °C using a Hemox Analyser (TCS Scientific, New Hope, PA, USA) as we have done previously (Borowiec et al., 2016). Following the manufacturer's recommendations, we used 5 ml of TES buffer, 20 µl of bovine serum albumin, 10 µl of anti-foaming agent (100× dilution of SAG-10, polydimethlysiloxane emulsion), and 10 µl of lysate from red blood cells. We calculated haemoglobin-O_2 affinity (P_{50}, the oxygen tension at which haemoglobin is 50% saturated) using Hemox Analytical Software (TCS scientific) at pH 7.4 and 7.0 for each sample. We measured haemoglobin pH sensitivity as the difference in P_{50} at pH 7.0 and 7.4 (normalized to a change of 1.0 pH unit).

2.5. Water and POCIS sampling

After we removed the POCIS samplers from the field, we transported them on ice to McMaster University where they were frozen at −20 °C. Water samples were also collected on the last day of POCIS sampling and were stored at −20 °C. We prepared water and POCIS samples for analysis of 24 target PPCP and endocrine active compounds at Trent University following methods described in Li et al. (2010) and Metcalfe et al. (2014). See Table 3 for full list of target compounds. Briefly, we rinsed POCIS samplers to remove debris from membrane surfaces before transferring sorbent powder into a glass chromatography column (1 cm × 30 cm) fitted with glass wool plugs and stopcocks. We then rinsed membranes with methanol to transfer any remaining sorbent to the column. After addition of the internal standard mixture, we eluted the sorbent with 50 ml methanol. The eluate was reduced in volume to about 1 ml by rotary evaporation, transferred to a conical centrifuge tube for evaporation to near dryness using a gentle nitrogen stream, and then transferred into an autosampler vial in 300 ml methanol for analysis. We extracted water samples using solid-phase extraction (SPE) cartridges and two multiresidue extraction methods to extract all analytes. We extracted the beta-blocker and antidepressant drugs, which are weak bases, with Oasis MCX cation exchange cartridges. All other compounds, including weakly acidic, phenolic, and neutral compounds, were extracted using Oasis MAX anion exchange cartridges (see Li et al., 2010 for further SPE extraction details).

We analyzed extracts from the POCIS samplers and water samples using liquid chromatography and tandem mass spectrometry (LC–MS/MS) with an electrospray ionization (ESI) source. All target pharmaceuticals and compounds from personal care products were analyzed by AB Sciex Q-Trap 5500 (Concord, ON, Canada) instrument operated either in positive or negative ion mode. The system was equipped with an Agilent 1100 series (Mississauga, ON, Canada) HPLC system. Following these analyses, the antibacterial agent triclosan was detected on our POCIS field blank from the downstream site and in one POCIS sample from that site. We therefore considered all samples to be contaminated by triclosan during handling at the downstream site, and triclosan was removed from calculating summary statistics at the downstream site.

Following POCIS analyses, we calculated the time-weighted environmental concentration (C_w) of each compound using the following equation:

$$C_w = \frac{N}{R_s t}$$

Where N is the amount of compound accumulated by each POCIS in ng/l, R_s is the sampling rate of each compound by the POCIS, and t is the duration of POCIS exposure in the field (14 days). We used POCIS sampling rates for each compound that were previously reported in the literature from static experimental conditions between 20 °C and 25 °C (sucralose, Metcalfe et al., 2014; all remaining compounds, Li et al., 2010), except for androstenedione and testosterone which have only been reported under flowing conditions (androstenedione, Bartelt-Hunt et al., 2011; testosterone, Morin et al., 2013).

2.6. Statistical analyses

All statistical analyses were conducted using R (version: 3.2.4, R Core Team, 2016). We used generalized linear mixed effects models (GLMM; glmmadmb package, Fournier et al., 2012) or linear mixed effects models (LMM; lme4 package; Bates et al., 2015) to analyze survival, behavioural, and physiological responses following exposure. In all analyses, we used likelihood ratio tests to test for main effects of our fixed factors, and followed these with Dunnet's *post-hoc* analyses.

We analyzed the proportion of round goby surviving each week using a binomial GLMM, where we included caging site and exposure week as fixed effects, and cage ID and deployment date as random effects. Due to high mortality at the highest exposure site (inside the WWTP), we excluded the WWTP fish (due to low sample size) from behavioural and physiological assays.

The number of aggressive acts in the mirror assay, as well as the number of movements and the number of chamber switches in the activity assay were all analyzed using negative binomial GLMMs for count data. The latency for fish to move towards the mirror, to move again after being startled, and to re-engage with the mirror after being startled were analyzed with LMMs. The behavioural response of fish (*i.e.* freeze or dart) to being startled was assessed using a binomial GLMM. For all these behavioural analyses, we included caging site and reproductive status of the fish as fixed effects, and cage ID and cage deployment date as random effects.

All physiological measures, including: resting metabolism (M_{O2}), hypoxia tolerance (P_{crit}), haematocrit, haemoglobin concentration, and mean cellular haemoglobin concentration were analyzed with LMMs, with ln-transformation when needed to meet parametric assumptions. We included caging site as a fixed effect, body mass as a covariate, and cage ID and deployment date as random effects. Haemoglobin P_{50} was measured for fish at both pH 7.0 and at pH 7.4, and was analyzed using a LMM with a random effect of fish ID and deployment week. Haemoglobin pH sensitivity was assessed using an LMM with a random effect of deployment week. See Table 1 for a summary of all sample sizes used in our analyses.

3. Results

3.1. Survival

Caging exposure site impacted round goby survival (Binomial GLMM: $N_{cages} = 20$; LRT_{site} $\chi^2 = 14.93$, $p = 0.0019$; Fig. 3). Fish caged at the highest exposure site had reduced survival compared to the reference site (Dunnet's *post-hoc*: WWTP *vs* reference, $Z = -4.47$, $p < 0.001$), fish caged at the wastewater outfall tended to have reduced survival compared to the reference site (outfall *vs* reference, $Z = -2.16$, $p = 0.078$), and fish caged at the downstream site had similar survival to fish at the reference site (downstream *vs* reference, $Z = -1.42$, $p = 0.34$). Mortality rate did not vary across weeks, indicating mortality was occurring consistently across the exposure period (LRT_{week} $\chi^2 = 3.57$, $p = 0.17$).

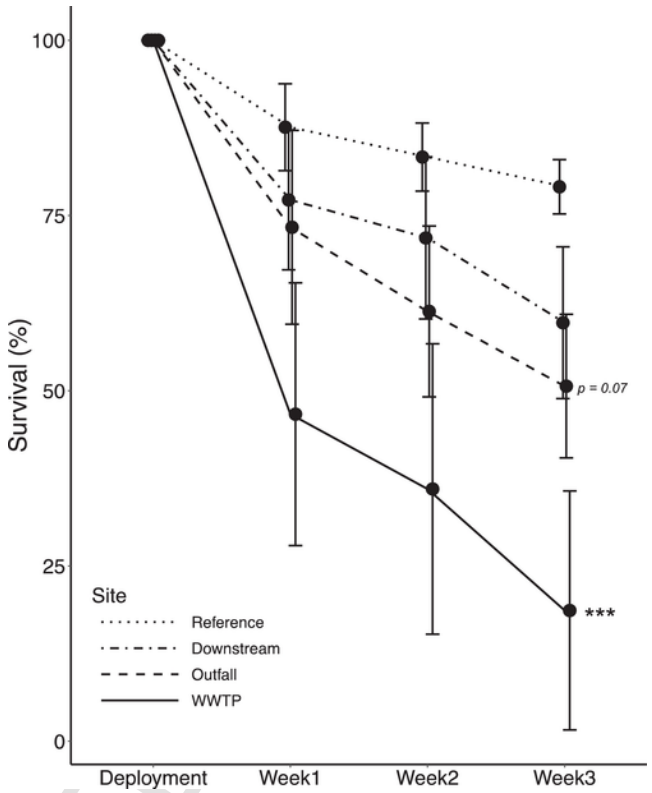

Fig. 3. Average percentage survival of round goby plotted by exposure week and exposure site. Error bars represent ± 1 standard error. *** $p < 0.001$, after comparison to reference site.

Table 1
Summary of sample sizes used for initial caging, and in the behavioural and physiological assays. Sample sizes vary depending on mortality throughout the experiment and experimental protocol.

		Site			
Caged	*N* Caged	WWTP	Outfall	Downstream	Reference
Deployed in the field	239	75	75	74	72
Behavioural assays	*N* Analyzed	WWTP	Outfall	Downstream	Reference
Mirror Aggression	68	–	22	20	26
Startle	80	–	26	25	29
Activity & dispersal	78	–	24	25	29
Physiology assays	*N* Analyzed	WWTP	Outfall	Downstream	Reference
Resting metabolism (MO_2)	27	–	9	8	10
Hypoxia tolerance (P_{crit})	28	–	9	9	10
Haematocrit (%)	37	–	12	8	17
Haemoglobin concentration	57	–	14	16	27
Mean cell haemoglobin	36	–	11	8	17
Haemoglobin P_{50}	28	–	10	7	11
Haemoglobin pH sensitivity	28	–	10	7	11

3.2. Behaviour

Exposure to wastewater effluent had little impact on round goby aggression, startle responses, or activity. The number of aggressive acts performed towards the mirror was similar across focal fish from all sites (Negative binomial GLMM: $N = 68$, LRT_{site}, $\chi^2 = 0.67$, $p = 0.72$; Fig. 2d), and the time taken for fish to move towards the mirror did not vary with exposure site (Linear mixed effects model ln-transform: $N = 68$, LRT_{site}, $\chi^2 = 2.16$, $p = 0.34$). After being startled, 75% of the round goby reacted by freezing while 25% of the fish darted away, but the degree of wastewater exposure (site) did not impact the type of behavioural response observed (Binomial GLMM; $N = 80$, LRT_{site}, $\chi^2 = 2.63$, $p = 0.27$). Fish from all sites also took similar amounts of time to begin moving after being startled (Linear mixed effects model: $N = 80$, LRT_{site}, $\chi^2 = 2.63$, $p = 0.27$), and to resume attacking the mirror (LMM: $N = 80$: LRT_{site}, $\chi^2 = 2.88$, $p = 0.24$; Fig. 2e). Exposure degree did not affect overall activity levels (Negative binomial GLMM; $N = 78$, LRT_{site}, $\chi^2 = 0.64$, $p = 0.73$; Fig. 2f), the time taken to disperse from the first compartment in the maze (LMM ln-transform: $N = 78$, LRT_{site}, $\chi^2 = 0.43$, $p = 0.81$), or the number of compartment switches in the maze (Negative binomial GLMM: $N = 78$, LRT_{site}, $\chi^2 = 0.49$, $p = 0.78$).

3.3. Physiology

Like behaviour, exposure to wastewater effluent had little effect on metabolism and respiratory physiology (see Table 2 for summary of physiological measures). Fish from all exposure sites had similar O_2 consumption rates at rest (LMM ln-transform; $N = 27$; LRT_{site}, $\chi^2 = 1.67$, $p = 0.43$; Fig. 4) and hypoxia tolerance (critical oxygen tensions, P_{crit}; LMM; $N = 28$; LRT_{site}, $\chi^2 = 0.31$, $p = 0.53$).

Exposure did not impact any haemoglobin-oxygen transport capacity or binding parameters, including: haematocrit (LMM: $N = 37$; LRT_{site}, $\chi^2 = 0.92$, $p = 0.63$), haemoglobin concentration (LMM: $N = 57$; LRT_{site}, $\chi^2 = 3.52$, $p = 0.17$), and mean cellular haemoglobin (LMM: $N = 36$; LRT_{site}, $\chi^2 = 1.20$, $p = 0.55$). Haemoglobin P_{50} was similar between exposure sites (LMM: $N = 28$, LRT_{site}, $\chi^2 = 0.91$, $p = 0.63$), but P_{50} was lower at pH 7.4 compared to pH 7.0 (LRT_{pH}, $\chi^2 = 118.60$, $p < 0.001$) due to the expected influence of the Bohr/Root effects on haemoglobin-O_2 binding. Haemoglobin pH sensitivity was also similar between exposure sites (LMM: $N = 28$, LRT_{site}, $\chi^2 = 0.36$, $p = 0.83$).

Table 2
Summary of average (± 1 SE) metabolic and respiratory physiology endpoints measured after the caging exposure.

	Site		
Measure	Outfall	Downstream	Reference
Resting metabolism (MO_2), mgO_2/hr	1.42 ± 0.15	1.31 ± 0.14	1.46 ± 0.18
Hypoxia tolerance (P_{crit}), kPa	2.95 ± 0.14	2.85 ± 0.23	3.04 ± 0.41
Haematocrit, %	31.94 ±2.24	32.61 ± 4.60	33.45 ± 2.72
Haemoglobin concentration, g/Dl	5.16 ± 0.41	4.47 ± 0.64	5.14 ± 0.50
Mean cell haemoglobin concentration	16.90 ± 2.36	19.13 ± 5.85	18.85 ± 3.23
Haemoglobin P_{50} pH 7.0, kPa	7.03 ± 0.26	7.09 ± 0.36	6.89 ± 0.18
Haemoglobin P_{50} pH 7.4, kPa	4.48 ± 0.20	4.43 ± 0.17	4.23 ± 0.09
Haemoglobin pH sensitivity	6.38 ± 0.44	6.63 ± 0.45	6.66 ± 0.32

3.4. Study site characteristics

The time-weighted estimated concentrations of PPCPs determined from the POCIS samplers and the measured concentrations determined from the water samples are reported in Fig. 5, and in Table 3. The POCIS and water samples generated roughly similar concentrations of the target analytes. Of the twenty-four compounds we assayed for in the POCIS samplers, we detected 20 in the WWTP, 19 at the outfall and downstream sites, and 10 at our reference site. Of these compounds, most were found at concentrations above the limits for accurate quantification: 19 in the WWTP, 17 at the outfall and downstream, and 6 at the reference site. Overall, concentrations of PPCPs were highest in the WWTP, slightly lower, but very similar between the outfall and the downstream sites, and lowest at our reference site. The similarity between the outfall and downstream sites suggests there is little degradation and/or dilution occurring between the sites (see Figs. 1 and 5). We did detect 6 compounds at our reference site, but the concentrations of these compounds were several orders of magnitude lower than at our downstream, outfall, and WWTP sites (Table 3, Fig. 5). We summarized water quality measures throughout the exposure period in Table 4. Similar to the PPCP trends, many water quality parameters were different in the WWTP than the similar values at the outfall and downstream sites, and all three wastewater-exposed sites were generally different than the reference site.

4. Discussion

Wastewater effluent is a complex mixture of various contaminants including PPCPs, and exposure to such contaminant mixtures may come at a metabolic cost that limits the aerobic scope for routine behaviours in fishes (Brown et al., 2004; Scott and Sloman 2004). We found that round goby exposed in the WWTP and at the outfall tended to experience higher mortality than fish caged at the reference site. Interestingly, we found that those round goby surviving the ex-

Table 3
Summary of average PPCPs using POCIS samplers ($N = 3$ replicates per site). Estimated time-weighted PPCP concentrations from the POCIS samplers were derived from sampling rates previously reported in the literature. Measured concentrations were determined from grab samples taken once at the end of the sampling period for comparison ($N = 1$).

		Estimated time-weighted concentration ng/L				Measured concentration ng/L			
Compound	Class	WWTP	Outfall	Downstream	Reference	WWTP	Outfall	Downstream	Reference
Caffeine	food	839.4	752.4	742.5	73.8	657.0	812.4	795.1	23.1
Sucralose	food	2500.5	3130.6	2996.0	9.9	789.0	991.1	709.9	46.7
Trimethoprim	anti-biotic	51.5	8.03	4.7	ND	43.4	20.0	19.5	ND
Sulfamethoxazole	anti-biotic	23.8	3.5	2.5	0.3	11.7	4.6	5.4	ND
Carbamazepine	anti-seizure	92.7	55.1	54.9	<LOQ	63.7	37.0	36.7	ND
Acetaminophen	analgesic	23.8	7.6	4.5	0.7	ND	ND	ND	ND
Ibuprofen	anti-inflammatory	92.4	31.0	20.3	6.0	100.6	64.8	74.7	ND
Gemfibrozil	lipid regulator	6.2	2.9	1.3	ND	15.0	9.1	ND	ND
Naproxen	anti-inflammatory	73.4	27.9	30.2	1.1	88.5	49.5	55.8	8.5
Triclosan	antibacterial	20.5	ND	–	ND	104.8	ND	ND	ND
Estrone (E1)	hormone	5.2	<LOQ	<LOQ	ND	8.2	ND	ND	ND
Estradiol (E2)	hormone	ND	N	ND	ND	ND	ND	ND	ND
Androstenedione	hormone	3.62	2.32	2.0	<LOQ	8.9	2.8	2.9	< LOQ
Testosterone	hormone	<LOQ	<LOQ	<LOQ	ND	3.1	ND	ND	ND
Venlafaxine	antidepressant	123.4	59.3	50.7	<LOQ	696.1	368.6	253.5	ND
O-dm-venlafaxine	metabolite	140.0	36.4	18.3	<LOQ	1594.8	671.2	289.7	ND
N-dm-venlafaxine	metabolite	9.6	6.9	4.3	ND	110.9	94.2	73.9	ND
Sertraline	antidepressant	11.1	1.9	0.4	ND	406.9	226.7	135.7	ND
dm-sertrailne	metabolite	ND	ND	ND	ND	70.8	11.6	ND	ND
Citalopram	antidepressant	ND	ND	ND	ND	ND	ND	ND	ND
Fluoxetine	antidepressant	ND	ND	ND	ND	ND	ND	ND	ND
Atenolol	beta-blocker	9.0	21.5	10.9	ND	125.3	65.6	51.5	ND
Metoprolol	beta-blocker	8.7	6.7	5.7	ND	42.0	31.8	24.2	ND
Propanolol	beta-blocker	59.9	3.3	4.7	ND	5.5	1.2	ND	ND

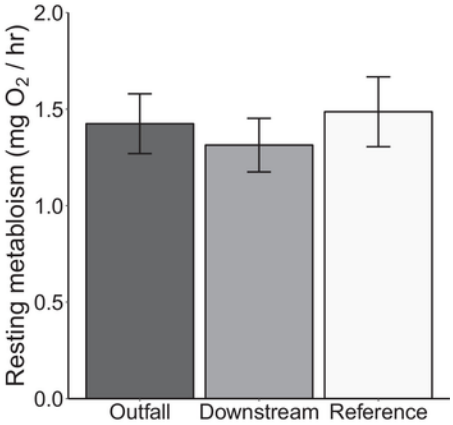

Fig. 4. Average resting metabolic rate, plotted by exposure site. Error bars represent ±1 standard error, finding was not significant (*ns*).

posure did not show any behavioural or physiological deficits. More specifically, exposure did not impact measures of fish aggression, their startle responses, or their overall activity. As well, we saw no exposure related differences in resting metabolism, hypoxia tolerance, or haemoglobin-oxygen transport.

To date, *in-situ* caging studies have shown inconsistent effects on fish survival following exposure to wastewater effluent. A select few studies have reported increased fish mortality after exposure to wastewater effluent (Kosmala et al., 1998; Mitz and Giesy, 1985; Nichols et al., 1999). For example, Nichols et al. (1999) found that fathead minnow (*Pimephales promelas*) survival was only 20% near a WWTP outfall, but 68% at an uncontaminated reference site following a three-week caging study. In contrast, most studies have reported no observable differences in survival following wastewater effluent exposure over similar durations to our study (3–4 weeks, Bernet et al., 2004; Burki et al., 2006; Giesy et al., 2003; Jasinska et al., 2015; Vermeirssen et al., 2005; Vincze et al., 2015). We observed reduced survival inside the WWTP and fish tended to have reduced survival at the outfall site, though this did not reach statistical significance. Generally, our survival results also followed the trends in water quality parameters and the concentrations of PPCPs. For example, we detected the highest concentrations of PPCPs in the WWTP, and slightly lower, but consistent concentrations at the outfall and downstream exposure sites. It is unlikely that the PPCPs we measured would singly cause fish mortality as their concentrations were much lower than concentrations that would cause lethality (Brausch and Rand, 2011; Brausch et al., 2012), moreover other compounds beyond what we measured may be present in the effluent. Another pollutant that is known to impact fish survival is ammonia (Canadian Council of Ministers of the Environment, 2010), and wastewater treatment plant effluents can contribute significant amounts of nitrogen to receiving environments (Carey and Migliaccio 2009). Previously, Nichols et al. (1999) linked fish mortality during a caging exposure downstream from a wastewater treatment plant outflow to concentrations of toxic ammonia (NH_3) in the treated effluent. During our exposure, ammonia was higher in the secondary effluent (mean: 0.19 mg/l, ±0.073 SE, Hamilton Water, 2015, *unpublished data*) at our WWTP site, than it was in the final treated effluent (mean: 0.056 mg/l, ±0.0087 SE, Hamilton Water, 2015, *unpublished data*) leaving the WWTP at the outfall site. Similarly, dissolved oxygen was lower in the WWTP than at the outfall, downstream, and reference sites (see Table 4). The combined effects of water quality parameters (*e.g.* ammonia, DO, temperature) alongside PPCPs and other wastewater contaminants could underlie the increased mortality we observed in our study.

We were surprised to find no evidence of behavioural or physiological deficits in round goby following our caging exposure. Even though only a few behavioural studies have been conducted to date exploring behavioural impacts following wastewater exposures, most have reported changes to fish behaviour (Garcia-Reyero et al., 2011; Martinović et al., 2007; Melvin, 2016; Saaristo et al., 2014; Sebire et al., 2011; but see Schoenfuss et al., 2002). We had also expected the fish to incur a metabolic cost from increasing exposure, as other researchers have noted changes to energy allocation and increased oxidative stress following wastewater exposures (Carney Almroth et al., 2008; Cazenave et al., 2014; Melvin, 2016). There are several possible reasons why we did not uncover an observable effect of our caging exposure on round goby behaviour, metabolism and respiratory physiology. One, the concentration of PPCPs or other pollutants may not have been high enough. Two, the exposure duration of our study may not have been not long enough, and had fish been exposed for longer they may have reached a "threshold" at which effects would become apparent. Three, the endpoints we evaluated may not have been particularly sensitive to wastewater effluent exposure. For example, the antidepressant venlafaxine was measured at 50 ng/l at the outfall, but only much higher concentrations (>200 000 ng/l) have been found to elicit behavioural effects in previous studies of hybrid striped bass (*Morone saxatilis x Morone chrysops*, Bisesi et al., 2014). In general, the effects of PPCP mixtures are still poorly understood, making it difficult to draw conclusions on behavioural and physiological effects in wastewater studies from exposure studies using individual compounds (Khetan and Collins, 2007; Backhaus, 2014).

Round goby, at least at the adult life stage that we tested, may also be tolerant of the effects of wastewater contaminants and poor water quality conditions. Round goby are known to tolerate a wide range of environmental conditions, and this tolerance has contributed to their widespread success as an invasive species in North America and Europe (Charlebois et al., 1997; Kornis et al., 2012). For example, this fish species can tolerate a wide range of salinities, water temperatures ranging −1 °C to 30 °C, and they have a critical threshold for dissolved oxygen as low as 0.4–1.3 mg/l (Arend et al., 2011; Charlebois et al., 1997; Cross and Rawding, 2009; Kornis et al., 2012). In a review by Moskal'kova (1996), the author connected round goby tolerance of adverse water conditions to their ability to settle in highly polluted environments such as industrial Harbours. Round goby are found in Hamilton Harbour (a large water body adjoining Cootes Paradise Marsh where our current study was conducted, see Fig. 1) and are common at locations that are highly contaminated with metals, polychlorinated biphenols (PCBs), and polyaromatic hydrocarbons (PAHs), where they are equally abundant in numbers to fish at comparatively cleaner sites (Marentette et al., 2010; McCallum et al., 2014). We are confident that the caging process itself did not give rise to our behavioural and physiological findings: round goby survived well in the reference location, and they are a small-bodied fish with a small home-range (5 m^2, Ray and Corkum, 2001) that would be well-suited to a caging experiment (Oikari, 2006; Palace et al., 2005). Despite the tolerance of round goby to wide environmental conditions, it remains possible that wastewater exposure eliminated sensitive individuals, or that wastewater exposure is stochastically eliminating a subset set of exposed individuals from the highest exposure groups (Fox, 1995; Newman and McCloskey, 2000). Further work would be needed to specifically test whether certain fish are more tolerant of wastewater effluent by using repeated exposures (Newman and McCloskey, 2000).

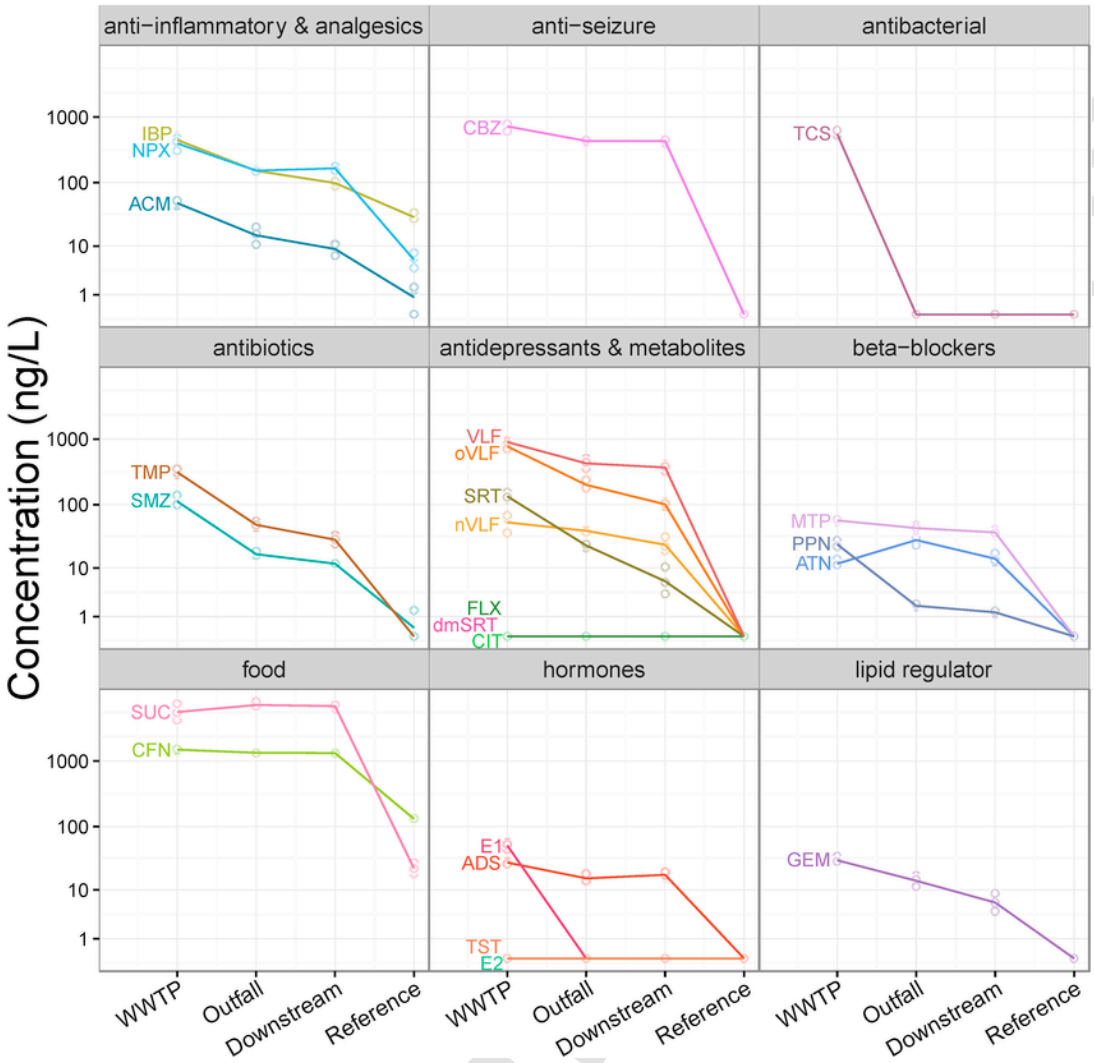

Fig. 5. Estimated time-weighted concentrations of assayed PPCPs measured with POCIS samplers, faceted by compound class. PPCP concentration (ng/l) is depicted on a log scale. Lines connect average concentrations from $N = 3$ POCIS samples per site, faded points represent individual observations per POCIS disk. IBP = Ibuprofen, NPX = naproxen, ACM = Acetaminophen, CBZ = carbamazepine, TCS = Triclosan, TMP = Trimethoprim, SMP = Sulfamethoxazole, VLF = Venlafaxine, oVLF = O-desmethyl venlafaxine, SRT = Sertraline, nVLF = N-desmethyl venlanfaxine, FLX = Fluoxetine, dmSRT = desmethyl sertraline, CIT = Citalopram, MTP = Metoprolol, PPN = Propanolol, ATN = Atenolol, SUC = Sucralose, CFN = Caffeine, E1 = Estrone, ADS = Androstenedione, TST = Testosterone, E2 = Estradiol, GEM = Gemfibrozil. See Table 3 for additional details.

Table 4
Summary of mean (±1 SE) water quality measures across the caging exposure period. Measures were taken once per week at all sites ($N = 7$, per site).

	Site			
Measure	WWTP	Outfall	Downstream	Reference
Temperature (°C)	18.21 (±0.47)	21.73 (±0.39)	22.95 (±0.41)	17.4 (±0.70)
Dissolved oxygen (mg/L)	1.98 (±0.44)	11.28 (±1.15)	8.84 (±0.82)	5.48 (±0.56)
pH	7.06 (±0.073)	7.95 (±0.17)	8.00 (±0.11)	8.00 (±0.16)
Conductivity (µS)	1276.00 (±68.08)	1243.37 (±41.82)	1283.87 (±40.42)	695.57 (±30.38)
Salinity (ppm)	592.67 (±31.35)	581.38 (±20.07)	600.50 (±19.33)	315.71 (±14.06)
TDS (ppm)	906.67 (±46.62)	883.38 (±30.31)	910.38 (±28.72)	494.71 (±21.19)
Flow (m/sec)	0.0050 (±0.0019)	0.016 (±0.0030)	0.017 (±0.0030)	0.021 (±0.0096)

To conclude, we found that exposure to wastewater effluent tended to reduce round goby survival within and immediately outside a wastewater treatment facility that releases effluent into an ecologically sensitive wetland. We found no discernable behavioural or physiological impacts of wastewater exposure on the surviving individuals. Locally, our work has implications for remediating Cootes Paradise Marsh, and may help inform the Remedial Action Plan for Hamilton Harbour, an International Area of Concern (International Joint Commission, 1999). Here, we have documented for the first time the presence and concentrations of a selection of the PPCPs that enter Cootes Paradise Marsh. When such contaminants are combined with poor wastewater effluent water quality, they may hinder the remediation of aquatic habitats for the wider range of less-tolerant taxa that inhabit the wetland. We recommend the continued use of caging techniques for studying real-world impacts of complex pollutants on fish survival, physiology, and behaviour. By combining environmental monitoring, with multiple measurements of fish physiology and

behaviour, we can more accurately begin to understand the effects of complex stressors, such as WWTP effluents, on wild fish species.

Uncited references

Handy and Depledge (1999) and Johnson and Sumpter (2014).

Acknowledgements

This research was conducted following approved protocols from the Animal Research and Ethics Board at McMaster University (AUP: 13-12-51) in accordance with the Canadian Council on Animal Care. We would like to thank B. Posedowski and M. Bainbridge from Hamilton Water for facilitating WWTP access and for data on the WWTP water chemistry; A. Bose for comments on this manuscript; B. Borowiec and N. Pranckevicius for technical assistance; T. Theÿsmeÿer and J. Bowman for assistance with cage deployment and site selection; B. Seaborn and C. D. Metcalfe for analytical support; and P. Saadat, R. Sangay, and K. Baliga for assistance with fish collections. This research was supported by RBC Blue Water and NSERC Discovery Grant to S. Balshine, NSERC Engage Grant and Early Researcher Award from the Ontario Ministry of Research and Innovation to G.R. Scott, and an NSERC PGS-D to E. McCallum. S. Balshine and G.R. Scott are supported by the Canada Research Chairs Program.

6.0

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.12.017.

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