PHARMACEUTICALS, WASTEWATER EFFLUENT, AND AGONISTIC BEHAVOIURS IN A WILD, INVASIVE FISH (*NEOGOBIUS MELANOSTOMUS*)

PHARMACEUTICALS, WASTEWATER EFFLUENT, AND AGONISTIC BEHAVIOURS IN A WILD, INVASIVE FISH (*NEOGOBIUS MELANOSTOMUS*)

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TITLE: Pharmaceuticals, wastewater effluent, and agonistic behaviours in a wild, invasive fish (*Neogobius melanostomus*)

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ABSTRACT

Anthropogenic contaminants in aquatic ecosystems are of widespread concern for ecosystem managers, scientists, and the organisms living in impacted habitats. Certain contaminants, like human pharmaceuticals and personal care products that are found in wastewater effluents, have been shown to have subtle but important effects on the physiology and behaviour of exposed organisms. Ecotoxicologists are therefore increasingly using behavioural endpoints to evaluate the impacts of treated wastewater effluents and pharmaceuticals on aquatic animals. However, few studies have evaluated whether behaviour is altered following exposure to wastewater effluents that wild fish are exposed to in their environments. Moreover, few studies have comprehensively evaluated the impacts of a given pharmaceutical or effluent exposure by testing behaviour over multiple behavioural contexts. In my thesis, I examined the effects of a pharmaceutical commonly reported in the environment, the antidepressant fluoxetine, as well as a complex mixture, wastewater effluent, on the behaviour of an invasive fish species, the round goby (*Neogobius melanostomus*). I focused on aggressive and social behaviours that are both critical for this species' survival and reproduction, and also explored these behaviours in the absence of any exposure. I showed that round goby are attracted to conspecifics, but do not preferentially associate with larger versus smaller groups of conspecifics (Chapter 2). Most of the "social" interactions were aggressive in nature, and I further explored aggression, showing that round goby were more aggressive over high quality (enclosed) shelters compared to low quality (open) shelters (Chapter 3). When round goby were exposed to the antidepressant fluoxetine, aggression declined rapidly and in multiple aggressive contexts, but only at concentrations higher than those reported in surface waters (Chapter 4). Intriguingly, fish exposed to wastewater effluent in the laboratory also showed reduced aggression towards a mirror (Chapter 5), but fish exposed to wastewater effluent in the wild showed little evidence of behavioural or physiological change following exposure (Chapter 6). Across experiments, I evaluated the relevance of mirror aggression assays as a surrogate for dyadic aggressive contests, and concluded that mirror aggression assays poorly predicted the outcome of more ecologically relevant paired interactions. Altogether, my findings suggest that adult round goby may be resilient to the exposure conditions I tested. My work further develops behaviour as a tool for investigating the impacts on environmental contaminants on aquatic organisms and broadens the species range used for such studies.

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Finally, I am very grateful that our passion for science and love of animals (aquatic and terrestrial) drew me and Aneesh Bose to apply for graduate work in Sigal's lab. His calm, level-headed encouragement has made even the craziest times across my PhD a manageable feat, and I am thankful for our endless discussions about fish and what they "do". For all of this, I cannot express enough appreciation.

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DECLARATION OF ACADEMIC ACHIEVMENT

This dissertation is organized in a sandwich format as approved by McMaster University. It consists of seven chapters and five appendices. **Chapter 1** provides an introduction to the use of behavioural ecology in studies of ecotoxicology, reviews the issue of wastewater effluent exposure impacts on wild aquatic animals, and introduces the study species and study sites that have been used in my research. **Chapter 2** is a published manuscript. **Chapter 3** is a manuscript currently under review. **Chapters 4, 5, and 6** are manuscripts that are in-preparation for journal submission. **Chapter 7** provides a discussion and synthesis of result from Chapters 2-6. I have included 2 additional published manuscripts as appendices, which are based on research I conducted while at McMaster University, but they are not thematically connected to my dissertation.

Chapter 1: General introduction

Author: Erin S. McCallum

Chapter 2: Aggression and sociality: conflicting or complementary traits of a successful invader?

Authors: Pauline M. Capelle, Erin S. McCallum, and Sigal Balshine.

Publication: *Behaviour, 152*, 127-146. Reprinted with permission.

Comments: PC and EM contributed equally to this manuscript and are co-first authors. PC, EM, and SB conceived of this study. PC and EM conducted behavioural trials, analyzed the data, and wrote the manuscript with input from SB.

Chapter 3: Contest dynamics in a territorial fish are dependent on prior resource experience, without in-contest updating

Authors: Erin S. McCallum, Sarah T. Gulas, and Sigal Balshine

Publication: Submitted to *Animal Behaviour (ANBEH-D-16-00587*)

Comments: EM, SG, and SB conceived of this study. EM and ST collected behavioural data. EM analyzed the data and wrote the manuscript, with input from SB and ST.

Chapter 4: The antidepressant fluoxetine decreases aggression across multiple behavioural contexts in round goby (*Neogobius melanostomus*)

Authors: Erin S. McCallum, Aneesh P. H. Bose, Theresa R. Warriner, and Sigal Balshine

Publication: In-preparation for submission to *Aquatic Toxicology*

Comments: EM and SB conceived of this study. EM collected and analyzed the data, and AB and TW assisted in data collection and statistical analyses. EM wrote the manuscript, with input from SB.

Chapter 5: Exposure to wastewater effluent reduces aggression in round goby (*Neogobius melanostomus*)

Authors: Erin S. McCallum, Emily Krutzelmann, and Sigal Balshine

Publication: In-preparation for submission to *Ecotoxicology*

Comments: EM and SB conceived of this study. EK collected behavioural data, with EM assisting with effluent exposures. EM analyzed the data and wrote the manuscript, with input from SB

Chapter 6: *In situ* exposure to wastewater effluent reduces survival but has little effect on the behaviour and physiology of an invasive Great Lakes fish

Authors: Erin S. McCallum, Sherry N. N. Du, Maryam Vaseghi-Shanjani, Jasmine A. Choi, Theresa R. Warriner, Graham R. Scott, and Sigal Balshine

Publication: In-preparation for submission to *Aquatic Toxicology*

Comments: EM, SD, GS, and SB conceived of this study. EM, SD, MV, JC, and TW conducted field exposures and collected data: EM conducted behavioural trials, MV conducted haemoglobin analyses, JC conducted gill morphometrics, SD conducted whole-body metabolic physiology. EM analyzed the data and wrote the manuscript, with input from SB, GS, and SD.

Chapter 7: General discussion

Author: Erin S. McCallum

Appendix A: The diet and foraging of Round Goby (*Neogobius melanostomus*) in a contaminated Harbour

Authors: Erin S. McCallum, Julie R. Marentette, Claire Schiller, Shagun Jindal, Kyle Empringham, Susan Marsh-Rollo, Harri Pettitt-Wade, Marten A. Koops, Aaron T. Fisk, and Sigal Balshine

Publication: *Aquatic Ecosystem Health and Management, accepted – in press.* Reprinted with permission.

Comments: EM, JM, and SB conceived of this study. EM, HP, and AF collected and analyzed stable isotope samples. EM, JM, and KE collected and analyzed diet samples. CS and JM collected measures of foraging behaviour. SJ and SMR collected and analyzed sediment samples. EM conducted statistical analyses. EM wrote manuscript, with input from SB, AF, and MK.

Appendix B: Persistence of an invasive fish in a contaminated ecosystem (*Neogobius melanostomus*)

Authors: Erin S. McCallum, Rachel E. Charney, Julie R. Marentette, Jennifer A. M. Young, Marten A. Koops, David J. D. Earn, Benjamin M. Bolker, and Sigal Balshine

Publication: *Biological Invasions*, *16*(11), 2449-2461. Reprinted with permission.

Comments: SB conceived of this long-term population monitoring study, supported by MK. EM and JR organized yearly data collection for this study. RC conducted statistical analyses, with assistance from JY, BB, DE, and EM. EM wrote this manuscript, with input from SB, BB, and RC.

Appendix C: Supplementary Table S1: Chapters 3, 4, 5, 6

Author: Erin S. McCallum

Comments: Round goby ethogram used to score all behavioural trials in Chapters 3, 4, 5, and 6.

Appendix D: Supplementary information for Chapter 4

Authors: Erin S. McCallum, Aneesh P. H. Bose, Theresa R. Warriner, and Sigal Balshine

Appendix E: Supplementary information for Chapter 6

Authors: Erin S. McCallum, Sherry N. N. Du, Maryam Vaseghi-Shanjani, Jasmine A. Choi, Theresa R. Warriner, Graham R. Scott, and Sigal Balshine

Chapter 1: General introduction

"Only within the moment of time represented by the present century has one species—man—acquired significant power to alter the nature of his world" – *Rachel Carson, Silent Spring*

1.1 Motivation for thesis

Human consumption and use of pharmaceuticals and personal care products (PPCPs) has been increasing around the world (OECD, 2013; Van Boeckel et al., 2014). For example, in Canada, spending on pharmaceuticals has increased each year since 1975, and between 1997 and 2007 pharmaceutical spending increased at an average annual rate of 5.7% (Canadian Institute for Health Information, 2012). Moreover, this spending was driven by increased pharmaceutical use and population growth, and not price inflation (Canadian Institute for Health Information, 2012). A consequence of rising use is that PPCPs are increasingly being reported in treated wastewater effluents and surface waters near effluent sources in Canada (Blair et al., 2013) and around the world (Kolpin et al., 2002; Vulliet & Cren-Olivé, 2011; Verlicchi et al., 2012). The potential impacts of PPCPs on aquatic animals inhabiting waters that receive wastewater inputs are only beginning to be realized (Corcoran et al, 2010; Boxall et al., 2012). Many pharmaceuticals are designed to alter human physiology and behaviour to treat human illnesses and ailments. Thus, there is growing concern over whether the reported low concentrations of pharmaceuticals present in the environment have similar, but unintended, therapeutic impacts on aquatic organisms, especially vertebrates that share evolutionarily conserved biological drug targets (e.g. receptors, enzymes; Gunnarsson et al., 2008).

Animal behaviour has emerged as a key tool or endpoint when assessing the sub-lethal effects of certain pharmaceuticals, especially for pharmaceuticals designed to treat human behavioural disorders (e.g. antidepressants, anxiolytics, mood stabilizers). While the development and use of behavioural assays has been growing for laboratory-based single-pharmaceutical exposure studies (for example: Brodin et al., 2013; Dzieweczynski & Buckman, 2013; Galus et al., 2014), it is hard to extrapolate these findings to the environmental conditions animals experience when exposed to complex wastewater effluents in the wild. To date, few studies have assessed behavioural changes in response to real-world wastewater effluents. Moreover, few studies have evaluated multiple behavioural contexts following an exposure, but this type of testing is necessary for identifying robust behavioural effects. In my PhD research and the chapters in this thesis, I studied the impact of two exposure regimes on the aggressive and social interactions of a wild fish species, the round goby (*Neogobius melanostomus*). These two regimes were: exposure to a single pharmaceutical contaminant (the antidepressant fluoxetine, Chapter 4) and exposure to the complex mixture of contaminants found in wastewater effluents (Chapters 5-6). I also studied round goby aggressive and social behaviours in the absence of any exposure to provide a starting context for my exposure work, and to better understand the behavioural ecology of this wild fish species (Chapters 2-3). In this introductory chapter, I will briefly review the theoretical underpinnings of my research program, which are described in greater detail in Chapters 2-6. I will also introduce the study species and study area that I used for my doctoral research.

1.2 Theoretical background

1.2.1 A brief introduction to behavioural ecotoxicology

Behavioural ecotoxicology is the assessment of how environmentally and ecologically relevant exposures to contaminants impact animal behaviour. The field has emerged from the intersection of three separate fields of study: *ethology*, the study of an organism's behaviour; *ecology*, the study of an organism's interactions with their environment; and *toxicology*, the study of toxic compounds and their effects on organisms (Dell'Omo, 2002). Before the emergence of behavioural ecotoxicology in the 1960's, the disciplines of ethology, ecology, and toxicology had relatively separate histories and these fields largely developed in parallel, each with its own unique methodologies and terminology. One of the first studies to combine these fields was conducted by Warner et al., (1966); these researchers showed that exposure to environmentally relevant doses of the pesticide toxaphene increased goldfish swimming activity. In the wild, animals are unlikely to be exposed to doses of pollutants that cause direct lethality (barring acute, unintended pollutant spills). Instead, exposure to chronic, low doses of a pollutant may cause sub-lethal changes in the physiology of wild animals, which may have far reaching impacts on an animal's behaviour, health, growth, and ultimately its reproductive success. As concern over the presence of pollutants in the natural environment began to rise in both the public and scientific communities in the 1970's, so too did a concern that pollutants may have sub-lethal effects on wild animals (Dunlap, 1991; Rattner, 2009).

Studying animal behaviour to assess the impacts of pollutants on wildlife is appealing to scientists for several reasons (see reviews: Clotfelter et al., 2004; Zala & Penn, 2004; Robinson, 2009; Hellou, 2011). First, changes to behaviour often become evident long before traditional lethality endpoints, and behavioural endpoints can sometimes be up to 10 to 100 times more sensitive than traditional LC_{50} measures (the lethal dose at which 50% of an exposed population is killed; Beitinger, 1990; Little & Finger, 1990). For example, Weis & Weis (1974) found that goldfish (*Carassius auratus*) exposed to 1 µg/l of the pesticide DDT had altered swimming and shoaling behaviour, while the DDT LC_{50} was reported to be much higher, 30-100 µg/l. Second, behavioural responses are relatively inexpensive to measure compared to certain molecular or physiological techniques (apart from the obvious expenses needed for housing animals, and the resources to run and record behavioural trails). Newer behavioural tracking technologies certainly add to cost, but greatly streamline data collection, e.g. Noldus Ethovision^{TM}. Third, behaviour can be measured without causing harm to the animal and is thus a non-invasive method to assess pollutant impacts. Behavioural measures can therefore be taken repeatedly to compare behaviour before and after a given contaminant treatment, or to observe behavioural impacts along a time-course of exposures, or to assess behavioural recovery after an exposure has ended. Fourth, and most importantly, behaviour is the result of an animal's current physiological state, which has been shaped by its evolutionary history, its developmental environment, and its current environment.

Behaviour is therefore an integration of internal, proximate processes (e.g. cellular signaling, hormonal process, metabolism) with ecological, ultimate consequences (e.g. selective pressures, historical events; Tinbergen, 1963; Grue et al., 2002). Behaviour can be used to investigate the potential mechanisms of pollutant action, while also having implications for understanding the survival and persistence of a population (Peakall, 1996; Grue et al., 2002).

Behaviour has an adaptive value that increases an individual's survival and reproductive success in a given environment (Tinbergen, 1963; Zala & Penn, 2004). For example, behaving appropriately in response to predators, foraging efficiently, successfully defending a territory, and attracting potential mates are all critical components of fitness. In studies of behavioural ecotoxicology, contaminants can impact animal behaviour and fitness directly or indirectly (Grue et al., 2002; Hellou, 2011). Behaviour is directly impacted when a contaminant-exposed organism displays a change in their behaviour that may lead to a change in their survival and/or reproduction. Indirect behavioural effects are harder to measure, and occur when *unexposed* organism's behaviour changes as a result of interactions with other contaminant-exposed organisms. Indirect effects cause down- or upstream changes in animal communities and ecosystems (e.g. predator-prey interactions, trophic cascades; Brodin et al., 2014; Kidd et al., 2014). For example, Kidd et al. (2014) noted an increase in zooplankton population growth following a contaminant exposure that eliminated their main predators. In Kidd and colleagues' experiment, an ecosystem was exposed to ethynilestradiol (EE2) and populations of fathead minnow (*Pimephales promelas*) and slimy sculpin (*Cottus* cognatus) collapsed due to reproductive failure. However, zooplankton populations increased after exposure to EE2 stopped (there was no effect on zooplankton during the exposure), strongly indicating an indirect effect on zooplankton population growth and reproduction through the removal of predation by fish. While direct effects are much more straightforward to measure, especially in the laboratory, the potential for indirect effects should not be ignored as they connect individual behaviour to the larger population, community or ecosystem.

1.2.2 Pharmaceuticals as environmental contaminants in wastewater effluents

The recent awareness of the presence of prescription, over-the-counter, and veterinary pharmaceuticals, as well as personal care products in the natural environment (collectively referred to as "PPCPs") has presented a new research focus for behavioural ecotoxicology (Daughton & Ternes, 1999). Ingested pharmaceuticals are excreted into the wastewater system only partially metabolized, or sometimes un-metabolized (Jjemba, 2006; Lienert et al., 2007). Likewise, pharmaceuticals can be improperly disposed of, and personal care products used in homes, businesses, and industry are partially washed down the drain (Ternes et al., 2004; Bound & Voulvoulis, 2005). An unintended consequence of increased PPCP use is the discharge of PPCPs, their byproducts, and metabolites in the environment (OECD, 2013; Van Boeckel et al., 2014). PPCPs enter water bodies around the world via wastewater treatment plant effluents, manufacturing and hospital discharges, and agricultural biosolids or aquaculture run-off (Pal et al., 2010). Wastewater treatment plants are a large contributor of PPCPs to the environment because they are poorly equipped to remove these compounds from wastewater. PPCPs are therefore only partially removed before treated effluent enters the aquatic environment (Jelic et al., 2012). There are several factors that will determine the concentrations of PPCP compounds in surface waters near a wastewater treatment plant, such as the size and demographics of the contributing population, the type of treatment technologies used, the time span over which treatment is implemented at the wastewater treatment facility, and the flow and dilution of wastewater effluent in the receiving water body (Pal et al., 2010; Jelic et al., 2012). Generally, in developed countries, PPCPs are measured at low concentrations in wastewater effluents and receiving waters (ng/l to µg/l: Kolpin et al., 2002; Blair et al., 2013).

Even at low concentrations, there is mounting concern over the effects that PPCPs, pharmaceuticals in particular, may have on wild aquatic animals (Boxall et al., 2012; Arnold et al., 2013). Pharmaceuticals are made to have therapeutic impacts on human physiology and behaviour. The biological targets that many drugs act upon (e.g. receptors, enzymes) are well-conserved across vertebrate taxa (Gunnarsson et al., 2008; Brown et al., 2014). For example, Gunnarsson et al. (2008) found that zebrafish (*Danio rerio*) had drug target orthologs for 86% of the 1318 human drug targets they investigated. If pharmaceuticals similarly affect animals as they affect humans, then the pharmaceuticals with the greatest potential to impact animal behaviour are psychiatric pharmaceuticals such as antidepressants, anxiolytics, and mood stabilizers. Psychiatric pharmaceuticals are prescribed to treat human behavioural disorders, and these drugs are frequently measured in the environment, though at low concentrations (Calisto & Esteves, 2009; Metcalfe et al., 2010; Klaminder et al., 2015). Researchers can assess the responses of aquatic animals to PPCPs in the laboratory by exposing them to the concentration of a PPCP that is reported in the environment; this is referred to as the environmentally relevant exposure concentration or dose. Recent laboratory investigations of fish behaviour following pharmaceutical exposures has indicated that psychiatric pharmaceuticals can change behaviours such as courtship, territory defense, and predator evasion, all important components of fitness (Brodin et al., 2013; Weinberger & Klaper, 2014; Greaney et al., 2015; Pelli & Connaughton, 2015). Behavioural effects following exposure to environmentally relevant concentrations of PPCPs are not always observed, and are sometimes only reported following exposure to concentrations that are higher than what is reported in the environment (Holmberg et al., 2011; Sebire et al., 2015).

Laboratory studies are essential to further our understanding of individual contaminants on animal behaviour and physiology. It is difficult to extrapolate laboratory findings to understand how wastewater effluents may impact fish in the wild, because wastewater effluent is a dynamic and complex mixture of contaminants. One way to understand environmentally relevant contaminant stressors, such as wastewater effluent, is to study the effects these realistic stressors under varying conditions of control (i.e. in the laboratory versus the field). Few studies have attempted to quantify behavioural responses following exposure to wastewater effluents (however, see: Martinović et al., 2007; Sebire, et al., 2011; Saaristo et al., 2014 for some exceptions). Also, few studies have compared laboratory exposures to field exposures while using the same study species. In this thesis, I have endeavored to address these research gaps by conducting exposure experiments with a single pharmaceutical pollutant (the antidepressant fluoxetine), but also with a complex contaminant mixture, wastewater effluent, that is being discharged into the environment. I focused on fluoxetine because it is a highly prescribed antidepressant (Milane et al., 2006; Milea et al., 2010) that is commonly reported in surface waters (Metcalfe et al., 2003; Christensen et al., 2009; Metcalfe et al., 2010; Blair et al., 2013), with a high potential to impact wild animal behaviour through its action on the serotonergic system (Brooks et al., 2003; Mennigen et al., 2011).

Since few investigations thus far have assessed multiple behaviours within an experiment, or tested behaviour repeatedly from the same individual (but see: Dzieweczynski & Hebert, 2012; Greaney et al., 2015), I evaluated the effects of my exposures on multiple behavioural endpoints. I have focused primarily on complex social and aggressive interactions between conspecifics, with additional assessment of more basic animal movement and startle reactions. I pursued this behavioural focus for several reasons: 1) Conspecific aggressive and social interactions are important for animals in the wild, allowing them to encounter potential mates and secure resources needed for their survival and reproduction (Clutton-Brock, 1988; Arnott & Elwood, 2008); 2) Psychiatric pharmaceuticals are often prescribed to treat human behavioural disorders that specifically modulate human social interactions (e.g. depression, social anxiety; Milea et al., 2010), making studying them in exposed fish especially relevant. I also investigated the social and aggressive behaviours of my study species in the absence of any exposure. Developing a thorough understanding of a species' behavioural ecology is essential to better understand how pollutants may affect their behaviour.

1.3 Study species: Round goby

Throughout this thesis, I have used round goby (Figure 1.1) to investigate the impacts of a pharmaceutical contaminant and wastewater effluents on fish behaviour. Round goby are a small, benthic, invasive fish species, native to the Ponto-Caspian region of Europe, that are now widespread throughout both the Laurentian Great Lakes and the waterways of Western Europe (Corkum et al., 2004; Kornis et al., 2012). Round goby were introduced to the Laurentian Great Lakes through ship ballast water discharge in the early 1990's, and quickly spread to all five Laurentian Great Lakes in under a decade (Corkum et al., 2004; Kornis et al., 2012). Many life-history, physiological, and behavioural factors have contributed to the success of round goby as an invasive species, their: tolerance for a wide-range of environmental conditions (Moskal'kova, 1996;

Figure 1.1
Adult round goby (*Neogobius melanostomus*). Photo credit: E. McCallum.

Charlebois et al., 1997); generalist diet (Johnson et al., 2008; Brush et al., 2012; McCallum et al. *in press* - Appendix A); rapid reproductive rates with multiple spawns each season (Corkum et al., 1998; MacInnis & Corkum, 2000); and aggressive nature (Balshine et al., 2005; Bergstrom & Mensinger, 2009).

Besides the obvious advantages of being small-bodied, these fish are relatively easy to collect and house in the laboratory, making the round goby a useful laboratory model system for assessing contaminant impacts. Moreover, aggressive behaviours are especially important for round goby survival and reproduction in the wild, making this species an intriguing system to study the impacts of pollutants on conspecific interactions.

Round goby occupy and prefer rocky, sheltered habitats in the littoral zone (Young et al., 2010), using these spaces to hide from many avian and fish predators (Somers et al., 2003; Reyjol et al., 2010). They also use these rocky shelters as an area in which to reproduce and care for offspring (Corkum et al., 1998; MacInnis & Corkum, 2000). Round goby will compete for and defend these highly valued shelters from both con- and heterospecifics (Dubs & Corkum, 1996; Balshine et al., 2005; Bergstrom & Mensinger, 2009). Monopolization of these shelters, which are often limited, is thought to be linked to declines in populations of small native fish species that occupy the same habitats, such as johnny darter (*Etheostoma nigrum*) and mottled sculpin (*Cottus bairdii*; Janssen & Jude, 2001; Lauer et al., 2004). In the laboratory, round goby readily display aggressive behaviours and will defend and occupy artificial shelter boxes (Stammler & Corkum, 2005; Sopinka et al., 2010). However, round goby are also incredibly abundant and are observed at high densities in the wild (Taraborelli et al., 2009), indicating that regular social interactions are likely to occur. Consequently, tolerance of nearby conspecifics may be a factor contributing to the widespread success of round goby, warranting further investigation into the social tolerance of these fish. An additional benefit to studying round goby is that their behavioural repertoire has begun to be characterized (Sopinka et al., 2010), increasing their usefulness as a model for assessing the behavioural impacts of environmental contaminants.

1.4 Study Location: Hamilton Harbour, ON, Canada

In this thesis, I report on my studies that examined how wastewater effluents that are discharged into Hamilton Harbour (ON, Canada; Figure 1.2) affect round goby behaviour. Hamilton Harbour is an International Joint Commission Area of Concern (International Joint Commission, 1999), and has been heterogeneously contaminated from both historical steel processing and a growing human population that created significant urban run-off, wastewater effluent inputs, and combined sewer overflows (Hamilton Harbour RAP, 1992). Hamilton Harbour currently receives wastewater effluent from three wastewater treatment facilities (Woodward, Burlington Skyway, and Dundas wastewater treatment facilities, Figure 1.2). In this thesis, I studied wastewater effluent from both the Woodward (serves ~ 400 000 people, Chapter 5) and Dundas (serves ~ 40 000 people, Chapter 6) wastewater treatment facilities. The Woodward wastewater treatment facility is a conventional activated sludge treatment plant with chlorine disinfection and secondary treatment (City of Hamilton, 2011). PPCPs such as carbamazepine and triclosan have already been measured in the surface waters downstream from this treatment facility's outflow (Metcalfe et al. 2003; Csiszar et al. 2011). The Dundas wastewater treatment facility is a conventional activated sludge treatment plant with chlorine disinfection and tertiary sand filtration (City of Hamilton, 2011). It is currently unknown whether and which PPCPs are present in surface waters downstream from the Dundas treatment facility's outflow.

The Harbour has been undergoing remediation since 1985 to improve water quality, reduce pollutant loadings, and enhance wildlife health and habitat quality (Hall et al., 2006; Hall & Connor, 2016). Because of the current conservation efforts, studying the behavioural impacts of wastewater and the contaminants within the Harbour, is especially pertinent and timely.

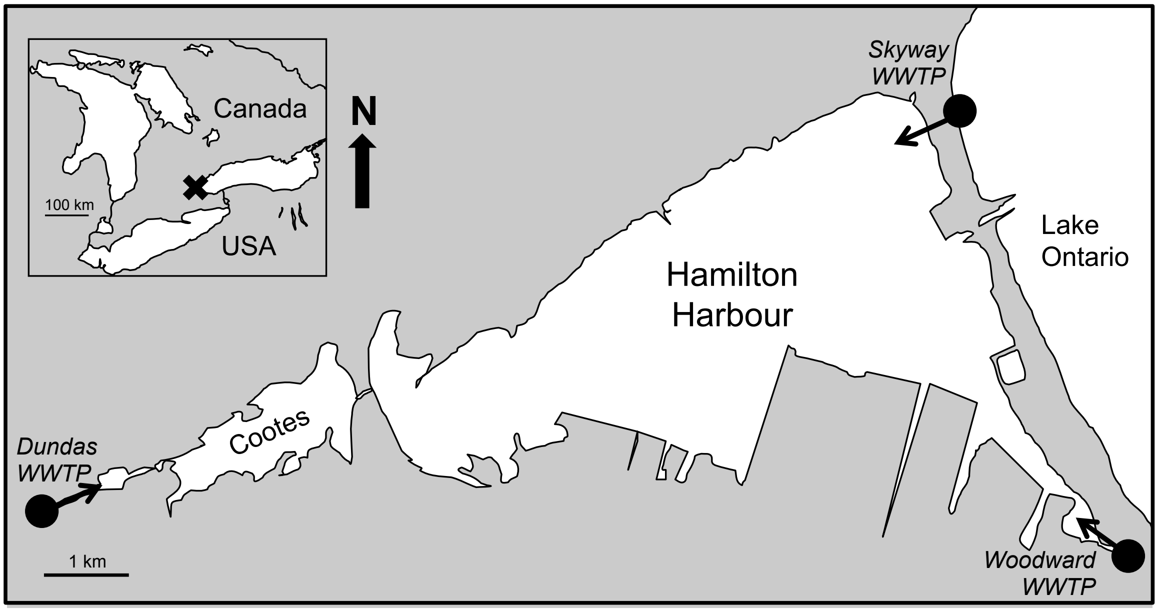

Figure 1.2
Map of Hamilton Harbour (ON, Canada). Black circles and arrows indicate the location and effluent inputs from wastewater treatment plants (WWTPs) into Hamilton Harbour. Inset depicts location of Hamilton Harbour at the western-most point of Lake Ontario, marked by the black cross. Map adapted from J. R. Marentette (2011).

1.5 Aims of the thesis

The specific aims of my thesis are as follows:

1) To further describe round goby social and aggressive interactions, focusing on round goby attraction to and tolerance of conspecifics, as well as the impact of valued shelter resources on the duration and intensity of their aggressive contests.
2) To address how a psychiatric pharmaceutical (the antidepressant fluoxetine) commonly reported in the environment, affects round goby social interactions and aggressive behaviours.
3) To assess the effect of complex effluents on round goby behaviours following exposure to treated wastewater effluents under both controlled laboratory conditions and *in situ* in the natural environment.

1.6 Thesis structure

In this section, I will provide an overview the structure of my thesis, briefly outlining the focus of each data chapter. In **Chapter 2**, I investigated round goby social interactions. Here, I assessed whether round goby exhibited a preference for interacting with a conspecific, whether they preferred larger groups of conspecifics, and whether they preferred to occupy a shelter to interacting with a conspecific. In **Chapter 3**, building off previous studies of round goby aggression (Stammler & Corkum, 2005; Sopinka et al., 2010; Groen et al., 2012), I further investigated round goby preference for shelter resources. By varying the quality of shelters, I explored how fish gathered information about resource value, and tested how resource value impacts aggressive contest dynamics.

In **Chapter 4**, I investigated how exposure to a pharmaceutical commonly reported in the environment, the antidepressant fluoxetine, impacts round goby movement, social interactions, and aggressive behaviours following an acute (3 day) and chronic (28 day) exposure. In **Chapter 5,** I investigated the impacts of complex wastewater effluent on round goby survival, movement, and aggressive behaviours following a chronic wastewater effluent exposure in the laboratory. In **Chapter 6**, I investigated the impacts of wastewater effluent exposure in the natural environment on round goby movement, aggression, and boldness in concert with measures of respiratory and metabolic physiology. In this study, I used an *in situ* caging exposure and experimentally placed fish at varying distances from a wastewater effluent source. In **Chapter 7**, I have reviewed and synthesized the findings of my thesis, and proposed a number of fruitful directions for continued research.

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Chapter 2: Aggression and sociality: conflicting or complementary traits of a successful invader?

2.1 Abstract

Invasion biology research has identified two juxtaposing behavioural traits, aggressiveness and sociality, that may both increase the success of species invasions. Highly aggressive invaders can out-compete native species for resources, while social gregarious invaders can tolerate high conspecific density. In order to tease apart the effects of aggressive versus social tendencies on the success of invasive species, we studied round goby (*Neogobius melanostomus*), a highly successful invasive fish species now common in the Laurentian Great Lakes. While round goby are well known for being aggressive, much less is known about their tendency to affiliate with conspecifics, in spite of the fact that they thrive in extremely high densities in many of their invaded habitats. We collected round goby from Hamilton Harbour, ON, Canada and conducted three separate experiments to explore group-forming behaviour by measuring preference for conspecifics. We found that round goby have a strong preference to associate with a single conspecific, and that both males and females showed this preference. No overall preference was detected for large versus small groups of conspecifics. Females chose the safety of a shelter over associating with a conspecific, but males were equally attracted to conspecifics as shelter. Our results provide new insight into how interactions between aggressive and social behaviours play a role in the rapid spread of invasive round goby.

2.2 Introduction

Behaviour has been identified as an important factor for understanding invasion processes (Holway & Suarez, 1999; Chapple et al., 2012). Studying how behaviours influence invasion dynamics can further our understanding of why particular species succeed to establish (Sol et al., 2008), but detailed information about an invasive species' behavioural repertoire is often missing. Sociality, or the tendency to live in groups, has been proposed as one behavioural attribute facilitating the colonization and establishment of an invasive species (Holway & Suarez, 1999; Chapple et al., 2012). Social species that are highly gregarious are thought to rapidly colonize and build up in population density, competitively displacing other species due to their numerical advantage (Holway & Suarez, 1999; Tsutsui et al., 2000). However, this idea has rarely been assessed empirically. Much more research has focused on aggressiveness, another classic behavioural characteristic of invasive species, with a number of studies showing that high interspecific aggression allows invasive species to out-compete and displace native species (Capelli & Munjal, 1982; Dick et al., 1995; Usio et al., 2001; Gherardi & Daniels, 2004; Duckworth & Badyaev, 2007; Weis, 2010). Both sociality and aggressiveness are proposed to propel invasion success (Chapple et al., 2012), yet these traits seem to stand in contrast. Successful invaders displaying high aggression to heterospecifics should also show high aggression towards conspecifics (Pintor et al., 2009), limiting the ability of a species to reach high densities. Exploring social and aggressive behaviours will help elucidate the mechanism of how successful invaders rapidly colonize new areas.

Most often, aggression is assessed in a given invasive species, rather than sociality. For example, among ants (Rowles et al., 2007; Carpintero & Reyes-López, 2008), crustaceans (Capelli & Munjal, 1982; Dick et al., 1995; Usio et al., 2001; Gherardi & Daniels, 2004; reviewed in Weis, 2010) and birds (Duckworth & Badyaev, 2007; Duckworth, 2009), invasive species are known to dominate in aggressive contests with native species. In invasive western bluebirds (*Sialia mexicana*), biased dispersal of aggressive individuals at the invasion front led to the displacement of less aggressive native species, but levels of aggression decreased in the established invasive population in subsequent generations (Duckworth & Badyaev, 2007). The few existing studies linking sociality and invasion success have focused on the influence of sociality across different stages of invasion. For example, asocial invasive mosquitofish (*Gambusia affinis*) were found to disperse further than social individuals, showing that the successful spread of an invader is associated with individuals who are not social (Cote et al., 2010, 2011). Successful establishment has been associated with high sociality and low intraspecific aggression in social insect species (Holway et al., 1998, 2002; Perdereau et al., 2011). Invasive Argentine ant (*Linepithema humile*) populations experienced a genetic bottleneck that facilitated the formation of large and dense colonies lacking intraspecific aggression while still displaying high levels of interspecific aggression, allowing them to out-compete native populations (Tsutsui et al., 2000). Taken together, the above work demonstrates the potential for sociality to facilitate invasion and establishment alongside aggression and underscores the need to empirically evaluate the importance of both aggression and social tendencies as traits in successful invasive species.

To this end, we examined social preferences in round goby (*Neogobius melanostomus*), an invasive fish species to the Laurentian Great Lakes that is well known to be highly aggressive (Charlebois et al., 1997; Corkum et al., 1998, 2004; Kornis et al., 2012). Round goby are native to the Black and Caspian Seas of Europe and were accidentally introduced into the St. Clair River via ballast ship water in 1990 (Jude et al., 1992) and rapidly spread to all five Laurentian Great Lakes (Corkum et al., 2004). Round goby are thought to be responsible for the decline of several native fish species that rely on similar resources (French & Jude, 2001; Janssen & Jude, 2001). The ability of round goby to out-compete native species has been attributed in part to their highly aggressive nature during interactions with native fish species (Dubs & Corkum, 1996; Janssen & Jude, 2001; Balshine et al., 2005; Bergstrom & Mensinger, 2009). To date, behavioural research on this species has focused mainly on interspecific competition between round gobies and native fish species, while far less research has explored round goby intraspecific interactions. Since round goby are a benthic, nest-guarding fish that are not thought to form tight social aggregations (Charlebois et al., 1997), it is surprising that they have been observed living at high densities in the Great Lakes Basin. They have been reported at densities of 0.80 to 7.76 fish/m^2 in western Lake Erie (Johnson et al., 2005), at densities of 3.88 to 9.64 fish/m^2 in the Bay of Quinte (Schaner et al., 2009; Taraborelli et al., 2009), and the highest densities have been reported in the Trent River at 9.6 and 17.0 fish/m^2 (Gutowsky et al., 2011; Brownscombe & Fox, 2012). Males have been reported sometimes nesting within centimetres of each other (Wickett & Corkum, 1998), indicating that round goby males in the Great Lakes may be highly tolerant of conspecifics even during the breeding season. In laboratory contests, round goby are able to perceive even very small (3%) size differences between themselves and a conspecific opponent, eliminating the need for prolonged or overt aggression (Stammler & Corkum, 2005; Groen et al., 2012). If round goby do indeed have a high tolerance of conspecifics, this may be another factor contributing to their rapid establishment and proliferation (Marentette & Corkum, 2008). However, a recent study examining the effects of density on competition found that at higher densities, round goby showed decreased growth and emptier digestive tracks (Kornis et al., 2014), suggesting that there is a cost to grouping and living at high density. This finding highlights the importance of studying intra-, as well as interspecific interactions to determine whether round goby simply tolerate conspecifics, or whether they have a natural tendency to move towards and affiliate with conspecifics because of possible benefits from grouping.

Using an established population of round goby, we sought to assess social aggregation decisions in this species and to provide an initial quantification of their potential for sociality. The specific aims of this study were to address three questions about grouping decisions in round goby: (1) Do round goby prefer to affiliate with a single conspecific or remain solitary? (2) Do round goby prefer to affiliate with small or large groups of conspecifics? (3) Do round goby prefer to affiliate with a conspecific more than inhabiting a shelter? We tested grouping preferences in three separate laboratory experiments using a well-established social preference assay (Svensson et al., 2000; Buckingham et al., 2007; Gomez-Laplaza & Gerlai, 2011; Reddon et al., 2011). Given the high density of round goby in the wild (Johnson et al., 2005), their apparent tolerance of conspecifics (Stammler & Corkum, 2005; Marentette & Corkum, 2008), and theoretical anti-predatory benefits from grouping (Hamilton, 1971; Foster & Treherne, 1981; Morgan & Godin, 1985), we predicted that round goby (of both sexes) would prefer to affiliate with a conspecific over remaining alone. Because large groups often provide better protection against predators compared to small groups (Foster & Treherne, 1981; Magurran & Pitcher, 1987), we also predicted that round goby would prefer a larger group of conspecifics compared to a smaller group. Although round goby may receive anti-predation benefits from grouping, they typically avoid predators by sheltering under rocks (Charlebois et al., 1997), and they have a strong preference for rocky substrate and will use and defend rock shelters year round (Ray & Corkum, 2001; Young et al., 2010, *personal observations*). Therefore, we predicted that round goby would prefer to spend time in a shelter versus affiliating with a conspecific. However, we anticipated a sex difference in the degree of shelter preference because although both males and females use shelters to hide from predators, males actively protect eggs in these shelters during the breeding season (Charlebois et al., 1997).

2.3 Methods

2.3.1 Fish collection and housing

We collected round goby from LaSalle Park Marina, in Hamilton Harbour, Ontario, Canada (43°18’1’’N, 79°50’47’’W). Round goby have been sampled at this site for over a decade (Young et al., 2010; McCallum et al., 2014 – Appendix B) and it has a mixture of rocky cobble and sandy substrate. Round goby were collected between 15 May and 20 August 2013 using minnow traps baited with frozen corn kernels (see Young et al., 2010; McCallum et al., 2014 – Appendix B, for additional details of the collection protocol). We transported the fish in lake water to the laboratory at McMaster University and housed them in 75-l tanks (61 × 46 × 30 cm) with dechlorinated tap water containing a static renewal filter (Aquaclear), a layer of natural gravel substrate (approx. 1 cm deep), and polyvinylchloride (PVC) tubes as shelters. Water temperature was maintained at 20–22°C. All fish were fed Nutrafin basix Staple Food once per day and were maintained on a 14:10 h light-dark schedule. Focal fish were housed in same-sex groups of three and were always housed separately from stimulus fish. After 24 h of acclimation to laboratory conditions, focal fish were individually marked with an injection of non-toxic acrylic paint (Wolfe & Marsden, 1998). Morphological measurements (total length, standard length and body mass) were taken at this time using calipers accurate to the nearest 0.01 cm and a digital scale accurate to the nearest 0.01 g (Scout Pro SP202).

2.3.2 Testing apparatus

To explore social preferences, round goby were tested in a 150-l tank (90 × 44 × 38 cm; Figure 2.1a and 2.1b) in Experiments 1 and 2, and in a 75-l tank (61 × 46 × 30 cm; Figure 2.1c) in Experiment 3. Testing tanks contained a layer of gravel substrate (approx. 1 cm deep) and a static renewal filter that was turned off before the start of each trial. Each trial was recorded with a video camera (Canon HD Vixia HFS100 8.0 Megapixel) concealed behind a blind with a hole for the camera that limited disturbance from the experimenter and the video camera. Video recordings were used later for behavioural scoring and analysis.

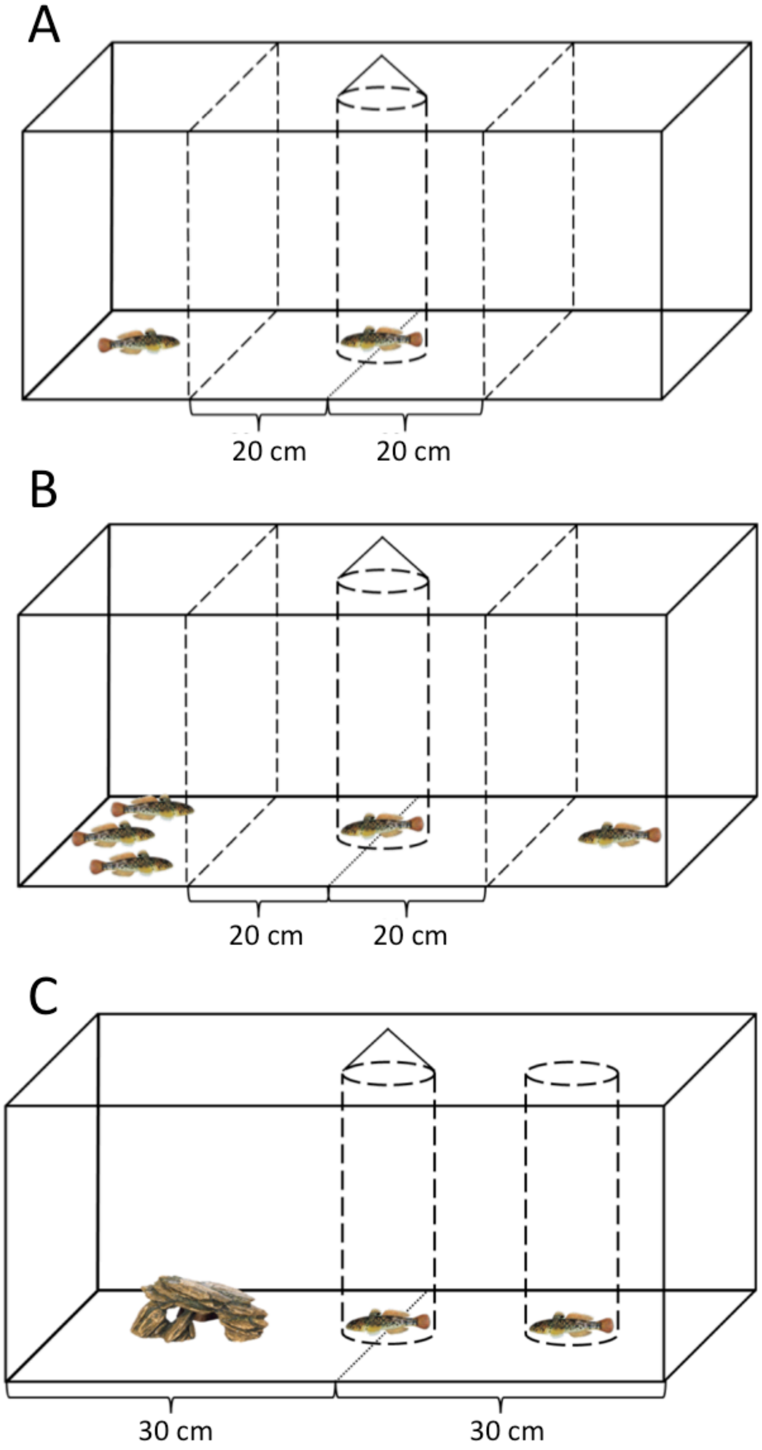

Figure 2.1

Experimental tank set-up for (A) one fish vs. empty chamber experiment; (B) three fish vs. one fish experiment; and (C) shelter vs. one fish experiment.

2.3.3 Testing procedure

All fish were housed in the laboratory for a minimum of 48 h before testing. We chose unfamiliar, size-matched, same-sex fish as stimulus fish to ensure that social preference reflected social partner choice and not mate choice (Reddon et al., 2011). Each stimulus fish was used for only three trials, ensuring that overall focal fish were exposed to many different stimulus fish. The side chosen to contain a particular stimulus was randomly assigned by a coin toss. Each focal fish was placed in a perforated cylindrical tube (13 cm diameter, 20 cm height) in the center of the testing tank and left to habituate for 15 min. During this habituation period the fish could see the rest of the tank and the stimuli on both sides of the tank. The central tube was then lifted remotely using a pulley from behind the blind, and the focal fish was free to navigate the exploration area for a 15-min period. Time spent in each half of the exploration area was recorded and used as a measure or index of preference for each stimulus. We also scored focal and stimulus fish movements as well as any behavioural acts performed across the barrier (Table 2.1).

2.3.4 Experiment 1: one fish versus an empty chamber

A total of 60 focal fish (30 males: mean ± SD total length = 6.3 ± 3.3 cm; 30 females: mean ± SD total length = 6.9 ± 2.9 cm) were used in this experiment. Two transparent perforated plastic barriers divided the testing tank into three compartments (two end compartments that were each 25 cm wide and a central exploration compartment that was 40 cm wide). The perforations allowed for the transfer of visual, olfactory, and

Table 2.1
Ethogram used to score focal fish and stimulus fish behaviours during sociality assays.

Behaviour		**Description**
Locomotor and maintenance	Hop (**H**)	Smooth locomotion on substrate driven by pectoral fins. Forward or sideways movement of distance less than one body length.
	Swim (**Sw**)	Sustained locomotion in the water column using all fins. Forward or sideways movement of distance greater than one body length.
	Dart (**D**)	A spontaneous, rapid swim along the substrate not directed at anything.
	Scrape (**Sc**)	Focal fish very quickly scrapes its side or underside against a surface.
	Glass Swim (**GS**)	Focal fish orients towards the side of the tank and repeatedly moves vertically, nose to the glass. Episode stops when fish comes to rest on bottom.
	Bury (**Bu**)	Focal fish vigorously wiggles its body into the substrate, partially or completely hiding its body.
	Dig (**Dg**)	Focal fish picks up object from the substrate, or pieces of substrate, in mouth and spits it out or moves it to a different location.
Aggressive	Ram (**R**)	Focal fish orients towards stimulus fish at transparent barrier and very quickly and forcefully rams nose at barrier. Ram is usually accompanied by a bite motion with puffing of the cheeks.
	Glass Ram (**GR**)	Ram is accompanied by an aggressive vertical glass swim with nose to the barrier.
	Parallel Display (**PD**)	Focal fish aligns itself parallel to barrier (usually during interaction with stimulus fish) and flaps tail against barrier using an S-curve body motion.

limited tactile cues. One stimulus fish was added to one of the testing tank's end compartments, while the other end compartment remained empty. To track each focal fish's preference and number of switches during the trial, a grid was drawn along the central exploration compartment dividing it into two equal zones measuring 20 cm each.

In addition, to track focal fish fine scale movement and activity rates, the central compartment was further divided into five equal grid zones measuring 8 cm each (average round goby length in our study population, Young et al. 2010). The focal fish was considered to have changed zones when at least 50% of its body—including its head—crossed a grid line and entered a new one on the grid. After testing, focal fish were euthanized using an overdose of benzocaine (0.025% in solution; Sigma Aldrich), and dissected to confirm reproductive status. Gonad weight was taken to the nearest 0.001 g (AcculabVicon Digital Scale), and used to calculate gonadosomatic index (GSI: (gonad mass/body mass) − gonadmass). Males were considered to be reproductive if their GSI was greater than 1%, and greater than 8% for females (MacInnis, 1997; Marentette & Corkum, 2008).

2.3.5 Experiment 2: large (3) fish versus small (1) fish groups

A total of 42 focal fish (18 males: mean ± SD total length = 7.4 ± 2.9 cm; 24 females: mean ± SD total length = 7.5 ± 3.9 cm) were used in this experiment. Testing tank set up was identical to Experiment 1, except that a group of three stimulus fish were added to one end compartment, and one stimulus fish was added to the other end compartment. As in Experiment 1, we scored focal and stimulus fish movement and activity as well as behavioural acts between the focal fish and stimulus fish. After testing, all fish were returned to their housing tanks for future experiments.

2.3.6 Experiment 3: one fish versus shelter

A total of 24 focal fish (12 males: mean ± SD total length = 6.0 ± 2.4 cm; 12 females: mean ± SD total length = 6.4 ± 3.6 cm) were used in this experiment. This tank was not divided into three compartments. A cylindrical, perforated tube (13 cm diameter, 20 cm height) containing one stimulus fish was placed on one side of the tank, while a plastic shelter (20 × 10 × 8 cm, see Figure 2.1c) was located on the opposite side of the tank. During the 15-min trial, each focal fish could interact with the stimulus fish across the perforated barrier of the tube as well as enter and explore the shelter. To track stimulus preference, lines were drawn along the front wall of the entire tank to divide the tank into two equal zones measuring 30 cm. We also tracked focal fish movement and activity by dividing the tank into six equal zones measuring 10 cm each. We scored focal fish and stimulus fish movements, behavioural acts between the focal fish and stimulus fish, time the focal fish spent in the shelter, and number of shelter visits. After testing, all fish were returned to their housing tanks for use in future experiments.

2.3.7 Statistical analyses

We assigned each focal fish a categorical stimulus preference on the basis of where they spent the majority of time and compared these patterns using a chi-square goodness of fit test. The magnitudes of the preferences were investigated by converting the raw time spent near each stimulus to a preference index value. In Experiment 1, the preference index was calculated as the time spent near the stimulus fish side/(time spent near the stimulus fish side + time spent near the empty side), and a preference index value of greater than 0.5 indicates that the focal fish preferred to affiliate with the conspecific. In Experiment 2, the preference index was calculated as the time spent near the three fish side/(time spent near the three fish side + time spent near the one fish side), and a preference index value of greater than 0.5 indicates that the focal fish preferred to affiliate with the larger group. In Experiment 3, the preference index was calculated as the time spent near the stimulus fish side/(time spent near the stimulus fish side + time spent near the shelter side), and a preference index value of greater than 0.5 indicates that the focal fish preferred to affiliate with the stimulus fish. Focal fish that never moved during the 15 min trial were excluded from analyses, resulting in a final sample size of 55 for Experiment 1 (27 males, 28 females), 35 for Experiment 2 (17 males, 18 females), and, 20 for Experiment 3 (9 males, 11 females). All statistical analyses were conducted using R version 3.0.2 (R Core Development team, 2013). Quantile–quantile and residuals-versus- fitted diagnostic plots were used to visually inspect preference index values for normality and homogeneity of variance. Preference index values from Experiment 1 and Experiment 3 were logit transformed (Warton & Hui, 2011) to meet normality criteria. Preference index values were split by sex for each experiment and tested against the null hypothesis of no preference (0.5) using a two-tailed one-sample t-test. Average number of tank half switches, grid line crossings (activity), and aggressive acts by the focal fish were compared between sexes and across experiments using a negative binomial logistic regression.

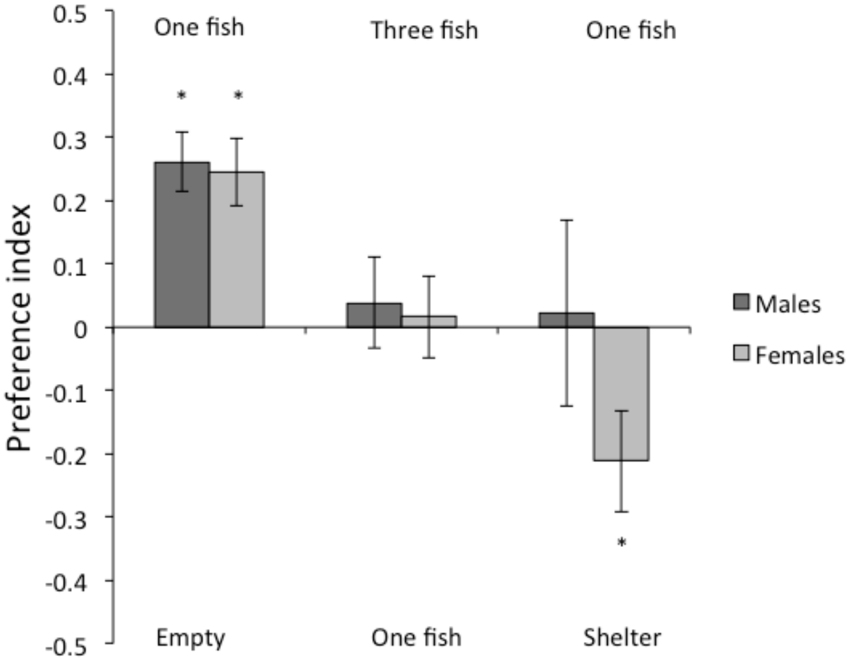

Figure 2.2

Average (±1 SE) proportion of time focal fish spent near the stimulus fish compared to the empty chamber in Experiment 1, three fish compared to one fish in Experiment 2, and the stimulus fish compared to a shelter in Experiment 3. For ease of interpretation, preference index values were scaled by 0.5 to produce absolute values where zero indicates no preference. Values above zero indicate a stronger preference for the stimulus fish in Experiment 1, large group of three stimulus fish in Experiment 2, and the stimulus fish in Experiment 3. Significant differences from null hypothesis of no preference (zero) are indicated by asterisks.

2.4 Results

2.4.1 Experiment 1: one fish vs. an empty chamber

Forty-seven fish preferred to affiliate with the conspecific while eight fish preferred the empty chamber (chi square test: $\chi^2_1 = 27.65$, $p < 0.001$). Both males and females spent more time in close proximity to the conspecific (one-sample t-test: $t_{\text{males}(26)} = 4.54$, $p < 0.001$; $t_{\text{females}(27)} = 4.14$, $p < 0.001$; Figure 2.2). Stimulus fish did not affect preference results, as when preference scores from focal fish experiencing the same stimulus fish were averaged and tested against the null hypothesis of no preference, we still found a preference for associating with a conspecific (one-sample t-test: $t_9 = 2.78$, $p = 0.021$). Fish were active in this experiment, crossing an average of 35 grid squares, and switching between sides of the exploration compartment an average of 7 times per trial. There were no sex differences in activity level (negative binomial logistic regression: $Z_{53} = 1.5$, $p = 0.13$; Table 2.2) or the number of side switches (negative binomial logistic regression: $Z_{53} = 1.3$, $p = 0.19$; Table 2.2).

Table 2.2
Average ±1 SE and *p*-values from negative binomial regression analyses for focal fish aggressive behaviours towards stimulus fish, tank half switches, and activity observed during each sociality assay.

		Aggressive displays		**Tank half switches**		**Total activity**	
One vs. empty	♂	10.48 ± 3.36	$p = 0.54$	8.93 ± 1.79	$p = 0.19$	42.00 ± 7.91	$p = 0.13$
	♀	14.57 ± 3.96		5.82 ± 1.24		28.25 ± 5.21	
Three vs. one	♂	8.35 ± 3.42	$p = 0.035$	15.47 ± 3.47	$p = 0.88$	53.41 ± 8.99	$p = 0.46$
	♀	24.50 ± 6.88		11.61 ± 2.29		44.06 ± 7.36	
Shelter vs. one	♂	15.89 ± 8.03	$p = 0.76$	1.78 ± 0.72	$p = 0.005$	17.22 ± 4.37	$p = 0.017$
	♀	12.36 ± 4.36		6.45 ± 1.60		34.55 ± 6.53	

Of the focal fish tested, 60% (33 out of 55 fish, 16 males and 17 females) displayed aggressive behaviours towards the stimulus fish. Males performed an average of 10 aggressive acts while females performed an average of 15 aggressive acts during the 15-min trial, and there was no overall sex difference in aggression (negative binomial logistic regression: $Z_{53} = -0.6$, $p = 0.54$; Table 2.2). Dissections confirmed that focal fish were mainly non-reproductive. Males had an average GSI of 0.45%, with 4 out of 27 males reaching the reproductive threshold of a GSI greater than 1%, while females had an average GSI of 3.86%, with 5 out of 28 females reaching the female reproductive threshold of a GSI of 8% or greater.

2.4.2 Experiment 2: three fish vs. one fish

Round goby did not show preference for larger groups. Twenty fish preferred to affiliate with the large group of conspecifics while 15 fish preferred to affiliate with the small group (chi square test: $\chi^2_1 = 0.71$, $p = 0.40$). Neither males or females showed a preference for the large group or the small group, spending approximately equal time in proximity to large and small groups (one-sample t-test: $t_{\text{males}(16)} = 0.54$, $p = 0.60$; $t_{\text{females}(17)} = 0.26$, $p = 0.80$; Figure 2.2). Focal fish were highly active in this experiment, crossing an average of 49 grid squares, and switched between sides of the exploration compartment an average of 14 times. Males and females showed similar activity levels (negative binomial logistic regression: $Z_{33} = 0.73$, $p = 0.46$; Table 2.2) and similar number of side switches (negative binomial logistic regression: $Z_{33} = 0.88$, $p = 0.38$; Table 2.2). Of the 35 focal fish, 27 or 77% (12 males, 15 females) behaved aggressively towards either the single or group of stimulus fish, and females performed more aggressive acts on average than males (negative binomial logistic regression: $Z_{33} = -2.10$, $p = 0.035$; Table 2.2).

2.4.3 Experiment 3: shelter vs. one fish

Twelve fish preferred to seek safety in the shelter while eight fish preferred to affiliate with the conspecific (chi square: $\chi^2_1 = 0.80$, $p = 0.37$). Females preferred to spend time in close proximity to the shelter over the conspecific (one sample t-test: $t_{10} = -2.68$,

$p = 0.02$; Figure 2.2), while males showed no such preference and spent equal time near the shelter and conspecific (one sample t-test: $t_8 = 0.47$, $p = 0.65$; Figure 2.2). Females made an average of three visits to the shelter while males made an average of only one visit to the shelter during the trial period (Table 2.3). Focal fish crossed an average of 27 grid squares and switched between tank halves an average of 4 times. In general, females were more active than males (negative binomial logistic regression: $Z_{18} = -2.39$, $p = 0.017$; Table 2.2) and made more switches between stimuli (negative binomial logistic regression: $Z_{18} = -2.83$, $p = 0.005$; Table 2.2). Out of the, 20 fish, 13 or 65% interacted aggressively with the conspecific (6 males, 7 females) and males and females displayed a similar number of aggressive acts (negative binomial logistic regression: $Z_{18} = 0.30$, $p = 0.76$; Table 2.2).

Table 2.3
Sex differences observed during Experiment 3. Average (±1 SE) number of visits to shelter, time spent inside shelter, and time spent interacting with conspecific across barrier.

	Shelter visits	Time in shelter (sec)	Time with conspecific (sec)
♂	1.11 ± 0.56	60.44 ± 33.25	69.00 ± 39.26
♀	3.36 ± 0.65	105.91 ± 24.81	34.82 ± 12.15

2.5 Discussion

Using three sociality assays, we demonstrated that round goby do exhibit social preferences for conspecifics. As predicted, in our first experiment, we showed that both male and female round goby have a strong preference for affiliating with a conspecific as opposed to remaining solitary. In our second experiment, we surprisingly found that round goby showed no preference for a larger group of conspecifics over a smaller group of conspecifics. Finally, in our third experiment we probed the value of conspecific affiliation against the value of protection in a shelter resource, and discovered that females preferred the shelter while males showed no such preference for the shelter over a conspecific.

In the laboratory, many fish species prefer to associate with conspecifics (Griffiths & Ward, 2011). Additionally, when given the choice, fish often prefer to affiliate with the larger of two groups (Hager & Helfman, 1991; Krause et al., 1998; Svensson et al., 2000; Agrillo et al., 2007; Buckingham et al., 2007; Reddon et al., 2011). Starting as low as 2:1, fish are capable of using the ratio of group size to make affiliation and group-joining decisions (Buckingham et al., 2007; Gomez-Laplaza & Gerlai, 2011). While we found that round goby affiliated with a conspecific instead of remaining solitary, they had no preference for group size at a 3:1 ratio of conspecifics. Since round goby avoid predation mainly by using rocks as shelter or burying into the substrate (Charlebois et al., 1997), it is likely that benthic round goby rely less on grouping to minimize predation risk compared to most pelagic shoaling fish species. Supporting this notion, Magoulick et al. (2004) found that many benthic fish species were less susceptible to predation than pelagic fish species due to their benthic habit and cryptic colouration. Therefore, round goby may not need to discriminate between groups of different sizes or join a large group of conspecifics to gain protection from predators. Though round goby did not make grouping decisions based on group size in our experiment, they may still discriminate between groups based on other criteria such as conspecific body size or under specific situational circumstances like predation risk (Pitcher & Paris, 1986; Lima & Dill, 1990; Krause & Godin, 1994; Hoare et al., 2000; Ward & Krause, 2001; Reddon et al., 2011).

We had expected that round goby — especially males — would prefer affiliating with a shelter over a conspecific. However, contrary to our prediction, female round goby preferred to be near the shelter, while males showed no such preference for the shelter. Sex differences in reproductive and predator avoidance behaviours are common in fish (Hanson et al., 2008). In teleost species with paternal care, such as rock bass (*Ambloplites rupestris*, Noltie & Keenleyside, 1987), three-spine stickleback (*Gasterosteus aculeatus*, Pressley, 1981), and sand goby (*Pomatoschistus minutus*, Lindström & Hellström, 1993), reproductive males spend more time in and around their nest preparing or caring for offspring (Blumer, 1979). Our shelter trials were conducted in August, which is at the end of the breeding season for round goby in the Laurentian Great Lakes (MacInnis & Corkum, 2000; Young et al., 2010). It is possible that the reproductive condition of the fish may be why males did not show a strong preference shelter. Only fish from Experiment 1 were dissected, and most often males were non-reproductive. Although fish were not dissected in Experiment 2 or 3, given the time in the season, their mottled body colour, small size and flaccid shape of their genitalia (Marentette et al., 2011), it is likely that most fish in both Experiment 2 and 3 were also not in reproductive condition. Consequently, non-reproductive males may have been less motivated to spend time near or inside a shelter. Alternatively, we had also predicted that round goby would seek shelter, as that is a typical behaviour when seeking refuge from predation. Though we collected our round goby from a site with known fish and avian predators (Somers et al., 2003; Brousseau & Randall, 2008), the absence of predation pressure in the lab may have led some fish in our sample to explore the entire tank and not take-up shelter during our trials.

In all three experiments, 60% or more of the interactions between focal and stimulus fish across the barrier were aggressive in nature, and most focal fish showed some level of aggression towards a lone conspecific or the group of conspecifics. Surprisingly, females showed similar levels of aggression as males, and even performed more aggressive acts than males on average in one of the experiments, revealing the importance of including females in future work on aggression. The high levels of aggression combined with the fact that round goby showed no preference for larger group sizes may indicate that although round goby tend to aggregate, high intraspecific competition will occur with increased densities due to the aggressive nature of round goby (Fitzsimons et al., 2006; Kornis et al., 2014). In this case, both social attraction and aggression towards conspecifics may combine to facilitate invasive behaviour. It is possible that round goby may aggregate until they reach a certain density threshold where high intraspecific aggression leads to the dispersal of asocial individuals, further facilitating the colonization of new populations (Cote et al., 2010; Fogarty et al., 2011).

We have assessed grouping preferences in invasive round goby, a fish known to be highly aggressive but also to thrive in high densities in the Great Lakes. Our work has shown that although round goby are a benthic, non-shoaling species, they have a tendency to affiliate with conspecifics but show no preference for large groups and following the initial approach may be aggressive to nearby conspecifics. Our results have important implications for understanding round goby behaviour, especially in terms of characteristics contributing to their invasion success. We demonstrate that round goby aggregate and this may account for their ability to thrive in high densities, and have played a role in their establishment and spread in the Great Lakes. It is likely that an interaction between high interspecific aggression and tolerance of conspecifics allowed round goby to competitively displace native species and spread rapidly. Future work will focus on cues that drive and motivate round goby intraspecific interactions, such as predator cues and reproductive status, in order to further understand the circumstances in which aggressive and social behaviours could lead to population growth and spread in this invasive species. We will continue to probe how round goby make group joining decisions by further manipulating stimulus group size and joining size-rank in the group, as both of these factors may contribute to social aggregation (Pitcher & Parish, 1993; Hoare et al., 2000; Griffiths & Ward, 2011). Our work has provided an initial assessment of the social tendencies of round goby in the Great Lakes, but it would be beneficial to explore whether these social behaviours vary across different stages of their invasion (or in fish from established areas versus the invasion front) and how they compare to fish from the native range. In sum, we have demonstrated that characterizing behaviours, like sociality, in invasive species can allow us to better understand the potential factors contributing to invasive species establishment and success.

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Chapter 3: Contest dynamics in a territorial fish are dependent on prior resource experience, without in-contest updating

3.1 Abstract

The effects of resource holding potential and resource value on animal contests have been the focus of many studies. Unlike opponent assessment, how competing animals assess resources has received less attention. An important question that still requires explicit testing is whether animals can assess resource value while in a contest, or whether they require experience with the resource to gauge their aggressive effort. We conducted a series of experiments using an invasive, territorial fish—round goby (*Neogobius melanostomus*)—to investigate the impact of resource quality on contest dynamics, and to test how fish gather information on resource value. First, we found that fish preferred an enclosed and defendable shelter ("high quality") to an open and less defendable shelter ("low quality"). In staged resource contests, we found that resource value did not affect contest dynamics when fish evaluated the resource for the first time during the contest. However, when fish were given experience with the resource before the contest, we found that contests over high quality resources began faster, were longer in duration, and had more aggressive acts, when compared to contests over low quality resources. We then switched the value of the resource, so that an individual's previous experience with a resource did not match the resource value encountered in the contest. We found that contest dynamics were not driven by the previous resource value, indicating that fish could recognize that the resource had changed from their previous experience. However, similar to the contests with no resource experience, the new

resource value did not affect contest dynamics. Together, our findings demonstrate that fish adjust their aggressive effort to reflect resource value, but previous experience with the resource is required. Our results suggest that round goby are unable to gather information about the resource during the contest.

3.2 Introduction

Animals commonly fight over resources such as food, mates, and territories, and such contests are more frequent when resources are limited in quantity or vary in quality (Enquist & Leimar, 1987, Hsu et al., 2011). A great deal of research has focused on what attributes an individual must possess to win a contest against a rival (see review: Arnott & Elwood, 2009). These attributes include an individual's body size, weaponry, and physiological scope for aggression (e.g., energy reserves). Larger individuals (Wells, 1988; Prenter et al., 2008; Reddon et al., 2011), with more developed weaponry (Sneddon et al., 1997; Kelly, 2006), greater energy reserves and higher anaerobic capacity tend to prevail (reviewed in: Briffa & Sneddon, 2007). For example, when sand gobies (*Pomatoschistus minutus*) fight over burrows for nesting, the larger individuals are more likely to win (Lindström & Pampoulie, 2005). Collectively, the attributes of a competitor that contribute to the probability of winning a contest, or "the absolute fighting ability of a given individual", are termed resource holding potential ("RHP" Parker, 1974).

In addition to possessing physical traits that increase the likelihood of winning a resource, aggressive contests often occur because of resource discrepancies. Therefore, the characteristics of the resource being contested over can also affect contest dynamics.

How valuable a resource is to each contestant will depend on resource quality, scarcity and the value of the resource for survival and reproduction (Enquist & Leimar, 1987; Arnott & Elwood, 2008). Opponents should use information about the resource to decide if and how to proceed with a fight. When the physical and physiological attributes of two contestants are similar, resource value can be a key determinant of contest dynamics (Enquist & Leimar, 1987). It is advantageous for individuals to assess resource value before fighting, and optimize their aggressive behaviour accordingly in order to minimize the costs (e.g., wasted energy, potential injury) associated with aggressive interactions (Parker, 1974). Therefore, resources that are strongly linked to reproductive success, such as high quality shelters and territories, receptive mates, or nutritious food, should provide a greater motivation for opponents to proceed with a contest. We would also expect that contests over high quality resources would be longer and more intense (Parker, 1974; Enquist & Leimar, 1987), and these predictions have been supported (reviewed in: Arnott & Elwood, 2008). For example, Bridge et al. (2000) found that resident male orb-weaving spiders (*Metellina mengei*) contesting with an intruder for access to a female mate had longer contests when the female was of higher value (larger body size, more fecund). Tibbetts & Shorter (2009) showed that resident queen paper wasps (*Polistes dominula*) fight longer against intruder queens for larger, high quality nests.

Investigations of resource value have often used resident-intruder experimental designs where one opponent is the resource owner and is familiar with its value, while the intruder has no such experience. In this design, it is difficult to disentangle the subjective resource value (i.e., perceived ownership) from objective resource value (i.e., the actual quality of the resource) during aggressive contests. Indeed, prior residency or resource ownership itself can affect contest outcomes. For example, Johnsson & Forser (2002) found that brown trout (*Salmo trutta*) that were residents over a territory for four days were more likely to win contests against size-matched intruders than residents who occupied the same territory for only two days. In this scenario, objective resource qualities (characteristics of the territory) are identical, but ownership itself makes the resource more valuable. Attributes of the resource itself could also make competitors more physiologically capable of winning a fight. For example, access to high quality food resources may give residents a competitive advantage. Ewald (1985) manipulated the nutritional quality of contested food resources on black-chinned humming bird (*Archilochus alexandri*) territories. The authors showed that birds with a high nutrition territory were more often contest winners, even when they were smaller in body size than their opponents.

It has been argued that owner-owner experimental designs will be more informative in revealing the importance of objective resource value (Arnott & Elwood, 2008; Elwood & Arnott, 2012). In this experimental design, both opponents become resident over their own resources, and are both familiar with the value of the resource before contesting, making the subjective resource value based on ownership approximately equal. Owner-owner contests have previously been used to investigate RHP during contests (e.g., Koops & Grant, 1993; Reddon et al., 2011; Groen et al., 2012), but much less frequently to investigate resource value. When this approach has been used, researchers have shown that animals tend to aggress longer and more intensely for high quality resources (Arnott & Elwood, 2008). In parasitoid wasps (*Goniozus nephantidis*), females that owned larger, more valuable hosts for egg-laying fought longer and harder when matched with females that were previously paired with a low quality host resource (Humphries et al., 2006). This example suggests that combating animals adjust their fighting effort to reflect their previous resource value experience.

While animals may adjust their fighting effort to reflect resource value when they are familiar with a resource, it is unclear whether animals are able to assess resource value during a contest. To date, few studies have attempted to understand if and how animals gather information about resources during contests. It is expected to be costly for an animal to simultaneously gather information about both their opponents and about resource quality during a contest (Enquist & Leimar, 1987). Indeed, certain studies have found no evidence for resource assessment, indicating animals are unable to evaluate the resource during the contest, or that gathering information might constitute a cost that outweighs the potential gains (Thornhill, 1984; Jennings et al., 2004). Certain resources may also be easier to evaluate than others. For example, males may be able to quickly evaluate the reproductive quality and resource value of a potential female mate using olfactory cues (e.g., Prenter et al., 1994; Sneddon et al., 2003). In contrast, we would expect that to evaluate the quality of a burrow, shelter or breeding territory, individuals would need to interact with the resource to be able to assess structural or spatial features. In some species of hermit crabs, individuals use both visual and tactile cues to assess shell volume and fit (Hazlett, 1996; Doake & Elwood, 2011). It has been speculated that trade-offs must occur during the information gathering process, especially if animals need to assess their opponent's ability along with the value of the resource at stake (Elwood & Arnott, 2012; Elwood & Arnott, 2013). However, investigations of resource assessment during contests in the literature are so far surprisingly limited, leaving much to be learned about this process.

To better understand how resource value can alter contest dynamics, and whether animals are able to update their information about resource value during contests, we conducted a series of experiments using the round goby (*Neogobius melanostomus*). This small, benthic fish species is native to the Ponto-Caspian region of Europe and is widely invasive in Western Europe and the Laurentian Great Lakes of North America (Kornis et al., 2012). This species is a useful model for studies of contest dynamics because its invasion success has been strongly attributed to its aggressive nature (Charlebois et al., 1997; Corkum et al., 2004). Round goby are known to outcompete similar-sized species for access to limited shelter spaces in the rocky littoral zone, and they will defend these shelters that are used to escape from predators, as sites for spawning, and for offspring care (Dubs & Corkum, 1996; Corkum et al., 1998; Belanger & Corkrum, 2003; Bergstrom & Mensinger, 2009; Janssen & Jude, 2001). In the laboratory, round goby will readily display defensive behaviour over artificial shelters, and are frequently aggressive to both con- and heterospecifics (Balshine et al., 2005; Stammler & Corkum, 2005; Sopinka et al., 2010; Groen et al., 2012).

Based on the knowledge that shelter is a highly valuable resource for round goby, we posed three questions. First, we sought to establish whether round goby could differentiate between shelters of varying quality. To do this, we provided fish with a binary choice between a shelter that was enclosed and easy to protect (a “high quality” shelter), and a shelter that was open, making it both less safe and more difficult to defend (a “low quality” shelter). We predicted that round goby would prefer the more defendable shelter, given their previously discussed habitat use (Dubs & Corkum, 1996; Janssen & Jude, 2001; Bergstrom & Mensinger, 2009).

Second, we assessed whether resource value (high quality versus low quality shelters) influenced contest dynamics between individuals of similar RHP (body size) when fish had no previous experience with either shelter. To address this question, we conducted owner-owner resource contests over high and low quality shelters, with resource naïve fish. Here, opponents needed to gather information about resource value during the contest and appropriately adjust their fighting effort to reflect this information. We evaluated contest dynamics by measuring motivation to begin a contest as the time taken to start aggressing, contest duration, and the total aggressive acts during the contest. We hypothesized that if round goby are able to evaluate resource value during a contest, and if they prefer high quality shelters, then fish fighting over high quality shelter would begin contests faster, have longer contests, and more intense contests than when fighting over low quality shelters.

Third, we evaluated the effect that prior resource experience had on contest dynamics using owner-owner contests where the fish had access to either a high or a low quality shelter for 24 hours before a contest over either a high or a low quality shelter. Thus, this third experiment created two contest scenarios: Experiment 3a) *Matched experience*: fish housed previously with high or low quality resources aggressed over resources of the same value (e.g. both housed with high quality and then contested over high quality), and Experiment 3b) *Mismatched experience*: fish housed previously with high or low quality resources aggressed over resources of opposing value (e.g. both housed with high quality and then contested over low quality). We hypothesized that if fish fight based on previous resource experience, and are unable to update their evaluation of resource value during the contest, then contest dynamics should reflect prior housing conditions. Fish previously housed with high quality resources would fight harder, regardless of resource value present in the actual contest. Contrarily, if fish were able to fully update their evaluation of resource value during the contest, we would expect contest dynamics to reflect the resource present during the contest itself and not prior housing. In all contest experiments, we described how round goby gathered information about resource value during aggressive contests. To do this, we monitored when each fish first entered the shelter resource and the amount of time each fish spent inside the shelter resource during the contest.

3.3 Methods

3.3.1 Fish collection and housing

We collected male round goby using baited minnow traps from LaSalle Park Marina (43°18'04 N 79°50'43 W) in Hamilton Harbour, Lake Ontario, Canada (see Young et al., 2010; McCallum et al., 2014 – Appendix B; for trapping and collection details). We transported the fish to the laboratory at McMaster University, and housed them in 75 l (61.0 x 33.0 x 43.2 cm) holding tanks. Tanks contained ~1 cm of natural gravel substrate, an airstone, and a static renewal filter. We maintained aquaria at 18°C to 20°C on a 16L: 8D hour light cycle. We fed fish flake food (Nutrafin Basix Staple food) daily.

3.3.2 Experiment 1: Shelter preference

Between May 19, 2014 and June 12, 2014, we conducted 14 shelter preference trials to ascertain whether round goby prefer an enclosed, defensible shelter to an open, less defensible shelter. We conducted these preference trials in 40 l experimental tanks (50.8 x 27.9 x 33.0 cm) equipped with ~0.5 cm of natural gravel substrate and a static renewal filter. Each experimental tank contained one enclosed, defensible shelter and one open, less defensible shelter on opposite sides of the tank, with the side counterbalanced between trials. The enclosed shelter was an enclosed black acrylic box (10.9 x10.9 x 5.0 cm, Figure 3.1a), with a single small entry/exit that would be easy to defend. The open shelter was identical in size and also built of black acrylic, but was open from the sides and the top with only two sidewalls (Figure 3.1a). This open shelter would be difficult to defend and would leave the fish more vulnerable to predation and challenges from rivals. We began each preference trail by releasing a focal fish into the center of the experimental tank and then recorded its shelter preference after 3 and 24 hours. Fish were considered to prefer a particular shelter if they were inside the shelter or were within a half a body length from the shelter entrance(s) at the time of observation. One trial was excluded from our analysis because the fish did not move during the trial and then died after 3 hours in the test tank.

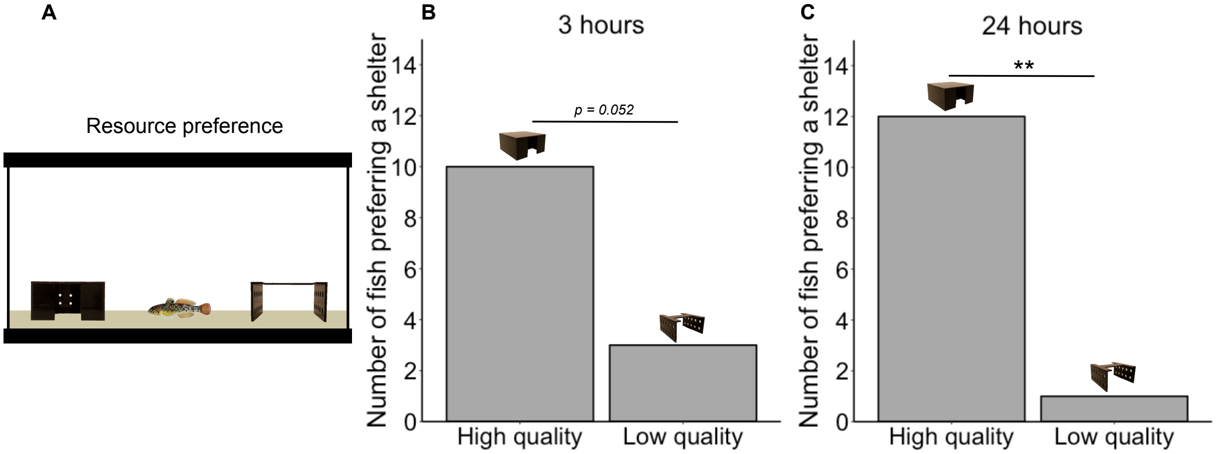

Figure 3.1
Resource preference experimental tank and results. **A)** Resource preference experimental tank set-up, depicting a choice between a high quality and low quality shelter. **B)** Resource preference after 3 hours of the preference trial. **C)** Resource preference after 24 hours of the preference trial. ** indicates $p < 0.001$.

3.3.3 Experiment 2: Resource contests with no previous experience

After we determined that round gobies prefer enclosed, defensible shelters ("high quality") to open, less defensible shelters ("low quality", see Results), we staged 26 owner-owner resource contests between August 1, 2015 and August 28, 2015. Three contests were excluded from scoring because fish did not interact during the trial. We staged ($N = 12$) contests over high quality resources, and ($N = 11$) over low quality resources. In all contests, we followed a three-day protocol. On day one, we selected two fish size-matched by body mass from laboratory stock tanks. We uniquely tagged each fish to facilitate identification during the resource contests by injecting non-toxic acrylic paint along the dorsal fin (Wolfe & Marsden, 1998). Fish were housed separately while recovering from tagging. On day two, we transferred the marked pair to opposite ends of an experimental tank (40 l aquaria, 50.8 x 27.9 x 33.0 cm) that was divided into three compartments by removable black opaque barriers (Figure 3.2). A shelter (either high or low quality) was placed into the central chamber. We conducted the resource contests on day three between 9:00 and 11:00. Contests began when an experimenter slowly removed the two barriers remotely from behind an opaque blind. The ensuing resource contest was video recorded for 30 minutes (Canon Vixia HF S100).

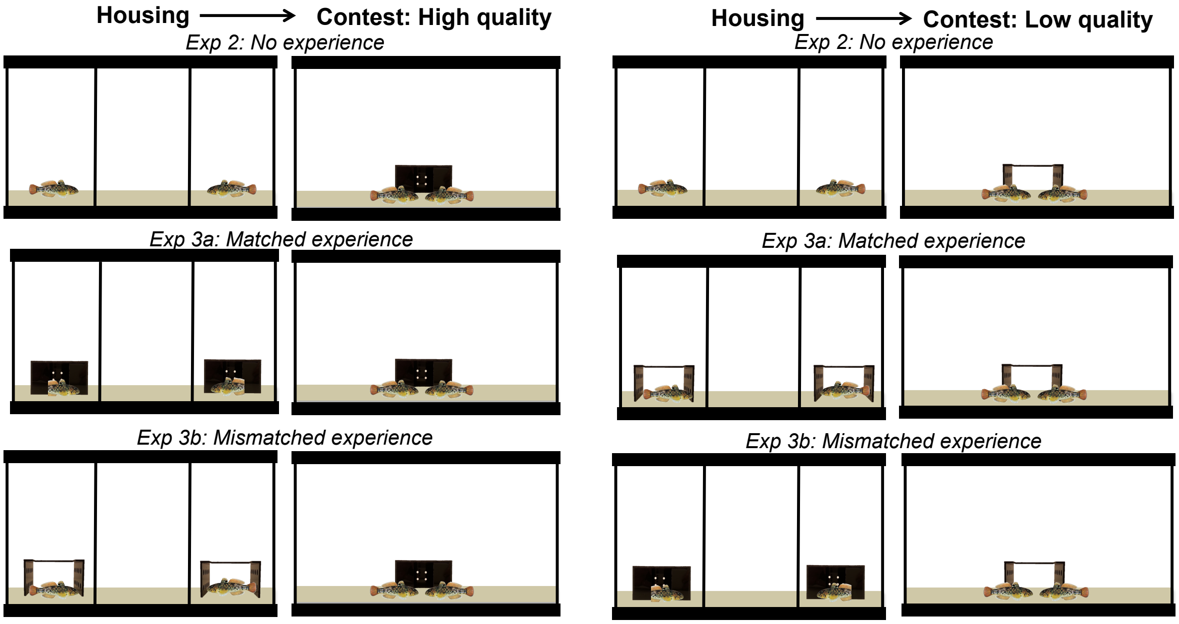

Figure 3.2
Example of owner-owner resource contest experimental tank set-ups. **A)** Housing conditions: opponents are separated by opaque barriers and either housed with or without resources, depending on experiment. **B)** Contest: pre-contest shelters are removed (if present), barriers are lifted, and opponents contest over a single remaining shelter resource.

3.3.4 Experiment 3a: Resource contests with matched prior resource experience

Between July 14, 2014 and August 28, 2014, we conducted 34 resource contests to assess how prior experience with a resource affected contest dynamics. Seven contests were excluded from scoring because fish did not interact during the trial. The contests were identical to those above, but on day two of our experimental protocol, we housed both opponents before a contest with either a high quality resource or a low quality resource for one day. On day three, the housing shelters and opaque barriers were removed to reveal either: a high quality shelter or a low quality shelter that matched their prior experience of shelter quality (Figure 3.2). This created two conditions: 1) fish housed with high quality shelters that contested over a high quality shelter (N = 12); and 2) fish housed with low quality shelters that contested over a low quality shelter (N = 15).

3.3.5 Experiment 3b: Resource contests with mismatched prior resource experience

Between July 14, 2014 and August 28, 2014, we conducted 32 resource contests, but excluded six contests from scoring because fish did not interact during the trial. We followed the same protocol as Experiment 3a, except there was a mismatch between then quality of the resource that fish were housed with and the quality of resource over which the fish contested (Figure 3.2). This protocol created two experimental conditions: 1) fish housed with high quality shelters that contested over a low quality shelter (N = 13); and 2) fish housed with low quality shelters that contested over a high quality shelter (N = 13).

3.3.6 Post-contest processing & behavioural scoring

After each contest, we euthanized both opponents using an overdose of benzocaine (0.025%, Sigma Aldrich) and re-measured each fish for standard length using calipers accurate to 0.01cm, and for body mass using a digital balance accurate to 0.001g.

We then measured gonad mass to confirm reproductive status using the gonadosomatic index (GSI: [gonad mass / (total mass – gonad mass)]*100), where males with a GSI over 1% are considered reproductive (Marentette & Corkum, 2008; Zeyl et al., 2014). All fish used in the following studies were confirmed to be non-reproductive.

We scored the video-recordings for aggressive motivation, contest intensity, contest duration, the time spent inside the shelter and the winner of the contest. The behavioural scorer could not be truly blind to resource value treatment (as the quality of the shelter resource present in the tank is clearly visible); however, their behavioural scores were corroborated by another scorer blind to the motivations of the experiment ($N_{\text{trials}} = 15$, $R^2 = 0.99$). We measured motivation to engage in aggression as the time taken from barrier removal for the fish to start an aggressive interaction. Contest intensity was evaluated by summing the total aggressive acts during the contest. The total number of aggressive acts performed and received by each fish during the contest was scored following an ethogram for this species (see Supplementary Table S1 – Appendix C, adapted from Sopinka et al., 2010). Contest length was measured as the time from the first aggressive act to the time when one opponent ceased to retaliate with aggression and fled. The fleeing fish was termed the losing, subordinate fish, while the other fish was considered to be the winning, dominant fish. To track resource assessment throughout the entire trial, we recorded the time each fish entered the shelter and the total time spent in the shelter resource.

3.3.7 Statistical analyses

All statistical analyses were conducted in R (Version 3.2.3 R Core Team, 2015). We assessed shelter preference after three and after 24 hours using chi-square tests. In our resource contest experiments, we size-matched pairs by body mass to control for RHP. For each pair, we calculated relative body mass difference as a percent: [(mass 1 – mass 2)/(mass 1 + mass 2)/2]*100 (Reddon et al., 2011; O'Connor et al., 2015). More accurate size matching was achieved was during Experiment 2 (mean difference ± SE: 2.43% ± 0.44, $N = 23$) than in Experiment 3a (10.69% ± 1.59, $N = 27$) and 3b (8.64% ± 1.34, $N = 26$; Kruskall-wallis, $\chi^2 = 21.75$, $N = 76$, $p < 0.0001$), but size matching was not different between treatments within each experiment (Kruskal-wallis: Experiment 2: $\chi^2 = 0.46$, $N = 23$, $p = 0.50$; Experiment 3a: $\chi^2 = 0.29$, $N = 27$, $p = 0.59$; Experiment 3b: $\chi^2 = 0.86$, $N = 26$, $p = 0.35$). Though contest outcome was not focal variable in this study, larger fish did tend to win contests in Experiment 3a and 3b (Binomial logistic regression: Experiment 3a: $Z = 1.82$, $N = 27$, $p = 0.068$; Experiment 3b: $Z = 2.36$, $N = 26$, $p = 0.018$), but not in Experiment 2 (Binomial logistic regression: $Z = 0.079$, $N = 23$, $p = 0.94$). To control for RHP in our subsequent analyses, we included absolute percent body mass asymmetry as a covariate in our models, and it did not predict time to start a contest, contest duration, or the number of aggressive acts within the contest in any of the following results described below (all analyses, effect of RHP, $p > 0.10$).

To evaluate the effect of resource value (high versus low quality shelter) on contest dynamics within our experiments (no prior experience, matched experience, mismatched experience), we assessed: 1) time to start a contest; 2) contest length; and 3) the number aggressive acts during the contest. Time to start a contest and contest length were analyzed using linear models. The number of aggressive acts during the contest was analyzed using a negative binomial regression appropriate for count data.

To better understand the use and evaluation of resource quality between opponents, we evaluated whether contest winners spent more time in the shelter than contest losers using a linear mixed effects model, where contest ID was included as a random effect. We assessed whether resource value affected the time winners spent in the shelter using linear models. We examined whether contests where a fish assessed the resource before the contest were more likely to start contests faster or have longer contests using linear models, and whether contests had more aggressive acts using a negative binomial regression. All time-variables (time to start a contest, contest duration, time in the shelter) were ln- or power-transformed to meet parametric assumptions.

3.3.8 Ethical note

The methods described for animal collection, handling, marking, and behavioural trials were assessed and approved by the Animal Research Ethics Board of McMaster University (Animal Utilization Protocol No. 13-12-51), in accordance with the Canadian Council for Animal Care, and adheres to ABS Guidelines for Use of Animals. We monitored all trials carefully by checking the video camera regularly. Had we observed any visible injury the trial would have been stopped, but no such trials occurred. Following each trial, we visually inspected each fish for injury (tattered fins, missing scales) and no damage was apparent. We followed the recommendation of Huntingford (1984), and minimally handled each fish, attempting to reduce stress by limiting the trials to a short duration, and we ended all contests after 30 minutes. Round goby are neither threatened, nor endangered, and are an invasive species in North America. Because they are invasive, they cannot legally be returned to the wild after collection.

3.4 Results

3.4.1 Experiment 1: Shelter preference.

Can fish differentiate between resources? Yes. When we provided round goby a choice between an enclosed, defensible shelter and an open, less defensible shelter, the fish tended to prefer the enclosed, defensible shelter after three hours, although this effect was marginally non-significant (Chi-square: $\chi^2 = 3.77$, $N = 13$, $p = 0.052$: Figure 3.1b), and strongly preferred the enclosed, defensible shelter after 24 hours (Chi-square: $\chi^2 = 9.31$, $N = 13$, $p = 0.0023$: Figure 3.1c).

3.4.2 Experiment 2: Resource contests without prior experience

Without prior resource experience, does resource value influence contest dynamics? No. Contests over enclosed, defensible ("high quality") and open, less defensible ("low quality") shelters did not differ when fish had no previous experience with the resource. Regardless of whether the shelters were of high or low quality, contests began after a mean of 366 (± 91 SE, $N = 23$) seconds (Linear model: $t = -0.02$, $N = 23$, $p = 0.98$; Figure 3.3a), the contests were of similar durations and lasted on average 46 (± 8 SE, $N = 23$) seconds (Linear model: $t = 0.93$, $N = 23$, $p = 0.36$, Figure 3.3b), and contained a similar number of aggressive acts (22 ± 3 SE; Negative binomial regression:

Z = -0.34, N = 23, p = 0.74). Together, these results suggest that fish did not fight according to the value of the resource present during the contest. All contests, regardless of resource type, were ended with a clear winner and loser. After the contest, winners always spent more time in the shelter than losers (Linear mixed effects model: Z = 4.24, N = 46, $p < 0.001$). In fights over high quality shelter, winners tended to spend more time in shelter than did the winners from fights over low quality shelter, although this effect was marginally non-significant (Linear model: t = 2.08, N = 23, p = 0.065, Figure 3.3c).

Figure 3.3
Results of Experiment 2, contests without prior resource experience. **A)** Time to start a contest plotted by resource value. **B)** Contest length plotted against resource value. **C)** Winner's time spent in the shelter resource, plotted by resource value. In all panels, error bars represent ± 1 standard error. *ns* = not significant.

3.4.3 Experiment 3a: Resource contests over with matched prior experience

Does experience with the resource influence contest dynamics? Yes. Fish previously housed with a high quality shelters that then fought over a high quality shelter, began contests faster (Linear model: $t = 2.38$, $N = 27$, $p = 0.026$, Figure 3.4a), had longer contests (Linear model: $t = -2.42$, $N = 27$, $p = 0.024$, Figure 3.4b), with more aggressive acts (Negative binomial regression: $Z = -2.53$, $N = 27$, $p = 0.0084$), compared to fish housed with and contesting over a low quality shelters. This indicates that prior experience with the resource allowed fish to adjust their fighting effort to reflect the value of the shelter resource. As in Experiment 2, all contests ended with a clear winner and loser after the contest. Winners always monopolized the shelter resource more than losers (Linear mixed effects model: $N = 54$, $t = 4.98$, $p < 0.001$), and winners spent more time in the high quality shelter than did winners of the low quality shelter (Linear model: $t = 2.52$, $p = 0.019$, Figure 3.4c).

3.4.4 Experiment 3b: Resource contests over with mismatched prior experience

Can fish update their assessment of resource value during a contest? No. Our evidence suggests that while fish can recognize that a resource is unfamiliar, they are not able to assess the unfamiliar resource during the contest. Fish with mismatched resource experiences did not fight according to the value of their current resource in the contest. However, they also did not fight in accordance with their previous resource experience. Fish took similar amounts of time to start a contest (Linear model: $t = 0.97$, $N = 26$, $p = 0.34$, Figure 3.4d), had contests of similar length (Linear model: $t = 1.21$, $N = 26$, $p = 0.24$, Figure 3.4e), with a similar number of aggressive acts per contest (Negative binomial regression: $Z = -0.44$, $N = 26$, $p = 0.66$) regardless of their previous or current resource quality. All contests ended with a clear winner and loser. Winners monopolized time in the shelter over loser fish (Linear mixed effects model: $t = 6.51$, $N = 52$, $p < 0.001$), but in this experiment, the time spent in the shelter did not different between winners of the high or low quality resource (Linear model: $t = 0.89$, $N = 26$, $p = 0.38$, Figure 3.4f).

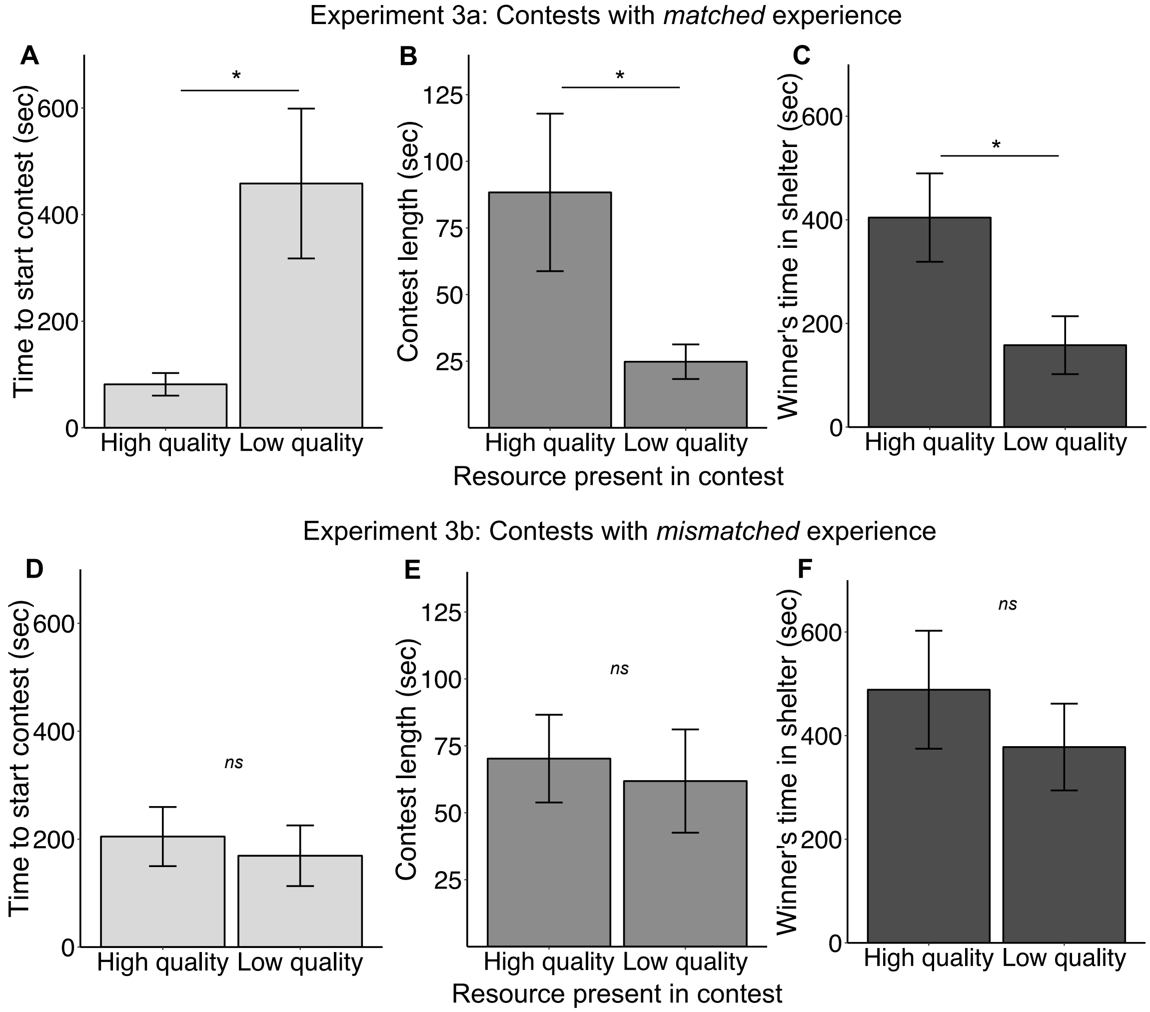

Figure 3.4

Results of Experiment 3a and 3b, contests over shelter resources that were matched or mismatched to prior resource experience. **A)** Time to start a contest plotted by resource value. **B)** Contest length plotted by resource value. **C)** Winner's time spent in the shelter resource plotted by resource value. **D)** Time to start a contest plotted by resource value. **E)** Contest length plotted against resource value. **F)** Winner's time spent in the shelter resource plotted by resource value. In all panels, error bars represent ± 1 standard error. * indicates $p < .05$, *ns* = not significant.

3.4.5 Information gathering across resource contest experiments

Did fish physically evaluate the resource during the contest? Mostly no. After the contest trial started, but before engaging in aggression, only 38 of the 152 fish used in Experiment 2 and Experiment 3 actually entered the shelter. A similar number of fish entered the shelter before fighting across experiments (Experiment 2: $N_{\mathrm{HQ}} = 4$, $N_{\mathrm{LQ}} = 6$; Experiment 3a Matched: $N_{\mathrm{HQ}} = 3$, $N_{\mathrm{LQ}} = 9$; Experiment 3b Mismatched: $N_{\mathrm{HQ}} = 7$, $N_{\mathrm{LQ}} = 9$). Both opponents entered the shelter before starting a contest in only two contest trials. Whether a fish entered the shelter resource before aggressing did not impact contest dynamics. Contests in which at least one opponent entered the shelter before aggressing started at similar times (Linear model: $t = 1.74$, $N = 76$, $p = 0.087$), lasted for similar durations (Linear model: $t = 0.25$, $N = 76$, $p = 0.80$), and had a similar number of aggressive acts (Negative binomial regression: $Z = -0.46$, $N = 76$, $p = 0.64$) as contests where fish did not enter the shelter before fighting. However, after the majority of fish (138 out of 152) entered the shelter at some time point during the 30-minute trial.

3.5 Discussion

We investigated resource assessment during aggressive contests, and how resource value affected contest dynamics in the round goby. We found that fish strongly preferred an enclosed (“high quality”) shelter resource to an open (“low quality”) shelter resource during our resource preference experiment. The high quality shelter would be more defendable from rival con- and heterospecifcs, and would provide better protection from predators than the open, low quality shelter. Moreover, the high quality shelter had only one entrance and could therefore offer a positional advantage to the owner, making them more prepared to defend the shelter resource. In the wild, round goby monopolize sheltered spaces in the rocky littoral zone, and such habitat provides protection from predation by larger fish species (Reyjol et al., 2010; Crane & Einhouse, 2016), water snakes (King et al., 2006), and avian predators (Hebert & Morrison, 2003; Somers et al., 2003). These rocky shelters are doubly valuable as they also create areas to reproduce and care for offspring during the breeding season (Corkum et al., 1998; MacInnis & Corkum, 2000).

In our staged contests, we asked whether resource value would affect contest dynamics and how experience with the resource influenced aggressive behaviours. We found that fish started contests faster, had longer contests, and contests with more aggressive acts when fighting over a high quality resource than over a low quality resource, but only when fish had experienced the shelter for 24 hours before the contest. When fish had no prior shelter experience, resource value did not influence contest dynamics. This indicates that round goby need to interact with the shelter to assess its resource value. Fish then appeared to use this information to gauge their effort in resource contests. That fish need time to assess the resource is supported by our initial resource preference experiment, where individual fish only exhibited a clear resource preference after 24 hours with the resource. Our findings where fish had no prior experience with the shelter are similar to those of Jennings et al. (2004; fallow deer, *Dama dama*) and Thornhill (1984; scorpionflies, *Harpobittacus nigriceps*), where animals may be unable to assess a resource during a contest, or are unable to modify their behaviour based on any gathered information. Likewise, when fish in our study had prior experience with the resource, our results are akin to those of Humphries et al (2006; parasitoid wasps *Goniozus nephantidis*), who demonstrated that prior resource experience leads individuals to fight harder for high quality resources.

Interestingly, when we further asked whether round goby could "update" their evaluation of the resource value during the contest (by switching the value of the resource present in the contest from that present in the prior housing period), we found no evidence that the new resource value present in the contest affected contest dynamics. Fish did not fight according to the value of the resource present during the fight (evaluating the resource present), but they also did not fight according to their prior experience of the resource (ignoring the new resource). This finding indicates that while fish could recognize that a resource was unfamiliar, they were not able to assess the new resource during the contest. We further explored this by investigating resource use throughout the contest trial, and our results suggest that information gathering about the resource mainly occurred after the aggressive contest was resolved. Fish were unlikely to enter the shelter before a contest started, but almost all fish entered the shelter at some point after the contest was decided. It is likely that fish are assessing their contest opponent at the start of each trial more than the resource present. More work will be needed to clarify the extent to which round goby are using visual information gathered at a distance to assess resource quality.

In our study, we used owner-owner resource contests, and comparatively few studies have employed this experimental design in the context of resource value. Instead, the most common form of contest is the resident-intruder paradigm. Resident-intruder studies provide valuable information on the effects that subjective resource value—perceived ownership—has on contest dynamics (e.g. Bridge et al., 2000; Tibbetts & Shorter, 2009; Mohamad et al., 2010; reviewed in Arnott & Elwood, 2008). However, owner-owner contests provide a clearer picture of objective resource value (the intrinsic quality of the resource) by making subjective resource value approximately equal between contestants (Arnott & Elwood, 2008; Elwood & Arnott, 2012). Of the owner-owner contests conducted thus far, most have given one opponent experience with a high quality resource and the other opponent experience with a low quality resource (e.g. Ewald, 1985; Humphries et al., 2006). These previous studies have yielded similar findings to our current results; animals that previously experienced a high quality resource fight longer and harder than those previously housed with a low quality resource. However, it can be unclear whether previous experience with the resource affects the owners' physiology to make them a better (or worse) competitor. For example, Ewald (1985) gave black-chinned hummingbirds access to high or low quality food sources, and found that those with access to the high quality source outcompeted those with previous access to the low quality source. In this design, the animals could be evaluating the quality of resource, or the resource itself could be altering their physiology to give them more energy for competition. The design we have employed here, where fish are housed with resources of identical value and resource value is manipulated between groups, allows us to further control for potential asymmetries in between contestants. Importantly, we have disentangled resource value from physiology, and we can draw firmer conclusions about the extent to which fish are evaluating the objective value of the resource and using this information during an aggressive interaction. An interesting future direction would be to assess contest dynamics in the absence of any resource. This would address whether fish remember previous resource experiences and continue to use this information in a contest that immediately follows with no resource currently present.

In summary, we present a novel investigation of resource assessment and information gathering abilities, and explore the effect that resource value has on contest dynamics in a territorial fish species, the round goby. We show that high quality resources are preferred over low quality resources, and that fish need prior experience with a resource to alter their contest behaviours. Moreover, fish appear to be limited in their ability to update their appraisal of resource value during a contest. We have clearly shown that the characteristics of a contested resource can impact contest dynamics, but we cannot definitively show that fish are willing to pay a higher cost for the high quality shelter, as would be predicted by traditional game-theory models of aggressive contests (Parker, 1974; Enquist & Leimar, 1987). It would be fruitful to follow our work with measures of the physiological costs of fighting to determine if longer contests are more energetically costly in this system (Briffa & Sneddon, 2007). We recommend the further use of owner-owner contests for understanding the impact of resource valuation on contest behaviours. Our findings contribute to a growing body of literature investigating how resource value affects animal contests, and helps to elucidate how animals gather information and assess the value resources during aggressive contests.

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Chapter 4: The antidepressant fluoxetine decreases aggression across multiple behavioural contexts in round goby (*Neogobius melanostomus*)

4.1 Abstract

The dramatic increase in pharmaceutical use has led to antidepressant drugs like fluoxetine (*Prozac*™) commonly being detected in water bodies downstream from wastewater effluent discharge sites. Fluoxetine is designed to alter human behaviour; however, because of conserved physiological pathways, it may affect the behaviour of aquatic organisms living in fluoxetine-polluted environments. While behavioural assays are increasingly being used to establish the sub-lethal effects of pharmaceuticals, the repeatability of these effects across experiments, behavioural contexts, and exposure durations have only rarely been considered. Here, we conducted two experiments and assessed how fluoxetine exposure affected a range of fitness-related behaviours in wild round goby (*Neogobius melanostomus*). We found evidence that fluoxetine impacts round goby behaviour at high (40 μg/l) doses, but not at low (1 μg/l), environmentally relevant, doses. In both experiments an acute, 3-day exposure to fluoxetine, reduced round goby aggression in multiple behavioural contexts, but had no detectable effect on activity or social affiliation. However, following a chronic 28-day exposure, fluoxetine exposure still reduced aggression, but this reduction was only detectable in one behavioural context. Our findings demonstrate the importance of repeated behavioural testing (both between and within experiments), and contribute to a growing body of literature evaluating the effects of fluoxetine and other pharmaceuticals on fish behaviour.

4.2 Introduction

Human use of pharmaceuticals and personal care products continues to escalate (OECD, 2013). Frequent use, ingestion and excretion, as well as improper disposal of these products burdens conventional wastewater treatment facilities that are rarely equipped to remove such compounds from the water they treat (Jelic et al., 2012). Consequently, small but measureable amounts of active pharmaceuticals are now found in urban watersheds with treated effluent acting as a major source of such contamination in the aquatic environment (Kolpin et al., 2002; Khetan & Collins, 2007; Metcalfe et al., 2010). Many pharmaceuticals are designed to modulate human physiology and behaviour (e.g. antidepressants, antibiotics, steroid hormones), and many of their biological targets (e.g. receptors, transporters, enzymes) and subsequent impacts are conserved across vertebrate taxa (Gunnarsson et al., 2008). Therefore, like humans, non-human vertebrates may experience physiological and behavioural changes when exposed to pharmaceuticals and personal care products, raising concern over the impacts of pharmaceuticals on aquatic species living near wastewater outfalls (Corcoran et al., 2010; Boxall et al., 2012; Arnold et al., 2013). While many pharmaceuticals may not be immediately lethal to organisms at concentrations found in the wild, their chronic effects may manifest through subtle alterations to individual physiology and behaviour that could directly or indirectly impact fitness (Brodin et al., 2014).

Antidepressants have a strong potential to alter wild fish behaviour, and these drugs are increasingly being prescribed in developed countries (OECD, 2013). Fluoxetine (commercial name, *Prozac*™) is an antidepressant commonly used for the treatment of human depression and anxiety disorders (Hemels et al., 2005; Paulose-Ram et al., 2007). Fluoxetine and its main active metabolite, norfluoxetine, are measured in treated wastewater effluents and have been recorded downstream in surface waters at concentrations ranging from 0.001 μg/l up to 1.3 μg/l in Europe and North America (Kolpin et al., 2002; Christensen et al., 2009; Metcalfe et al., 2010). Fluoxetine has been found to bioconcentrate in the blood and tissues of fish sampled downstream from wastewater outfalls (Brooks et al., 2005; Ramirez et al., 2009). Fluoxetine causes mortality in fish at concentrations much higher than those reported in the environment (e.g. 48 hr LC_{50} 705 μg/l, for fathead minnow, *Pimephales promelas,* Brooks et al., 2003; 96 hr LC_{50} 2000 μg/l, for sheepshead minnow, *Cyprinodon variegatus,* Winder et al., 2009), but is designed to have therapeutic effects on humans at lower doses.

Fluoxetine is a selective serotonin reuptake inhibitor that functions by increasing serotonin concentrations in the brain and blocking serotonin's reuptake in the synaptic cleft (Stahl, 1998). Specifically, fluoxetine targets the serotonin transporter, and this transporter is well-conserved across vertebrates, including fish (Mennigen et al., 2011). The serotonergic system is integral to many biological processes (e.g. appetite and metabolism, cardiovascular functioning, reproduction). For example, fluoxetine exposure reduced food intake in fathead minnow (*Pimephales promelas,* Gaworecki & Klaine, 2008) and goldfish (*Carassius auratus*, Mennigen et al., 2010a), and reduced growth and glucose metabolism (Mennigen et al., 2009; 2010a). Moreover, fluoxetine has also been found to disrupt reproductive physiology in male fish by reducing testosterone and milt production (Mennigen et al., 2010b), and increasing circulating estradiol and egg-yolk precursor protein, vitellogenin (Mennigen et al., 2010b; Schultz et al., 2011). Fluoxetine is also a well-known modulator of fitness related behaviours in vertebrates, including aggression, predator evasion, and cooperation (Winberg & Nilsson, 1993; Berger et al., 2009; Kiser et al., 2012). In fishes, high brain serotonin is generally correlated with submission, increased sociability, and a muted stress response, while the opposite is true of low brain serotonin (Bell et al., 2007; Loveland et al., 2014; reviewed in: Schjolden & Winberg, 2007; Lillesaar, 2011). Similar submissive behavioural phenotypes have been observed when serotonin levels are experimentally elevated with serotonin receptor agonists or serotonin transport inhibitors (Perreault et al., 2003; de Abreu et al., 2014; Paula et al, 2015). Pharmacological alterations to the serotonergic systems of wild fish may therefore have repercussions for behaviours important for survival and reproduction.

Laboratory methods for assaying the effects of fluoxetine on fish behaviour have involved exposing fish to various concentrations of fluoxetine and then quantifying ecologically relevant behaviours after a given exposure duration. At environmentally relevant levels (< 1 μg /l), researchers have shown that fluoxetine can alter behaviour rapidly; for example, after only 48 hours of exposure, fluoxetine reduced aggressive displays in male Siamese fighting fish (*Betta splendens,* Dzieweczynski & Hebert, 2012). Additionally, after 6 to 7 days of fluoxetine exposure, brood defense during parental care and aggression towards a conspecific decreased in male Siamese fighting fish (Forsatkar et al., 2014; Greaney et al., 2015), and in Arabian killifish (*Aphanius dispar,* Barry, 2013). However, animals in the wild are likely to be exposed to pharmaceuticals over much longer durations.

Research to date has shown that animals chronically exposed to fluoxetine for 21 to 28 days are less adept at avoiding predators (fathead minnow *Pimphales promelas*, Weinberger & Klaper, 2014; and guppy *Poecilia reticulata,* Pelli & Connaughton, 2015), and chronic 21-day fluoxetine exposure also reduced nest quality in three-spine stickleback (*Gasterosteus aculeatus*) without altering aggression towards a mirror (Sebire et al., 2015). Given such sometimes contradictory findings, there is a need to understand the effects of fish behaviour of both short- and long-term exposures to low doses of fluoxetine on fish behaviour. It is important to repeatedly examine relevant behaviours in multiple contexts and over multiple time points within an experiment (e.g. Dzieweczynski & Hebert, 2012). It is also important to replicate findings between experiments (Sumpter et al., 2014) if we are to ascertain whether effects are robust. In addition, there is an urgent need to develop robust and reliable behavioural assays for more species, specifically for wild, non-model fish species that inhabit affected waterways (Brooks, 2014).

To this end, we conducted two experiments to identify the behavioural effects of exposure to fluoxetine in a wild fish, the round goby (*Neogobius melanostomus*). This benthic fish species is widespread throughout the Laurentian Great Lakes, Western Europe, and the Ponto-Caspian region of Eastern Europe (Corkum et al., 2004; Kornis et al., 2012). We first conducted an acute, 3-day exposure to fluoxetine and assessed how contest aggression over a resource, social interaction with a conspecific, and activity in an open tank were affected. We predicted that fluoxetine exposure would reduce aggression in resource contests and increase the time spent interacting with conspecifics, as has been observed in other fish species exposed to fluoxetine (Dzieweczynski & Hebert, 2012; Barry, 2013; Forsatkar et al., 2014; Greaney et al., 2015). We then conducted a second experiment, in which we assessed the effects of fluoxetine exposure on round goby behaviour after three days, replicating our first experiment, and then tested these same fish again after 28 days of exposure. Thus, we repeated our measurements both within and between experiments to assess the repeatability of certain assays, and determine how fluoxetine's effects may change with exposure duration. In the second experiment, we again assessed aggression in a resource contest and also assessed aggression using a mirror assay, a standard method to gauge individual differences in aggressiveness (Balzarini et al., 2014; Elwood et al., 2014). We predicted that we would again see reduced aggression in the resource contest and in the mirror aggression assay after 3 days of exposure. Furthermore, we expected that aggression would remain low after 28 days of exposure if fluoxetine similarly reduces aggression after a chronic exposure, as it has in shorter exposure experiments (< 10 days; Dzieweczynski & Hebert, 2012; Forsatkar et al., 2014; Greaney et al., 2015). In both exposure experiments, we monitored non-aggressive activity to ensure any reductions in aggression observed were not simply a result of an overall reduction in activity.

4.3 Methods

4.3.1 Fish collection and housing

We collected round goby between May 10, 2013 and June 10, 2013 (Experiment 1) and between July 10, 2014 and July 30, 2014 (Experiment 2) from LaSalle Park Marina, Hamilton, ON, Canada (43°18’1 N, 79°50’47 W) using baited minnow traps. For collection methods details see McCallum et al. (2014 – Appendix B) and Young et al. (2010). We transported the fish to McMaster University where we housed them in same-sex groups of three to six fish in 75 l aquaria (H30 cm x W62 cm x D46 cm). We equipped the housing aquaria with ~1cm of natural gravel substrate, an airstone, plastic PVC tubes for shelter, and a static renewal filter (Aquaclear). We fed fish Nutrafin Basix Staple Food once daily and kept a 14L:10D light schedule. After 24 hours acclimation to the laboratory, we weighed each fish to the nearest 0.01g, measured their standard length to the nearest 0.01cm, and uniquely tagged them using non-toxic acrylic paint (Wolfe & Marsden, 1998; Groen et al., 2012; Capelle et al., 2015) before returning them to their housing tanks. The visual tag was used to identify individuals throughout behavioural trials and later sampling. We monitored water quality daily, checking: dissolved oxygen, temperature, pH, conductivity, total dissolved solids, and salinity (LaMotte Pocket Tracer, Oakton PCTestr 35).

4.3.2 Fluoxetine exposures and experimental protocol

Experiment 1: Acute exposure only

We exposed 88 round goby for 72 hours to three fluoxetine treatments: a 0 µg/l control treatment, a 1µg/l environmentally relevant low treatment, and a 40 µg/l high treatment. We used 44 males ($N_{control}$=15, N_{low} = 14, N_{high} = 15) and 44 females ($N_{control}$=15, N_{low} = 14, N_{high} = 15). We first prepared a fluoxetine 1mg/ml stock solution by dissolving fluoxetine hydrochloride (99.9% purity, Sigma Aldrich) in anhydrous ethanol. Then, we prepared individual dosing aliquots for each treatment by dilution with ultrapure water (MilliQ). Control doses contained only ethanol and ultrapure water. We controlled for the amount of ethanol used across all doses, and was kept well below toxicity levels for fishes (24 hr LC_{50} rainbow trout: 11,200,000 μg/l, Majewski et al., 1978). All doses were relabeled (A-C) by a member of the research group not involved in the experiment, as this ensured we remained blind to treatment while conducting exposures and behavioural trials. We froze the individual dosing aliquots at -20°C until their use at the beginning of an exposure period. We exposed fish in a static-renewal exposure in their 75 l housing tanks in the same-sex, groups of three. We removed the activated carbon inserts from the filter of each tank during exposures. No fish died during the exposure period.

Experiment 2: Acute and chronic exposure

We exposed 144 round goby for 28 days to the same three fluoxetine treatments used in Experiment 1. We used 69 females ($N_{control}$=24, N_{low} = 24, N_{high} = 21), and 75 males ($N_{control}$=24, N_{low} = 24, N_{high} = 27) in this experiment. We prepared fluoxetine doses as described above, and the experimenters were similarly blind to treatment. Fish were exposed in groups of eight in their 75 l housing tanks, and again the activated carbon was removed from the filters. Following the first exposure dosing, we re-dosed each tank every 72 hours with half the original dose concentration (the half-life of fluoxetine in a stocked tank, following Gaworecki & Klaine, 2008). In addition, we conducted two water changes occurred across the 28-day exposure period, every 12 days coinciding with a re-dosing day where we replaced 30% of the tank water with de-chlorinated tap water.

We quantified fluoxetine by taking grab water samples from three tanks of each treatment one hour after dosing, and again at 72 hours immediately before re-dosing. One low exposure one-hour sample broke during transport for analysis and we excluded it from further analysis. Fluoxetine samples were quantified following Metcalfe et al. (2010). Briefly, 20 ml samples were extracted using Oasis MCX SPE cation-exchange cartridges (Waters Scientific). The eluant from the SPE cartridge was collected in a centrifuge tube, evaporated just to dryness, and then reconstituted in methanol. The sample was then transferred to an autosampler vial with an insert for analysis. All samples were analyzed by LC-MS/MS using a Q-Trap LC-MS/MS System. After one hour of exposure, we found fluoxetine concentrations to be an average (± SE) of 0.00 (± 0.00) µg/l for the 0 µg/l control treatment, 0.55 (± 0.15) µg/l for the 1 µg/l low treatment, and 35.43 (± 4.44) µg/l for the 40 µg/l high treatment. After 72 hours of exposure, we found fluoxetine concentrations to be an average (± SE) of 0.00 (± 0.00) µg/l for the 0 µg/l control treatment, 0.00 (± 0.00) µg/l for the 1 µg/l low treatment, and 22.60 (± 6.65) µg/l for the 40 µg/l high treatment.

Five fish died from unknown causes during the exposure. But, as these fish came from different tanks and treatment groups (1 control, 3 low, 1 high), it is highly unlikely that the mortality was related to the fluoxetine exposure.

4.3.3 Behavioural assays

We equipped the tanks used for behavioural testing, described in detail below, with ~1 cm of natural gravel substrate, a static renewal filter, and an airstone. Unless otherwise stated, we video-recorded all trials on a Canon HD recorder (Vixia HFS100 8.0 Megapixel) and we conducted all trials from behind opaque barriers to limit external influence. In Experiment 1, we conducted a contest aggression assay, a social interaction assay, and an activity assay. In Experiment 2, we again conducted a contest aggression assay, as well as a mirror aggression assay (Figure 4.1).

Experiment 1: Acute exposure only

We first conducted the contest aggression and social interaction assays. We conducted these trials before 12:00 pm, counterbalancing which assay occurred first. In our contest aggression assay, we staged contests over a resource: a black, square shelter (H5 cm x W15 cm x D15 cm deep) with only one defendable entrance (Figure 4.1a). Round goby will reliably fight over and defend such shelters from both conspecifics (Sopinka et al., 2010; Groen et al., 2012) and heterospecifics (Balshine et al., 2005), because they use shelter for breeding and as protection from predators (MacInnis & Corkum, 2000; Belanger & Corkum, 2003). We assessed aggression in a 75 l tank, and first we gave the resident had a 20-minute habituation period to take-up residency in the shelter before an intruder was added. Each exposed fish acted as the resident and was paired with a slightly smaller, same-sex, unexposed intruder fish (average body mass difference: 2.54g ± 0.27 SE). This would ensure the resident was likely to win the contest, allowing us to measure their territorial defense across the entire trial (Taylor & Elwood, 2003). After the intruder was added, we then recorded a 20-minute aggression trial.

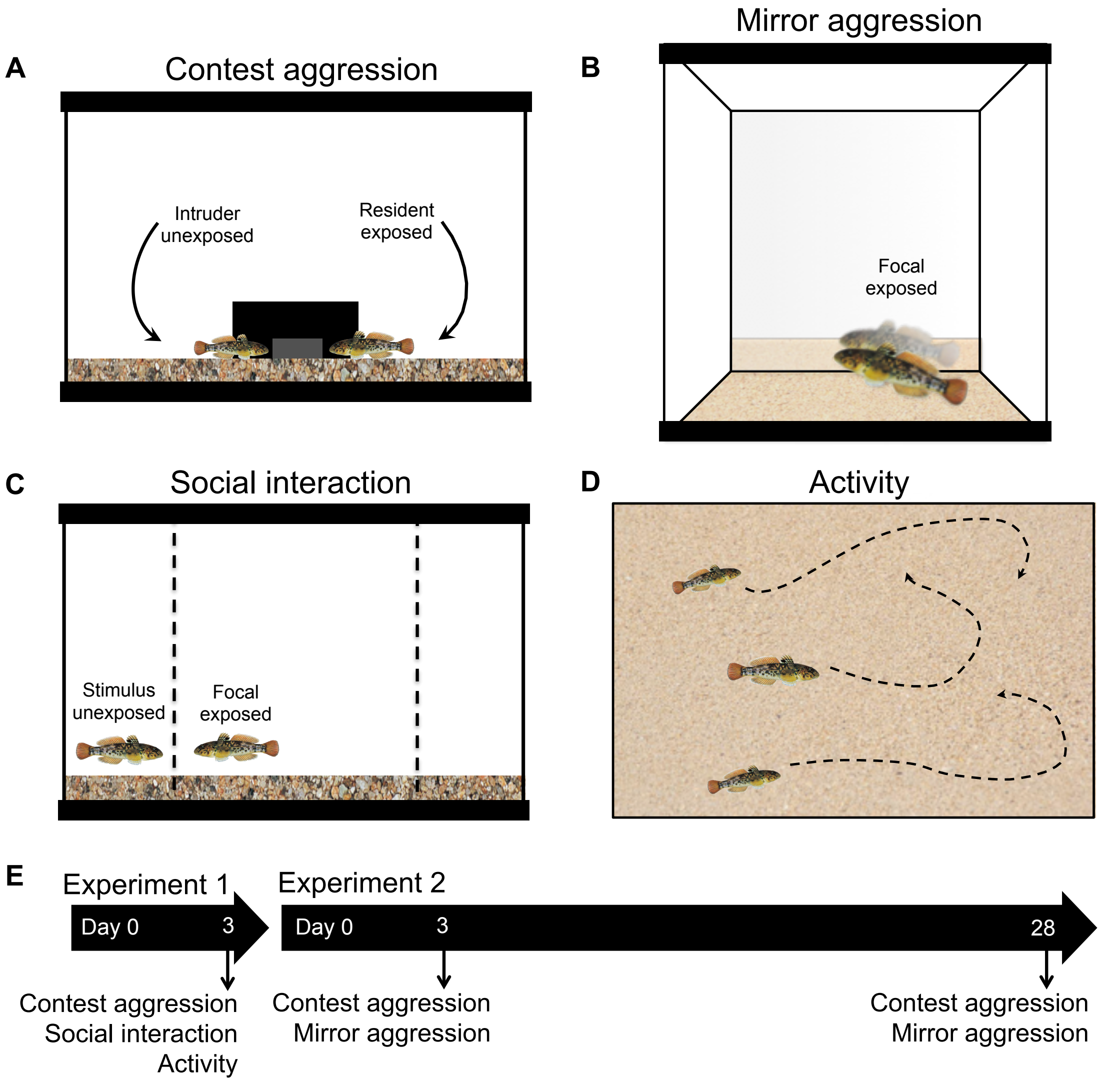

Figure 4.1

Tank set-ups for all behavioural assays and an experimental timeline. **A)** Testing tank for contest aggression assay, depicting the shelter resource and resident and intruder fish interacting. **B)** Testing tank for mirror aggression assay, showing a focal fish interacting with its mirror image. **C)** Testing tank for the social interaction assay. Dashed lines represent the two end compartments where the conspecific stimulus fish would have been placed. **D)** Testing tank for activity assay, an open field without shelters as viewed from above. **E)** Experimental timeline for both Experiments 1 and 2 and the behavioural assays conducted.

Following an established ethogram for this species (Supplementary Table S1 – Appendix C, adapted from Sopinka et al., 2010), an observer blind to a fish's exposure history recorded all aggressive acts given by the resident to the intruder and all aggressive acts received by the resident from the intruder across the 20-minute trial. They also recorded the time from the beginning of the trial to the first aggressive act from either resident or intruder. They then scored contest length (when bi-directional aggression between opponents is occurring) as the time from the first aggressive act to when a clear winner or dominant fish was established. A dominant fish is established when either resident or intruder fish performed three or more consecutive aggressive acts with no retaliation from the other fish. If a contest winner could not be assigned to resident or intruder, the trial was scored as a tie.

Our social interaction assay was conducted in a 150 l tank (H44 cm x W90 cm x D38 cm; Figure 4.1c). The tank was divided into three compartments by two perforated, clear, plastic barriers. A same-sex (average focal-stimulus body mass difference: 0.99g ± 0.32 SE) non-exposed stimulus fish from laboratory stock tanks was placed in one of the two end compartments, while the opposite chamber always remained empty. The side of the tank containing the stimulus fish was counterbalanced across trials. The exposed fish was then added to the central compartment, and isolated in a perforated clear plastic tube (13 cm diameter, 20 cm high) for a 15-minute habituation period. The tube was then lifted remotely and the focal fish could freely move around the central compartment. An observer blinded to fluoxetine treatment recorded all behaviours (aggressive and non-aggressive) of the exposed fish during the 15-minute trial (Supplementary Table S1 –

Appendix C). The 8 cm area in front of either end compartment was considered to be an 'association zone' (the average body length of round goby in our collection area, McCallum et al., 2014 - Appendix B), the observer also recorded the time spent by the focal fish in each association zone. This was used to create a preference index ([time spent near the stimulus fish side / (time spent near the stimulus fish side + time spent near the empty side)]: Svensson et al., 2000; Reddon et al., 2011; Capelle et al., 2015 – Chapter 2 of this thesis). A preference index > 0.5 indicates a preference for interacting with the conspecific, while a preference index < 0.5 indicates an aversion.

After the contest aggression and social interaction assays, we returned fish to their exposure tanks until we conducted our activity assay after 15:00, under low light or dusk conditions (when round goby are known to be most active; Johnson et al., 2008; Marentette et al., 2011). We assessed overall activity using a shallow open field tank (H15 cm x W50 cm x D75cm; 4.1d; adapted from Marentette et al., 2011). Three fish, all from the same exposure tank, were transferred to the activity assay tank, held together in a habituation tube for 10 minutes, and tested as a group. Previous work has shown that round goby are more active when assessed as a group than in isolation (Marentette et al., 2011). Once we removed the tube, the three fish could then freely explore the entire compartment for a 15-minute trial. All behaviours (Supplementary Table S1 – Appendix C) of each fish were live-tallied by an observer blind to treatment, for 5 minutes each in random order (total trial time was 15 minutes). No aggression was observed between the three fish in this activity assay. No aggression likely occurred because the fish were familiar from being housed in the same exposure/housing tank, and the activity assay tank did not contain any shelters over which the fish might fight.

Experiment 2: Acute and chronic exposure

We performed both the mirror and contest aggression assays on the same day and in the same 40 l (H33 cm x W51 cm x D28 cm) behavioural testing tank to limit handling disturbance. We first assessed mirror aggression (Figure 4.1b) to evaluate aggressive tendencies and the motivation to fight in the absence of a live opponent. We used mirrors because opponent motivation can vary considerably and introduce undesired variation into fight dynamics (Balzarini et al., 2014; Elwood et al., 2014). Exposed fish were given a 30-minute habituation period before an opaque barrier was remotely lifted to reveal a mirror and a 30-minute mirror aggression assay was recorded. We had each mirror aggression assay scored by two observers blinded to fluoxetine treatment (Supplementary Table S1 – Appendix C). The observer recorded the time taken for the fish to move towards the mirror, the total number of aggressive acts performed towards the mirror, and the total number of non-aggressive acts conducted while away from the mirror (as proxy measure of activity). Fish were considered to be interacting with the mirror when they were within one body width of the mirror and oriented towards their reflection. We calculated inter-rater reliability scores by having the two observers score the same 10 videos: the observations were highly correlated (Aggressive acts $R^2 = 0.98$; Non-aggressive activity $R^2 = 0.99$).

After the mirror aggression trial, we lifted the mirrored barrier to reveal a shelter resource (used in Experiment 1). The contest aggression trial followed a similar protocol to that described for Experiment 1. Except, we gave residents a 90-minute habituation period to take-up residency with the shelter before a same-sex, unexposed intruder fish from the laboratory stock was added to the tank (Residents were again bigger than intruders: mean body mass difference 1.02 g ± 0.07 SE). We then recorded a 30-minute contest. We used new fish as intruders between the acute and chronic time point to ensure that no resident encountered the same intruder at both time points. Aggressive interactions were scored following the same procedure as in Experiment 1 by two observers blind to treatment scored the mirror and contest assays. The inter-rater reliability between the two observers was highly correlated ($N_{\text{videos}} = 10$, aggressive acts $R^2 = 0.98$).

4.3.4 Post-behavioural processing

After each experiment, we euthanized all exposed fish with an overdose of benzocaine (0.025%, Sigma Aldrich), and dissected them to confirm sex and reproductive status. We measured standard length (snout to caudal peduncle) using calipers accurate to the nearest 0.01cm, and we measured body mass, liver mass, and gonad mass to the nearest 0.001g. We calculated gonadosomatic index (GSI: gonad mass / body mass – gonad mass), and classified males as reproductive if their GSI was greater than 1%, and females as reproductive if their GSI was greater than 8% (Marentette & Corkum, 2008; Zeyl et al., 2014). In Experiment 1, the majority of the males (33 of 44) and the females

(31 of 44) were non-reproductive, and the reproductive fish were evenly distributed across treatments (N_{High} = 8, N_{Low} = 8, $N_{Control}$ = 8). In Experiment 2, all fish 144 tested were non-reproductive.

4.3.5 Statistical analyses

All statistical analyses were conducted using R (version: 3.2.2, R Core Team, 2015). Residual analysis, Shapiro-Wilk, and Breusch-Pagan tests were used to assess parametric model assumptions. Any non-significant interactions were removed from the models. Two inclusion criteria were employed before data analysis: 1) we only analyzed the data from fish that interacted with the intruder at least once during the aggressive contest, as this would ensure the resident was aware of the intruder fish, and 2) we only analyzed data from fish that moved at least once during the social interaction and mirror aggression assay, trials to ensure that all fish were actively sampling their environment. (See Table 4.1 for a description of the sample sizes used before and after these inclusion criteria were applied). In all analyses, fluoxetine treatment and sex were included as fixed factors. In Experiment 2, all analyses used mixed effects models where exposure time (acute = 3 days, chronic = 28 days) was included as repeated measures factor, and fish ID was included as a random effect to account for the non-independence between time points (package lme4, Bates et al., 2015; package glmmADMB, Fournier et al., 2012).

Table 4.1

Summary of fish used in all behavioural assays before and after the inclusion criteria was applied. -- Indicates no inclusion criterion was applied before data analysis.

	N **exposed**	***N*** **scored**	**Inclusion criteria**	***N*** **analyzed**			
Experiment 1 – Acute only				**Control**	**Low**	**High**	**Total**
Resident-intruder contest	88	82	how many interacted?	18	17	21	56
Social interaction	88	88	how many moved once?	25	21	20	66
Activity	88	88	--	30	28	30	88
Experiment 2 – Acute & chronic							
Acute: Mirror aggression	144	139	how many moved once?	42	39	37	118
Acute: Resident-intruder contest	144	137	how many fish interacted?	39	40	41	120
Chronic: Mirror aggression	144	137	how many moved once?	41	42	36	119
Chronic: Resident-intruder contest	144	139	how many fish interacted?	41	42	37	120

Because body size difference is known to have a large impact on contest dynamics (Arnott & Elwood, 2009), resident-intruder body mass difference was also included as a covariate in all contest aggression analyses. Differences in body mass between exposed residents and non-exposed intruder fish did not vary across treatments (ANOVA: Experiment 1: $F_{(2,53)} = 0.13$, $p = 0.88$; Experiment 2 Acute: $F_{(2, 113)} = 0.97$, $p = 0.38$; Experiment 2: Chronic: $F_{(2, 111)} = 1.80$, $p = 0.17$), or between sexes (Experiment 1: $F_{(2,53)} = 1.59$, $p = 0.21$; Experiment 2 Acute: $F_{(1, 113)} = 3.27$, $p = 0.073$; Experiment 2 Chronic: $F_{(1, 111)} = 3.11$, = 0.08).

In the contest aggression assays, we tested for effects on: (1) the time to begin the aggressive contest using a linear model after ln-transformation to meet parametric assumptions; (2) contest length using a linear model after power-transformation; (3) total contest aggression across the trial using a negative binomial generalized linear model; and (4) the likelihood of the exposed resident fish winning the contest using a binary logistic regression. We also specifically tested for an interaction between fluoxetine treatment and total contest aggression (all aggression given from the resident, and all aggression received from the intruder) as that would indicate if exposure was altering aggressive interactions between resident and intruder fish. In Experiment 2, we tested for a three-way interaction between total contest aggression, fluoxetine treatment, and exposure duration. This would indicate whether exposure duration either exacerbates or diminishes the effects of fluoxetine on aggressive interactions between resident and intruder fish.

In the mirror aggression assay analyses, we tested for effects on: (1) the time to move towards the mirror using a linear model after a ln-transformation; (2) the number of aggressive acts performed towards the mirror, and (3) the number of non-aggressive movement behaviours (used as proxy for activity level) performed away from the mirror; 2 and 3 were both analyzed using a negative binomial generalized linear model for count data.

For sociality assays, we tested for effects on: (1) the conspecific preference index for the stimulus fish, and (2) amount of aggression performed towards the stimulus fish across the transparent barrier. Effects on preference were assessed with a beta regression for proportion data (betareg package: Cribari-Neto & Zeileis, 2010). Effects on aggression were assessed using a negative binomial generalized linear model for count data.

For our activity assay, effects on total activity were analyzed for all fish using a negative binomial generalized linear model. As fish were tested in groups of three, this analysis was conducted as a mixed effects model, where exposure group ID was included as a random effect to account for any shared variance among activity levels within a group.

4.4 Results

4.4.1 Experiment 1: Acute exposure only

In the contest aggression assay, we found an interaction between fluoxetine treatment and total contest aggression between resident and intruder fish. Contests where a resident had been exposed to the high treatment of fluoxetine involved fewer aggressive acts from the resident, and more aggressive acts from the intruder, when compared to control treatment contests. (Negative binomial GLMM: Treatment-by-contest aggression interaction, $N = 56$, High vs Control $Z = 2.07$, $p = 0.039$; High vs Low $Z = 0.53$, $p = 0.60$; Low vs Control, $Z = 1.53$, $p = 0.13$; Figure 4.2a). Fluoxetine treatment did not impact how quickly aggression was initiated (Linear model: $N = 56$, High vs Control, $t = 0.57$, $p = 0.57$; High vs Low: $t = 0.54$, $p =0.59$; Low vs Control: $t = 0.050$, $p = 0.96$). Although fish exposed to the high dose of fluoxetine displayed less aggression towards the intruder, this did not affect how long it took to establish a dominant contest winner (Linear model: $N = 56$, High vs Control, $t = 0.41$, $p = 0.68$; Low vs Control: $t = 0.97$, $p = 0.34$; High vs Low: $t = -0.63$, $p = 0.53$).

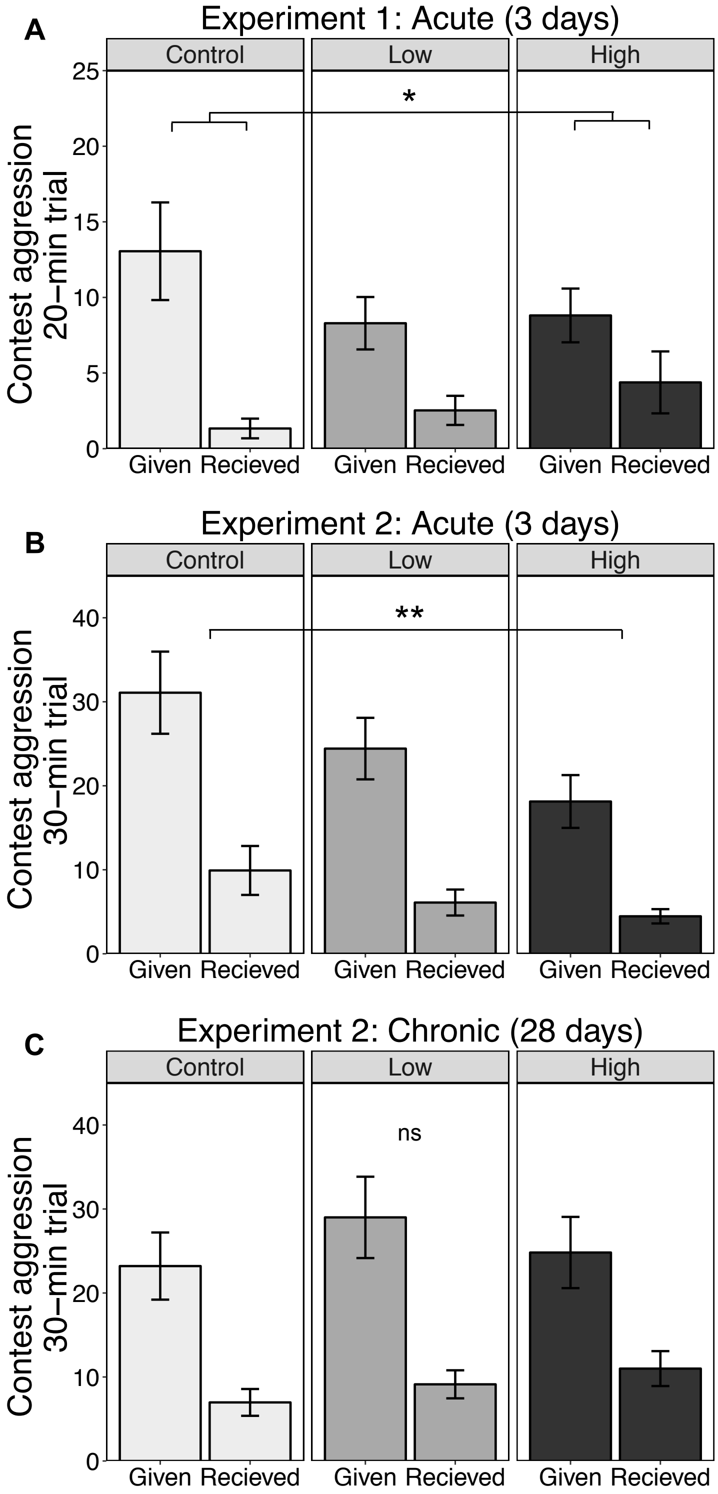

Figure 4.2

Contest aggression in Experiments 1 and 2. * $p < 0.05$, ** $p < 0.01$, ns = no significant difference in contest aggression in relation to treatment. Error bars represent ± SE

A) Contest aggression plotted as aggression given and aggression received, by fluoxetine treatment. Brackets denote an interaction between treatment and the aggression performed by residents and intruders during the contests.

B) Contest aggression plotted as aggression given and aggression received, by treatment. Brackets show a reduction in contest aggression for both residents and intruders.

C) Contest aggression plotted as aggression given and aggression received, by treatment.

Fluoxetine treatment also did not impact the likelihood of the exposed resident fish winning the contest (Binary logistic regression: $N = 56$, High vs. Contol $Z = -1.68$, $p = 0.09$; Low vs. Control Z = -0.55, $p = 0.58$; High vs. Low $Z = -1.16$, $p = 0.25$). Resident fish won the majority of contests (taking 88% of control, 81% of low dose, and 65% of high dose contests). There was no effect of sex in the above analyses, and all subsequent sex-effects are summarized in Table 4.2.

In our social interaction assay, fish spent on average 72% (± 3.9% SE) of the total trial time associating with the stimulus fish. However, fluoxetine treatment did not affect the amount of time fish spent interacting (Beta regression: $N = 66$, High vs Control $Z = 1.01$, $p = 0.31$; High vs Low $Z = 0.42$, $p = 0.67$; Low vs Control, $Z = 0.57$, $p = 0.56$). Fish mostly displayed aggression across the barrier towards the stimulus fish, and those fish exposed to the high fluoxetine dose were less aggressive than the control fish (Negative binomial regression: $N = 66$, $Z = -2.12$, $p = 0.03$: Figure 4.3). High treatment fish were not statistically different from low treatment fish ($Z = -0.72$, $p = 0.47$), and low treatment fish did not differ from controls ($Z = -1.39$, $p = 0.16$).

The activity assay revealed that fish moved in the open tank an average of 17 times (± 2 SE) over a 5-minute period. Activity levels of fish were not influenced by acute exposure to fluoxetine at any dose (Negative binomial GLMM: $N = 88$: High vs Control $Z = 0.81$, $p = 0.42$; High vs Low $Z = -0.29$, $p = 0.77$; Low vs Control $Z = 1.09$, $p = 0.28$).

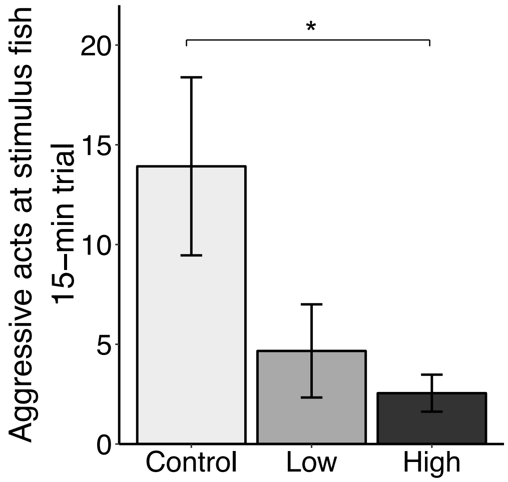

Figure 4.3
Number of aggressive acts towards the conspecific stimulus fish in the social interaction assay, plotted by treatment. Brackets show a reduction in aggression towards the stimulus fish following an acute, 3-day fluoxetine exposure. * $p < 0.05$. Error bars represent ± SE.

4.4.2 Experiment 2: Acute and chronic exposure

We found no evidence for a three-way interaction between fluoxetine treatment, resident-intruder contest aggression (aggression given and aggression received), and exposure time (Negative binomial GLMM: $N = 140$, High vs. Control: $Z = 1.03$, $p = 0.30$; Low vs. Control: $Z = 0.77$, $p = 0.44$; High vs. Low, Z = 0.27, p = 0.79). We therefore analyzed the acute and chronic time points separately to simplify the interpretation of the analysis.

Unlike Experiment 1, we found no interaction between fluoxetine treatment and contest aggression between the residents and intruders (Negative Binomial GLMM: Treatment-by-contest aggression interaction: $N = 120$, High vs Control, $Z = 0.23$, $p = 0.82$; Low vs Control, $Z = -0.60$, $p = 0.55$; High vs Low: $Z = 0.84$, $p = 0.40$). However, after the acute exposure (3 days), contests after the high fluoxetine treatment were less intense, and involved fewer aggressive acts for *both* the resident and the intruder fish, when compared to contests from the control treatment (Control vs High: $Z = -2.31$ $p = 0.021$; Control vs Low: $Z = -0.71$, $p = 0.48$; High versus Low: -1.61, $p = 0.11$, Figure 4.2b).

After the chronic (28 day) exposure, we again found no interaction between fluoxetine treatment and contest aggression between the residents and intruders (Negative Binomial GLMM: Treatment-by-contest aggression interaction, $N = 120$, High vs. Control: $Z = 0.97$, $p = 0.33$; Low vs. Control: $Z = 0.21$, $p = 0.84$, High versus low: $Z = 0.78$, $p = 0.43$). Furthermore, after 28 days, aggressive contests under high fluoxetine treatment were just as intense, i.e. involved similar numbers of aggressive acts by *both* resident and intruder fish, when compared to other treatment conditions (High vs. Control $Z = 0.36$, $p = 0.72$; Low vs. Control $Z = 0.95$, $p = 0.34$, High vs Low $Z = -0.56$, $p = 0.58$, Figure 4.2c).

Fluoxetine treatment did not impact how quickly aggression was initiated in the contest assay (Linear mixed effects model: $N = 140$; High vs Control $t = 1.76$, $p = 0.082$; Low vs Control $t = 1.01$, $p = 0.31$; High vs Low: $Z = 0.76$, $p = 0.45$). However, the fish took longer to begin contests after 28 days of exposure than after 3 days of exposure (effect of exposure time: $t = 2.53$, $p = 0.013$). Fish took on average 498 seconds (± 40 SE) seconds to begin fighting after 3 days and 585 seconds (± 40 SE) to start aggressing after 28 days. As in Experiment 1, fluoxetine exposure did not impact how quickly a dominant contest winner was established (LMM $N = 140$, High vs Control, $t = -0.65$ $p =$

0.52; Low vs Control *t* = 0.08, *p* = 0.94; High vs Low: *t* = -0.74, *p* = 0.46; effect of exposure time: *t* = 0.70, *p* = 0.49). Residents won more contests than intruders (taking 82% of control, 80% of low dose, and 75% of high dose contests), and treatment with fluoxetine did not affect the likelihood of the resident winning the contest (Binary logistic GLMM: *N* = 140, High vs Control, *Z* = -1.21, *p* = 0.23; Low vs Control *Z* = -0.48, *p* = 0.63; High vs Low: *Z* = -0.77, *p* = 0.44). Residents were equally likely to win contests at the acute and chronic time points (effect of exposure time: *Z* = -0.60, *p* = 0.55).

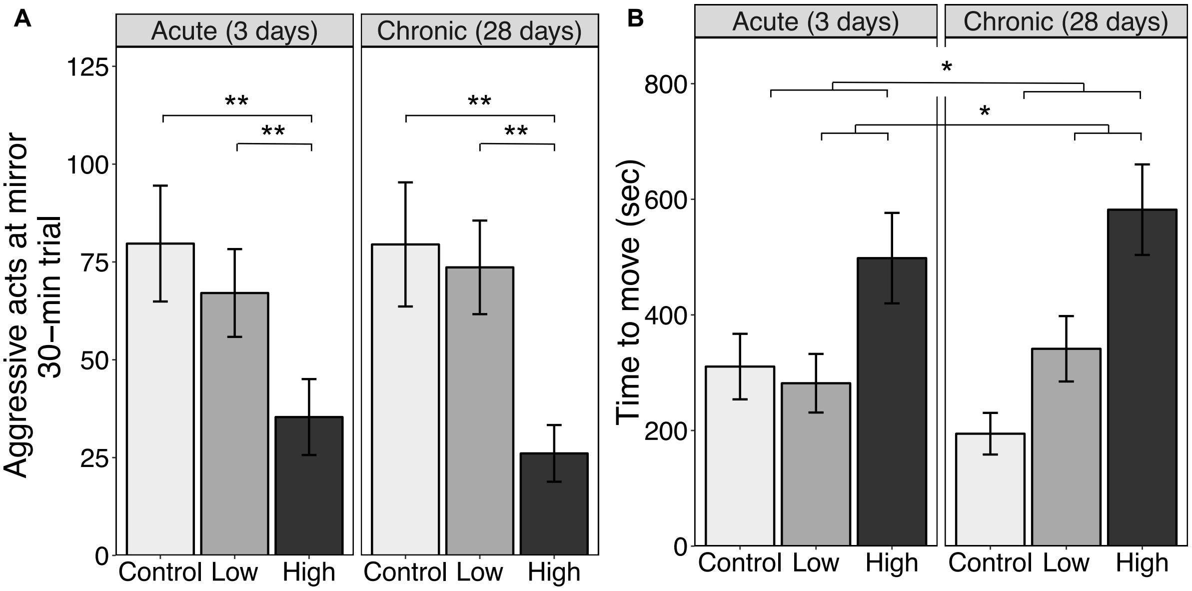

Figure 4.4
Mirror aggression results from Experiment 2. **A)** Aggressive acts towards the mirror plotted against treatment and exposure duration. Brackets show reduction in aggression at both acute and chronic exposure durations. **B)** Time to move towards the mirror and begin aggression plotted against treatment and exposure duration. Brackets indicate an interaction effect between treatment and exposure duration. * p < 0.05, ** p < 0.01. Error bars represent ± SE.

In the mirror aggression assay, fish treated with the high dose of fluoxetine were less aggressive after exposure for 3 days, but in contrast to the contest assays with live opponents, the fish exposed to fluoxetine were also less aggressive after 28 days (Negative binomial GLMM: $N = 135$, High vs Control $Z = -3.40$, $p = 0.00067$; High vs Low $Z = -3.16$, $p = 0.0016$; Low vs Control $Z = -0.24$, $p = 0.81$; Figure 4.4a; effect of exposure time: $Z = -0.21$, $p = 0.84$). Fish exposed to the high dose and to the low dose of fluoxetine took longer to begin moving towards their mirror image and initiating aggression compared to controls, but this effect was only apparent after 28 days of exposure (Linear mixed effects model: $N = 135$: Time-by-treatment interaction for High vs Control: $Z = 2.31$, $p = 0.045$; Low vs Control: $Z = 1.96$, $p = 0.050$; High vs Low: $Z = 0.07$, $p = 0.95$, Figure 4.4b). Consistent with our other activity assays there was no effect of fluoxetine treatment on the overall non-aggression related activity in this mirror assay at either time point (Negative binomial GLMM: $N = 135$, High vs Control $Z = -1.34$, $p = 0.18$: Low vs. Control: $Z = 0.46$, $p = 0.65$; High vs Low $Z = -1.79$, $p = 0.073$; effect of exposure time $Z = -0.93$, $p = 0.53$).

Table 4.2
Summary of sex-effects from all statistical models.

Assay	**Statistical test**	**Statistic**	**p-value**	**Mean (± SE)**
Experiment 1				
Contest aggression				
Total contest aggression	Negative binomial GLMM	$Z = 1.13$	$p = 0.26$	
Time to start contest	Linear model	$t = -0.53$	$p = 0.60$	
Time to establish a contest winner (sec)	Linear model	$t = 1.01$	$p = 0.32$	
Contest winner	Binomial GLM	$Z = -1.70$	$p = 0.089$	
Social interaction				
Conspecific preference	Beta regression	$Z = 0.71$	$p = 0.78$	
Aggression	Negative binomial GLM	$Z = -0.005$	$p = 0.99$	
Activity				
Number of movements	Negative binomial GLM	$Z = -1.97$	$p = 0.049$	♂: 12 ± 2.96, ♀: 21 ± 3.03
Experiment 2				
Contest aggression				
Acute: contest aggression	Negative binomial GLMM	$Z = 2.25$	$p = 0.024$	♂: 28 ± 3.23, ♀: 18 ± 2.97
Chronic: contest aggression	Negative binomial GLMM	$Z = 1.69$	$p = 0.091$	♂: 28 ± 3.84, ♀: 19 ± 2.74
Time to start contest (sec)	Linear mixed model	$t = -1.93$	$p = 0.056$	♂: 472 ± 33.8, ♀: 619 ± 45.1
Time to establish a contest winner (sec)	Linear mixed model	$t = -0.11$	$p = 0.91$	
Contest winner	Binomial GLMM	$Z = 0.08$	$p = 0.94$	
Mirror aggression				
Aggressive acts	Negative binomial GLMM	$Z = 1.95$	$p = 0.061$	♂: 72 ± 7.72, ♀: 46 ± 6.45
Time to move to mirror	Linear mixed model	$Z = 0.10$	$p = 0.92$	
Non-aggressive activity	Negative binomial GLMM	$Z = 0.72$	$p = 0.84$	

4.5 Discussion

4.5.1 Behavioural responses to exposure concentrations

Through its role in modulating the serotonergic system, fluoxetine exposure alters multiple physiological and behavioural processes important for fitness (Gaworecki & Klaine, 2008; Mennigen et al., 2009; 2010a; 2010b; 2011; Schultz et al. 2011). In our study, we found that fluoxetine impacted round goby aggression after exposure to the high dose (40 µg/l), and found little evidence that exposure impacted aggression after exposure to an environmentally relevant low dose (1 µg/l). However, low-dose exposed fish were often behaviorally intermediate between control and high exposure fish, indicating that we may have lacked power to identify a behavioural effect. This pattern of results (impact observed only at the high dose) was consistent between experiments (Experiment 1 and Experiment 2) and between exposure durations within Experiment 2 (3 days versus 28 days).

It is possible that a 1µg/l dose was not high enough to cause changes to the behavioural endpoints we measured, or that a 28-day exposure was not long enough to elicit an effect in round goby. Recently, Margiotta-Casaluci et al. (2014) showed that fathead minnow (*Pimphales promelas*) displayed altered behaviours only after exposure to fluoxetine for 28 days and only at a concentration greater than 30 µg/l. Furthermore, these researchers showed that only at these high doses did the minnows have plasma concentrations of fluoxetine similar to the plasma concentrations needed to elicit therapeutic responses in humans (Human therapeutic plasma fluoxetine concentrations 0.09 µg/l - 0.30 µg/l; Amsterdam et al., 1997; de Freitas et al., 2010). Round goby may be similar to fathead minnow in that only above-environmental doses of fluoxetine might elevate blood concentrations to therapeutic levels. Using calculations from the Fish Plasma Model (Huggett et al., 2003; see Supplementary Materials – Appendix D, for calculations), we estimated that the steady state fluoxetine plasma concentration in fish from our study was 0.011 µg/l and 0.42 µg/l for the exposure to our low 1 µg/l dose and high 40 µg/l dose, respectively. Therefore, while the high treatment in our study is predicted to be above the plasma concentration of fluoxetine for human therapeutic action, our low treatment exposure was lower than that observed in humans. In studies with other fish species, however, doses as low as our 1 µg/l or lower have influenced behaviour (e.g guppy *Poecilia reticulata* Pelli & Connaughton, 2015; Siamese fighting fish, *Betta splendens* Dzieweczynski & Hebert, 2012; Greaney et al., 2015; fathead minnow *Pimephales promelas* Weinberger & Klaper, 2014). However, in a current review of fluoxetine exposures, Sumpter et al. (2014) noted that most of the documented behavioural effects of fluoxetine occur at water concentrations of 30 µg/l to 100 µg/l. Our consistent results between and within experiments suggest that an exposure concentration of 1 µg/l is unlikely to cause negative effects on its own in round goby, at least on adult fish and within the exposure scenario that we tested.

4.5.2 Effect of fluoxetine on aggression

Aggressiveness is a trait commonly associated with dominance and can be associated with reproductive success in many vertebrate species (Clutton-Brock, 1988), and often correlates with an individual’s ability to secure resources for breeding or for protection from predation (Arnott & Elwood, 2009). Round goby use shelter for protection from aquatic and avian predation, and for reproduction (MacInnis & Corkum, 2000; Somers et al., 2003; Reyjol et al., 2010; Kornis et al., 2012). Fluoxetine acted quickly to reduce aggression in round goby, as we found that an acute, 3-day exposure to the high dose (40 µg/l) of fluoxetine reduced round goby aggression in multiple behavioural contexts: aggression towards a mirror reflection, aggression towards an intruder in a resource contest, and aggression towards a conspecific in a social interaction assay. Dzieweczynski & Hebert (2012) and Greaney et al. (2015) have documented a similar acute reduction in aggression with male Siamese fighting fish (*Betta splendens*); fish exposed to 0.5 µg/l displayed reduced territorial aggression after only 48 hours and also after 6 days. Likewise, Barry (2013) found reduced chasing behaviours in Arabian killifish after an exposure to 3 µg/l for seven days. At a much higher exposure, (Kohlert et al., 2012) noted that *Betta splendens* exposed to 350µg/l and 705 µg/l decreased aggression towards a mirror after 11 days of exposure.

The acute reduction in aggressive behaviour we observed in our experiments, and in these other studies described here, may be attributed to the immediate actions of fluoxetine on the serotonergic system. By blocking the reuptake of serotonin by the serotonin transporter, fluoxetine acutely increases serotonergic signaling, which appears to have a highly conserved effect of reducing aggression (Gaworecki & Klaine, 2008; Winder et al., 2009; Mennigen et al., 2011). In rodent models of aggression following fluoxetine treatment, researchers have similarly observed a decline in aggression after 3 days of treatment (Dulawa et al., 2004; Mitchell & Redfern, 2005).

In comparison to acute exposures, few studies have addressed whether fluoxetine similarly reduces aggression in fish after chronic exposure time (> 21 days). Interestingly, Sebire et al. (2015) found no evidence that fluoxetine affected three-spine stickleback aggression after a 21-day exposure to 3.2 µg/l, 10 µg/l, and 32 µg/l doses. There is growing evidence, especially in mammals, that individuals exposed chronically (~ 1 month or more) can exhibit a full behavioural recovery or even have behavioural effects in the opposite direction from those of acute exposure (Mitchell & Redfern, 2005). Such "recovery process" could be mediated by a negative feedback process in which serotonin autoreceptors decrease serotonin production in order to return serotonin to pre-treatment or lower levels (Mitchell & Redfern, 2005).

At the acute, 3-day testing time in our study, we found that fish exhibited reduced aggression in *both* the mirror assay and the contest assay. However, when we re-assessed these fish at the 28-day testing time point, exposed fish showed reduced aggression *only* in the mirror assay. Because round goby were still less aggressive towards the mirror after a chronic exposure, similar to the acute time point, we find it unlikely that a homeostatic serotonin recovery process is driving the lack of treatment effects in the chronic contest aggression assay. Instead, we would suggest that this inconsistency in aggression reduction was more likely to be driven by variability from the actions of the unexposed intruder fish. Intruder behaviour varied between the two acute exposures in Experiment 1 and Experiment 2: intruders *increased* their aggression towards the resident in Experiment 1, while intruders *decreased* their aggression towards the resident in Experiment 2. The use of different intruders with different motivations likely introduced some degree of variability in the aggressive interactions between residents and intruders. Intruders were not exposed to fluoxetine and were randomly selected amongst intruders of a similar size class. It is very difficult to control for intruder variability between testing time points without re-using the exact same intruder (Arnott & Elwood, 2009). Mirror aggression assays may therefore provide more consistent and reliable measures of how an environmental contaminant like fluoxetine affects individual aggressiveness.

4.5.3 Effect of fluoxetine on social interaction and activity

The effects of fluoxetine on social grouping behaviours have rarely been assessed. We found that acute exposure to the highest dose of fluoxetine did not change the amount of time round goby spent interacting with a conspecific; regardless of dose, the fish spent over 70% of the trial interacting with the novel stimulus fish. Instead, the high fluoxetine dose changed the quality of the interactions by reducing aggression during the time interacting. Round goby are not considered to be a social, group-living, or cooperative species (Kornis et al., 2012). And in a recent investigation of their social decision-making, we found that round goby do not make conspecific association choices based on group size (Capelle et al., 2015 – Chapter 2 of this thesis). Interestingly, a similar result was also documented in the bluestreak cleaner wrasse (*Labroides dimidiatus*; Paula et al., 2015). In this symbiotic species, fluoxetine treatment increased the number of interactions with other fish species (potential cleaning clients), but did not alter the number of social interactions the wrasse had with conspecifics. Hence, fluoxetine exposure could alter round goby interspecific interactions, and his suggestion of indirect effects would be interesting to investigate given the widespread presence of this invasive species in both the Laurentian Great Lakes and Western Europe.

To assess sub-lethal behavioural effects of contaminant exposures, most research has focused on changes in fish activity while changes in complex social behaviours like aggression or conspecific affiliation are not commonly studied (reviewed in: Little & Finger, 1990; Bayley 2002). Here, we found that an acute fluoxetine exposure had no effect on round goby activity in an open tank (Experiment 1), nor did acute and chronic exposure to fluoxetine alter non-aggressive activity levels in a mirror assay (Experiment 2). Most of the research to date indicates that fluoxetine does not affect fish activity levels at environmentally relevant, and low to mid exposure ranges (>100ug/l; but see Perreault et al., 2003; Clements & Schreck, 2007 for activity following fluoxetine injection). Fluoxetine appears to only impact activity at doses higher than those tested in this experiment (e.g. Winder et al., 2012 > 300ug/l; Kohlert et al., 2012 > 350ug/l), or at environmentally relevant doses but only in developing, larval or juvenile fish (Henry & Black, 2008; Painter et al., 2009). It would be advantageous to further investigate the sensitivity of developing round goby to fluoxetine exposure, as it is appears effects on movement in developing fish may be more prominent.

4.5.4 Conclusions

Our research shows that exposure to a high dose of fluoxetine decreases aggression in wild round goby, while any effect on behaviour at the environmentally relevant exposure was less apparent. Fluoxetine exposure had no detectable effect on activity or social interactions. As behavioural assays are increasingly incorporated into ecotoxicology studies, we emphasize the need for reliable and highly repeatable assays. Ideally, these assays will be suited to test behavioural effects in a standardized manner across a wide variety of organisms. We suggest that mirror assays are likely to provide a more consistent indicator of aggressive motivation when assessing specifically the effects of contaminants on aggression. However, aggressive contests between two fish will better capture the effects of exposures on the outcomes of aggressive interactions (i.e. acquiring resources). While adult round goby in our study appeared unaffected by a low, environmentally relevant dose, future research testing a wider range of fluoxetine concentrations would help elucidate at what exposure concentrations behavioural effects become apparent. Our research can be added to a growing body of literature indicating that fluoxetine does not affect fish behaviour at doses lower than ~30 µg/l (Sumpter et al., 2014), with a few specific species exceptions (Siamese fighting fish *Betta splendens*; Dzieweczynski & Hebert, 2012; Greany et al., 2015; and Arabian killifish *Aphanius dispar,* Barry, 2013). Overall, more chronic exposure studies are now needed to improve our understanding of how long-term exposures might affect fish behaviour. Chronic exposures would show whether a homeostatic serotonergic "recovery process" works to counter the effects of fluoxetine in fishes as it does in mammals. Future work will incorporate more exposure doses to measure effective concentrations (EC_{50}) for the behavioural assays that we have developed for round goby, and should address the potential for effects at earlier life-stages in round goby. Partnering future behavioural work with measurements of serotonin will be especially informative for ascertaining a mechanism of action for altered behaviours.

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Chapter 5: Exposure to wastewater effluent reduces aggression in round goby (*Neogobius melanostomus*)

5.1 Abstract

Pharmaceuticals and personal care products (PPCPs) 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 PPCPs will similarly affect aquatic organisms. Though laboratory studies are beginning to address the effects of individual PPCPs on fish behaviour, comparatively few studies have assessed the behavioural impacts of exposure to complex, realistic wastewater effluents. To address this gap we conducted an in-laboratory exposure to treated wastewater effluent using a wild, invasive fish species, the round goby (*Neogobius melanostomus*). We exposed fish for 28-days to 0%, 50% or 100% wastewater effluent, and then tested the effects of this exposure on aggression in two scenarios: aggression towards a mirror and aggression towards an unexposed intruder fish in a resource contest. Survival was not linked to wastewater concentrations but fish exposed to wastewater had larger gonads after controlling for body mass. We found that increasing concentrations of wastewater effluent reduced round goby aggression towards a mirror, but did not impact aggression in a resident-intruder contest. We discuss our findings in relation to concentrations of antidepressants measured in the wastewater effluent, water quality parameters of the wastewater effluent, and measured ethoxyresorufin-*O*-deethylase (EROD) activity in liver tissue. Altogether, our findings

suggest the exposure to wastewater effluent reduces aggression, but that these effects may be context specific.

5.2 Introduction

There has been rising concern in recent years over the effects that wastewater treatment plant (WWTP) effluents have on wild aquatic animals (Sumpter, 2009; Strayer & Dudgeon, 2010). Part of this concern stems from the fact that WWTP effluents contain anthropogenic, endocrine-active contaminants like plasticizers such as bisphenol-A (BPA), steroid hormones, and pharmaceuticals and personal care products (PPCPs; Environment Canada, 2001; Klecka et al., 2010). Traditional WWTPs are ill-equipped to remove most of these compounds before wastewater effluent is discharged into the environment, and reports of PPCPs in natural water bodies have been increasing (Kolpin et al., 2002; Verlicchi et al., 2012; Blair et al., 2013). For pharmaceuticals specifically, human consumption has been increasing in developed countries over several decades (Hemels et al, 2005; OECD, 2013). Pharmaceuticals in the environment are of special concern for aquatic animals because most drugs are designed to modulate human physiology and behaviour, and vertebrates have well conserved biological targets (e.g. receptors, enzymes) on which certain pharmaceuticals may act (Gunnarsson et al., 2008; Brown et al., 2014). Even though most PPCPs are present in the environment at low concentrations (ng/l to μg/l) that do not cause lethal toxicity, they may cause sub-lethal changes to animal physiology and behaviours important for survival (Söffker & Tyler, 2012; Brodin et al., 2014). Behaviours such as predator avoidance, territorial defense, foraging, and reproduction are crucial for animal fitness (Zala & Penn, 2004; Smith & Blumstein, 2008). The effects that wastewater effluent has on wild animal behaviour are only beginning to be understood.

One type of PPCP with strong potential to alter animal behaviour in the wild is psychiatric pharmaceuticals, such as anxiolytics or antidepressants, prescribed for treatment of human behavioural disorders. These drugs are commonly reported in wastewater effluents and in surface waters in low concentrations (10 - 5000 ng/l, Metcalfe et al., 2010; Verlicchi et al., 2012; Klaminder et al., 2015). In response to reports of psychiatric pharmaceuticals in the environment, researchers have been investigating whether exposure to environmentally relevant concentrations of psychiatric pharmaceuticals affect fish behaviour in the laboratory. For example, it has been found that the anxiolytic oxazepam increased boldness in European perch (*Perca fluviatilis*), making exposed fish more active and exploratory in a new environment (Brodin et al., 2013). Predator avoidance behaviours have been disrupted after exposure to the antidepressant fluoxetine, causing delayed predator reactions in guppy (*Poecilia reticulate*; Pelli & Connaughton, 2015) and in fathead minnow (*Pimephales promelas*; Weinberger & Klaper, 2013). Similarly, the antidepressant sertraline was found to reduce foraging efficiency in European perch (Hedgespeth et al., 2013).

While the number of studies connecting psychiatric pharmaceutical exposure to changes in fish behaviours is growing (see additional: Painter et al., 2009; Dzieweczynski & Hebert, 2012; Olsén et al., 2014; Greaney et al., 2015) it is important to note that some studies have found no evidence for behavioural changes following environmentally relevant exposures (e.g. Holmberg et al., 2011; Sebire et al., 2015). Controlled laboratory exposures are critical for understanding the behavioural impacts and mechanisms of action for PPCPs in aquatic animals. However, any behavioural changes observed in laboratory experiments with single-compound exposure may not easily generalize to the wild, because wastewater effluent contains a complex mixture of PPCPs and other compounds.

Relatively few studies have assessed behavioural endpoints following exposure to complex mixtures such as wastewater effluents in either the laboratory or the wild. In the laboratory, Garcia-Reyero et al. (2011) and Martinović et al. (2007) found that male fathead minnow (*Pimphales promelas*) exposed to 100% wastewater effluent were less successful at securing a nesting site against unexposed competitors. Sebire et al. (2011) found similar results in male three-spine stickleback (*Gasterosteus aculeatus*) exposed to 50% and 100% wastewater, where exposed fish built fewer nests and spent less time courting female mates. In one of the only studies on fish collected directly from and exposed field site, Saaristo et al. (2014) found that male mosquitofish (*Gambusia holbrooki*) from downstream of a WWTP outfall courted females *more* than males from a reference location. The few studies reviewed here have focused primarily on reproductive behaviours, but many other behaviours impact animal survival (e.g. foraging, territory defense). A recent study by Melvin (2016) showed that short-term exposure to wastewater effluent also impacted non-reproductive behaviours in fish. The author showed that empire gudgeons (*Hypseleotris compressa*) exposed to effluent had reduced activity and swimming performance.

Comparing behavioural effects across different wastewater exposure studies is challenging because researchers often use different endpoints, different study species, and most importantly wastewater from different treatment facilities. However, such studies are locally important for evaluating the potential impacts that wastewater effluents have on environments and aquatic communities that receive wastewater effluent discharge. Studies investigating the effect of wastewater effluent on fish behaviour would be a natural next-step to connect controlled laboratory exposures to realistic field exposures.

In this study, we assessed how exposure to wastewater effluent affected aggression in an invasive fish species, the round goby (*Neogobius melanostomus*). As an invasive species that is native to the Black and Caspian seas of Europe, round goby have been very successful in invading the Laurentian Great Lakes and waterways of Western Europe (Corkum et al., 2004; Kornis et al., 2012). Part of the round goby invasion success has been attributed to their aggressive behaviour (Corkum et al., 2004; Kornis et al., 2012). Round goby have been shown to outcompete heterospecifics for sheltered and rocky habitats in the littoral zone (Dubs & Corkum, 1996; Balshine et al., 2005; Bergstrom & Mensinger, 2009). Competition over resources, such as shelter, is an important behaviour for round goby survival, as without a shelter round goby are susceptible to many avian and aquatic predators (Belanger & Corkum, 2003; Somers et al., 2003; King et al., 2006; Reyjol et al., 2010). Moreover, round goby use sheltered spaces to reproduce and care for offspring in the breeding season (Corkum et al., 1998; MacInnis & Corkum, 2000).

To investigate how wastewater effluents affect competitive behaviour, we exposed fish to 0%, 50% or 100% wastewater for four weeks in the laboratory, and then tested fish with two competitive aggression assays: a mirror aggression assay and a contest aggression assay. Mirror aggression is increasingly being used to investigate animal aggression: mirror assays reduce the number of fish needed for a study, provide more experimental control without the presence of non-standardized stimulus fish, and also reduces the potential harm to test animals that may be elicited in dyadic contests (Balzarini et al., 2014; Elwood et al., 2014). However, it is important to realize that paired contests have more ecological relevance and better capture the nature of complex interactions over territory ownership and resources, as such interactions would occur in the wild (Arnott & Elwood, 2009).

We collected wastewater for our exposures from the Woodward Wastewater Treatment Facility in Ontario, Canada. The effluent from the facility discharges into Hamilton Harbour, an International Joint Commission Area of Concern (International Joint Commission, 1999). The Harbour has been undergoing remediation since its designation in 1985 to improve water quality, pollutant loadings, aquatic habitat, and fish and wildlife health that were degraded from historical industrial activities, urban run-off, wastewater effluent inputs, and combined sewer overflows (Hamilton Harbour RAP, 1992; Hall et al., 2006). The wastewater effluent from the Woodward facility has been previously characterized for pharmaceuticals such as carbamazepine, ibuprofen and gemfibrozil, and personal care products like triclosan (Metcalfe et al., 2003; Csiszar et al., 2011), and the antidepressants fluoxetine, venlafaxine and citalopram have been reported in the surface waters at this facility's outfall (Metcalfe et al., 2003).

We characterized the wastewater effluent used in our exposures in three ways to better connect our behavioural measures to attributes of the effluent. First, we quantified the concentrations of antidepressant pharmaceuticals found in the effluent during the exposure period. Antidepressants have previously been reported to reduce aggression in fish by acting on the serotonergic system and increasing serotonergic signaling (Perreault et al., 2003; Mennigen et al., 2011; Dzieweczynski & Hebert, 2012; Forsatkar et al., 2014). We predicted, as others have found, that if antidepressants were present in the wastewater, we would observe less aggression towards both a mirror and a conspecific rival (Perreault et al., 2003; Forsatkar et al., 2014; see Chapter 4 of this thesis). Second, we monitored basic water quality properties of the effluent during the exposure period (including: ammonia, nitrate, pH, dissolved oxygen, dissolved solids). Third, though we have focused on PPCPs because of their potential to impact behaviour, the effluent from this facility receives wastewater from homes, businesses, industry, and urban run-off through a partially combined sewer system. Organic pollutants like polyaromatic hydrocarbons (PAHs) are more commonly reported in the wastewater effluent from combined sewer systems as a product of petroleum combustion (Gasperi et al., 2010; Jones et al., 2012). We therefore assayed for ethoxyresorufin-*O*-deethylase (EROD) expression to determine whether fish are also exposed to this class of contaminants. EROD induction indicates CYP1A activity, cytochrome P450 enzymes involved in metabolizing aromatic hydrocarbons (Whyte et al., 2000).

5.3 Methods

5.3.1 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 Marina, Hamilton, ON, Canada. We collected fish using baited minnow traps (for detailed collection methods see McCallum et al., 2014 – Appendix B), and transported the fish live to McMaster University. We housed fish in same-sex groups of six on a 14L:10D light schedule in 75 l aquaria equipped with ~1cm of natural gravel substrate and a static renewal filter (Aquaclear). All fish were fed Nutrafin Basix Staple Food once daily. After 24 hours acclimation to the lab, we uniquely tagged fish using non-toxic acrylic paint (Wolfe & Marsden, 1998) along their dorsal fin to facilitate later identification in behavioural trials.

5.3.2 Wastewater effluent collection and exposure

The Woodward Avenue Wastewater Treatment Facility is a secondary conventional activated sludge treatment facility serving the populations of Hamilton, Stoney Creek, and Ancaster, Ontario (~ 400 000 population). Woodward treats an average of 409 Ml of wastewater per day from residences, businesses, industry, and storm sewers throughout the city, and handles wastewater from a combined (40%) and separated (60%) sewer system (City of Hamilton, 2011). We collected wastewater effluent every three days after the final stage of treatment, but immediately before it would be returned to the watershed. We transported the effluent to McMaster University in opaque plastic containers and kept it in cold storage at 4°C in dark conditions to prevent degradation.

We exposed round goby to wastewater effluent in the laboratory for 28 days. Exposures were conducted between September 25, 2013 and October 31, 2013, in 20 l aquaria equipped with 1cm of natural gravel substrate, black PVC tubes for shelter, an airstone, and a static renewal filter with the carbon insert removed. Fish were exposed in same-sex groups of six fish to one of three exposure treatment concentrations: 0% wastewater effluent (control), 50% wastewater effluent (low), or 100% wastewater effluent (high). Prior to adding the effluent, we allowed it to equilibrate with room temperature overnight under dark conditions. 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 hours, 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. During exposures, we fed fish once daily (Nutrafin Basix Staple Food) and monitored fish survival, noting any mortalities.

5.3.3 Behavioural assays

We assessed the effect of the 28-day wastewater effluent exposure on round goby survival and aggression. We assessed aggression with two behavioural assays: a mirror aggression assay and a resident-intruder contest. We conducted both aggression assays sequentially on day 28 of exposure, in 20 l experimental tanks equipped with ~1cm layer of natural gravel substrate and an airstone. Each tank contained two removable barriers (the first was made of opaque black acrylic, and the second barrier was a mirror mounted on black acrylic) positioned in the middle of the tank, splitting the tank into two compartments (Figure 5.1). The front compartment was devoid of shelter but the second compartment behind the two barriers contained an opaque box shelter (14.5cm x 14.5cm x 5cm) with a small entrance (5cm x 3cm). Round goby will readily take-up and defend such shelters in the laboratory (Sopinka et al., 2010; Groen et al., 2012). We tested fish in the same wastewater effluent exposure conditions as their actual exposure. We filled the experimental tanks on the day of testing with the appropriate concentration of wastewater effluent (50% or 100%) or dechlorinated tap water (0%), and thoroughly cleaned tanks in between testing days. We conducted video recording of the experimental tanks from behind opaque blinds to limit experimenter influence on the fish.

Behavioural trials began when we transferred a focal fish from its exposure tank to the front compartment of the experimental tank, with both barriers in place. We gave each fish 30-minutes to recover from handling and habituate to the testing tank, and then we conducted the mirror aggression assay. This assay began by remotely removing the black opaque barrier to reveal the mirror to the focal fish. We video-recorded the subsequent 15-minute trial (Canon HD Vixia HFS100 8.0 Megapixel). We then raised the mirrored barrier to reveal the second compartment and allowed the focal fish to access and interact with the shelter box in the back of the tank. The focal fish was given 90-minutes to habituate and to take up residence in the shelter. After this habituation period, a resident-intruder contest was initiated by adding unexposed intruder fish to the experimental tank. Intruders were taken from laboratory stock tanks, and were always larger (in body mass) than the focal fish. Larger intruders created asymmetric contests, where residents would be expected to lose the shelter, providing us with the ability to assess a “giving-up” time for the contest and also allowed us to measure how willing or motivated the residents were to fight for their shelter resource (Taylor & Elwood, 2003). The resident-intruder contest was video-recorded for 15-minutes. After behavioural testing, all focal fish were returned to their exposure tanks and all intruder fish were returned to their stock tanks.

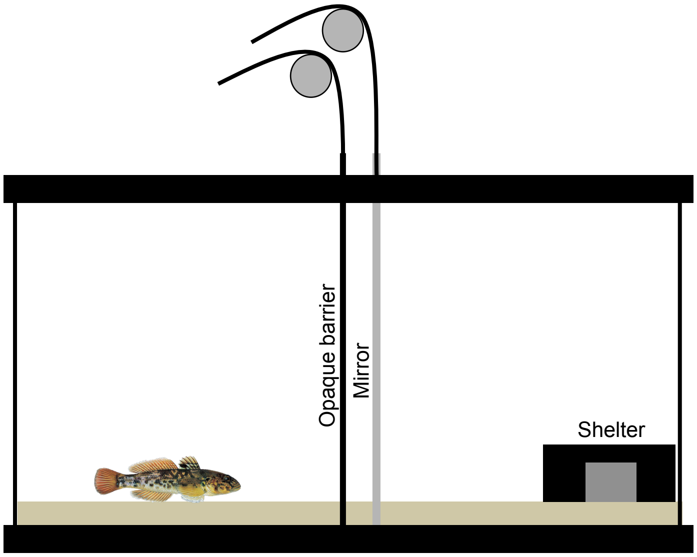

Figure 5.1
A diagram of the experimental tank for behavioural assays showing the removable barriers in place at the start of a trial. Fish were habituated for 30 minutes before any barrier was lifted. Then the opaque barrier was lifted to reveal the mirror for the 15-minute mirror aggression assay, then the mirror was removed to reveal the shelter resource for 90 minutes before the 15-minute resident-intruder contest assay began.

All videos were scored for aggressive behaviours towards the mirror and towards the intruder fish. The researcher scoring the behavioural videos was blind to wastewater exposure treatment, and based their behavioural scores on an ethogram designed for the round goby (Supplementary Table S1 – Appendix C). For the mirror aggression assay, we recorded: 1) *time taken for the focal fish to move towards the mirror,* this was measured as the time elapsed from barrier removal to first movement towards the mirror; 2) *the number of aggressive acts towards the mirror*; 3) *the number of aggressive bouts with the mirror*. Round goby aggressive interactions often involve bouts of aggressive activity separated by a period of resting or non-aggressive activity (e.g. tank exploration or 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 behaviours* (other activity) when not interacting with the mirror. Fish were considered to be interacting with the mirror when they were oriented towards the mirror and were within one body length of the mirror.

For the resident-intruder contest assay, we recorded: 1) *the time taken for the contest to begin*, this was measured as the time that had elapsed from the addition of the intruder until the first aggressive act occurred by either resident or intruder; 2) *contest duration*, as the time that had elapsed from the first aggressive act until the emergence of a contest winner (winners were assigned when the other fish fled the contest and ceased aggressive retaliation); 3) *the number of aggressive acts* given by the exposed resident to the intruder, and the number of aggressive acts the exposed resident received from the intruder across the entire trial.

5.3.4 Morphological measures and tissue collection

Following the behavioural assays, we euthanized all focal fish with a cerebral concussion and spinal severance to measure body condition and reproductive status. We measured standard length (snout to caudal peduncle) using calipers accurate to 0.01cm.

We measured body mass using a digital balance accurate to 0.001g (Ohaus Adventurer Pro). 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 gonadosomatic index (GSI) using the gonad mass as: [gonad mass / (body mass – gonad mass)]*100. We used GSI to assign reproductive status, where males are considered reproductive if they had a GSI > 1% and females were considered reproductive if they had a GSI > 8% (Marentette & Corkum, 2008; Zyel et al., 2014). By these established criteria of reproductive status, no females in our study were in reproductive condition and only one male fish had a GSI high enough to be classified to be in reproductive condition. Liver tissue was then frozen in liquid nitrogen and stored at -80°C for later EROD analyses.

5.3.5 Characterization of wastewater effluent & EROD activity

We characterized the water quality parameters of the wastewater effluent in three ways. First, we summarized water quality parameters of the final treated effluent across our collection period that was measured by the Hamilton Water wastewater treatment facility (Hamilton Water, 2013, *unpublished data*). These parameters included: total suspended solids, total phosphorus, total nitrogen, ammonia, nitrate, nitrite, carbaceous oxygen demand (cBOD), E. coli, and conductivity. All parameters were measured five days per week, except E. coli and conductivity, which were measured once per week. All measures were taken from composite samples of the final effluent over a 24-hour period.

We also took water quality measurements from a randomly selected exposure tank of each treatment type, these measures included: dissolved oxygen and temperature (La Motte Pocket Tracer), pH, conductivity, total dissolved solids and salinity (Oakton Multiparameter PCS Testr 35). Second, we collected wastewater samples (N = 8) immediately after collection from the treatment facility and froze these at -20°C and later quantified antidepressants and their metabolites in the effluent. From each wastewater collection, we prepared samples for analysis in triplicate following the protocol described by Metcalfe et al., (2010). Briefly, we filtered 100mL of sample through glass fiber filters. Samples were then extracted using Oasis MCX SPE cation-exchange cartridges (Waters Scientific). The eluant from the SPE cartridge was collected in a centrifuge tube, evaporated just to dryness, and then reconstituted in 400 mL of methanol. The sample was then transferred to an autosampler vial with an insert for analysis. All samples were analyzed by LC-MS/MS using a Q-Trap LC-MS/MS System.

We assessed 7-ethoxyresorufin-*O*-deethylase (EROD) activity in round goby liver to identify exposure to aromatic hydrocarbon contaminants in the wastewater effluent. We used a subset of N = 24 livers from exposed males ($N_{\text{Control}} = 7$, $N_{\text{Low}} = 8$; $N_{\text{High}} = 9$) following a protocol adapted from Marentette et al., (2010). Larger exposed males were selected for this part of our study because of the volume of liver tissue available for analyses. Each liver was thawed and homogenized in buffer (50mM Tris HCl, 0.15M KCl, pH 7.4, 4 ml/g tissue). Liver homogenates were then centrifuged for 10 min at 750 *g* and 10 min at 12,000 *g*, at 4°C (Eppendorf, Centrifuge 5904 R). The supernatant S9 fraction (containing cytosol and microsomes with cytochrome P450 isoforms) was drawn off and the protein content of the S9 fraction (in mg/ml) was determined with 5 ul of the homogenized sample, from a Bradford protein assay (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 96-well microplate fluorometer (SpectraMax Gemini XPS, Molecular Devices). EROD activity was calculated as pmol resorufin per min, per mg of liver protein.

5.3.6 Statistical analyses

All statistical analyses were conducted in R (version 3.2.4: R Core Team, 2016). We analyzed the effect of wastewater exposure on fish survival using a binomial logistic regression. We used ANOVAs to analyze the effect of sex and wastewater exposure on hepatosomatic index, gonadosomatic index, and body condition. In the mirror aggression behavioural assay, we only analyzed data from fish that moved at least once during the trial to ensure focal fish had recovered from handling stress. We used a linear model to assess the latency to move towards the mirror, and negative binomial regressions appropriate for 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 (our measure of activity). To assess whether effluent exposure affected aggressive behaviours during resident-intruder contests, we analyzed only fish that had interacted with the intruder at least once to ensure the fish were aware of an intruder fish in their experimental tank. We used a linear model to assess the time taken to the first aggressive act and contest duration. We used a negative binomial generalized linear mixed effect model to assess total contest aggression performed by the resident and intruder across the trial, including fish ID as a random effect. We specifically tested for an interaction between wastewater treatment and total contest aggression, as this would indicate if wastewater exposure was altering aggressive interactions between the resident and intruder fish. We used a Fisher test to assess the contest winner. When necessary, we transformed the data (using ln- or power transformations) to meet the assumptions of parametric tests. See Table 5.1 for a summary of sample sizes used in the following analyses. We pooled results from both sexes as a result of our small sample.

Table 5.1
Summary of sample sizes used in exposures, behavioural analyses, and morphology and EROD assays

	Total *N*	**Control (0%)**	**Low (50%)**	**High (100%)**
Exposed	72	24	24	24
Survived	57	17	19	21
Behavioural assays	***N* Analyzed**	**Control (0%)**	**Low (50%)**	**High (100%)**
Mirror aggression	48	15	17	16
Resident-intruder contest	49	14	17	18
Morphology and EROD	***N* Analyzed**	**Control (0%)**	**Low (50%)**	**High (100%)**
HSI, GSI, & Body condition	57	17	19	21
EROD	22	7	6	9

5.4 Results

5.4.1 Survival and morphological measures

On average, 79% of exposed fish survived. Fish survived to the same extent in all exposure groups across the 28-day exposure (Binomial regression: $N_{tanks} = 12$; high vs control $Z = -0.49$, $p = 0.63$; low vs control $Z = -0.25$, $p = 0.80$; high vs low $Z = -0.23$, $p = 0.81$). Exposure did not change hepatosomatic index (ANOVA: $F_{\text{Dose}(2,\,53)} = 0.48$, $p = 0.62$), but females had higher hepatosomatic indices than males (ANOVA: $F_{\text{Sex}(1,\,53)} = 32.06$, $p < 0.0001$). Body condition was similar across exposures (ANOVA: $F_{\text{Dose}(2,\,53)} = 0.089$, $p = 0.92$), and between males and females (ANOVA $F_{\text{Sex}(1,\,53)} = 1.27$, $p = 0.27$). As expected, females had higher gonadosomatic indices than males (ANOVA $F_{\text{Sex}\,(1,\,52)} = 145.67$, $p < 0.0001$). As stated above, all fish of both sexes were in non-reproductive condition, except one male. Even with this one reproductive male removed from the sample, the remaining fish showed increased gonadosomatic investment with increased wastewater effluent exposure (ANOVA: $F_{\text{Dose}\,(2,\,53)} = 11.59$, $p < 0.0001$; Tukey HSD: high vs control $p = 0.026$; low vs control $p = 0.00042$; high versus low $p = 0.62$).

5.4.2 Behavioural assays

During the mirror aggression assay, fish exposed to the highest dose (100%) of wastewater effluent took longer to move towards the mirror compared to control fish (Linear model on ln-tranformed values: $N = 48$; high vs control $t = 2.17$, $p = 0.035$; low vs control $t = 1.01$, $p = 0.32$; high vs low $t = 1.21$, $p = 0.23$; Figure 5.2a). On average, fish exposed to 100% wastewater took 292 (± 59 SE) seconds to start moving, while those exposed to 50% wastewater took 202 (± 50 SE) seconds, and those exposed to no wastewater took 106 (± 24 SE) seconds to start moving. Fish exposed to the high dose to effluent were less aggressive towards the mirror, preforming fewer aggressive acts towards the mirror (Negative binomial regression: $N = 48$; high vs control $Z = -2.95$, $p = 0.003$; low vs control $Z = -0.92$, $p = 0.35$; high vs low $Z = -2.12$, $p = 0.034$; Figure 5.2b). Fish exposed to the high dose of wastewater effluent also had fewer aggressive bouts with the mirror (Negative binomial regression $N = 48$; high vs control $Z = -2.25$, $p = 0.024$; low vs control $Z = 0.22$, $p = 0.82$; high vs low $Z = -2.52$, $p = 0.012$; Figure 5.2c). On average, fish exposed to the highest concentrations of wastewater had only 1 (± 0.32 SE) aggressive bout with their mirror image before giving up, while fish exposed to the low dose and to the control water had 3 (± 0.71 SE / ± 0.79 SE) aggressive bouts with the mirror. In contrast to aggression towards the mirror, wastewater exposure did not affect non-aggressive activity during the trial (Negative binomial regression $N = 48$; high vs control $Z = -1.43$, $p = 0.15$; low vs control $Z = 0.36$, $p = 0.72$; high vs low $Z = -1.84$, $p = 0.066$).

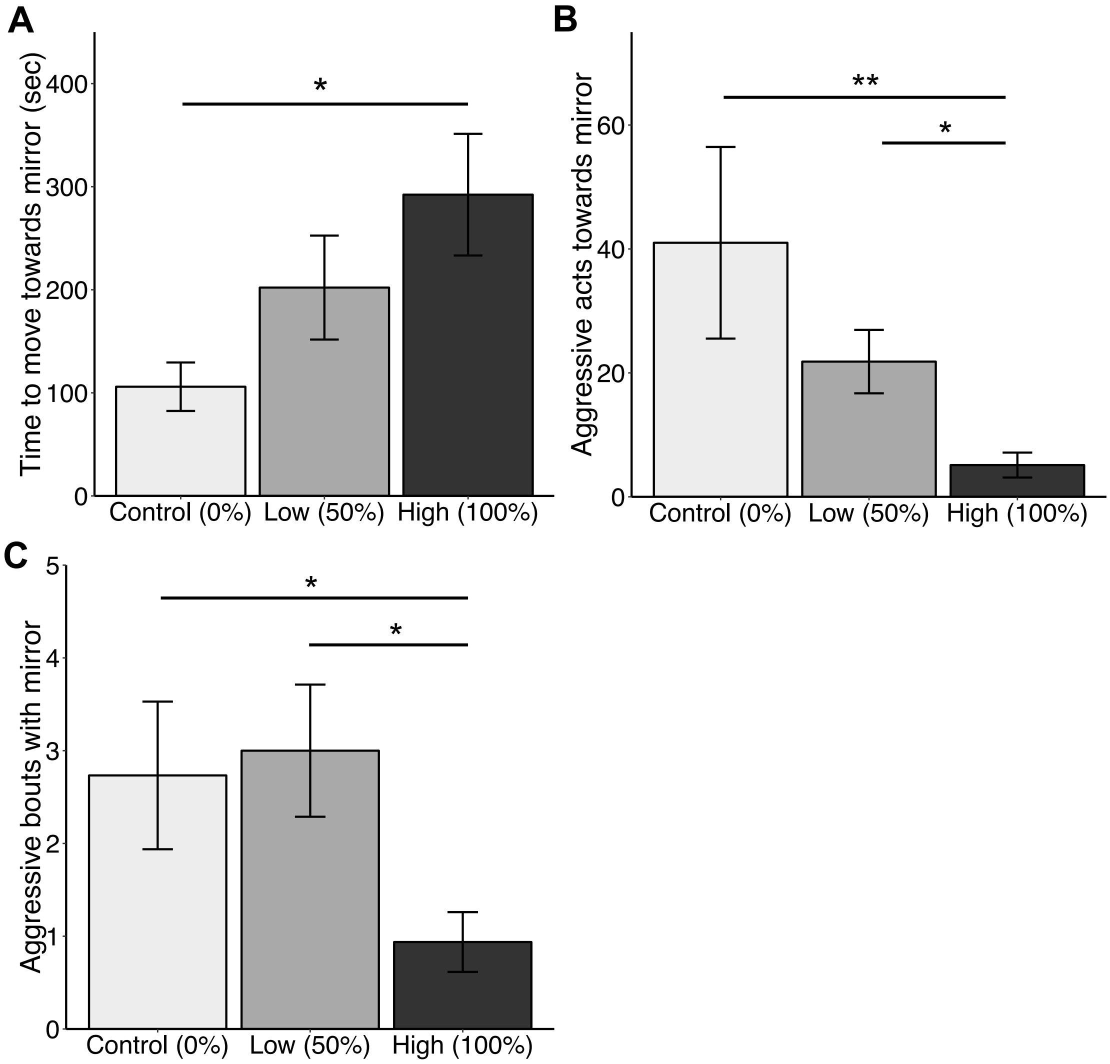

Figure 5.2

Results of the mirror aggression assay, plotted by wastewater exposure treatment. **A)** Average time taken for fish to move towards the mirror in seconds. **B)** Average aggressive acts performed towards the mirror. **C)** Average number of aggressive bouts fish had with their mirror image. Error bars represent ± 1 standard error. * indicates $p < 0.05$, ** indicates $p < 0.01$.

In general, exposure to wastewater effluent had little effect on aggressive interactions in the resident-intruder contests. Of the 57 contest trials conducted, residents and intruders interacted in in only 49 trials. On average, an aggressive interaction began after 206 (± 33) seconds, and there was no effect of wastewater exposure on the time until to the first aggressive act by either resident or intruder (Linear model on ln-transformed values: $N = 49$; high vs control $t = -0.19$, $p = 0.85$; low vs control $t = -0.34$, $p = 0.73$; high vs low $t = 0.20$, $p = 0.85$; Figure 5.3a). Aggressive contests lasted on average for only 84 (± 17 SE) seconds, and wastewater exposure had no impact on contest length or the time taken for residents to "give-up" dominance (Linear model on power-transformed values: $N = 49$; high vs control $t = 0.47$, $p = 0.64$; low vs control $t = 0.49$, $p = 0.63$; high vs low $t = -0.08$, $p = 0.94$; Figure 5.3b). We found no evidence for an interaction between wastewater effluent dose and total contest aggression between the resident and intruder fish, indicating that the number of aggressive act given and received between the competing fish did not change depending on wastewater treatment (Negative binomial GLMM: Treatment-by-contest aggression interaction, $N = 49$, high vs control $Z = -1.66$, $p = 0.097$; low vs control, $Z = -0.52$, $p = 0.60$; high vs low $Z = -1.18$, $p = 0.24$; Figure 5.3c). Exposure to wastewater did not impact contest outcome (Fisher test: $N = 49$, $p = 0.30$), across all treatments intruders won 31 of the 43 contests, residents won seven contests, and five contests ended in a tie.

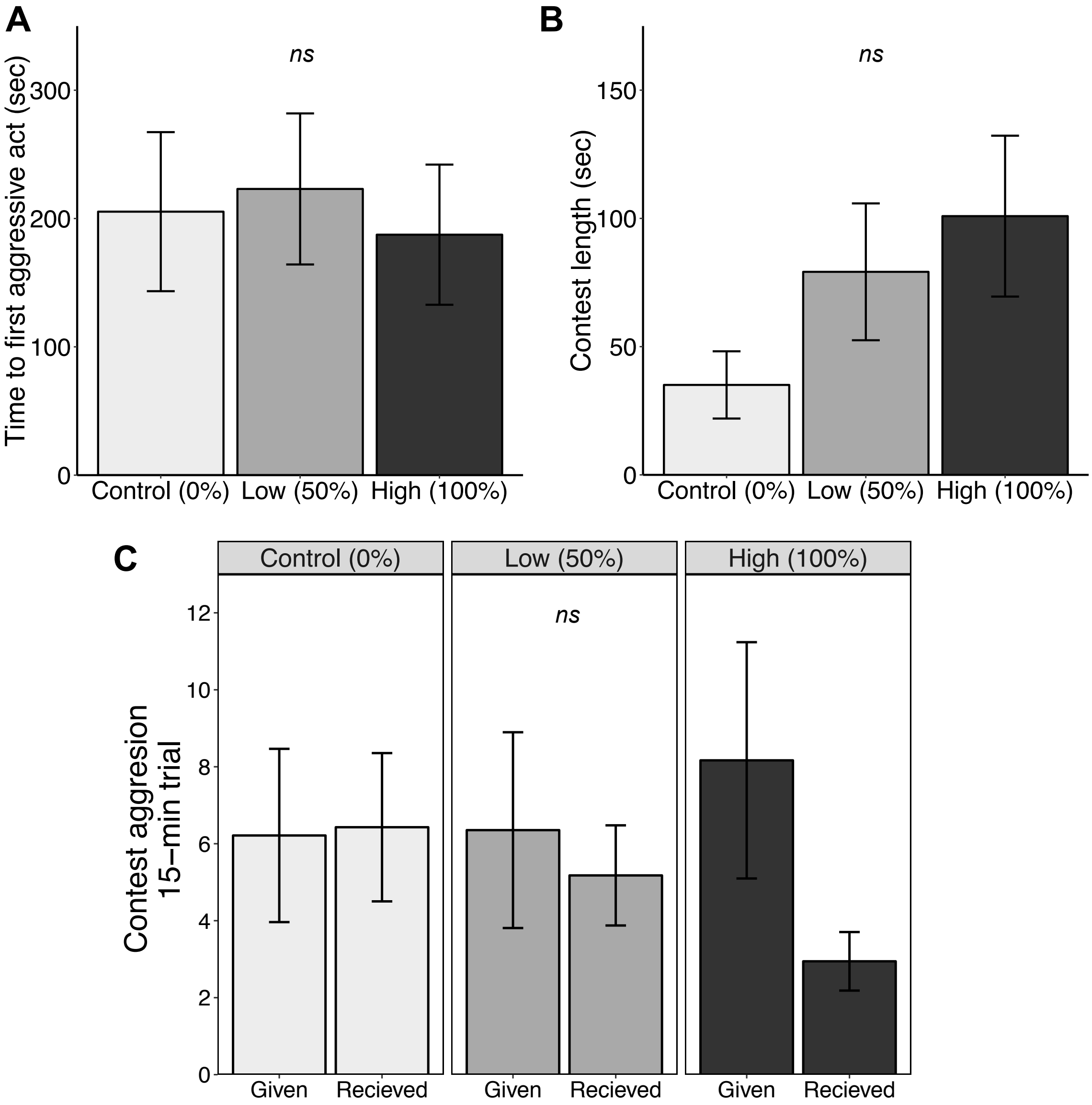

Figure 5.3

Results of resident-intruder contest assay, plotted by wastewater exposure treatment. **A)** Average time until the first aggressive act by either fish. **B)** Average contest length (time from the first aggressive act until a winner is established). **C)** Average contest aggression, plotted as aggressive acts given and received. Error bars represent ± 1 standard error. *ns* = not significant at α = 0.05.

5.4.3 Characterization of the wastewater effluent & EROD activity

The water quality parameters measured in the final treated effluent and in our exposure tanks are summarized in Table 5.2 and Table 5.3, respectively. In our exposure tanks, fish were exposed to similar temperature and dissolved oxygen conditions, however pH was higher in the 100% effluent high exposure tanks. Conductivity and total dissolved solids both increased with wastewater exposure dose. We have summarized the average concentrations of antidepressants and antidepressant metabolites detected in the wastewater effluent in Table 5.4. We detected six of the nine antidepressants and metabolites that we assayed for in our effluent samples, moreover all six compounds were detected in each of the eight effluent samples.

Table 5.2

Average water quality measures of the final treated effluent discharged from the Woodward Wastewater Treatment Facility during the exposure period. Sample sizes vary depending on frequency of testing. All measures were taken in mg/l, unless otherwise stated. Data provided by the water quality laboratory at Hamilton Water. cBOD = carbaceous oxygen demand, CFU = colony-forming unit.

	Final effluent (100%)		
	mean	range	*N*
Total suspended solids	10.28	(0.76 – 44)	27
Total phosphorus	0.51	(0.33 – 0.85)	27
Total nitrogen	2.11	(1.05 – 4.2)	27
Ammonia	0.88	(0.17 – 2.65)	27
Nitrate	13.15	(9.58 – 17.2)	27
Nitrite	0.23	(0.1 – 0.38)	27
cBOD	4.33	(2.5 – 9.0)	27
EColi, CFU/100mL	10	(10 -10)	3
Conductivity, µS/cm	1032.4	(927 – 1190)	5

Table 5.3

Average daily water quality measures from exposure tanks in the laboratory. All measures were collected from a randomly selected tank per treatment, each day. Upper case letter indicate statistical difference following ANOVA tests with Tukey Post-Hoc.

	Control (0%)	Low (50%)	High (100%)
	mean (range)	mean (range)	mean (range)
Temperature °C	21.38^a (20.1 – 23.4)	21.14^a (19.4 – 24.0)	20.89^a (19.4 – 23.5)
Dissolved oxygen mg/l	9.96^a (8.46 – 10.75)	9.54^a (7.05 – 10.95)	9.63^a (7.53 – 10.86)
pH	8.33^a (7.99 – 8.51)	8.20^b (7.90 – 8.90)	8.13^b (7.74 – 8.60)
Total dissolved solids ppm	275.74^a (250 – 299)	610.27^b (561 – 673)	891.16^c (823 – 956)
Conductivity µS/cm	387.09^a (352 – 416)	857.06^b (791 – 948)	1253.56^c (1160 – 1346)

Table 5.4

Summary of antidepressants and active metabolites in the final treated wastewater effluent from *N* = 8 collection dates. ND = not detected.

	Concentration (ng/l) in final effluent (100%)	
Compound	mean	range
Venlafaxine	70.51	(5.60 – 113.80)
O-desmethyl venlafaxine	135.44	(52.60 – 212.0)
N-desmethyl venlafaxine	9.00	(4.93 – 15.40)
Citalopram	18.13	(4.02 – 26.48)
Desmethyl citalopram	44.75	(21.80 – 105.0)
Fluoxetine	2.75	(1.65 – 4.07)
Norfluoxetine	ND	ND
Desmethyl-sertraline	ND	ND
Setraline	ND	ND

Exposure to wastewater effluent increased hepatic EROD activity in fish exposed to the high dose of wastewater compared to control and low dose fish (Linear model: *N* = 22; high vs control *t* = 2.77, *p* = 0.012; low vs control *t* = 0.49, *p* = 0.63; high vs low *t* = 2.13, *p* = 0.047; Figure 5.4).

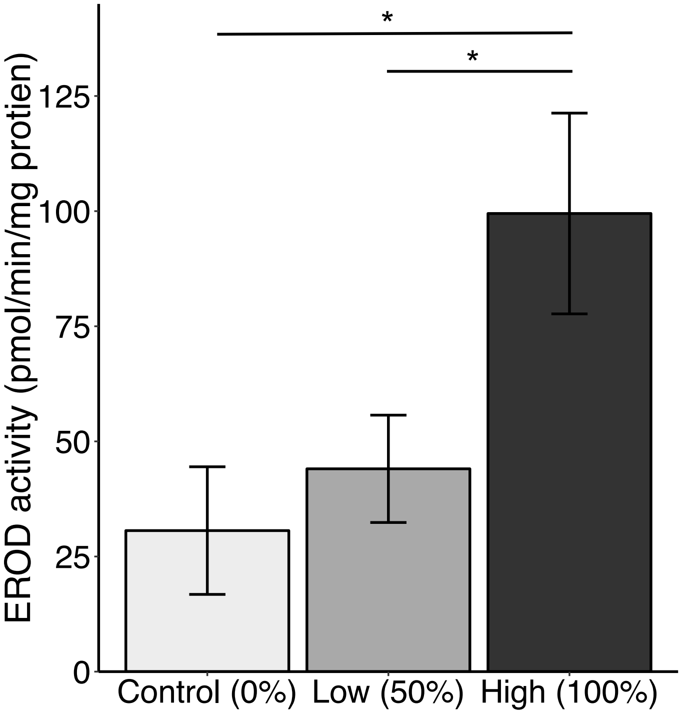

Figure 5.4
EROD expression in the liver tissue, plotted against wastewater exposure treatment. Error bars represent ± 1 standard error. * indicated $p < 0.05$.

5.5 Discussion

We exposed round goby for 28 days to 0%, 50% or 100% treated wastewater effluent, and assessed the effects of this exposure on competitive behaviours in a mirror aggression assay and a resident-intruder contest assay. We found that increasing concentrations of wastewater effluent reduced round goby aggression towards a mirror. Exposed fish took longer to move towards the mirror, performed fewer aggressive acts at mirror, and had fewer discrete bouts of aggressive interaction with the mirror. Although the main focus of our study was not to definitively identify contaminants causing behavioural disruption, we did investigate concentrations of antidepressants in the effluent used in our exposures. Antidepressants are known to reduce aggressive behaviours in a number of fish species when tested in the laboratory (Perreault et al., 2003; Dzieweczynski & Hebert, 2012; Forsatkar et al., 2014). We detected three antidepressants and three biologically active antidepressant metabolites in the effluent, but the concentrations were low for each compound individually (range: 1.65 – 212 ng/l, Table 5.3). The concentrations of antidepressants we measured are consistent with previously published work on the effluent from this treatment facility (Metcalfe et al., 2003). Because antidepressant concentrations as low as we measured in our samples have not been linked to behavioural disruption in fish in the laboratory, separately, each antidepressant is unlikely to have caused the reduced aggression we observed. For example, fluoxetine reduced Siamese fighting fish (*Betta splendens*) aggression, but only at concentrations of 500 ng/l, (no lower concentrations were tested, Dzieweczynski & Hebert, 2012). Venlafaxine has yet to be tested for its effects on fish aggression specifically, but only exposures above at 200 000 ng/l were found to affect feeding on live prey in hybrid striped bass (*Morone chrysops x Morone saxatilis* (Bisesi et al., 2014). In general, very few, if any, investigations on the effects of antidepressants on fish behaviour have uncovered effects on aggression below exposure concentrations of 500 ng/l (Sumpter et al., 2014), but exposure concentrations below 500 ng/l are also infrequently used in laboratory studies.

It is also possible that the measured antidepressant compounds acted in mixture. The impacts of pharmaceuticals mixtures on fish behaviour are only now beginning to be studied. Interestingly, Painter et al. (2009) found that a mixture of environmentally relevant concentrations of four antidepressants (fluoxetine, venlafaxine, sertraline, and bupropion) reduced predator avoidance behaviours (c-start reflexes) in larval fathead minnow (*Pimephales promelas*). Because of the mixture of compounds present in wastewater effluent, there may be more pronounced effects on fish behaviour during the developing juvenile or larval growth period, and this should be a focus for future research.

Beyond mixtures of antidepressants in the wastewater, there are other potential contaminants that may have acted to reduce round goby aggression towards the mirror. One set of compounds that has received a great deal of focus in wastewater effluents is estrogenic endocrine disrupting compounds and endogenous steroid estrogens (Tyler & Jobling, 2008). For example, in the laboratory, environmentally relevant concentrations (> 50 ng/l) of the synthetic estrogen, ethynilestradiol (EE2) have been found to reduce aggressive behaviours in many fish species including: three-spine stickleback (*Gasterosteus aculeatus*; Bell, 2001), sand goby (*Pomatoschistus minutus*; Saaristo et al., 2010), zebrafish (*Danio rerio*; Colman et al., 2009), and fathead minnows (*Pimephales promelas*; Salierno & Kane, 2009). Moreover, Garcia-Reyero et al. (2011) and Martinović et al. (2007) found that fathead minnows exposed to wastewater effluents that had strong estrogenic activity were less aggressive towards competitor fish. Steroid estrogen, estrone and estrogenic bisphenol-A (BPA) have both been previously reported in the effluent from the treatment facility in our study, but both compounds were reported at low concentrations (0.04 ng/l and 63 ng/L, respectively; Galus et al. 2013). Additional updated analyses for natural and synthetic estrogen compounds in the wastewater effluent from this treatment facility would be beneficial to better understand the changes to aggressive behaviours we observed in our study.

In contrast to our mirror aggression assay, we found little evidence that exposure to wastewater effluent affected round goby aggression in staged dyadic contests between exposed resident fish and unexposed intruder fish. There are several possible reasons why we observed little difference in aggression following wastewater exposure in the contest assay. First, contest assays involve complex interactions where two individuals respond to one another's behaviours; therefore, the unexposed intruder fish may have added an additional source of variation to our behavioural measures. We used intruder fish that were larger than the exposed resident fish, and this created asymmetric contests where the resident fish was expected to lose. This allowed us to measure how long an exposed fish will fight before "giving up" and allowing the intruder to be the dominant competitor. However, the resident fish in our study performed extremely few aggressive acts (6.98 ±1.54 SE) before surrendering their shelter resource to the intruder. This was especially obvious when we compared to the number of aggressive acts performed towards the mirror in this study (22.25 ± 5.50 SE acts), and in a previous study (60.60 ± 5.06 SE acts, over 30-minute trial, see Chapter 4 of this thesis). It is possible that the size difference between residents and intruders was too great for a reasonable contest to occur (on average the two fish varied in body mass by 17% ± 1.66 SE). Body size differences, however, did not dictate contest length or intensity, and fish regardless of treatment resolved conflict with very little interaction, limiting our scope for detecting the effects of exposure on aggression. A second reason that we may have failed to observe an effect of wastewater exposure on contest aggression is that we conducted our behavioral tests in the exposure concentration of wastewater effluent in which the fish were treated. While this ensured that any behavioural changes we observed in the residents was not due to sudden changes in water quality between their exposure tank and behavioural testing tank, the effluent may have affected the unexposed intruder fish added from laboratory stock tanks. We did observe that intruders added to a contest with the high dose of effluent (100% effluent) gave less aggression to the residents than intruders in the control group (0% effluent) (Negative binomial GLM with Tukey Post-Hoc, High vs. Control, $Z = -2.50$, $p = 0.03$), potentially suggesting that the intruders were less motivated to fight.

While we have focused primarily on PPCPs in wastewater in this discussion, we also assayed for EROD expression to assess whether fish were being exposed to planar hydrocarbon contaminants (e.g. polyaromatic hydrocarbons, PAHs). Such compounds are commonly measured in combined sewer systems where industrial, business, and residential sewage mixes with and urban road run-off (Welker, 2007; Gasperi et al., 2010; Barco-Bonilla et al., 2013). Fish exposed to 100% wastewater effluent in our study had increased hepatic EROD activity compare to fish exposed to 0% or 50% wastewater effluent. EROD induction has been assayed in a number of other studies on wastewater effluents (Kosmala et al., 1998; Sole et al., 2002; Lahti et al., 2012; Jasinska et al., 2015). Kosmala et al., (1998) found that EROD was induced on mirror carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) after a two-week exposure to 100% wastewater effluent. However, EROD has not been induced in the majority of other studies on the effects wastewater effluent exposure on fish (Sole et al., 2002; Lahti et al., 2012; Jasinska et al., 2015). The EROD induction we observed in this study indicates that fish are being exposed to aromatic hydrocarbon contaminants in the wastewater, but further work will be needed to identify the precise concentrations of these compounds in the wastewater and any potential biological effects they may have on fish. We also observed a slight increase in gonadosomatic index (GSI) for both 50% and 100% wastewater exposure groups in our study. While this increase in GSI is intriguing, we believe it is not likely to be biologically relevant as all fish were still in non-reproductive condition.

In conclusion, we show that exposure to a high dose (100%) of wastewater effluent reduced round goby aggression in our mirror aggression assay, but not in a contest aggression assay against a live unexposed intruder rival. More precise size-matching between resident and intruders should be attempted in future work to ensure a contest between opponents can be observed. Our work has local relevance for Hamilton Harbour, an International Joint Commission Area of Concern (International Joint Commission, 1999), which has been undergoing remediation to improve water quality and fish and wildlife habitat (Hamilton Harbour RAP, 1992; Hall et al., 2006). Regardless of potential contaminant cause, we demonstrate that complex wastewater effluents can impact round goby aggression, an important behaviour linked to their success as an invasive species (Kornis et al., 2012). Our study also underscores the importance of assessing multiple behavioural contexts when studying the impacts of pollutants or effluents, as our findings indicate that aggression may not be affected by effluent exposure in all behavioural contexts where aggression can be elicited. Our contest aggression assay may have been less sensitive to effluent-induced changes in aggression when compared to our mirror aggression assay findings. We suggest that mirror aggression assays may provide a clearer or cleaner picture of the effects of contaminants on fish aggression, but caution extrapolating results to dyadic contests. 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.

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Chapter 6: *In situ* exposure to wastewater effluent reduces survival but has little effect on the behaviour or physiology of an invasive Great Lakes fish

6.1 Abstract

Treated effluents from wastewater treatment plants (WWTP) are a significant source of endocrine disrupting compounds, 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 metabolism and non-reproductive 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 a wild invasive fish species (round goby, *Neogobius melanostomus*), at different distances from wastewater outflow for three weeks. We then evaluated the effects of this exposure on fish behaviour, metabolism, and respiratory physiology. Fish caged inside the WWTP and close to the outfall experienced higher mortality than fish from the reference site. Interestingly, the fish that survived the exposure performed similarly to fish caged at the reference site in aggression, startle-response, and dispersal assays. Moreover, the fish caged near the WWTP displayed similar resting O_2 consumption rates, hypoxia tolerance, blood haemoglobin contents, haematocrit, blood-oxygen binding affinities, and gill morphology as the fish from the reference site. Our results suggest that round goby that persisted through the wastewater exposure were relatively insensitive to the wastewater effluent exposure. Environmental contamination may therefore select for resilient individuals that are better equipped to cope with the physiological costs of wastewater effluent exposure.

6.2 Introduction

Wastewater treatment plant (WWTP) effluents are one of the largest and most ubiquitous sources of aquatic pollution (Environment Canada, 2001; Strayer & Dudgeon, 2010). WWTP effluents reduce dissolved oxygen, contribute to eutrophication *via* nutrient inputs, and increase anthropogenic contaminants like endocrine active substances in receiving waters (Environment Canada, 2001; 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 (Kolpin et al., 2002; 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 (Jelic et al., 2012), a number of these compounds are regularly measured in low but consistent quantities in the environment (i.e. ng/l to μg/l; Kolpin et al., 2002; Pal et al., 2010). The presence of such 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 in the wild (Environment Canada, 2001; Johnson & Sumpter, 2014). Endocrine-active substances that are found in treated wastewater effluent have the potential to act upon and disrupt the regulation of the variety of physiological systems that have conserved functions across aquatic vertebrates (Gunnarsson et al., 2008; Brown et al, 2014). While researchers have begun to quantify the effects of WWTP effluents on reproductive physiology, there has been comparatively little research on the effects that effluents have on fish behaviour or aspects of non-reproductive physiology like metabolism. Behaviour and metabolic physiology can both influence fitness (Scott &

Sloman, 2004), so understanding the impact that wastewater effluents on these processes is crucial for informing wastewater remediation efforts.

The problem of endocrine active substances in WWTP effluents has become more widely recognized as the number of studies reporting the presence of these compounds in the environment has increased (Boxall et al., 2012). In addition to understanding the effects of exposure to wastewater effluent on fish reproduction (Liney et al., 2006; Vajda et al., 2008; Harris et al., 2011; Tetreault et al., 2012; Fuzzen et al., 2015), there has been a recent push to understand the effects on other indices of fitness such as fish behavior and metabolic physiology (Söffker & Tyler, 2012; Brodin et al., 2014). Behaviour plays an important role in animal fitness through successful reproduction, territory defense, predator evasion, and foraging (Söffker & Tyler, 2012; Brodin et al., 2014). A growing number of studies have assessed how single endocrine active contaminants affect fish behaviour in the laboratory (e.g. Brandão et al., 2013; Brodin et al., 2013; Hedgespeth et al., 2013; Galus et al., 2014), but comparatively few have addressed the impacts of complex WWTP effluents. A few notable exceptions do exist. 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 after a three-week exposure to 100% wastewater effluent in the laboratory. Similarly, male three-spine stickleback (*Gasterosteus aculeatus*) exposed to 50% or 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 that male mosquitofish (*Gambusia affinis*) collected downstream from a wastewater treatment plant outfall actually courted females *more* than fish collected from a pristine site.

Metabolic physiology (such as energy utilization and respiration) is a major contributor to fitness, and provides the cellular energy needed to support behaviour (Brown et al, 2004; Scott & Sloman, 2004; Biro & Stamps, 2010). Not surprisingly, previous studies have demonstrated a tight link between behaviour and metabolism (Biro & 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 detoxification and routine bodily and behavioural processes (Scott & Sloman, 2004). Handy et al. (1999b) 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). Consequently, contaminant exposure may increase routine metabolic costs, which could in turn limit 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., 1999b; Scott & Sloman 2004; Killen et al., 2013).

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. Caging exposure provides certain experimental advantages over collecting exposed fish from the wild. For example, with caging we can control for exposure duration and fish mobility, allowing us to better-connect measured effects to the exposure. Moreover, field exposures allow us to integrate ambient environmental conditions into the exposure regime, something laboratory studies are unable to replicate (Palace et al., 2005; Oikari, 2006). 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. Theysmeyer, Head of Natural Lands, Royal Botanical Gardens, *personal communication*). The impact of wastewater effluent on aquatic organisms is especially important in effluent-dominated streams where there is little dilution of contaminants or water quality to reduce any potential impacts (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 & Blumstein, 2008), and these behaviours have been previously shown to be impacted by wastewater exposure (Martinović et al., 2007; Garcia-Reyero et al., 2011). Our physiological assays measured resting O_2 consumption rate and critical O_2 tension (an index of hypoxia tolerance that reflects the amount of dissolved O_2 needed to maintain bodily metabolic processes) to evaluate how fish cope metabolically with wastewater effluents. We then partnered these measures with assessments of several respiratory traits, including blood haemoglobin-oxygen transport characteristics and gill morphology, to assess whether changes to metabolism or hypoxia tolerance are facilitated by changes to oxygen transport *via* the gills and blood. If there is a trade-off between metabolism and behaviour (Handy et al., 1999b; Scott & Sloman, 2004), we expected that fish exposed to wastewater effluent would have higher resting metabolic rates that would be associated with dampened reactivity to startle stimuli and less activity. If resting metabolic rates are higher, we also predicted that increased oxygen extraction and transport would facilitate this metabolic cost (Weber & Jensen, 1988; Jensen et al., 1993; Perry & Laurent, 1993).

6.3 Methods

6.3.1 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 – Appendix B; Young et al., 2010). Fish were transported live to McMaster University where they were housed in groups of 10 to 20 fish in 150 l housing tanks (H44cm x W80 cm x 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 hours under laboratory conditions to ensure health and regular feeding before we deployed them in cages for field exposures.

6.3.2 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, Figure 6.1). 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 (Figure 6.1), and is the main source of flow to the canal aside from a small run-off ditch (Hamilton Water, *unpublished data*; T. Theysmeyer, *personal communication*). Characterizing the effluent from this canal is of special interest because it flows into Cootes Paradise Marsh, 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 introductions, combined sewer overflows, and wastewater effluents drastically reduced water quality and aquatic biodiversity in the early 1900's (Mayer et al., 2008;

Thomasen & Chow-Fraser, 2012). We caged fish inside the secondary clarifiers of the Dundas Wastewater Treatment Facility (our highest exposure site, Figure 6.1). 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 at a site 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 protected lands and is part of the same watershed; specifically, it is part of the headwaters for Spencer Creek that flows into the marsh. It does not receive wastewater effluents from any wastewater treatment facilities (Hamilton Conservation Authority, 2009).

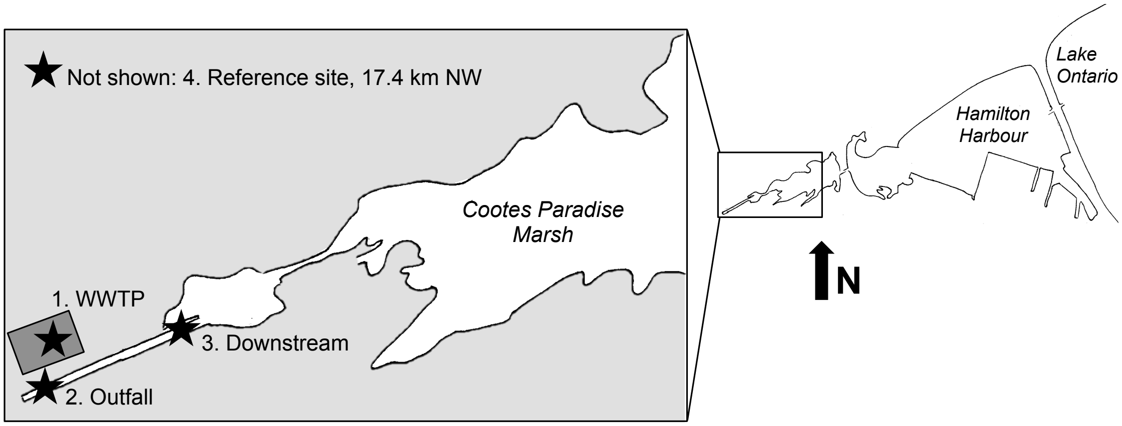

Figure 6.1
A diagram of the caging locations (indicated with stars) near Cootes Paradise Marsh, connected to Hamilton Harbour, Lake Ontario. The outfall site was located 50 m downstream from the effluent discharge. The downstream site was located 830 m downstream from the effluent discharge. The reference site was located in the headwaters to the Spencer Creek watershed that empties into the marsh.

We caged fish in groups of 14 – 16 fish for 21 days at each location. The cages were 114 l plastic totes (Rubbermaid: H51cm x W81cm x D44.5cm), each with approximately 200, 0.5 cm drilled 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: $F_{(3,147)} = 1.89$, $p = 0.13$).

6.3.3 Behavioural assays

In the laboratory, we housed previously caged fish in site-matched, groups of three in 40 l tanks (H33 cm x W51 cm x D28 cm) for 24 hours before beginning behavioural testing. We conducted three behavioural assays: 1) a mirror aggression assay, 2) a startle response assay, and 3) an activity and dispersal assay. 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 minutes. We then remotely lifted the removable opaque barrier to reveal the mirror, and a 20-minute mirror aggression trial was video recorded (Figure 6.2a, 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 (Figure 6.2b). The fish's startle response and any movement after the drop was recorded for an additional 20 minutes. 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 x 175 cm wide x 75cm deep) was separated into seven compartments (Figure 6.2c, 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 of the dispersal tank in a start-box where they remained for 10 minutes. The start box was then removed and the fish were allowed to freely explore the first compartment for 5 minutes. We live-scored total activity (all individual behaviours exhibited, see Supplementary Table S1 – Appendix C) for each fish for 5 minutes 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-minute trial. We live-scored the time and direction of each compartment switch (Figure 6.2c). An observer blind to exposure site later scored the behaviour from the video recordings of the aggression and startle-response assays. The observer recorded the latency for fish to move towards the mirror, the number of aggressive acts towards the mirror (following Supplementary Table S1 – Appendix C), 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.

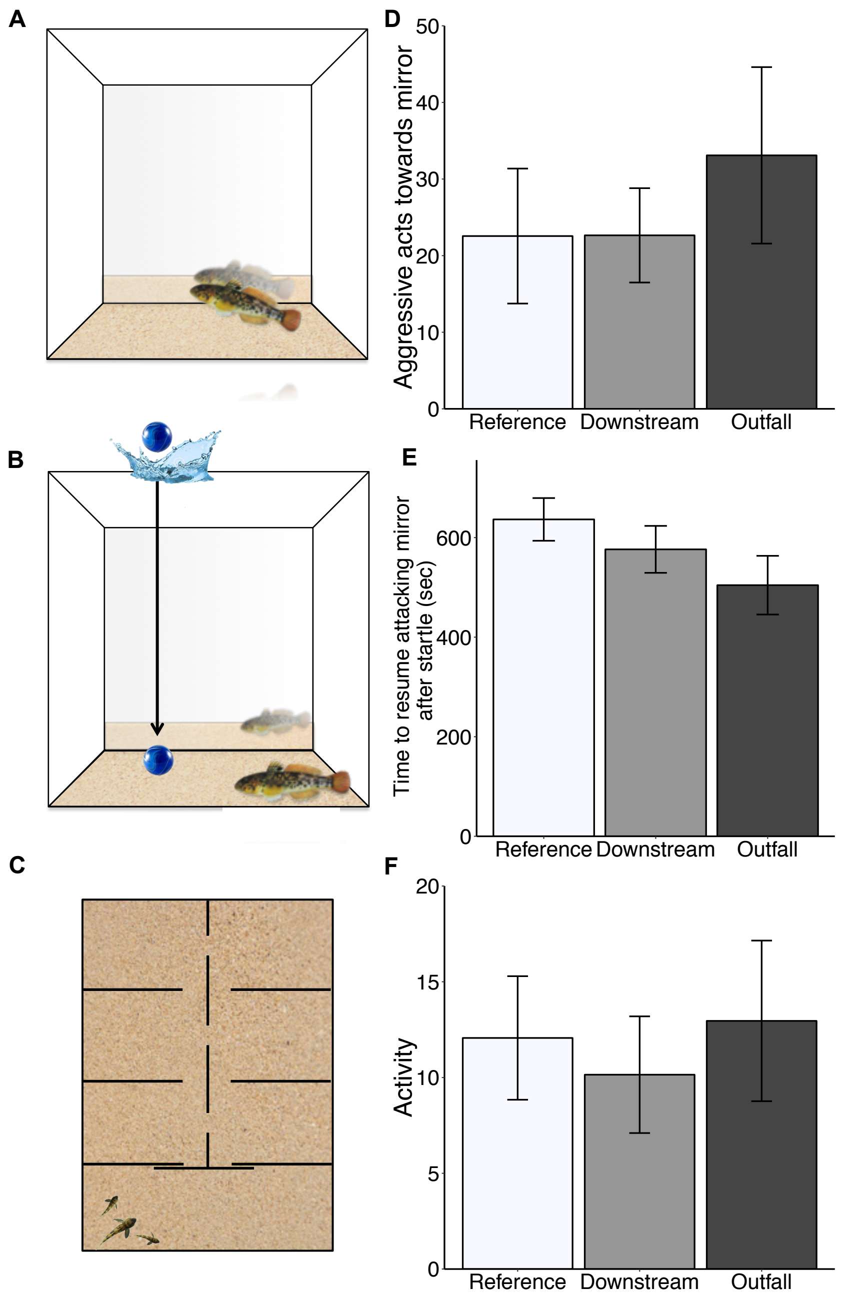

Figure 6.2

Behavioural assays and results. **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. **D)** Average activity during the dispersal trials plotted against exposure site. Error bars represent ± 1 standard error. All findings were not significant (*ns*).

6.3.4 Physiological assays and fish sampling

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) 10 hours 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, Regensburg, 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 minutes. At 10% air saturation, we closed the chamber and fish were allowed to consume the reaming oxygen until 0.5% air saturation was reached. We the 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 minute measurement periods. We then used regress software (Yeager & Ultsch, 1989) was used to determine P_{crit} from the M_{O2} data.

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 0.01cm. We measured total body mass using a digital scale accurate to 0.001g. 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 minutes. Samples collected *via* capillary tubes were centrifuged at 14,000 rpm for 5 minutes 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 removed the right and left gill arches from each fish, and stored one arch at -80°C and fixed the other at 4°C (in 2% paraformaldehyde and 2% glutaraldehyde in phosphate-buffered saline, PBS). 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 & 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%). Unless otherwise stated, reproductive status did not impact behaviour or physiology in all statistical analyses (all analyses, $p > 0.1$).

Haemoglobin-oxygen binding

We used the lysate from frozen red blood cells to evaluate haemoglobin-O_2 binding in the presence of 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 (100x 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 saturation at which hemoglobin 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).

Gill morphology

We removed the four arches of one side of the gills and fixed them in PBS for morphometric analyses. Each arch was isolated, and we took images at 10x magnification using a stereomicroscope to measure the total filament length (mm) as the sum of all filament lengths, the number of filaments from all gill arches, and lamellar density (the number of lamellae per filament) on every twentieth filament on the first gill arch. All gill measures were taken using ImageJ software.

6.3.5 Water and POCIS sampling

After we removed the POCIS samplers from the field, we transferred them on ice to McMaster University where they were immediately frozen at -20°C for later analysis. Water samples were also collected on the last day of POCIS sampling and were immediately stored at -20°C. We prepared water and POCIS samples for analysis of target PPCP and endocrine active compounds at Trent University following methods described in Li et al. (2010) and Metcalfe et al. (2014) for sucralose (an indicator of presence of wastewater effluent). Briefly, we rinsed POCIS samplers to remove debris from membrane surfaces before transferring sorbent powder into a glass chromatography column (1 cm x 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. Sulfonamide antibiotics, neutral pharmaceuticals, and antidepressants were analyzed by LC- APCI-MS/MS using an Agilent 1100 HPLC coupled to an Applied Biosystems Q-Trap tandem mass spectrometer (MDS Sciex) equipped with an APCI source. All other analytes were analyzed by LC-ESI-MS/MS using a Waters 2695 HPLC coupled to a Micromass Quattro LC tandem mass spectrometer fitted with a Z-spray electrospray interface (Micromass). 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, 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).

6.3.6 Statistical analyses

All statistical analyses were conducted using R (version: 3.2.4, R Core Team, 2016). We used QQ plots, residuals-versus-fitted, Shapiro-Wilk, and Breusch-Pagan tests to test models for parametric assumptions. We used generalized linear or linear mixed effects models (GLMM or LMM lme4 package; Bates et al., 2015) to analyze survival, behavioural, and physiological responses following exposure. We analyzed round goby survival using a binomial generalized linear mixed effects model (GLMM; glmmadmb package, Fournier et al., 2012), where we included caging site and weeks since deployment as fixed effects, and cage ID and deployment date as random effects. Due to high mortality leading to very low samples sizes at the highest exposure site (inside the WWTP), we excluded the WWTP fish from behavioural and physiological assays.

For 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. The number of aggressive acts in the mirror assay, 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.

All physiological measures, including: M_{O2}, 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 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 a subset of 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 6.1 for a summary of all sample sizes used in our analyses.

We descriptively summarized water quality measures, contaminant uptake in the POCIS samplers, and contaminant concentrations in water grab samples using means and standard errors.

Table 6.1

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

Caged	***N* Caged**	**Control**	**Downstream**	**Outfall**	**WWTP**
Deployed in the field	239	72	74	75	75

Behavioural assays	***N* Analyzed**	**Control**	**Downstream**	**Outfall**	**WWTP**
Mirror Aggression	68	26	20	22	--
Startle	80	29	25	26	--
Activity & dispersal	78	29	25	24	--

Physiology assays	***N* Analyzed**	**Control**	**Downstream**	**Outfall**	**WWTP**
Resting metabolism	27	10	8	9	--
Hypoxia tolerance, P_{crit}	28	10	9	9	--
Hematocrit (%)	37	17	8	12	--
Mean cell hemoglobin	36	17	8	11	--
Hemoglobin concentration	57	27	16	14	--
Hemoglobin P_{50}	28	11	7	10	--
Hemoglobin pH sensitivity	28	11	7	10	--
Gill morphometrics	26	11	8	7	--

6.4 Results

6.4.1 Survival

Across the exposure period, fish had the lowest survival at the highest exposure sites (within the Dundas WWTP and at the outfall site in Desjardins Canal), when compared to the reference site (Binomial GLMM: $N_{cages} = 20$; WWTP vs reference, $Z = -4.47$, $p < 0.001$; outfall vs reference, $Z = -2.16$, $p = 0.031$; Figure 6.3). However, fish caged at the downstream site (830 m away from the outfall) had statistically similar survival to fish at the reference site (downstream vs reference, $Z = -1.42$, $p = 0.16$), and the outfall site (downstream versus outfall, $Z = -0.76$, $p = 0.45$). Mortality rate did not differ across weeks, indicating mortality was occurring consistently across the exposure period (week 2 vs week 1, $Z = 1.83$, $p = 0.067$; week 3 vs week 1, $Z = 0.32$, $p = 0.75$; Supplementary Table 6.S1 – Appendix E).

6.4.2 Behaviour

Exposure to wastewater effluent had little impact on round goby behaviour. The number of aggressive acts that focal fish performed towards the mirror was similar between all sites (Negative binomial GLMM: $N = 68$, Likelihood Ratio Test $(\text{LRT})_{\text{site}}$, $\chi^2 = 0.67$, $p = 0.72$; Figure 6.2d), 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 site or the degree of wastewater exposure did not impact the type of behavioural response observed (Binomial GLMM; $N = 80$, LRT_{site}, χ^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$_{site}$, χ^2 = 2.63, *p* = 0.27), and to resume attacking the mirror (LMM: *N* = 80: LRT$_{site}$, χ^2 = 2.88, *p* = 0.24; Figure 6.2e).

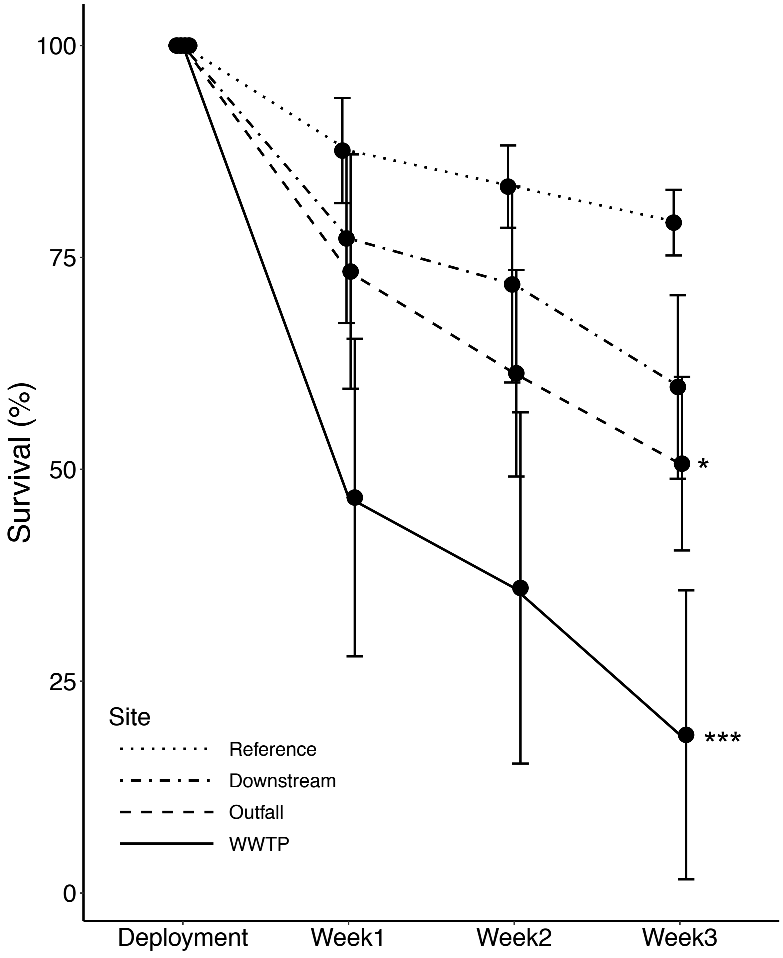

Figure 6.3
Average percent survival of round goby plotted by exposure week. Fish caged in the wastewater treatment plant and at the outfall site had lower survival compared to fish caged at the reference site. Error bars represent ± 1 standard error. * $p < 0.05$, ** $p < 0.01$

Exposure did not affect overall activity levels (Negative binomial GLMM; *N* = 78, LRT$_{site}$, χ^2 = 0.64, *p* = 0.73; Figure 6.2f), the time taken to disperse measured as the time taken to exit 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: $N = 78$, $\mathrm{LRT}_{\mathrm{site}}$, $\chi^2 = 0.49$, $p = 0.78$).

6.4.3 Physiology

Like behaviour, exposure to wastewater effluent had little effect on metabolism and respiratory physiology (see Table 6.2 for summary). Fish from all exposure sites had similar O_2 consumption rates at rest (LMM ln-transform; $N = 27$; $\mathrm{LRT}_{\mathrm{site}}$, $\chi^2 = 1.87$, $p = 0.39$; Figure 6.4) and critical oxygen tensions (P_{crit}; LMM; $N = 28$; $\mathrm{LRT}_{\mathrm{site}}$, $\chi^2 = 0.25$, $p = 0.88$).

Table 6.2

Results summary of average (± 1 SE) measures of respiratory physiology, haematology, and gill morphometric collected after the caging exposure

	Site		
Measure	**Reference**	**Downstream**	**Outfall**
Respiratory physiology			
P_{crit}, kPa	3.04 ± 0.41	2.85 ± 0.23	2.95 ± 0.14
Haematology			
Haematocrit, %	33.45 ± 2.72	32.61 ± 4.60	31.94 ±2.24
Haemoglobin concentration, g/Dl	5.14 ± 0.50	4.47 ± 0.64	5.16 ± 0.41
Mean cell hemoglobin, g/Dl	18.85 ± 3.23	19.13 ± 5.85	16.90 ± 2.36
Haemoglobin P_{50} pH 7.0, kPa	6.89 ± 0.18	7.09 ± 0.36	7.03 ± 0.26
Haemoglobin P_{50} pH 7.4, kPa	4.23 ± 0.09	4.43 ± 0.17	4.48 ± 0.20
Haemoglobin pH sensitivity	6.66 ± 0.32	6.63 ± 0.45	6.38 ± 0.44
Gill morphometrics			
Total gill filament length, mm	597.41 ± 26.98	632.73 ± 51.03	715.34 ± 88.64
Total gill filament number	286.09 ± 7.40	304.88 ± 13.29	302.14 ± 10.12
Mean lamellar density	17.10 ± 0.27	18.03 ± 0.54	17.48 ± 0.35

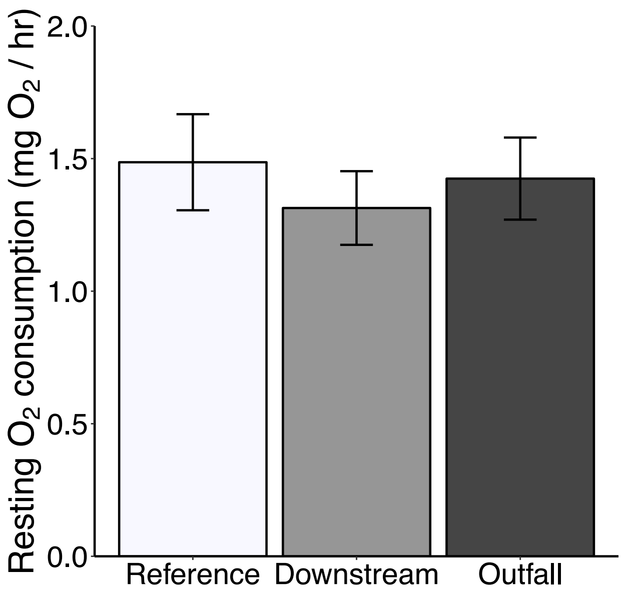

Figure 6.4
Average resting metabolic rate, plotted by exposure site. Error bars represent ± 1 standard error, finding was not significant (*ns*).

Exposure did not impact any haemoglobin-oxygen transport capacity or binding parameters, including: haematocrit (LMM: $N = 37$; LRT_{site}, $\chi^2 = 0.99, p = 0.61$), haemoglobin concentration (LMM: $N = 57$; LRT_{site}, $\chi^2 = 2.49, p = 0.29$, $\text{LRT}_{\text{ReproductiveStatus,}}$ $\chi^2 = 5.51, p = 0.019$), and mean cellular haemoglobin concentration (LMM: $N = 36$; LRT_{site}, $\chi^2 = 0.86, p = 0.65$). 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. Hemoglobin pH sensitivity was also similar between exposure sites (LMM: $N = 28$, LRT_{site}, $\chi^2 = 0.36, p = 0.83$).

Fish from the downstream site had slightly longer total gill filament lengths than fish from the reference site (LMM: $N = 26$; LRT_{site}, $\chi^2 = 10.76, p = 0.0051$; Tukey HSD:

reference vs outfall Z = 1.83, p = 0.16; reference vs downstream Z = 3.38, p =0.0021; outfall vs downstream Z = -1.29, p = 0.40; Supplementary Figure 6.S1 – Appendix E). The average gill filament length of fish from the downstream site was 70.21mm (± 20.78 SE) longer than reference site fish. This difference in gill filament length was likely caused by a modest lengthening of individual filaments, because there was no effect of exposure site on gill filament number (LMM: N = 26; LRT_{site}, χ^2 = 5.26, p = 0.072), nor were there any effects of exposure site on lamellar density (LMM: N = 26; LRT_{site}, χ^2 = 3.13, p = 0.21).

6.4.4 Study site characteristics

The time-weighted concentrations of PPCPs determined from the POCIS samplers and the concentrations determined in water samples are reported in Figure 6.5, Table 6.3 and Table 6.4. 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 (Figure 6.5).

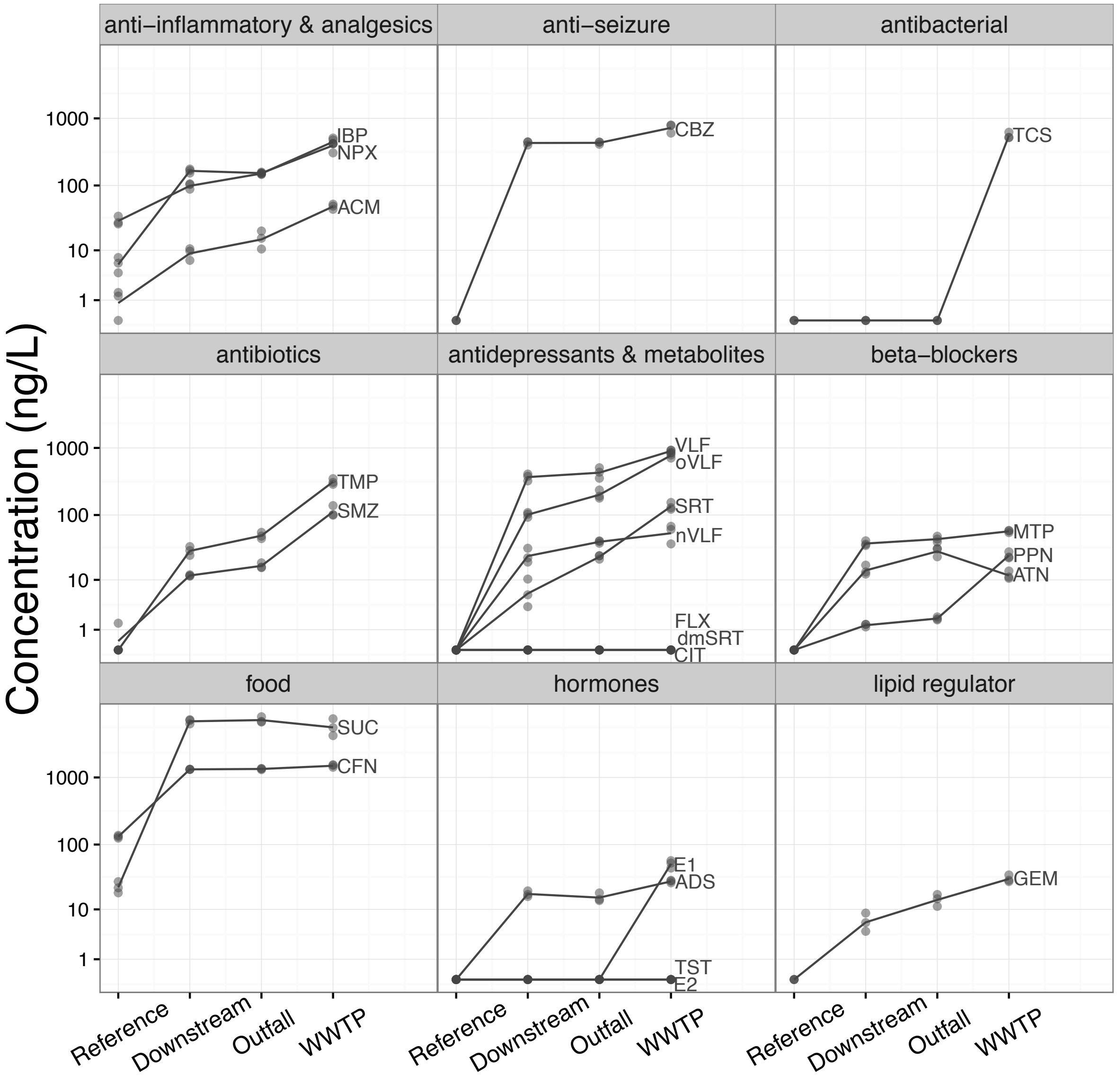

Figure 6.5

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 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

Table 6.3

Summary of average PPCPs using POCIS samplers (*N* = 3 replicates per site). Time-weighted PPCP concentrations from the POCIS samplers were derived from sampling rates previously reported in the literature. -- indicates sample was not analyzed.

		Time-weighted concentration ng/l			
Compound	**Class**	**Reference**	**Downstream**	**Outfall**	**WWTP**
Caffeine	food	73.8	742.5	752.4	839.4
Sucralose	food	9.9	2996.0	3130.6	2500.5
Trimethoprim	anti-biotic	ND	4.7	8.03	51.5
Sulfamethoxazole	anti-biotic	0.3	2.5	3.5	23.8
Carbamazepine	anti-seizure	<LOQ	54.9	55.1	92.7
Acetaminophen	analgesic	0.7	4.5	7.6	23.8
Ibuprofen	anti-inflammatory	6.0	20.3	31.0	92.4
Gemfibrozil	lipid regulator	ND	1.3	2.9	6.2
Naproxen	anti-inflammatory	1.1	30.2	27.9	73.4
Triclosan	antibacterial	ND	--	ND	20.5
Estrone (E1)	hormone	ND	<LOQ	<LOQ	5.2
Estradiol (E2)	hormone	ND	ND	N	ND
Androstenedione	hormone	<LOQ	2.0	2.32	3.62
Testosterone	hormone	ND	<LOQ	<LOQ	<LOQ
Venlafaxine	antidepressant	<LOQ	50.7	59.3	123.4
O-dm-venlafaxine	metabolite	<LOQ	18.3	36.4	140.0
N-dm-venlafaxine	metabolite	ND	4.3	6.9	9.6
Sertraline	antidepressant	ND	0.4	1.9	11.1
dm-sertrailne	metabolite	ND	ND	ND	ND
Citalopram	antidepressant	ND	ND	ND	ND
Fluoxetine	antidepressant	ND	ND	ND	ND
Atenolol	beta-blocker	ND	10.9	21.5	9.0
Metoprolol	beta-blocker	ND	5.7	6.7	8.7
Propanolol	beta-blocker	ND	4.7	3.3	59.9

Table 6.4

Summary of average PPCPs using grab water samples. Samples were taken once at the end of the sampling period for comparison to POCIS samplers (*N* = 1).

		Water sample concentration ng/l			
Compound	**Class**	**Reference**	**Downstream**	**Outfall**	**WWTP**
Caffeine	food	23.1	795.1	812.4	657.0
Sucralose	food	46.7	709.9	991.1	789.0
Trimethoprim	anti-biotic	ND	19.5	20.0	43.4
Sulfamethoxazole	anti-biotic	ND	5.4	4.6	11.7
Carbamazepine	anti-seizure	ND	36.7	37.0	63.7
Acetaminophen	analgesic	ND	ND	ND	ND
Ibuprofen	anti-inflammatory	ND	74.7	64.8	100.6
Gemfibrozil	lipid regulator	ND	ND	9.1	15.0
Naproxen	anti-inflammatory	8.5	55.8	49.5	88.5
Triclosan	antibacterial	ND	ND	ND	104.8
Estrone (E1)	hormone	ND	ND	ND	8.2
Estradiol (E2)	hormone	ND	ND	ND	ND
Androstenedione	hormone	<LOQ	2.9	2.8	8.9
Testosterone	hormone	ND	ND	ND	3.1
Venlafaxine	antidepressant	ND	253.5	368.6	696.1
O-dm-venlafaxine	metabolite	ND	289.7	671.2	1594.8
N-dm-venlafaxine	metabolite	ND	73.9	94.2	110.9
Sertraline	antidepressant	ND	135.7	226.7	406.9
dm-sertrailne	metabolite	ND	ND	11.6	70.8
Citalopram	antidepressant	ND	ND	ND	ND
Fluoxetine	antidepressant	ND	ND	ND	ND
Atenolol	beta-blocker	ND	51.5	65.6	125.3
Metoprolol	beta-blocker	ND	24.2	31.8	42.0
Propanolol	beta-blocker	ND	ND	1.2	5.5

Interestingly, at our reference site, we detected caffeine and sucralose, the antibiotic sulfamethoxazole, and pain-relievers acetaminophen, ibuprofen, and naproxen, but the concentrations of these compounds at the reference site were much lower than at our downstream, outfall, and WWTP sites (Table 6.3, Table 6.4, Figure 6.5). We did not detect the antidepressants fluoxetine and citalopram, the antidepressant metabolite desmethylsertraline, or the endogenous hormone estradiol on the POCIS samplers at any of our sites, and testosterone was only present below the limit of quantification. We summarized water quality measures throughout the exposure period in Table 6.5. 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 contaminated sites were generally different than the reference site.

Table 6.5
Summary of average (± 1 SE) water quality measures across the caging exposure period. Measures were taken once per week at all caging sites (*N* = 7, per site).

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

6.5 Discussion

Wastewater effluent is a complex mixture of various contaminants including PPCPs, and exposure may come at a metabolic cost that limits the aerobic scope for routine behaviours in fish. We found that round goby exposed to effluent in the WWTP and at the outfall had higher mortality than fish caged in the reference site. Interestingly, we found that the round goby that survived the exposure did not show any behavioural or physiological deficits. More specifically, exposure did not impact measures of aggression, startle responses, or overall activity. As well, we saw no exposure related differences in resting metabolism, hypoxia tolerance, hemoglobin-oxygen transport, or gill morphology.

In-situ caging studies have shown inconsistent effects on fish survival following exposure to wastewater effluent. A select few studies have reported increased mortality in fish exposed to wastewater effluent (Mitz & Giesy, 1985; Kosmala et al., 1998; 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, Giesy et al., 2003; Bernet et al., 2004; Vermeirssen et al., 2005; Burki et al., 2006; Jasinska et al., 2015; Vincze et al., 2015). We observed reduced survival inside the WWTP and at the outfall site, but not at the downstream site when compared to the reference site. It is important to also note that while survival at the outfall site was statistically different from the reference site, survival was not different from the downstream site. Generally, our survival results also followed the concentrations of PPCPs and water quality parameters. For example, we detected the exact same nineteen contaminants in similar concentrations at the downstream and outfall sites. The water quality parameters we measured (temperature, dissolved oxygen, pH, conductivity, salinity, total dissolved solids) were also similar between the outfall and downstream sites. Taken together, our contaminant and water quality monitoring data would suggest that if these parameters in combination were causing the mortality we observed, then we would also expect mortality to be similar at the outfall and downstream site when compared to the reference site. A final parameter that may impact survival is ammonia, as wastewater treatment plant effluents can contribute significant amounts of ammonia to receiving environments (Environment Canada, 2001). 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. Unfortunately, we did not measure ammonia directly at our exposure sites, but the amount in the final treated effluent leaving the WWTP during our exposure period was very low, 0.056 mg/l (range: 0.04 – 0.07mg/l, Hamilton Water, 2015, *unpublished data*). Similarly, nitrite (mean: 0.092mg/l, range: 0.05 – 0.14mg/l, Hamilton Water, 2015, *unpublished data*), and nitrate (mean: 14.64mg/l, range: 13.10 – 16.70mg/l, Hamilton Water, 2015, *unpublished data*) were low and within the Canadian Water Quality Guidelines for the protection of freshwater aquatic life (Canadian Council of Ministers of the Environment, 2010; 2012). It is plausible that the combined effects of ammonia, nitrate, nitrite, other water quality parameters, and contaminant loads compounded to increased fish mortality in the outfall site compared to the downstream site.

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 (Martinović et al., 2007; Garcia-Reyero et al., 2011; Sebire et al., 2011; Saaristo et al., 2014; 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 may be several reasons why we documented no observable effect of our caging exposure on round goby behavior, metabolism, or various respiratory physiology traits. First, it is possible that the amount of PPCPs present at our sampling locations or the duration of exposure were insufficient to cause changes in our behavioural and physiological assays. For example, the antidepressant venlafaxine was measured at ~50 ng/l at the outfall, but only much higher concentrations (> 200 000 ng/l) were found to elicit behavioural effects in previous studies of hybrid striped bass (*Morone saxatilis x Morone chrysops*, Bisesi et al., 2014). However, the effects of PPCP mixtures on fish are still poorly understood, especially for mixtures of compounds with different mechanisms of action (Khetan & Collins, 2007; Backhaus, 2014). This makes it difficult to draw conclusions on behavioural and physiological effects from studies on individual compounds.

Second, it is possible that round goby, at least in the adult life-stage that we tested, are more 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, they can tolerate water temperatures ranging -1°C to 30°C, dissolved oxygen as low as 0.4 to 1.3 mg/l, and a wide range of water salinities (Charlebois et al., 1997; Cross &

Rawding, 2009; Arend et al., 2011; 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 (the larger water body adjoining Cootes Paradise Marsh where our current study was conducted) at locations that are highly contaminated with metals, polychlorinatedbiphenols (PCBs), and polyaromatic hydrocarbons (PAHs), where they are equally abundant to the numbers of fish in comparatively cleaner sites (Marentette et al., 2010; McCallum et al. 2014 – Appendix B). 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 (~5m^2, Ray & Corkum, 2001) that would be well-suited to a caging experiment (Palace et al., 2005; Oikari, 2006). Instead, we expect that wastewater exposure may be eliminating sensitive individuals from the population, leaving only those that can behaviorally and physiologically cope with the environmental conditions (Fox, 1995).

To conclude, we found that exposure to wastewater effluent reduced 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. More broadly, our results suggest that exposure to effluent may be selecting for individual round goby that are able to cope with exposure, and the tolerance of round goby to a wide range of water quality conditions may contribute to their continued persistence and invasion success. 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.

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Chapter 7: General Discussion

7.0 Thesis aims revisited:

My thesis aimed to: 1) describe the social and aggressive interactions of the invasive round goby; 2) assess how the antidepressant fluoxetine (a pharmaceutical commonly found in surface waters), impacts round goby affiliative and aggressive behaviours; and 3) ascertain the behavioural impacts of exposure to complex wastewater effluents on round goby. Altogether, these aims allowed me to further develop fish behaviour as a measure of contaminant exposure, and locally evaluate the impacts of Hamilton Harbour (ON, Canada) wastewater effluents on round goby. In the following chapter, I will briefly summarize my main thesis findings, review my contributions to the literature, and end by proposing several possible directions for future research.

7.1 Thesis summary

Though the aggressive interactions of round goby with hetero- and conspecifics have been previously described (Balshine et al., 2005; Bergstrom & Mensinger, 2009; Stammler & Corkum, 2005), very little has been done to understand their non-aggressive, social interactions. In **Chapter 2**, I showed that round goby are attracted to conspecifics, but they do not have an obvious preference for group size. Moreover, I showed that while females preferred spending time in a shelter to associating with a conspecific, male round goby showed no such preference. In **Chapter 3**, I further investigated round goby preferences for shelter resources and explored their ability to collect information on resource value during aggressive contests. I showed that round goby prefer enclosed, defendable ("high quality") shelters. Moreover, I found that size-matched pairs of fish had longer and more aggressive contests over these high quality shelters than contests over open, less easily defended ("low quality") shelters. I also showed that fish required previous experience with the shelter to adjust their aggressive effort and fight based on the value of the resource.

In the remaining chapters of my thesis, I investigated the impact of a pharmaceutical contaminant, fluoxetine, and of a complex mixture, wastewater effluent, on round goby behaviour. This allowed me to address how these emerging contaminants of concern may be affecting fish behaviours important for survival and reproduction. In **Chapter 4**, I investigated how the antidepressant fluoxetine impacted round goby associative and aggressive behaviours after an acute (3 day) and chronic (28 day) exposure. I demonstrated that fluoxetine reduced round goby aggression in multiple behavioural contexts after an acute exposure, but aggression was reduced only in one aggressive context (towards a mirror) after a chronic exposure. In **Chapter 5** and **Chapter 6**, I investigated the effect of a chronic (21 – 28 days) exposure to complex wastewater effluents on round goby behaviour in the laboratory (**Chapter 5**) and in the field (**Chapter 6**). In these two final chapters, I showed that a laboratory exposure to wastewater from the Woodward Wastewater Treatment Facility (Hamilton, ON, Canada) reduced round goby aggression towards a mirror, but not towards a conspecific in a paired contest over a valued shelter resource (**Chapter 5**). However, in **Chapter 6**, when round goby were exposed to wastewater effluent in the field from the Dundas Wastewater Treatment Facility (Dundas, ON, Canada), I found no evidence that the exposure impacted round goby behaviour or measures of their metabolic physiology. Instead, fish survival near the wastewater effluent outfall was reduced.

7.2 Social and aggressive interactions in round goby

Social tolerance and aggressiveness are often viewed as orthogonal behavioural traits, but both can facilitate the establishment and persistence of invasive animal populations (Holway & Suarez, 1999; Chapple et al., 2012). Tolerance of conspecifics is expected to allow new populations to grow and high interspecific aggression allows dispersing invaders to outcompete native species for access to resources (Holway & Suarez, 1999; Chapple et al., 2012). My primary contribution to expanding our understanding of round goby social interactions is detailed in **Chapter 2**, where I provide one of the first analyses of social attraction and conspecific preferences in the highly invasive round goby. I found that while round goby are attracted to conspecifics, they are not sensitive to group size, and many of the interactions that round goby had with other conspecifics were aggressive in nature. This finding is at odds with long-stated predictions that invasive species should be socially gregarious to conspecifics (Holway & Suarez, 1999; Chapple et al., 2012). It is possible that while round goby exhibit aggression towards conspecifics in the wild, their aggressive interactions are resolved quickly and without extensive harm, allowing round goby to coexist and possibly lead to stable dominance hierarchies among neighboring fish. Indeed, in all my studies of dyadic round goby aggressive contests (**Chapter 3, Chapter 4, Chapter 5**) I have never observed overt harm (torn fins, missing scales, abrasions) or death during aggressive contests. However, Balshine et al. (2005) found that round goby were highly aggressive towards native log perch (*Percina caprodes*) in the laboratory, and despite being well size-matched, round goby sometimes killed and consumed log perch in several of these trials. Interestingly, Kornis et al. (2014) found that when round goby were housed at high densities (10.7 round goby/m^2 – a density within those observed in the Laurentian Great Lakes) they suffered reduced growth. The authors attributed this reduction in growth to high conspecific competition, but they did not record any aggressive interactions. It would be particularly interesting to further explore the limitations and conditions surrounding round goby social tolerance, especially since these fish are observed at such high densities in the wild (7.76 fish/m^2, Johnson et al., 2005; 9.64 fish/m^2, Taraborelli et al., 2009).

In **Chapter 3**, I endeavored to further understand round goby aggressive behaviour by exploring round goby shelter preference and aggression over shelter resources. I focused on resource preference for several reasons. First, in the context of my overall thesis, I used resource contests (**Chapter 4** and **Chapter 5)** to understand the impacts of anthropogenic pollution on aggressive interactions between fish, making it important to understand whether these resources were actually valued by competing fish. Second, shelter resources are essential for round goby survival and reproduction in the wild (Corkum et al., 1998; Belanger & Corkum, 2003). Third, there is interest among behavioural ecologists to understand how animals gather information about resource value and use this information to modulate their aggressive effort (Arnott & Elwood, 2008; Elwood & Arnott, 2012). For my thesis, I confirmed that fish prefer an enclosed, defendable shelter to an open, less easily defended shelter. I used the enclosed shelter as a critical resource over which to stage aggressive contests during my subsequent analyses of aggressive behaviour. I was able to provide one of the first studies to investigate the ability of animals to assess resource value during aggressive interactions. An important contribution from my work inn **Chapter 3** was that I was able to disentangle subjective resource value (i.e. perceived ownership) from objective resource value (i.e. intrinsic qualities of the resource) by using owner-owner resource contests. Previously, this owner-owner experimental design been used infrequently to investigate resource value, and I have called attention to its usefulness for future studies on resource assessment.

7.3 Behaviour as an indicator of exposure to environmental contaminants

In the remaining chapters of my thesis, I investigated how the antidepressant fluoxetine (**Chapter 4**) and complex wastewater effluents (**Chapter 5**, and **Chapter 6**) affected the round goby behaviour. I aimed to further develop round goby behaviour as a tool for evaluating the impacts of contaminant exposure on wild fish (building upon previous work: Marentette & Balshine, 2012; Marentette et al., 2012), especially in response to wastewater effluents entering the highly impacted Hamilton Harbour (Hamilton Harbour RAP, 1992; Hall et al., 2006). As previously outlined in my introduction and thesis chapters, I focused on aggressive interactions, because securing a shelter and territory is highly relevant for round goby reproductive success and survival. I will now synthesize across my findings by revisiting concepts that I originally introduced and reviewed in **Chapter 1**.

7.3.1 Is round goby behaviour a sensitive indicator of exposure?

Behavioural endpoints are reported to be more sensitive to contaminant exposure than traditional measures of lethality (Beitinger, 1990; Zala & Penn, 2004; Robinson, 2009). My findings from **Chapter 4** and **Chapter 5** support this generalization. In the laboratory, I observed that exposure to a high dose of fluoxetine (40 µg/l) or to a high dose of wastewater effluent (100% wastewater) reduced round goby aggressive behaviours, but I found no association between either exposure and fish mortality. However, this pattern of findings (observing behaviour changes before large scale mortality changes) did not generalize to the most ecologically relevant exposure I conducted when fish were caged in the field at different distances from effluent discharge (described in **Chapter 6)**. In **Chapter 6**, I found that fished caged in close proximity to the wastewater outfall had increased mortality, but I documented no evidence for behavioural or physiological effects of the exposure on the surviving fish. It is understandably difficult to directly compare my separate exposure experiments because the wastewater effluent I used was from different treatment facilities. These facilities will have different mixtures of pollutants stemming from the different human populations they serve and differences in the wastewater treatment process (Woodward is a secondary treatment facility, Dundas is a tertiary treatment facility). However, my findings from **Chapter 6** challenge this broad generalization that behaviour is always more sensitive than lethality in the wild. My findings also highlight the complexity of generalizing from a laboratory-based exposure (**Chapter 5**) to a field-based exposure scenario (**Chapter 6**).

It is possible that the fish first exhibited altered behaviour before succumbing to the exposure in **Chapter 6**; however, I was unable to observe this in the field.

Focusing specifically on the sensitivity of round goby behaviour to the environmentally relevant exposure conditions I tested, my behavioural findings from **Chapters 4, 5,** and **6** generally suggest that adult round goby are fairly resilient to environmentally relevant exposures. In **Chapter 4**, round goby exposed to the 1 μg/l environmentally relevant dose of fluoxetine were often behaviourally intermediate between the control and high dose fish, and I found that they were not statistically different from either control or high dose fish. The impacts of fluoxetine exposure on round goby behaviour became obviously apparent at the high dose (40 μg/l), and that was the exposure that we estimated to create an internal fluoxetine concentration in round goby that would be close to the therapeutic dose in human plasma. Similarly, in **Chapter 5**, when fish were exposed to wastewater effluent, we found that aggressive behaviours towards a mirror were reduced at the highest wastewater effluent dose (100% wastewater), while round goby aggression towards the mirror following the low dose (50% wastewater) was intermediate between the control (0% wastewater) and high dose. Finally, in **Chapter 6** we recorded no behavioural (or physiological) differences between fish caged at a reference location and fish caged downstream from a wastewater treatment plant outfall. This apparent resilience of round goby to wastewater effluent and fluoxetine may stem from the fact that round goby are highly tolerant of a wide range of environmental conditions (reviewed in: Moskal'kova, 1996; Kornis et al., 2012). Round goby tolerate and persist in highly polluted locations, such as industrial Harbours (Janssen

& Jude, 2001; Dermott et al., 2012; Marentette et al., 2012; McCallum et al., 2014 – Appendix B), and originally invaded from areas of poor water quality in Europe in the early 1990's (i.e. the Black Sea: Maldonado et al., 1999; Kideys et al. 2002; Brown & Stepien, 2009). Though my findings may suggest round goby are fairly resilient, caution must be taken when interpreting a lack of evidence ("absence of evidence is not evidence of absence"). It would be beneficial to assess behaviour following exposure to additional fluoxetine concentrations to those tested in **Chapter 4** (e.g. 0, 0.5, 1, 5, 10, 20, 40 μg/l). Likewise, assessing the behavioural effects of exposure to additional wastewater dilutions to those tested in **Chapter 5** (e.g. 0, 25, 50, 75. 100% effluent). Testing additional exposure conditions with larger sample sizes would allow for more accurate investigations of which exposure concentrations lead to changes in behaviour. Moreover, additional exposure conditions would allow us to replicate and further study the intermediate effects of our exposures on round goby behaviour noted in **Chapter 4** and **Chapter 5** (i.e. those not statistically different from control or high doses, 1μg/l of fluoxetine and 50% wastewater effluent).

The results and findings from my thesis can only be applied to the adult life-stage that I tested in my experiments. However, round goby (and other aquatic organisms) are potentially exposed to PPCPs in wastewater effluents across an entire lifespan. Early-life is a particularly sensitive developmental period that can be prone to disruption by external stressors like contaminants (Iguchi et al., 2001; Hotchkiss et al. 2008). For example, guppies (*Poecilia reticulate*) exposed to environmentally relevant concentrations of fluoxetine had reduced growth, reduced survival, and abnormal movement behaviours, while adult guppies exposed in similar conditions only showed altered anti-predator behaviour (Pelli & Connaughton, 2015). Exposure during early life may therefore cause more noticeable or exaggerated effects of fish morphology, physiology, and behaviour. It would be advantageous to further investigate the effects of fluoxetine and wastewater effluents on developing fish to better characterize the impacts of exposure across the lifecycle. Round goby may not be an ideal study species for investigating developmental effects of contaminant exposure, at least under laboratory conditions, as round goby are very difficult to consistently breed in captivity (Meunier et al. 2009). Instead, established laboratory model species such as fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), or Japanese medaka (*Oryzias latipes*) would be better suited to lifecycle exposure experiments because of their high reproductive output and ease of care and breeding in the laboratory.

7.3.2 Are effects on round goby behaviour consistent? Were the behavioural changes generalizable between contexts?

A central focus throughout my thesis was measuring round goby behaviour multiply in response to fluoxetine and wastewater effluent exposures. I did this by either measuring the same endpoint within or between experiments (i.e. acute versus chronic effects; repeated experiments), or measuring multiple behavioural contexts for one behavioural phenotype (i.e. mirror versus dyadic contests to measure aggression).

I will first discuss behavioural measurements between and within experiments. In **Chapter 4,** I provide one of the first studies to repeatedly test the behavioural impacts of a pharmaceutical exposure between individual exposure experiments and across multiple behavioural contexts (but see: Dzieweczynski & Hebert, 2012; Brodin et al., 2013). I showed that fluoxetine exposure acutely reduced round goby aggression towards a mirror, towards a conspecific, and during a social interaction assay. I repeated this effect in two different acute exposure experiments, and showed that a chronic 28-day exposure still reduced aggression towards a mirror (though the impact on contest aggression was less clear). I have consistently demonstrated that fluoxetine dampens aggressive behaviours in round goby at a higher dose than what is observed in the environment (40 μg/l). The robustness of my findings are especially pertinent and timely because the repeatability of behavioural effects following pharmaceutical exposures, and pollutant exposures more generally, has been raised as an issue by a number of researchers (Peakall, 1996; Sumpter et al., 2014). Tests of the repeatability of behavioural findings are still widely lacking in studies on the behavioural impacts of pharmaceutical exposures in ecotoxicology.

Next, I will discuss measuring multiple contexts for the behavioural phenotype that I principally focused on during my thesis: aggression. In **Chapter 4** and **Chapter 5**, I evaluated the effects of exposure to fluoxetine and wastewater effluent on two commonly used assays of fish aggression: mirror aggression and dyadic contest aggression. I wanted to establish whether a simple assay (mirror aggression) would produce similar effects to more complex and realistic dyadic aggressive interactions with a real competitor. Mirror assays are simpler to conduct than dyadic contests because they do not require the presence of a novel competitor fish that is appropriately matched in size. The degree of body size difference between competitors in dyadic contests can play a large role in determining the outcome and intensity of aggressive interactions (Arnott & Elwood, 2009). These body mass differences between competitors must therefore be carefully controlled, especially between treatment groups in a pollutant exposure experiment. Logistically, it may be unfeasible for researchers to have access to a sizeable stock of potential competitor fish when working with wild-collected animals. Therefore, mirror assays would be hugely beneficial for scientists desiring to quickly establish the effect of a given pharmaceutical or effluent pollutant on animal aggression, and would be doubly useful if the results predicted an ecologically relevant outcome (such as resource ownership after an aggressive contest).

In **Chapter 4** and **Chapter 5**, I found that even though exposure to fluoxetine and wastewater effluent reduced round goby aggression towards a mirror**,** this did not always translate to a reduction in aggression during the aggressive contest. What might have caused this discrepancy between aggression assays? Aggressive responses to a mirror may not be reflective of the aggressive interactions between two live opponents. In both chapters, irrespective of exposure treatment, fish gave almost twice the number of aggressive acts towards a mirror than they did towards an opponent. Mirror interactions provide focal fish no indication of relative rank or fighting ability, and do not allow the focal fish to resolve an aggressive interaction or establish dominance. Teles et al. (2013) and Teles & Oliveira (2016) also found that zebrafish (*Danio rerio*) spent more time being aggressive towards a mirror than a live opponent. Interestingly, Oliveira et al. (2005) found that male Mozambique tilapia (*Oreochromis mossambicus*) fighting against their mirror image failed to mount an androgen response, something that has been commonly documented during aggressive interactions in fish and other vertebrates (reviewed in: Oliveira et al., 2002). The aggressing individual may therefore perceive mirror interactions differently than dyadic contests, and there are a growing number of investigations aimed at uncovering the hormonal and neural mechanisms behind the behavioural differences following mirror and contest assays (Desjardins & Fernald, 2010; Teles et al., 2013). Integrating my results from **Chapter 4** and **Chapter 5**, I would recommend the use of mirror assays for a simple, fast measure of individual aggressiveness (as suggested by: Wilson et al., 2011; Elwood et al., 2014). However, I would caution extrapolating the results to live interactions between opponents. Balzarini et al. (2014) reached a similar conclusion when they tested whether mirror interactions predicted outcomes in aggressive contests for three cichlid fish species (*Neolamprologus pulcher, Telmatochromis vittatus, Leipiolamprologus elongatus*). The authors concluded that mirror assays accurately predicted aggression in a paired contest for one of the fish species (*Neolamprologus pulcher*), underscoring the need to validate the predictive ability of mirror assays before generalizing the findings from mirror assays to dyadic contests in different taxa. Mirror tasks may therefore have limited ecological relevance, but could be used as a screening tool to investigate whether exposure to a given pharmaceutical or wastewater warrants further investigation.

7.3.3 What are the implications of exposure-related changes to round goby behaviour and survival following exposure?

Behavioural endpoints are often measured because of behaviour's ecological relevance for an organism's survival and reproductive success in the wild. One could hypothesize that changes to an individual's behaviour will have important fitness impacts, and may therefore scale-up to have potential effects on animal populations (Brodin et al., 2014; Hamilton et al., 2015). I measured aggressive interactions between round goby over a shelter resource because resource ownership is an ecologically relevant consequence of aggressive interactions in this species. In both studies, I found no evidence that fluoxetine exposure impacted the ability of round goby to retain ownership over a shelter (**Chapter 4**), or that wastewater exposure affected the speed at which they "gave-up" the shelter to a larger intruder (**Chapter 5**), even when overall aggression decreased with high dose exposures. These findings would indicate that even though individual aggressiveness was altered, exposure did not have an impact on which fish (exposed resident or unexposed intruder) was expected to own the resource. My findings from **Chapter 3** might help inform this discrepancy. Here, I found that round goby needed time to form preference for a shelter resource, and fish fought harder for the high quality shelter (also used in **Chapter 4** and **Chapter 5**) only after having previous experience with the shelter. It is possible that if exposed fish had experienced the shelter resource for longer (> 90 minutes, the habituation period I used), it may have intensified their territorial aggression during the resource contests. This connection between chapters demonstrates the utility of first investigating the behavioural ecology of a species of interest, in the absence of pollutants, to better understand how a given exposure might impact their behaviour.

If round goby exposed to fluoxetine or wastewater effluent do *not* experience a change in their ability to access and hold resources, then I could hypothesize that this would also be unlikely to change with exposure in the wild. This may indicate that exposed fish would have equal chances of accessing shelter for protection from predation and for reproduction, suggesting that round goby populations would continue to persist in locations receiving wastewater effluent. Of course, there are many aspects of this hypothesis that would need to be explicitly tested and validated with field studies (see section 7.6, Future directions). Especially given that my results from the laboratory exposure to fluoxetine (**Chapter 4**) and laboratory exposure to wastewater effluent (**Chapter 5**) are in contrast to my findings from the field wastewater effluent exposure in **Chapter 6**. Here, exposure reduced survival, without having a measureable impact on round goby behaviour. Based on my findings from **Chapter 6** alone, I could hypothesize that exposure to wastewater effluent under natural conditions selects for fish that are behaviourally and physiologically tolerant of the environmental conditions and pollutants present in close proximity to a wastewater outfall. This could potentially change the phenotypic and genetic characteristics of the round goby population in an exposed habitat across generations if certain adults fail to reproduce before being eliminated from the reproductive population (Fox, 1995; Hamilton et al., 2015). One drawback of using the experimental caging protocol I employed in **Chapter 6** is that it did not allow me address whether animals behaviourally avoid (or are potentially attracted to) wastewater effluent discharge sites.

7.4 Future directions

There are many possibilities for future research based on the results of my thesis. These suggested studies would help us to 1) better understand round goby aggressive and social affiliative interactions, 2) shed light on the impacts of pharmaceutical pollutants on round goby behaviour, and 3) more broadly assist in the development of behavioural tools for better use in ecotoxicology.

7.4.1 Round goby social tolerance and aggressive behaviour

There is much to be learned about round goby aggressive interactions and social tolerance that could inform their biology as an invasive species, and provide a better understanding of how pollutants will affect this species in the wild. If round goby are more aggressive than socially tolerant towards conspecifics—as I observed across the barrier in **Chapter 2**—then it would be interesting to understand how round goby resolve aggressive conflicts. In my studies, I carefully controlled for differences in body mass between competitors in contest aggression trials, but another focus could be to carefully titrate differences in body mass between opponents and explore how round goby establish social dominance. The effects of body mass on social dominance could be first investigated in dyads, and then in triads or larger groups to understand multi-fish hierarchies. Shelter resource proximity could also be manipulated to test the spatial tolerance of round goby for conspecifics; for example, whether larger fish always gain access to resources when they are limited in number, or whether aggressive interactions occur more frequently in densely populated areas. An interesting tool that could be applied to these investigations would be social network analyses that record interactions among groups of individuals to characterize dominance hierarchies (Wey et al., 2008; Krause et al., 2009). Social network tools have proven to be a useful tool for understanding complex animal interactions in the laboratory and in the wild (Dey et al., 2013; Dey & Quinn, 2014).

While laboratory investigations of aggressive interactions allow for careful control of contest scenarios, laboratory studies should be matched with observations of fish in the wild. We currently know that wild round goby are observed caring for offspring in sheltered locations (Corkum et al., 1998; MacInnis & Corkum, 2000), that round goby are present in high densities (Taraborelli et al., 2009; Gutowsky et al., 2011), and that round goby tethered in exposed locations are more likely to be predated (Belanger & Corkum, 2003; Brownscombe & Fox, 2012). Observing aggressive or social interactions over sheltered territories in the wild would elucidate whether dominant fish have greater access to shelter resources, and enhance their fitness through increased reproduction and better survival from predation. This type of field investigation could be conducted with SCUBA or by snorkeling, or with the use of underwater cameras (e.g. GoPros) to track interactions between fish holding shelter resources.

7.4.2 Round goby, wastewater effluent, and the Hamilton Harbour ecosystem

A necessary next step for investigations of the effects of wastewater effluents on round goby in Hamilton Harbour would be to establish whether round goby are naturally exposed to wastewater effluent in receiving environments in the Harbour. We recently documented round goby (and other fish species, e.g. green sunfish *Lepomis cyanellus*, goldfish *Carassius auratus*) in close proximity to the Dundas WWTP outfall (McCallum et al., 2016, *unpublished data*), but still need to investigate their presence near the Woodward WWTP. It would be beneficial to collect fish downstream from wastewater outfalls and test the effects of exposure to wastewater effluent in the field with similar behavioural tasks that I employed in the laboratory (**Chapter 5** and **Chapter 6**). This would provide a full comparison of the behavioural impacts of wastewater exposure on round goby after exposure in the laboratory, in the field using experimental caging, with those fish exposed in the environment.

A study that progresses naturally from documenting whether round goby are close to wastewater outfalls in the field, is establishing whether round goby act as a vector for pharmaceutical transfer to higher trophic levels. Concern for round goby as a contaminant vector has been previously expressed because round goby tolerate highly contaminated locations and prey on dreissenid mussels, which are known to readily accumulate pollutants (Kwon et al., 2006; Marentette et al., 2010; Hebert et al., 2014; McCallum et al., 2014 – Appendix B, McCallum et al., 2016 – Appendix A). Moreover, round goby are concerning for contaminant transfer because they are preyed upon by aquatic vertebrates (water snakes: King et al., 2006, fish: Reyjol et al., 2010), and piscivorous birds (Hebert & Morrison, 2003; Somers et al., 2003). For example, Kwon et al. (2006) showed that polychlorinated biphenyls (PCBs) are transferred from dreissenid mussels to round goby and then from round goby to smallmouth bass. Currently, the degree to which larger aquatic vertebrates and terrestrial vertebrates are exposed to pharmaceutical contaminants is largely unknown (Arnold et al., 2013; Shore et al., 2014). As an ecosystem in transition, and with round goby predicted to play a key role in the diets of many Harbour animals (Hossain et al., 2012), understanding the role that round goby play in mobilizing pharmaceutical contaminants will be especially important for Hamilton Harbour remediation.

7.4.3 Field validation of laboratory behavioural tools

If behavioural assays are to be used to help legislate water policy in order to protect aquatic animals from sub-lethal effects of pharmaceutical exposure, behavioural assays need to be field-validated (Arnold et al., 2014; Robinson, 2009). Currently in Canada, there are no regulations for concentrations of pharmaceutical products in natural waters (Canadian Council of Ministers of the Environment, 2016). Part of the issue associated with regulating pharmaceuticals in the natural environment is linking any observed behavioural (or physiological) effects in the laboratory to the complex field environment. Besides a growing body of literature connecting ethynilestradiol (EE2) exposure to fish feminization and population collapse (Robinson et al., 2003; Kidd et al., 2007; Tyler & Jobling, 2008; Salierno & Kane, 2009), there are limited examples of pharmaceutical-caused deficits to behaviour impacting animal populations.

There is a great need for increasing the "realism" of investigations of pharmaceuticals and wastewater effluent. Increasing realism can be thought of from two perspectives; increasing the realism of the <u>exposure</u> itself, as I have done in my thesis (i.e. single compound exposures → known mixtures of compounds exposures → realistic effluent exposures), and by increasing the realism of <u>ecological context</u> in which these exposures are evaluated (i.e. controlled laboratory → semi-natural settings → field collected/exposed/monitored). See Figure 7.1 for a schematic and review potential methods to evaluate behaviour in more naturalistic settings, with suggestions for more realistic exposure environments. Moving from smaller, laboratory-based testing environments to semi-natural mesocosms or larger flumes that mimic field settings would be a feasible intermediate step between the field and the laboratory for many investigators. Monitoring behaviour in the field is challenging and some options for monitoring include: SCUBA or snorkeling to collect observations of fish or aquatic organisms in the wild, or the use of underwater cameras. However, SCUBA or snorkeling may be undesirable or dangerous in locations contaminated with wastewater effluents.

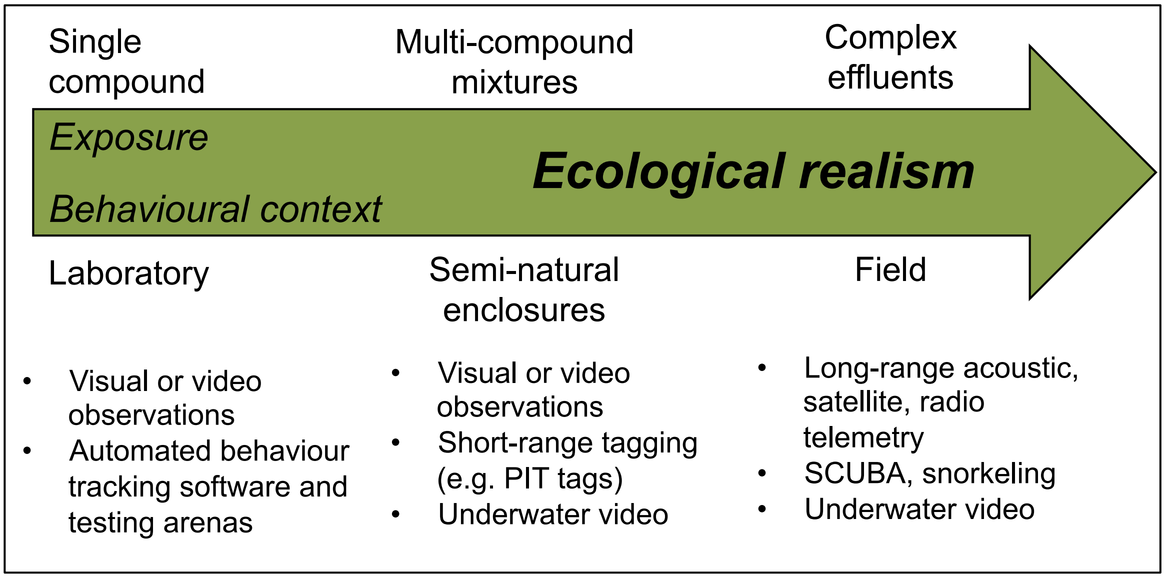

Figure 7.1
Summary of possible exposure scenarios and methods to measure fish behaviour in various environmental testing contexts.

Moreover, placing cameras can also require significant contact with pollutants, and the turbidity often observed in environments receiving wastewater outfalls may make underwater videography less useful (Environment Canada, 2001). A final option for tracking animal behaviour in the wild that has great promise is the use of telemetry technology. This tracking technology has been employed to monitor animals for conservation purposes for over a decade (Hussey et al., 2015). Telemetry technology has recently been applied in studies of aquatic toxicology (Hellström et al., 2016), and is becoming ever-more accessible as prices for tags and equipment decrease. Additionally, the size of tags has also been decreasing to allow them to be implanted in smaller and smaller animals, and the number of parameters a tag can monitor is increasing (e.g. location, depth, acceleration, heart rate: Donaldson et al., 2014; Hellström et al., 2016; Metcalfe et al., 2016).

7.5 Conclusions

My thesis research has investigated the aggressive and social behaviours of the invasive, benthic round goby, and how anthropogenic pollutants modulate these behaviours. I have demonstrated that even though round goby are attracted to conspecifics, they tend to interact with them aggressively. These fish highly value enclosed shelter resources, but require time to assess the true value of a shelter before being capable of responding to this information in an aggressive contest. I have provided an initial investigation into the effects of a commonly used pharmaceutical and of complex wastewater effluents on the behaviours of round goby. More work is now needed to evaluate how common round goby are near wastewater outfalls to determine the local relevance of exposure to fish in highly impacted Hamilton Harbour, Ontario, Canada. I show that pollutant-induced changes in aggression towards a mirror did not reliably predict similar changes in aggression towards a live conspecific. My work underscores the need to validate the ecological relevance of behavioural assays. I encourage the continued use of behavioural endpoints for monitoring the effects of pharmaceuticals and wastewater effluents. Importantly, to protect water quality and create conservation policies that will be protective of wild fish behaviour and aquatic habitat health, we must assess fish behaviour in ecologically relevant settings that will connect exposures in the laboratory to exposures in the wild.

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Appendix A: Diet and foraging of Round Goby (*Neogobius melanostomus*) in a contaminated Harbour

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ABSTRACT

Anthropogenic pollution and the introduction of invasive species are two contributing factors to ecosystem degradation. Although Hamilton Harbour (ON, Canada), a highly impacted ecosystem, is well-studied, the diet, trophic position, and foraging behaviour of the invasive Round Goby (*Neogobius melanostomus*) in this area is not well understood. In this study, we compared digestive tract contents, foraging behaviour, and stable isotope values of Round Goby from sites of low and high sediment contamination in Hamilton Harbour. We also assessed prey availability by conducting sediment invertebrate abundance analyses at these sites. Regardless of site, chironomids, cladocerans, copepods and dreissenids were the most common food items found in Round Goby digestive tracts, and females always had heavier gut contents compared to males. Fish from the high contamination site consumed fewer prey items, had lower gut fullness scores, and fed at a lower trophic level based on lower δ^{13}C and δ^{15}N values. Our results suggest that Round Goby living in highly contaminated areas are feeding less than Round Goby from areas of lower contamination, but that these diet differences do not reflect differences in prey availability. Fish from the high contamination site also typically moved more slowly while foraging. Taken together, these results provide an analysis of the main prey items of Round Goby in Hamilton Harbour, and demonstrate how polluted environments can impact diet, trophic position, and foraging of an introduced fish species.

1.0 INTRODUCTION

Freshwater habitat degradation is often caused by human activities such as pollution or invasive species introductions (Strayer and Dudgeon, 2010). Hamilton Harbour (Ontario, Canada) – the western-most embayment of Lake Ontario – is a highly impacted ecosystem and an International Joint Commission Area of Concern that has been undergoing remediation for the past 30 years (Hamilton Harbour Remedial Action Plan, 1992; IJC, 1999). Remediation efforts in the Harbour have resulted in significant social, economic, and environmental improvements (Hall et al., 2006). One important ongoing remediation goal is the restoration of fish and wildlife populations. Urban runoff, wastewater effluent discharge and combined sewer overflows, as well as historical inputs from industrial steel processing has resulted in habitat degradation and decline in fish populations in Hamilton Harbour (Poulton, 1987; Hamilton Harbour Remedial Action Plan, 1992; Curran et al., 2000). Many fish species in the Harbour have been observed with morphological abnormalities, and fish consumption advisories have been issued for 21 different species due to high concentrations of PCBs, mercury, and pesticides in fish tissues (Hamilton Harbour Remedial Action Plan, 1992; OMOE, 2015). Increased nutrient inputs have made the Harbour eutrophic, which along with water quality fluctuations are thought to contribute to fish population declines (Minns et al., 1994; Hiriart-Baer et al., 2009). Hamilton Harbour's invertebrate community has similarly been degraded by pollution and poor water quality (Dermott and Bonnell, 2010). Both fish and invertebrates have begun to recover with remediation, but still do not meet delisting goals (Dermott and Bonnell, 2010; Brousseau and Randall, 2008). In addition, fish and invertebrate populations have been challenged by repeated introductions of invasive species, such as the Common Carp (*Cyprinus carpio*), and Zebra and Quagga Mussels (*Dreissena polymorpha and D. rostriformis bugensis*; Holeck et al., 2004). Invasive species introductions are of special concern when an ecosystem is unstable, as they impose an extra stressor for native species experiencing already poor conditions (Strayer, 2010).

The Round Goby (*Neogobius melanostomus*) is an invasive species that poses a challenge to Hamilton Harbour ecosystem health and remediation. Originating in the Ponto-Caspian area of Europe, Round Goby are a benthic fish that were introduced to the Laurentian Great Lakes via ship ballast discharge (Jude et al., 1992). Round Goby are extremely successful invaders; they spread quickly throughout all five Great Lakes and continue to invade the surrounding streams and tributaries (Poos et al., 2010). Deterministic back-calculations show that Round Goby likely arrived in Hamilton Harbour in 1994-1995 and had reached establishment densities by 1998-1999 (Vélez-Espino et al., 2010). They were first observed in the Harbour in 1999 (Balshine et al., 2005). Round Goby have had negative impacts on native species for several reasons. As an aggressively territorial species, Round Goby outcompete native fish for food and shelter (Balshine et al., 2005; Bergstrom and Mensinger, 2009). Round Goby aggression has been linked to population declines of native benthic species such as the Johnny Darter (*Etheostoma nigrum*) and Mottled Sculpin (*Cottus baird~~i~~i*) (Janssen and Jude, 2001; Lauer et al., 2004). Round Goby have also been implicated in declines in invertebrate quantity and species richness in the Great Lakes (Kuhns and Berg, 1999; Lederer et al., 2008). Finally, because they may consume contaminated benthic organisms or have constant physical contact with contaminated environments, Round Goby may also play a role in contaminant cycling, facilitating transfer of pollutants to higher trophic levels through their diet (Charlebois et al., 2001). This has been recorded for polychlorinated biphenyls (Kwon et al., 2006), perfluorinated compounds (Kannan et al., 2005) and Type E Botulism (Hebert et al., 2014).

Many of the negative impacts exerted by Round Goby result from their foraging and diet. Understanding the feeding ecology of an invasive species like the Round Goby can inform ecosystem managers of potential paths for further environmental disruption. To date, diet studies of Round Goby in the Great Lakes have revealed a generalist benthic feeder with a diet composed of invertebrates, especially chironomids, cladocerans and dreissenids (Johnson et al., 2008). Studies have also shown an ontogenetic shift in diet to foraging on dreissenid mussels, at approximately 6.0 cm standard length, with larger fish more easily and readily consuming mollusks (Ray and Corkum, 1997). Additionally, because Round Goby can tolerate a wide range of ecological conditions, they can be found in both pristine and degraded areas such as industrial harbours (Roche et al., 2013; McCallum et al., 2014). Indeed, in Hamilton Harbour, Round Goby are equally abundant at sites of high and low sediment contamination (Marentette et al., 2010; McCallum et al., 2014). However, there is little knowledge of their diet in this well-studied ecosystem, even though Round Goby have been identified as an abundant and central species in the Hamilton Harbour food-web (Hossain et al., 2012). The use of diet analyses partnered with stable isotope analyses can provide detailed information on the trophic position of this invasive species (Vander Zanden et al., 1997).

To address how contaminated environments affect Round Goby diet, trophic position, and foraging behaviour, we compared fish from an area of relatively low sediment contaminant burdens (La Salle) and another one of extremely high sediment contaminant burdens (Pier 15, near Randall Reef) in Hamilton Harbour. We quantified gut fullness, identified prey items in gut contents, and assessed prey availability from sediment samples. Based on Round Goby diet studies from other Great Lake locations (Barton et al., 2005; Lederer et al., 2008; Johnson et al., 2008), and invertebrate prey abundance in the Harbour (Dermott and Bonnell, 2010), we predicted that Round Goby would mainly consume chironomids, copepods, cladocerans and dreissenids. Second, we expected to observe the same ontogenetic diet shift reported in other studies with more dreissenid mussels found in larger individuals (Ray and Corkum, 1997). Third, because exposure to toxicants have been shown to decrease general activity, food consumption, and prey capture in fishes (Kasumyan, 2001; Weis et al*.,* 2001; Candelmo et al., 2010), and because fewer organisms might be present in contaminated sediment (Beasley and Kneale, 2002), we predicted that fish from the low contamination site would have fuller guts and more prey items than fish from the high contamination site. We would expect to see this reflected in the stable isotope values, where fish from the low contamination site would have higher trophic position. We also examined feeding behaviour in the laboratory, and predicted that fish from the high contamination site would approach food more slowly and have lower foraging rates relative to fish from the low contamination site (Marentette et al., 2010).

2.0 METHODS

Round Goby were collected from two sites in Hamilton Harbour (Figure 1): Pier 15 (43° 16' N, 79°50' W) and La Salle (43° 18' N, 79° 50' W). Both sites are embayments with a rocky substrate and underlying sand and silt. Across collection years, both sites had similar mean water clarity, dissolved oxygen, temperature, and pH (Supplementary Table 1); however, they differ in the degree of sediment contamination. Sites were selected based on established sediment contamination studies (Hamilton Harbour Remedial Action Plan, 1992; Zeman, 2009). The high contamination site (Pier 15) has a long history of sediment contamination resulting from close proximity to Randal Reef, an area of historic coal tar deposits with high concentrations of polycyclic aromatic hydrocarbons (Hamilton Harbour Remedial Action Plan, 1992; Zeman, 2009). Previous work has shown that total PAHs and total PCBs were higher at the high contamination site, as were sediment concentrations of arsenic, chromium, cobalt, copper, iron, lead, mercury, vanadium, and zinc, exceeding provincial guidelines for probable effect levels (PELs; Milani and Grapentine, 2006; Zeman, 2009), compared to our low contamination sampling site, La Salle. Moreover, Round Goby collected from this high contamination site have higher tissue burdens of copper and cadmium, evidence of fin erosion, higher levels of EROD expression, and males with high vitellogenin levels, feminized external genitalia and higher levels of intersex when compared to fish from the site with lower contamination (Bowley et al., 2010; Marentette et al., 2010).

2.1 Diet and sediment analyses of benthic organisms

A total of 213 fish were collected from La Salle (N = 145) and Pier 15 (N = 68) between June 24 and July 26, 2010 (see Supplementary Table 2 for detailed fish demographics, basic gut contents). Minnow traps were set 1.5 h before sunset (7:30pm), and collected 1.5 h after sunset (10:30pm), as Round Goby are most actively feeding during crepuscular periods (Johnson et al., 2008). Traps were baited with frozen corn enclosed in a nylon bag to ensure no bait was eaten. Fish were euthanized immediately by immersion in a 0.025% benzocaine solution (Sigma Aldrich) and preserved in a 70% ethanol solution, with an incision made in the abdominal cavity to permit ethanol to rapidly penetrate the body wall. In the laboratory, fish were measured with calipers to the nearest 0.01 cm for standard length (SL). The total body mass, liver mass and gonad mass were measured to the nearest 0.001 g using a digital balance (Acculab Vicon). The gut was removed from esophagus to anus, weighed, and then visually rated on a five-point gut fullness scale (adopted from Puvanendran and Brown, 2002). On this scale, 0 = 0% fullness; 1 = 25% fullness; 2 = 50% fullness; 3 = 75% fullness; 4 = 100% fullness. The gut contents were then removed, weighed and the mass of the empty gut was also measured. Gut contents were preserved in 70% ethanol and stored in scintillation vials. All vials were visually inspected for the presence of dreissenids by an observer who was blind to sampling site. The gut contents of 50 randomly selected fish (counterbalancing for site and sex) were examined under a dissecting scope at 2x magnification (Leica MZ75). Items in the gut were counted and identified by taxonomic group.

Sediment samples were collected at La Salle and Pier 15 on June 24 and July 10, 2012. Three samples were collected at each location within 1 m of the shore, 10 m apart, and placed in a 500 ml glass container and preserved with 70% ethanol. In the laboratory, samples were passed through a stack of mesh sieves of 1 mm, 250 µm and 63 µm sizes. Sorted samples were examined under a Luxo KFM magnifier (120V, 220W, 60Hz) and a stereo microscope at 0.63x – 2.5x magnification (Leica MZ75). Samples were sorted and organisms were identified to lowest possible taxonomic grouping. Each sediment sample was placed in a glass dish, dried in an oven (Lab-Line L-C Oven) at 105°C for 24 h, and then cooled for 5-6 h. A top-loading balance (Mettler Toledo, AB204-S/FACT) was used to take the mass of the sample, which was then transferred into a graduated cylinder to record volume.

2.2 Stable isotope analyses

Between June 1 and July 30, in both 2012 and 2013, 119 Round Goby were collected from La Salle and Pier 15 for stable isotope analyses ($N = 52$ fish in 2012 and $N = 67$ fish in 2013; Supplementary Table 2). Additionally, in 2012, we collected 15 dreissenids from La Salle and 20 dreissenids from Pier 15 to serve as baseline primary consumers in the stable isotope analyses. Round Goby were collected using minnow traps as above, but deployed for 24 h. Upon retrieval, fish were euthanized by ice bath immersion followed by cerebral concussion and spinal severance before being transported on ice to the laboratory. Fish were measured with calipers to the nearest 0.01 cm for standard length (SL), and the total body mass, liver mass and gonad mass were measured to the nearest 0.001 g using a digital balance (Acculab Vicon). Then a muscle (dorsal axial) section was taken from each fish, which was placed in a glass scintillation vial, and frozen at -20°C. The dreissenids were transported live to the laboratory, where they were shucked to remove their shells. Dreissenids were placed in individual glass scintillation vials and frozen at -20°C until stable isotope analyses. Frozen tissue samples were freeze-dried and ground to homogeneity using a mortar and pestle. Dreissenid tissues were then lipid extracted using Solvent Distillation with 2x agitation of tissue in 2:1 chloroform/methanol solution at 85°F for 24 h, solvent decanted and then sample air-dried. Round Goby muscle tissues were not lipid extracted because they have a low C:N ratio (< 3.5). Individual samples were then weighed into tin cups (5 mm x 9 mm). Samples and standards were then run for $\delta^{13}C$ and $\delta^{15}N$, C% and N%, using a Delta V IRMS (Thermo Electron Corporation, Waltham, Massachusetts, USA) equipped with an elemental analyzer (Costech, Santa Clarita, California, USA). The abundance of carbon and nitrogen stable isotopes within samples was expressed in delta notation (relative to standard materials) and calculated using the following equation:

$$\delta X\ (‰) = [R_{sample}/R_{standard} - 1]\ \mathrm{x}\ 1000$$

where R is the ratio of nitrogen ($^{15}N/^{14}N$) or carbon ($^{13}C/^{12}C$) isotopes. Pee Dee Belemnite (PDB) carbonate and atmospheric nitrogen were standard reference materials. To assess repeatability every 10th sample was run in triplicate. Precision of analysis from internally run standards run every 12th sample was 0.15 ‰ for $\delta^{15}N$ and 0.1 and 0.08 ‰ for $\delta^{13}C$ (internal fish muscle standard and NIST bovine muscle 8414, $N = 30$). Accuracy based on the difference between standards run internally and certified NIST standards (2 year average ± SE) was 0.03 and 0.02 for $\delta^{13}C$ (NIST 8542, $N = 97$ and 8573, $N = 96$, respectively), and 0.03, 0.10 and 0.17 ‰ for $\delta^{15}N$ (NIST 8573, 8549 and 8548, respectively, $N = 118$-120). The following equation was used to estimate the effect of sampling site on fish trophic position:

$$Trophic\ position = [(\delta^{15}\mathrm{N}_{fish} - mean\ \delta^{15}\mathrm{N}_{mussel})/3.4] + 2$$

Where 3.4 is the diet tissue discrimination factor for $\delta^{15}N$ and represents the change in $\delta^{15}N$ for each trophic position, assuming that dreissenids, as a filter feeder occupy a trophic position of 2 (Post, 2002).

2.3 Foraging behaviour experiment:

Fish for this experiment ($N = 45$; Supplementary Table 2) were collected between September 3 and October 24 2008 as described above. Fish were transported live to McMaster University, and placed in 60 L laboratory stock tanks (60 x 45 x 30 cm) for 48 h in sex and site matched groups. The stock tanks contained ~2.0 cm of aquarium gravel substrate, and a static renewal filter. Fish were fed Nutrafin© fish flakes ad libitum, daily. Experimental tanks (60 L) were similarly set up but were divided in half with a removable, opaque acrylic barrier. One half contained a PVC half-cylinder shelter, while the other half contained a food stimulus placed there before a trial started (the side with the shelter was counter-balanced across the trials). Water temperature in both experimental and stock tanks was maintained at 20-22°C. Experimental foraging trials began by removing a fish from the stock tank, placing it on the side of the experimental tank with the shelter for a 48 h habituation period. Fish were not fed during this habituation period. Before the foraging trial, commercially-available lumpfish eggs were placed on a 6 cm petri dish in the empty half of the experimental tank. For every 5 g of fish mass, 2.5 g of eggs were provided. Foraging observations by an observer blind to sex and collection site began when the opaque barrier was removed and the fish on the shelter side was followed continuously for 15-minutes. We recorded the time the fish spent on each side of the tank, the time taken to enter the food compartment, and the time taken to until the first feed. All subsequent feeds were also recorded. Since Round Goby are more active during dusk and night (Johnson et al., 2008), the trials were conducted during the dark phase of the light cycle using red lights. Following each trial, the fish was removed from the experimental tanks, euthanized with a benzocaine solution, dissected, and measured with calipers and a scale as above.

2.6 Statistical analyses

All statistical analyses were performed using R (version 3.1.2, R Core Team, 2014). In all analyses, site and sex were included as fixed factors. Gut content mass was log transformed to meet model assumptions and analyzed using an ANCOVA, where body mass was included as a covariate. Our gut fullness index was analyzed using an ordinal regression. Abundance of items in the guts was analyzed using a negative binomial regression for count data, with standard length as a continuous covariate. Taxon richness in gut samples was assessed using an ANOVA. The effect of fish size (standard length) on the probability of dreissenids being present in gut contents was analyzed using a logistic regression, with standard length as a continuous predictor. Pearson's chi-square tests were used to test the effect of site and sex on dreissenid mussel presence or absence in the gut. A linear mixed effects model was used to assess Round Goby and dreissenid $\delta^{13}C$ and $\delta^{15}N$, with sampling year was included as a random effect. Additionally, for the $\delta^{15}N$ and trophic position models, the variance was weighted by site due to uneven variances between sampling sites. For the sediment samples, taxon richness and item abundance were scaled by sample volume before analysis. The effect of site on taxon richness and item abundance was assessed using T-tests for estimating model parameters, and permutation tests of the same models to extract accurate p-values, using 10 000 random permutations of the data. Three measures were used to quantify foraging behaviour: latency to enter the food compartment, latency to first feed, and total feeds during the trial. Latency to enter the food compartment and latency to the first feed were analyzed using ANOVA on log-transformed values. Total feeds were analyzed using a generalized linear model assuming a quasi-Poisson error distribution appropriate for count data.

3.0 RESULTS

3.1 Diet and sediment analyses for benthic invertebrates

Of the 213 fish collected for basic diet analyses, three were excluded from further analyses due to poor preservation. Round Goby at the high contamination site (Pier 15) tended to have lighter gut content mass than fish from the low contamination site (La Salle), but this difference did not reach significance (ANCOVA, $F_{Site}(1, 206) = 3.46$, $p = 0.064$), and female Round Goby had heavier gut content mass than males at both sites ($F_{Sex}(1, 206) = 12.26$, $p = 0.00057$: Figure2a). Fish from the high contamination site also had lower gut fullness scores than fish from the low contamination site (ordinal regression, $Z_{Site} = -2.79$, $p = 0.0053$: Figure 2b). Again, females had higher gut fullness scores than did males (ordinal Regression, $Z_{Sex} = -2.35$, $p = 0.019$). Not surprisingly, gut fullness scores and gut content mass were positively correlated (Spearman's rank correlation: $\rho = 0.16$, $p = 0.018$). Larger fish were more likely to have dreissenid mussels present in their gut contents compared to smaller fish (Logistic regression: estimate (± SE): 0.47 (± 0.11), $N = 213$, $Z = 4.37$, $p < 0.0001$). The smallest fish to consume a dreissenid mussel was 5.80 cm standard length. Of fish over 5.80 cm standard length, 26% of them had consumed dreissenids. There was no effect of sex (33% females, 24% males: Pearson's Chi-square: $\chi^2 = 1.38$, $p = 0.24$) or collection site (25% La Salle, 28% Pier 15: Pearson's Chi-square: $\chi^2 = 0.030$, $p = 0.86$) on the total number of dreissenids consumed.

Across the 50 fish sampled for detailed gut content analyses, we identified 13 different types of items. Item richness in the gut samples ranged from 0 – 12 (mean ± SD: 3.48 ± 2.21). Chironomids, cladocerans and copepods were the most common items in the gut samples, and were identified in 74%, 56% and 46% of the samples, respectively. The distribution of the ten most common types of items in the guts is plotted in Figure 3, and a detailed summary can be found in Supplementary Table 3. There was no effect of sampling site or sex on item richness in the gut samples (ANOVA: $F_{Site}(1, 47) = 0.10$, $p = 0.75$; $F_{Sex}(1, 47) = 0.58$, $p = 0.45$). Item abundance in the gut samples ranged from 0 – 95 items per gut, with an average of 11 items being identified per gut sample. Fish scales were the most abundant item, but resulted from many scales being found in only a few gut samples. Copepods and chironomids were the next most abundant items, with a total of 136 and 115 being counted across all the samples, respectively. On average, fish from the high contamination site had fewer items in their guts than fish from the low contamination site (Negative Binomial Regression: estimate(± SE): -0.83(± 0.36), N =50, Z =-

2.31, p =0.021), and there was no effect of body size (estimate(± SE): -0.099(± 0.11), N =50, Z =0.84, p =0.40) or sex on prey item abundance in the guts (estimate(± SE): 0.041(± 0.31), N =50, Z =0.13, p =0.90).

We identified 18 different prey items in the sediment samples. Item richness in the sediment samples ranged from 9 – 16 item types (mean ± SD: 12.9 ± 2.8). Ostracods, copepods, and gastropod shells were the three most common types found in the sediment samples, all being found in 100% of samples. Nematodes and cladocerans were the next most common item types, being identified in 90% of the samples. See Supplementary Table 3 for a detailed summary of sediment analyses. There was no effect of sampling site on item richness (t = 1.27, N =10, permutation p = 0.10), on item abundance (t = 0.0036, N = 10, permutation p = 0.53) in our sediment samples.

3.2 Stable isotope analyses

Male and female Round Goby had similar $\delta^{15}N$ (Linear mixed effects model: estimate(± SE): -0.07(± 0.23), N = 119, t = -0.32, p = 0.76) and $\delta^{13}C$ values (estimate(± SE): -0.06(± 0.28), N = 119, t = -0.23 p = 0.81), and we therefore pooled the data from both sexes and compared these to the baseline values from dreissenids of the same sites. Dreissenids had lower $\delta^{15}N$ values than Round Goby at both sampling sites (estimate(± SE): -4.91(± 0.20), N = 156, t = -24.08 $p < 0.001$: Figure 4a). Both Round Goby and zebra mussels had lower $\delta^{15}N$ values at the high contamination site than the low contamination site (3.3 and 1.4 % difference, respectively; estimate(± SE): -2.77(± 0.22), N = 156, t = -12.36 $p < 0.001$: Figure 4a). Dreissenids had higher (less negative) $\delta^{13}C$ values than did Round Goby at both sampling sites (estimate(± SE): 0.88(± 0.29), N = 156, t = 3.06 p = 0.0026: Figure 4b). Both Round Goby and dreissenids had lower (more negative) $\delta^{13}C$ values at the high contamination site compared to the low contamination site (1.8% and 2.4% lower, respectively; estimate (± SE): -1.87(± 0.23), N = 156, t = -8.17, $p < 0.001$: Figure 4b). Round Goby from the high contamination site had a lower trophic position (estimate(± SE): -0.96(± 0.081), N = 119, t = 60.30, $p < 0.001$: Figure 4c), and trophic position did not differ between the sexes (estimate(± SE): -0.04(± 0.061), N = 119, t = -0.61, p = 0.54).

3.3 Foraging behaviour experiment

Fifteen of the tested fish were excluded because they did not move during the 15-minute trial. Compared to fish from low contamination site, high contamination site fish tended to enter the food compartment later (ANOVA: F(1, 27) = 3.47, p = 0.07; Supplementary Figure 1a), and tended to take longer to make their first feeding attempt (F(1, 27) = 3.01, p =0.094; Supplementary Figure 1b), however, these effects did not reach statistical significance. Total feeding strikes taken did not differ between fish from the different sites (Quasi-Poisson generalized linear model: estimate (standard error): -0.018 (0.46), t = -0.039, p = 0.97; Supplementary Figure 1c). There was no effect of sex on foraging behaviour (effect of sex, all comparisons $p > 0.10$)

4.0 DISCUSSION

We found that chironomids, cladocerans, copepods and dreissenids were the most abundant diet items in Round Goby from Hamilton Harbour, and this fits well with results from other Round Goby diet studies from coastal areas of Lake Michigan and Lake Huron (Barton et al., 2005; Lederer et al., 2008; Cooper et al., 2009), and from eastern Lake Ontario (Johnson et al., 2008;

Brush et al., 2012). In our study, 26 % of the fish consumed dreissenids: all fish that ate dreissenids were larger than 5.8 cm (standard length), supporting the size-dependent diet shift that has been documented in the past (Ray and Corkum, 1997). Many Round Goby in our study did not consume any dreissenids possibly because Hamilton Harbour is one of the few shallow areas of the Laurentian Great Lakes that has not been heavily invaded by dreissenids (Gerlofsma et al., 2007). Additionally, Round Goby have been shown to prefer other invertebrate prey types over dreissenids in laboratory experiments (Diggins et al., 2002), and fish fed exclusively dreissenids had reduced growth (Coulter et al., 2011). Given the abundance of other prey items consumed by the fish in our study, and the potential cost of consuming only dreissenids, Round Goby may be favouring other invertebrates that are easier to handle (Brush et al., 2012). We also found a sex difference in Round Goby diet, where females had fuller and heavier digestive tracts. While female Round Goby are not restricted to any specific territory and can continue feeding throughout the breeding season, males defend a territory and offspring (Corkum et al., 1998). Paternal care is energetically costly for males and restricts their foraging opportunities during the breeding season (Bose et al., 2014). Similar sex-differences in gut fullness have been reported in Round Goby from their invasive range in Europe and in other goby species with male-only parental care (Salgado et al., 2004; Brandner et al., 2013).

Round Goby were consuming benthic prey items that were abundant in their environment. Our sediment analyses revealed chironomids, cladocera, copepods, ostracods, dreissenids and gastropods to be the most abundant prey items available across sites. Our results confirm findings from earlier, detailed analyses of Hamilton Harbour sediments across multiple years by Gerlofsma et al. (2007) and Dermott and Bonnell (2010) showing these invertebrate groups to be very abundant in the Harbour. Some notable potential prey items that were present at high frequencies in the sediment, but were *not* present or common in the digestive tracts, included nematodes, Turbellaria, bryozoan statoblasts, and oligochaetes (e.g. oligochaetes were present in 67% of sediment samples versus 8% of gut samples). These items all tend to be soft-bodied compared to other prey items that were observed, and thus could have been digested before identification. Alternatively, Round Goby may avoid these prey items in favour of other prey that may be easier to handle or find. Lastly, if these prey items are patchy in the environment, then it is possible that we would need an even more intensive sampling study to capture the complete range of the prey items consumed by Round Goby at each site.

Round Goby from our high contamination site had fewer prey items in their guts and lower gut fullness scores compared to fish from our low contamination site. These findings are not a result of lower prey availability at the high contamination site. In contrast to our predictions, and in contrast to findings of previous studies (Beasley and Kneale, 2002), benthic invertebrate abundance and diversity were not lower at the site with high contamination (Pier 15). Fewer prey in the guts and lower gut fullness scores at the high contamination site also do not appear to be caused by Round Goby being more selective of the types of prey items consumed, as we found similar prey item richness in the guts and in the sediment samples from both sites. Moreover, the top five types of prey in the guts were similar between Round Goby from each site. Our observations of lower gut fullness scores at the high contamination site could be the result of more direct effects of contaminants on foraging behaviour. We observed that fish from the high contamination site tended to initiate feeding more slowly. Though this trend did not reach statistical significance, previous studies have shown that Round Goby from this high contamination site had decreased activity levels (Marentette et al., 2012). Moreover, exposure to contaminants such as polycyclic aromatic hydrocarbons and metals like those documented at our high contamination site are known to decrease activity in other fish species (Kasumyan, 2001; Weis et al., 2001; Candelmo et al., 2010) and in Round Goby (Leonard et al., 2014).

We found that Round Goby had higher $\delta^{15}N$ and higher $\delta^{13}C$ at the low contamination site, and similar trends were also observed in the dreissenids. Round Goby and dreissenids at both sites had very similar $\delta^{13}C$ values, which suggests similar carbon sources. Round Goby had much higher $\delta^{15}N$ then dreissenids at both sites, as would be expected given their higher position in the ecosystem. When $\delta^{15}N$ was used to calculate tropic position, we found that Round Goby from the low contamination site had a trophic position estimate of 3.5, while Round Goby from the high contamination site had a much lower trophic position estimate of ~2.5. This difference does *not* match the gut content findings, and would suggest that Round Goby at the low contamination site are feeding on different items than Round Goby from the high contamination site. This may be a result of the isotope values reflecting differences in feeding over longer time periods compared to the "snap-shot" nature of stomach contents. However, a trophic position estimate of ~2.5 at the high contamination site would suggest Round Goby are partially feeding on primary production. This is highly unlikely given the results of our study and other published diet studies (Barton et al., 2005; Lederer et al., 2008; Cooper et al., 2009; Brush et al., 2012). This trophic value for Round Goby may also be driven by the trophic position assigned to our baseline dreissenids that was taken from an established average in the literature (Post, 2002). As there is always variation around this average, it is possible that the dreissenids in Hamilton Harbour occupy a higher trophic position than the literature average, which would also increase the trophic position of Round Goby. It is not possible to address the above issues without additional stable isotope analyses, and future work will use multiple sampling spatially and temporally (Syväranta et al., 2006).

The difference in stable isotope values observed between sites for both dreissenids and Round Goby likely stem from proximity to point sources of nitrogen input, such as wastewater treatment plant effluent (Carey and Migliaccio, 2009). The low contamination site is located slightly closer (~3.5 km) to a wastewater treatment effluent source than the high contamination site, ~5.1 km. Interestingly, when compared with stable isotope values obtained for Round Goby elsewhere in the Great Lakes (Barton et al., 2005; Brush et al., 2012, Pettit-Wade et al. 2015), Round Goby in Hamilton Harbour possess very high $\delta^{15}N$, consistent with previous stable isotope values reported for the food web of Hamilton Harbour (Ryman, 2009). Even Round Goby measured directly outside the Harbour entrance in Lake Ontario had lower $\delta^{15}N$ than those within the Harbour (Pettitt-Wade et al., *in-prep*), suggesting that proximity to sources of nutrient input can influence stable isotope values even at small scales (< a few kms). The eutrophic nature of Hamilton Harbour (Hiriart-Baer et al., 2009) is likely due to multiple nitrogen-rich wastewater effluent sources and combined sewer overflow input across the Harbour (Hamilton Harbour Remedial Action Plan, 1992). Yet, as our isotope measurements suggest, eutrophication within Hamilton Harbour is likely to be heterogeneous and centered on sites of high nutrient input. We therefore stress the importance of accounting for proximity to environmental sources of nitrogen and carbon when measuring stable isotopes.

In this first diet and foraging analysis of Round Goby from a highly contaminated ecosystem, we show that fish from a contaminated site consumed fewer prey, had emptier digestive tracts, and occupied a lower trophic position. These results were not driven by prey availability, and instead may be related to foraging behaviour of fish exposed to contaminants. As remediation goals for Hamilton Harbour include improving aquatic biodiversity, our results indicate that the abundant Round Goby could negatively impact (via predation pressure) the invertebrate community in Hamilton Harbour, with similar impacts elsewhere in the Great Lakes (Kuhns and Berg, 1999; Lederer et al., 2008). We show that benthic organisms comprise a large portion of the Round Goby diet. Because these benthic organisms, especially dreissenids, accumulate toxicants and chemicals from the sediments and the water column (Reynoldson, 1987), this fish in a key position in the food web may be crucial in mobilizing contaminants to higher trophic levels (Hossain et al., 2012). Hence, future research assessing contaminant burdens in invertebrates and dreissenids from highly contaminated sites, and their possible transfer to Round Goby and larger predators is a necessary step to identify contaminant transfer in the Hamilton Harbour ecosystem. Stable isotope analyses will certainly continue to be an important tool for understanding these trophic relationships and monitoring eutrophication in the aquatic community of Hamilton Harbour. Our study demonstrates how diet, trophic, and foraging analyses can provide a rich understanding of the aquatic community in Hamilton Harbour, and shows that the abundant and invasive Round Goby can be used as an indicator of ecosystem health.

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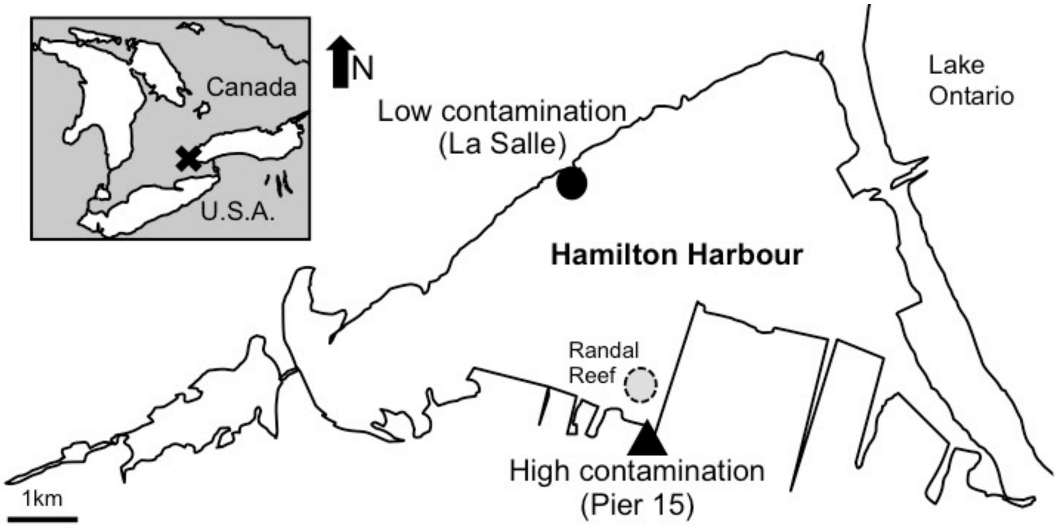


Figure 1: Hamilton Harbour map indicating the low contamination site (La Salle – circle) and high contamination site (Pier 15 – triangle). Randal Reef (grey circle), a historic coal tar deposit, is also marked.

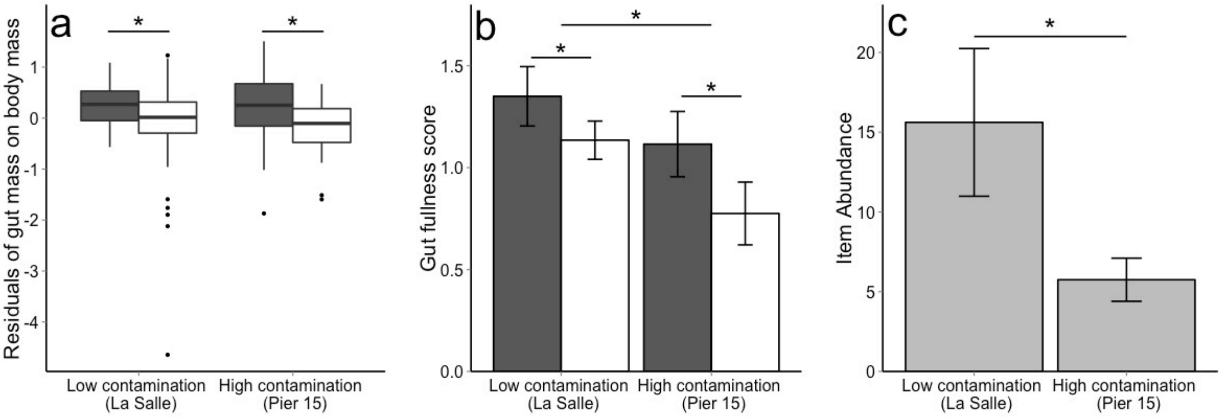


Figure 2 **a)** Residuals of gut content mass on body mass plotted by sampling site and sex (females: dark grey, males: white). Box hinges represent the first and third quartile, whiskers show 1.5 * inter-quartile range from hinges, and points show outliers. **b)** Average gut fullness scores (as rated from 0 – 4) plotted by sampling site and sex (females: dark grey, males: white). **c)** Average prey item abundance found in guts plotted by site, with sexes combined. In all panels * indicates $p < .05$, error bars indicate ± SE.

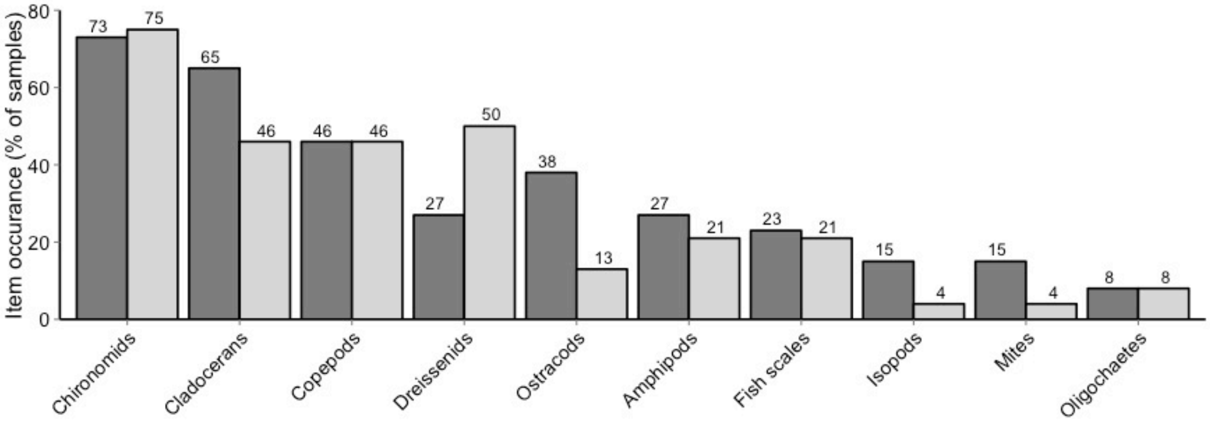


Figure 3: Occurrence of the top ten prey items in Round Goby gut samples plotted by site, where darker bars = low contamination site (La Salle), and lighter bars = high contamination site (Pier 15).

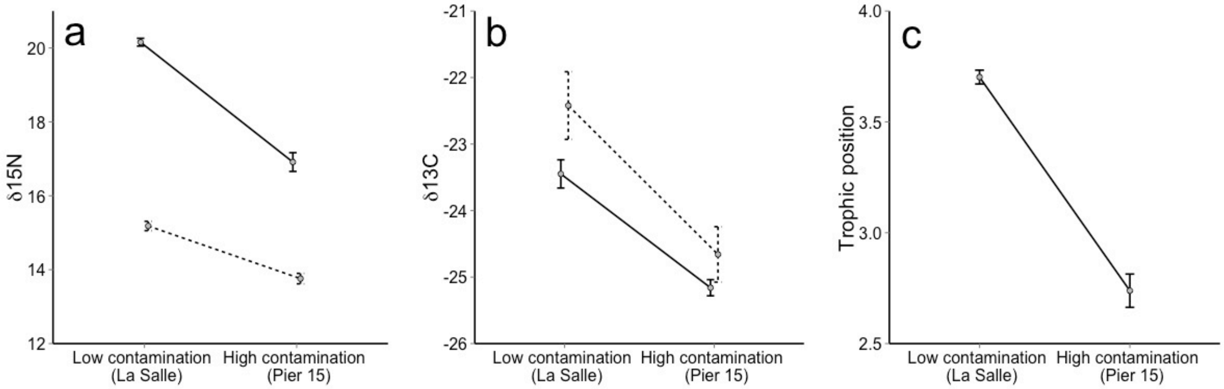
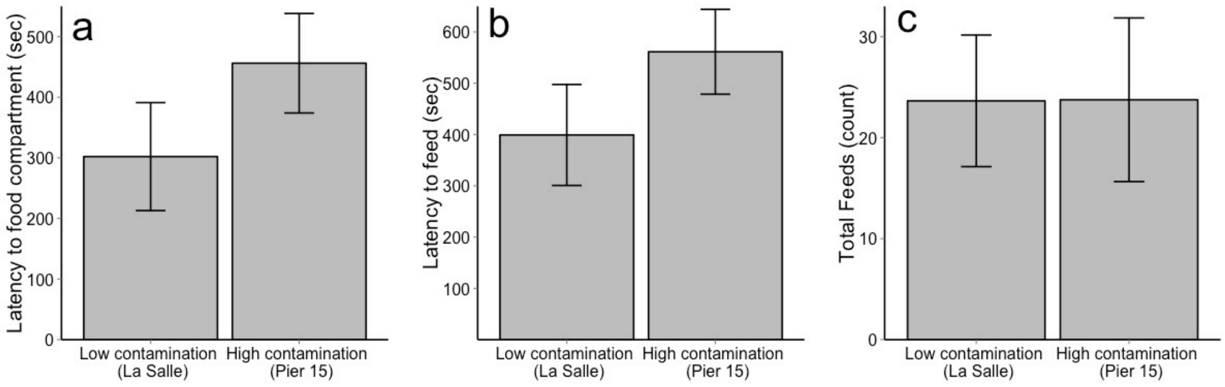


Figure 4. **a)** Average δ^{15}N (%) plotted by sampling site and species. **b)** Average δ^{13}C (%) plotted by sampling site and species. **c)** Average trophic position for Round Goby plotted by sampling site. In all panels: Round Goby: solid line, dreissenids: dashed line, error bars indicate ± SE

ONLINE APPENDIX

Supplementary Figure 1: In all panels, error bars indicate ± 1 standard error. **a)** Average latency found Round Goby to enter the food compartment plotted by sampling site. **b)** Average latency for Round Goby to begin feeding plotted by sampling site **c)** Average number of feeding strikes at the food stimulus plotted by site.



Supplementary Table 1: Average water quality measures (mean ± SE) at both sampling sites for years 2008 – 2013. Samples were taken twice monthly, between May – August each year. Clarity was recorded using a secchi disk, temperature, dissolved oxygen with a YSI 550A field meter, and pH was recorded using a Oakton Multiparameter PT Testr 35.

	Low contamination	**High contamination**	**ANOVA**	***p* value**
Clarity	0.92 m ± 0.21	0.96 m ± 0.08	$F_{site}(1, 60) = 1.32$	$p = 0.26$
Temperature	21.1°C ± 4.2	21.6°C ± 4.3	$F_{site}(1, 62) = 0.42$	$p = 0.52$
Dissolved oxygen	13.0 mg/L ± 6.7	12.4 mg/L ± 6.9	$F_{site}(1, 62) = 0.13$	$p = 0.72$
pH	8.5 ± 0.5	8.5 ± 0.3	$F_{site}(1, 52) = 0.29$	$p = 0.59$

Supplementary Table 2: The number of Round Goby used in each experiment grouped by sex and sampling site, with average standard length and mass of for each group.

	Low contamination				**High contamination**			
	Male		Female		Male		Female	
Experiment	*N*	SL (cm) Mass (g)	*N*	SL (cm) Mass (g)	*N*	SL (cm) Mass (g)	*N*	SL (cm) Mass (g)
Diet Analyses								
Basic gut contents	105	9.05 17.51	40	7.70 10.22	42	6.64 6.99	2 6	6.84 7.97
Detailed gut contents	13	8.75 14.49	13	8.26 12. 43	12	6.98 7.95	1 2	6.76 8.05
Stable Isotopes	32	7.61 14.71	16	5.59 4.90	39	5.29 4.01	3 2	4.49 2.35
Foraging Behaviour	12	7.38 10.30	12	6.64 7.61	10	7.29 9.49	1 1	6.37 6.21

Supplementary Table 3: The percent occurrence of all prey items categorized in Round Goby gut samples and sediment samples, grouped by phylum and sub-grouped by the next level of identification. (-) indicates that prey item was not identified in any sample.

	Gut Samples			**Sediment Samples**		
	Low contamination (La Salle)	High contamination (Pier 15)	Total (*N* = 50)	Low contamination (La Salle)	High contamination (Pier 15)	Total (*N* =8)
Crustacea						
Amphipoda	27	21	24	75	33	54
Ostracoda	38	13		100	100	100
Cladocera	65	46	56	100	83	92
Copepoda	46	46	46	100	100	100
Isopoda	15	4	10	25	66	46
Mysida	-	-	-	75	16	46
Arthropoda						
Chironomidae	73	75	74	100	50	75
Mite	15	4		100	100	100
Coleoptera (beetle)	-	-	-	25	50	38
Misc. insect	1	-	1	-	-	-
Mollusca						
Dreissena	27	50	39	75	100	88
Fingerling clam	1	0	1	75	50	63
Gastropoda	3	1	2	100	100	100
Annelida						
Oligochaeta	8	8	8	50	83	67
Nematoda						
Nematoda	-	-	-	75	100	88
Platyhelminthes						
Turbellaria	-	-	-	100	50	75
Bryozoa						
Plumatellidae (statoblasts)	-	-	-	100	66	83
Tardigrada						
Tardigrada	-	-	-	50	50	50
Chordata						
Fish scales	23	21	22	75	50	63

Appendix A: Persistence of an invasive fish in a contaminated ecosystem (*Neogobius melanostomus*)

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ORIGINAL PAPER

Persistence of an invasive fish (*Neogobius melanostomus*) in a contaminated ecosystem

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Abstract Post-establishment dynamics of invasive species have been under-studied. However, understanding these dynamics is particularly important for the management of invasive species known to impact native communities. Following the invasion of a highly invasive species, the round goby (*Neogobius melanostomus*), we document long-term population changes after establishment and address how population dynamics of a successful invader change through persistence and integration. Round goby present a threat to the areas they invade by out-competing native species for resources. Furthermore, as a pollution tolerant species, round goby present a second threat by acting as a possible vector for contaminant transfer to higher trophic levels in invaded ecosystems with areas of contamination. We sampled round goby for 11 years (2002–2012) at four low contamination sites and two high contamination sites within Hamilton Harbour ON, Canada, an International Joint Commission Area of Concern. Across sampling years, we show that round goby abundance has declined at low contamination sites, while remaining stable at high contamination sites. Moreover, we show that average body size decreased and reproductive investment increased both across sampling years and between sites of low and high contamination. Our results document population demographic shifts in a persisting invasive species, and underscore the importance of management practices for this species in contaminated environments.

Keywords Population dynamics · Round goby · Hamilton harbour · Contamination · International area of concern

Introduction

Most research on the introduction and establishment of an invasive species in a new environment has focused on documenting the early stages of species invasions (i.e. the spread and establishment phases), while potential for eradication is still viable.

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Understanding the persistence and integration of invasive species into an invaded area over a longer time-scale has received less attention (Puth and Post 2005). Although eradication becomes less feasible, documenting how an invasive species integrates into a new environment can help to inform management of the species, and aid in controlling damage to the invaded environment (Andersen et al. 2004). This is especially important when a well-established invasive species is known to have an extensive and profound impact on native species, potentially leading to long-term and significant declines in the ecological and economic productivity of an invaded ecosystem (Davis 2009; Lockwood et al. 2009).

The round goby (*Neogobius melanostomus*) invasion of the Laurentian Great Lakes is one example of a well-established invasive species with widespread impacts on the environments it has invaded. This small, benthic fish—native to the Black and Caspian Seas of Europe—was introduced to the Great Lakes via ship ballast water discharge in the early 1990s (Jude et al. 1992). As a multiple spawner with a long breeding season (~3–4 months), invading round goby spread quickly (Corkum et al. 1998). Since 1990, they have spread more rapidly throughout all five Great Lakes than any previous aquatic invader (Corkum et al. 2004; Kornis et al. 2012). In addition to their reproductive habits, a number of behavioural and physiological characteristics have contributed to the success of their invasion, and the resultant concern for the invaded environments. Round goby are opportunistic foragers, and are known to consume the eggs of larger fish species important for local fisheries (Fitzsimons et al. 2006; Roseman et al. 2006; Steinhart et al. 2004). Round goby are highly aggressive in interspecific interactions, and are associated with the decline of native species using the same habitat (Bergstrom and Mensigner 2009; Balshine et al. 2005; Janssen and Jude 2001; Dubs and Corkum 1996). Taken together, these characteristics suggest that round goby will pose a threat to the balance and health of the ecosystems they invade, and the productivity of fisheries in these areas.

In addition to their interactions with and impacts on native species, round goby present another pressing concern for invaded areas, they are a potential vector for contaminant transfer to higher trophic levels (Poste and Ozersky 2013; Kwon et al. 2006; Hanari et al. 2004). As a mussel specialist, round goby readily ingest contaminants sequestered in tissue of filter-feeding mollusks (Lederer et al. 2006; Gossiaux et al. 1998). Moreover, as a benthic species, with a small home-range (Marentette et al. 2011; Ray and Corkum 2001) round goby have the potential to accumulate contaminants directly from sediment and the water column in highly impacted areas (Bowley et al. 2010; Marentette et al. 2010). These contaminants can then be passed to higher trophic levels via multiple predator pathways, as round goby are a known prey species for water birds (Jakubas 2004; Somers et al. 2003), water snakes (King et al. 2006), and larger fish species (Reyjol et al. 2010; Taraborelli et al. 2010; Dietrich et al. 2006; Truemper and Lauer 2005). Round goby are often reported to exist in polluted, as well as pristine, aquatic environments, and are thought to be a pollution tolerant species (Pinchuk et al. 2003). Consequently, it is feasible for round goby to act as a sentinel species to ascertain how invasive species demographics may be affected in contaminated habitats, and assess the potential for contaminant transfer in the ecosystem.

We have monitored round goby in Hamilton Harbour, ON, Canada for 11 years (2002–2012) to address two inter-related questions about species invasions. First, by monitoring round goby population demographics after their establishment in Hamilton Harbour (Vélez-Espino et al. 2010), we addressed how the population demographics of a successful invader are altered as they integrate and persist in a non-native ecosystem. Second, as Hamilton Harbour is an area with long-term heterogeneous contamination from industrial steel production, urban run-off and combined sewer overflows (RAP 1992, 2002), we can use round goby as a sentinel species to assess how a stressor, contamination from multiple sources, affects the population demographics of an established invasive species. Moreover, we assess the potential for this invasive species to be an ecosystem stressor by acting as a vector for mobilizing contaminants up trophic levels. To answer these questions we assessed a suite of demographic parameters, including: fish abundance, body size, body condition, proportion of the population in reproductive condition, gonadosomatic index (GSI), and the relative frequency of male alternative reproductive tactics (round goby males come as one of two morphs, guarding males and sneaker males; Marentette et al. 2009).

A previous study tracked the round goby population in Hamilton Harbour over a shorter period (Young et al. 2010), and found a decline in round goby abundance across time. We therefore predicted that round goby abundance in the Harbour would continue to decline and possibly stabilize as this species integrate into the ecosystem through predator–prey interactions. As mentioned previously, diet shifts to include more round goby have been documented in a number of predator species (Reyjol et al. 2010; Taraborelli et al. 2010; Dietrich et al. 2006; Truemper and Lauer 2005; Jakubas 2004; King et al. 2006; Somers et al. 2003), but a complete population crash of round goby would be highly unlikely without an extreme weather or disease event because this species is such a well-established invader with a high and rapid reproductive capacity (Davis 2009; Bomford and O'Brien 1995). As a predation response strategy, we predicted that on average body size and size-at-first-reproduction would decrease over time in the round goby population. This decrease in body size in response to predators is predicted to occur based on classic life-history theory models (Stearns 1976), and such shifts have been abundantly documented in aquatic invertebrates, (Ball and Baker 1996; reviewed in Riessen 1999), and in aquatic vertebrate species (Hernaman and Munday 2005; Johnson 2001; Reznick et al. 2001; Chivers et al. 1999). Again, we expected these patterns to stabilize over time as predator–prey interactions equilibrated. In addition, we assessed the relative frequency of round goby male alternative tactics over time, as it was previously predicted the guarding male morph would be more abundant earlier in the invasion process (Marentette et al. 2009). Finally, as a result of physiological contaminant burdens and endocrine disruption observed in fish from contaminated sampling areas (Marentette et al. 2010; Bowley et al. 2010), we predicted that round goby from sites with higher contamination would be less abundant, smaller, and have altered reproductive investment patterns compared to the fish from sites with lower contamination. Such trends have been observed in round goby and other fish species from contaminant burdened environments (Marentette et al. 2010; Kruitwagen et al. 2006; Canli and Atli 2003; Rowe 2003).

Methods

Sampling sites and collection methods

The data for this study extends the collections described in Young et al. (2010). Between 2002 and 2012, we sampled round goby in Hamilton Harbor, ON, Canada (43°N, 70°W), twice per month, from May through October of each sampling year. We collected round goby from the following sites: La Salle Park, Grindstone Creek, Desjardins Canal, Fisherman's Pier, Pier 27, and Sherman Inlet (43°N, 79°W; Fig. 1). The first four sites represent sites of lower contamination in Hamilton Harbour, while the latter two sites represent sampling sites with higher contamination (Zeman 2009). Sampling was conducted at low contamination sites from 2002 to 2012, while the high contamination sites were sampled only from 2006 to 2012. Choice and categorization of these sites was based on their proximity to contaminant sources (Marentette and Balshine 2012; Marentette et al. 2010). Hamilton Harbour is an International Joint Commission Area of Concern (International Joint Commission 1999), but contamination within the Harbour is heterogeneously distributed, and areas of highest contamination are associated with Randal Reef and the Windermere Arm (Zeman 2009; Pozza et al. 2004; RAP 2002, 1992; Fig. 1). These areas contain pollutants from historical industrial steel processing, extensive urban run-off, as well as combined sewer overflows and wastewater effluent discharge. The most prominent and concerning contaminants in these areas are polycyclic aromatic hydrocarbons, polychlorinated biphenyl, and metals such as lead, zinc, and cadmium (Zeman 2009; RAP 1992).

At each site, we sampled round goby using minnow traps baited with approximately 25 g of frozen corn kernels. Two traps were set at each site from the years 2002–2004, and four traps were set at each site from the years 2005–2012. Traps were set at least 10 m apart, each at a depth of 1 m, and approximately 5 m from the shoreline. Traps were recovered 24 h after being set, any traps that had washed up on shore, been accidentally opened, or intentionally tampered with were excluded. All fish were counted per trap and sexed by examining the urogenital papilla (Miller 1984). Any fish that were unable to be sexed were recorded as juveniles. Water

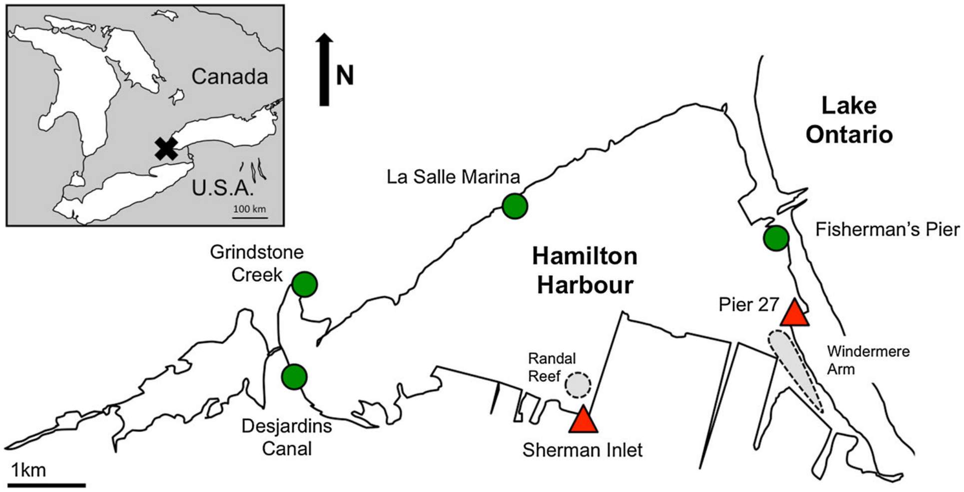

Fig. 1 A map of Hamilton Harbour, ON, Canada (43°N, 79°W), the western-most embayment of Lake Ontario, with sampling sites and areas of high contamination undergoing remediation plotted. *Circle* site markers show low contamination sampling sites, and *triangle* site markers show high contamination sampling sites. *Gray with black-hatched borders* show two highly contaminated areas of Hamilton Harbour undergoing remediation (RAP 1992, 2002). A *scale bar* depicts distance in kilometers

quality was assessed on each sampling date, and at each sampling site, by measuring water temperature, dissolved oxygen (Lamonte tracer probe), pH (YSI 550A and a multi-parameter 35 probe), and water clarity (Secchi disk). Fish were then euthanized and brought to the laboratory on ice for further analysis.

Morphological measurements

In the laboratory, round goby morphological parameters were measured. Standard length (snout to caudal peduncle), head width, and papilla length were taken using calipers measuring to the nearest 0.1 cm. Body mass, liver mass, and gonad mass were measured to the nearest 0.001 g using a digital balance (Acculab Vicon Digital Scale). Body condition was then determined using Fulton's body condition index ($10^5 \times$ [body mass (g)/standard length (mm)^3]) (Ricker 1975). Gonad mass was taken from 2004 onwards, allowing the GSI to be calculated for each fish as 100 × [gonad mass (g)]/[body mass (g) − gonad mass (g)] (Schreck and Moyle 1990). Round goby were classified as reproductive if their GSI exceeded 1 % for males and 8 % for females (Marentette and Corkum 2008; MacInnis 1997).

Statistical analyses

Statistical tests were performed using R version 2.15.2 (R Core Team 2012). Quantile–quantile plots were used to visually check normality. Population abundance, standard length, body mass, body condition, GSI, proportion reproductive, and the proportion of male reproductive tactics were analyzed by fitting data to linear mixed effects models using the "nlme" package (Pinheiroet al. 2013). We controlled for unknown among-site differences by allowing for sampling site to act as a random effect in our models. We included a linear effect of time, and the categorical effects of sex (male and female) and site type (low or high contamination) in our models. The model for population abundance also included a quadratic effect of time in order to test whether the rate of decrease of round goby population abundance was changing over time. Generally, year was centered at 2006, the first year of data collection at contaminated sites. However, the population abundance model was centered at 2002 when it was run for low contamination sites only. Any non-significant interactions were subsequently removed from further analyses. For each model, sample size for number of individuals (n) and either number of sites by year combinations (n_{s*y}), or number of sites by year by sex combinations (n_{s*y*s}) is given. With the exception of the analysis of population abundance, juveniles were not included in any of our models because sex cannot be assigned to juvenile fish.

We used number of fish per trap as a measure of population abundance, as this helped account for

Table 1 Total number of round goby collected, partitioned by sex, site, and year

	DC		GC		LS		FP		P27		SI	
	M	F	M	F	M	F	M	F	M	F	M	F
2002	226	128	90	64	185	88	287	118	–	–	–	–
2003	52	13	17	16	61	27	76	14	–	–	–	–
2004	77	72	46	58	51	26	90	57	–	–	–	–
2005	157	66	77	30	226	73	131	72	–	–	–	–
2006	180	56	59	24	193	98	116	64	9	6	83	45
2007	116	57	20	19	142	47	97	47	156	99	80	89
2008	78	35	16	4	71	29	42	21	65	16	62	51
2009	42	31	13	10	68	40	32	19	–	–	–	–
2010	41	14	30	6	180	69	125	41	192	68	69	58
2011	93	65	18	8	191	73	137	80	150	101	218	166
2012	88	64	32	10	136	48	161	78	202	109	108	64

sampling irregularities such as trap theft or breakage. Occasionally, extra traps were set at the four sites (away from the population sampling study traps) to collect fish for other experiments. These fish were excluded from the population abundance counts, but were measured and included in analysis of morphological characters. Hence, the sample sizes for population abundance analyses and morphological characteristic analyses are not identical. Male reproductive morph (guarding male or sneaker male) was assigned based on a linear discriminant analysis that included the following variables: seminal vesicle mass, seminal vesicle mass to testes mass ratio, and head width to standard length ratio. Guarding males typically have larger seminal vesicles and head widths (Marentette et al. 2009). Male round goby with reproductive tactics that could not be predicted with 80 % confidence were labeled as 'unknown'.

Ethical note

All methods for handling round goby were approved by McMaster University's Animal Research Ethics Board and adhere to the standards of the Canadian Council on Animal Care.

Results

Over the 11-year study, 9,052 round goby were collected from Hamilton Harbour. Of the fish caught, 8,666 could be sexed, and 363 fish were classified as sexually immature juveniles. An additional 23 fish were not recorded as males, females or juveniles, and were excluded from subsequent analyses. Overall, many more males ($n_{\text{male}} = 5{,}715$) were caught than females ($n_{\text{female}} = 2{,}951$), and when we examined the sex ratio on a trap basis, more traps were male-biased than female-biased [effect size (mean ± standard error) = 1.30 ± 0.20, $n = 8{,}666$, $n_{s*y} = 112$, $p \leq 0.001$: see Table 1 for abundance summary].

When we fitted a quadratic trend model to the population abundance data (from lower contamination sites only, allowing for among-site variation both in population abundance and in the quadratic trend), this model revealed a decrease in population abundance across time. The initial slope of the decline was 1.20 ± 0.40 fish/trap/year, but the magnitude of the decline decreased over time by 0.08 ± 0.03 fish/trap/year/year (i.e. a positive quadratic term). However, due to the small number of sites (only four) and the large variation among sites, these trends were not significant ($p_{\text{linear}} = 0.08$, $p_{\text{quadratic}} = 0.12$). We then fitted a model to the combined abundance data from low contamination and high contamination sites (Fig. 2) and incorporated differences in the time trends between low contamination and high contamination sites, removing these when non-significant. This created a model with linear and quadratic fixed effects of time and random among-site variation in these terms, with an additional term quantifying the

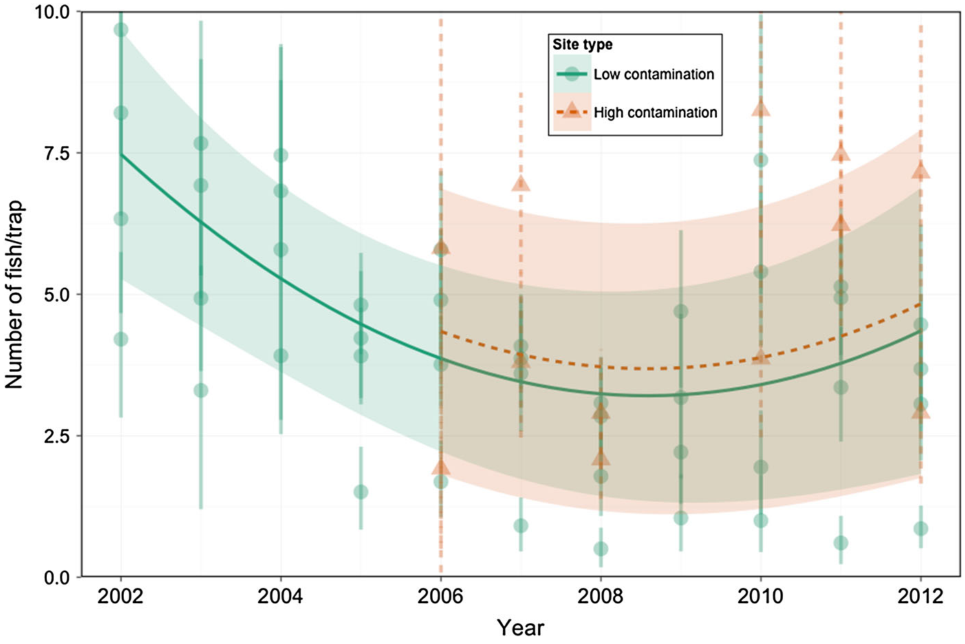

Fig. 2 Population abundance with *smooth lines* showing the predictions of a linear mixed effects model separated by site type (i.e. high or low contamination) with a quadratic trend in time. *Ribbons* indicate 95 % confidence intervals of the model predictions. *Background points* show mean number of fish per trap for each individual sampling site (*triangles* denote high contamination sites, *circles* denote low contamination sites), while *bars* around these *points* show 95 % confidence intervals for the mean value at each site

difference between low contamination and high contamination site types. With the augmented data sets, both the linear and quadratic terms were significant, and we found an estimated decrease of 0.51 ± 0.12 fish/trap/year in 2006 ($p = 0.01$), with the magnitude of this decline decreasing by 0.10 ± 0.03 fish/trap/year ($p = 0.03$). The estimated difference in population abundance between low and high contamination sites was small, and not statistically significant (0.48 ± 1.50 fish/trap, $p = 0.80$).

Next, we fitted linear trend models to data for body condition (Fig. 3a), body length (Fig. 3b) and body mass. Average body length was 7.00 ± 0.02 cm (range 3.40–13.20 cm), and average body mass was 10.10 ± 0.08 g (range 0.90–64.80 g), where males were longer and heavier than females (length: effect size = 1.10 ± 0.08 cm, $n = 9{,}438$, $n_{s*y*s} = 112$, $p \leq 0.001$; mass: effect size = 5.10 ± 0.30 g, $n = 9{,}439$, $\mathrm{n}_{s*y*s} = 112$, $p \leq 0.001$). Males were also in better body condition than females (effect size = 0.05 ± 0.02 g/mm^3, $n = 9{,}434$, $n_{s*y*s} = 112$, $p = 0.02$). Across years, body length and body mass decreased (length: effect size = −0.10 ± 0.02 cm, $n = 9{,}438$, $n_{s*y*s} = 112$, $p = 0.004$; mass: effect size = −0.40 ± 0.10 g, $n = 9{,}439$, $n_{s*y*s} = 112$, $p = 0.01$), while in contrast, body condition increased over time (effect size = 0.015 ± 0.004 g/mm^3, $n = 9{,}434$, $n_{s*y*s} = 112$, $p = 0.03$). Fish from areas of low contamination were longer and heavier than the fish in high contamination areas (length: effect size = 0.80 ± 0.20 cm, $n = 9{,}438$, $n_{s*y*s} = 112$, $p = 0.008$; mass: effect size = 2.70 ± 0.50 g, $n = 9{,}439$, $n_{s*y*s} = 112$, $p = 0.008$), but body condition did not differ statistically between fish from low and high contamination sites (effect size = 0.09 ± 0.04 g/mm^3, $n = 9{,}434$, $n_{s*y*s} = 112$, $p = 0.10$).

A linear trend model was fit to GSI data and reproductive data. Investment in reproduction as measured by GSI did not change over time (effect size: 0.13 ± 0.10, $n = 7{,}808$, $n_{s*y*s} = 96$, $p = 0.26$), but fish from more contaminated sites had higher GSI than those from lower contamination sites (effect size: 1.43 ± 0.34, $n = 7{,}808$, $n_{s*y*s} = 96$, $p = 0.01$). However, when this model was run with only reproductive round goby (i.e. males with a GSI >1 %, and females with a GSI >8 %, see Methods section), the GSI difference between low and high contamination sites was no longer present (effect size: 0.63 ± 0.45, $n = 2{,}225$, $n_{s*y*s} = 94$, $p = 0.24$). Overall, 32 % of the males caught, and 24 % of the females caught were in reproductive condition. The proportion of reproductive fish did not change over time (effect size = 0.01 ± 0.01, $n = 8{,}118$, $n_{s*y*s} = 96$, $p = 0.22$; Fig. 4), but there was a larger proportion of reproductive males at high contamination sites, compared to males at the low contamination sites, and females at both site types (effect size = 0.10 ± 0.02, $n = 8{,}118$, $n_{s*y*s} = 96$, $p = 0.02$).

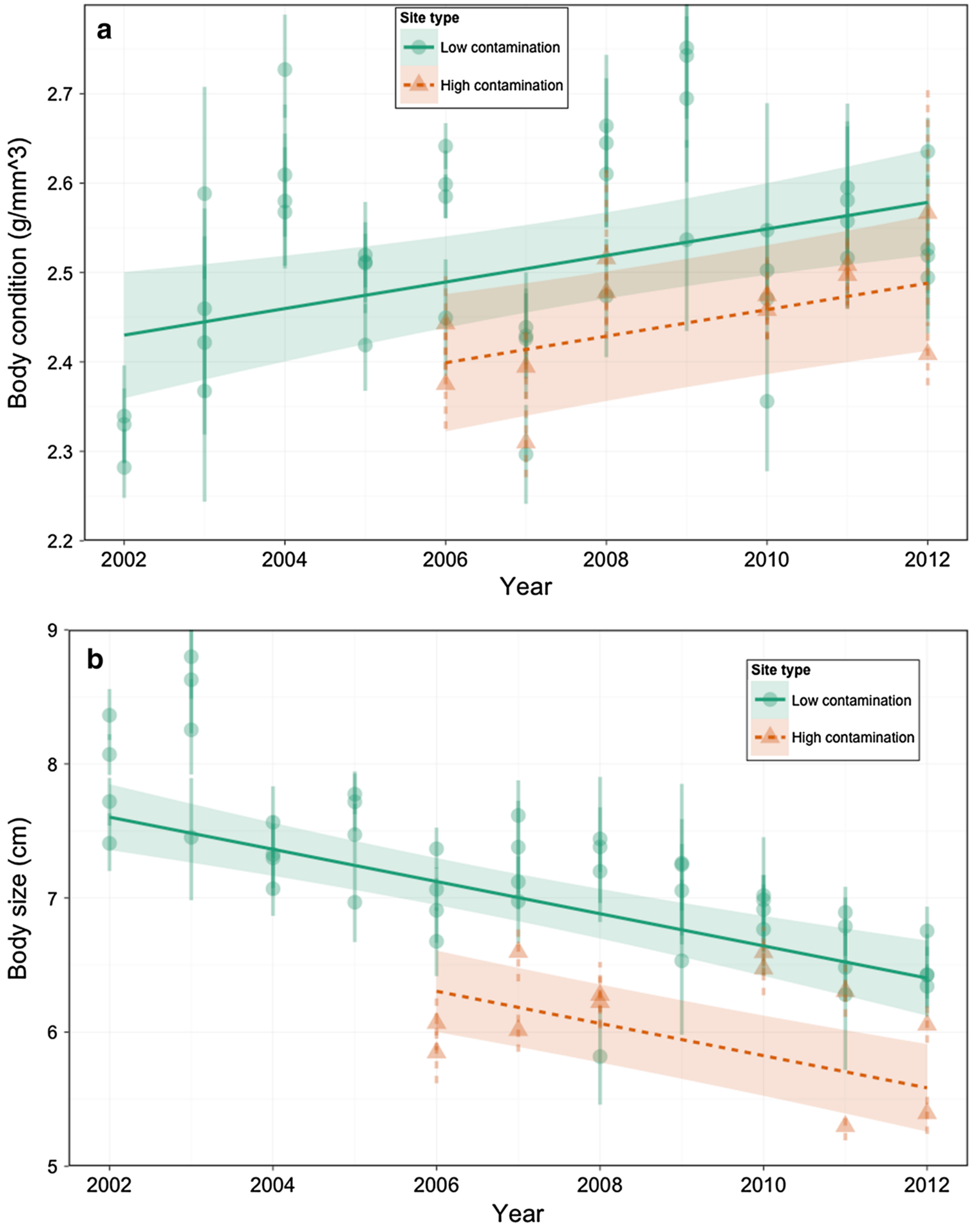

Fig. 3 **a** Body size with *smooth lines* showing the predictions of a linear mixed effects model separated by site type (i.e. high or low contamination). **b** Body condition with *smooth lines* showing the predictions of a linear mixed effects model separated by site type. For both panels, *ribbons* indicate 95 % confidence intervals of the model predictions. *Background points* show mean values for individual sites (*triangles* denote high contamination sites, *circles* denote low contamination sites), while *bars* around these *points* show 95 % confidence intervals for each mean value

Lastly, we fit a linear trend model to the male reproductive tactic data, and found that the proportion of guarding males did not change over time (effect size = 0.001 ± 0.01, $n = 1{,}172$, $n_{s*y} = 42$, $p = 0.93$), nor did it vary between low and high contamination sites (effect size = −0.1 ± 0.09, $n = 1{,}172$, $n_{s*y} = 42$, $p = 0.25$; Fig. 5).

Discussion

Summary

In support of our predictions, round goby abundance and body size declined across time. However, we found that round goby were equally abundant at low

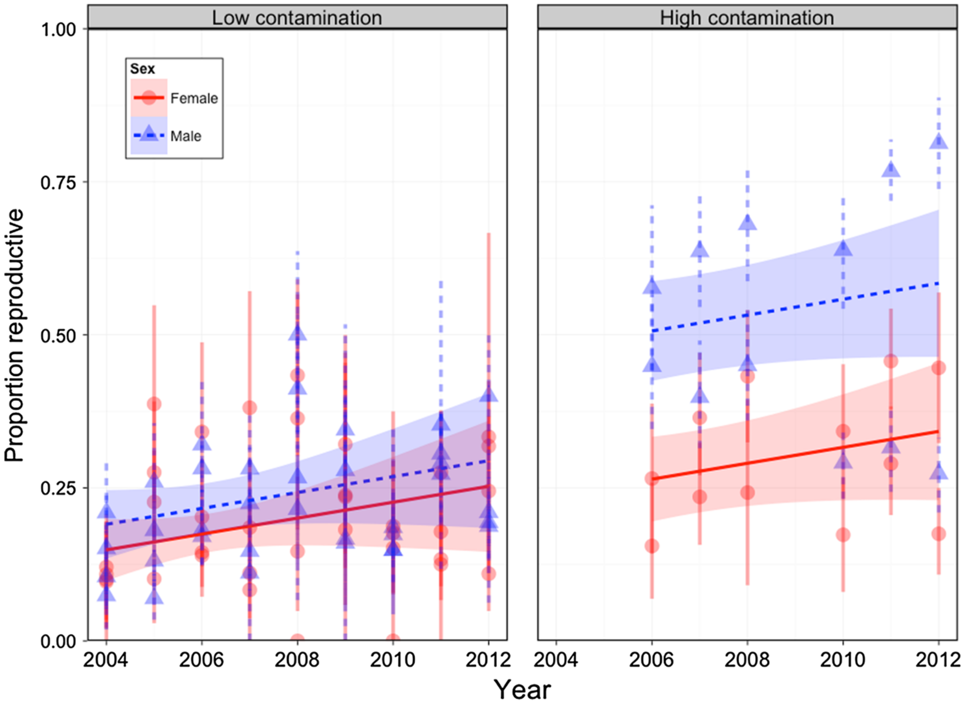

Fig. 4 Proportion of reproductive round goby across sampling years faceted by site type (i.e. high or low contamination). *Smooth lines* show the predictions of a linear mixed effects model separated by sex. *Ribbons* indicate 95 % confidence intervals of the model predictions *Background points* show the mean proportion of reproductive round goby at individual sites (*triangles* denote males, *circles* denote females), while *bars* show 95 % confidence intervals for each mean value

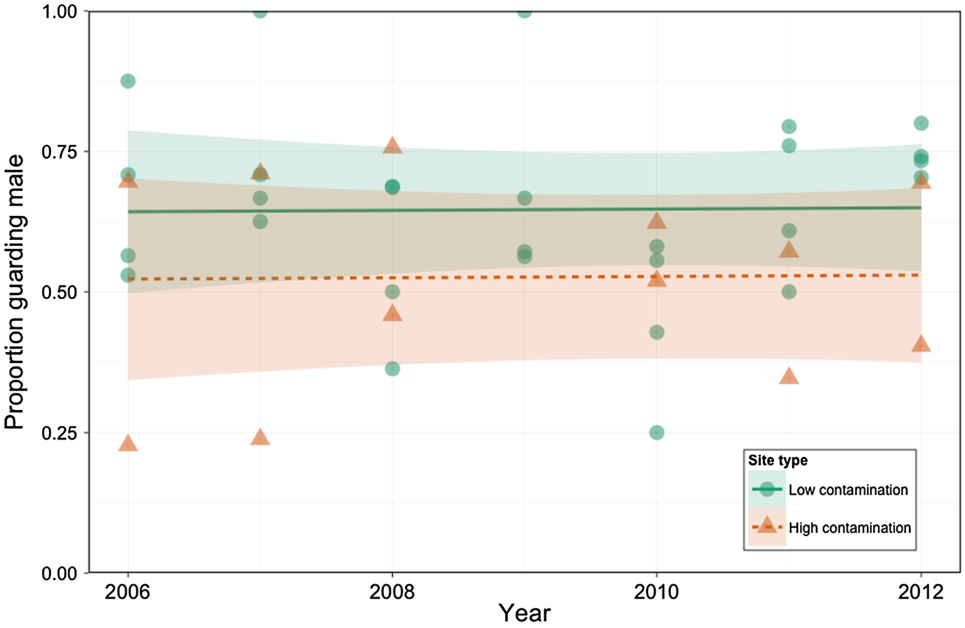

Fig. 5 Proportion of guarding males across sampling years with *smooth lines* showing the predictions of a linear mixed effects model separated by site type (i.e. high or low contamination). *Ribbons* indicate 95 % confidence intervals of the model predictions, while *background points* show mean proportion of parental males at individual sites (*triangles* denote high contamination sites, *circles* denote low contamination sites)

and high contamination sites within the Harbour. Again, in support of our predictions, round goby were smaller at contaminated sites, but surprisingly fish at these sites had higher investment in reproduction, and a greater proportion of reproductive males were observed. In contrast to our original predictions, we found no difference in the relative abundance of male alternative reproductive morphs across sampling years, or between sites of varying contaminant load. The implications of these findings will be discussed in detail below.

Trends across sampling years (2002–2012)

While round goby abundance declined across the years we sampled, the rate of decline stabilized in recent years. Additionally, we observed a similar decline in body size (both length and mass) across years. Taken together, these findings strongly indicate that round goby in Hamilton Harbour may have initially increased beyond their carrying capacity and density saturation threshold (Vélez-Espino et al. 2010), but once high intra- and interspecific competition for food and shelter ensued this may have selected for slower growth, smaller overall body size, and even an eventual reduction in overall abundance (Blanckenhorn 2000; Peters 1983). The declines in body size and abundance may have also been the result of predators such as double-crested cormorants (Somers et al. 2003), and larger fish species such as yellow perch, largemouth and smallmouth bass, northern pike, and walleye (Reyjol et al. 2010; Taraborelli et al. 2010; Dietrich et al. 2006; Truemper and Lauer 2005) recognizing and consuming the round goby as a prey source as the goby became established in the Harbour. Indeed, these predator species have been surveyed in close proximity to our sampling sites (Bowlby et al. 2010; Brousseau and Randall 2008; Somers et al. 2003), and round goby are known to be a substantial prey source for these species (Hossain et al. 2012). In support of this idea, Brownscombe and Fox (2013) showed that round goby tethered in established locations receive more predation events than round goby tethered in newly invaded areas with naïve predators. Conversely, body condition increased across sampling years, and this may be associated with the declines in abundance. Increased predation may lead to lower intraspecific competition for food and shelter resources, facilitating improved body condition. Though our sampling and subsequent removal of round goby from Hamilton Harbour occurred frequently (twice per month), it did not affected population abundance trends; had this had occurred we would have observed a continuous decline across years and within each sampling season, and this pattern was not observed. These findings highlight the equilibration process that occurs between native species and an invading species when they persist beyond establishment and integrate into the ecosystem of an invaded area. This phenomena of population decline after initial population expansion has been documented in other invaders, including the zebra mussel invasion of the Great Lakes (Petrie and Knapton 1999; Schloesser and Nalepa 1994), and in pike, killifish and black acara invasions of the wetlands of Florida, United States (Trexler et al. 2000). It has been theorized that population saturation and increased predation pressure interact to cause the observed cases of invasive species boom-and-bust dynamics (Davis 2009; Simberloff and Gibbons 2004).

The relative abundance of male reproductive morphs remained constant across sampling years. Marentette et al. (2009) had predicted, based on theory of alternative reproductive tactics (Gross 1996), that the guarding male reproductive morph would be more abundant during the earlier stages of an invasion when there would have been less male–male competition for defendable nests and mating opportunities, followed by a subsequent rise in the relative abundance of the sneaker male reproductive morph as the population density and competition for nests increased. In our population monitoring of round goby in Hamilton Harbour, we may not have captured the earliest stages of the round goby invasion, and it is possible that the proportion of each male reproductive tactic had already settled into an equilibrium state, as the population was established by the start of our sampling regime.

Trends between sites of low and high contamination

Round goby were equally abundant at both low and high contamination sites suggesting that highly contaminated areas are not barriers to round goby establishment. The presence of round goby at these contaminated sites over a long time-span, and at similar densities to low contamination sites, supports previous claims that the round goby is a pollution tolerant species (Pinchuk et al. 2003). These results underscore the management concerns for this invasive fish species which provides a potential pathway for the transport of contaminants up trophic levels in invaded ecosystems. Indeed, contaminant transfer from zebra mussel, to round goby, to smallmouth bass has already been documented in Lake Erie (Hogan et al. 2007; Kwon et al. 2006), making this issue a present and serious concern for Lake Ontario.

Though contaminant load did not prevent round goby from residing in areas of high contamination, fish collected from these sites were smaller overall. Smaller body size has been documented in marine and freshwater fish when collected from areas of prolonged contamination (Kruitwagen et al. 2006; Canli and Atli

2003), and reduced growth has been observed when fish were raised on contaminated sediments (Rowe 2003). Previous work has shown that round goby collected from our high contamination sampling sites in Hamilton Harbour were younger when aged using otolith analyses (JR Marentette, unpublished data), perhaps indicating earlier mortality in high contamination sites, reduced recruitment at low contamination sites, or a habitat-use shift with increasing age. The round goby collected from contaminated sites had a higher proportion of males in reproductive condition (~50 %, compared to ~25 % at low contamination sites), and had a larger relative investment in reproductive tissue across both sexes (as measured by gonadosomatic index). Evidence of endocrine disruption and intersex have been reported in other aquatic species from contaminated sites Hamilton Harbour (Kavanagh et al. 2004; Arcand-Hoy and Metcalfe 1999; de Solla et al. 1998), and in round goby (Bowley et al. 2010; Marentette et al. 2010). It is possible that these differences in reproductive characteristics are linked to the presence/absence of contaminants at our high contamination versus low contamination sampling sites (Marentette et al. 2011; Zeman 2009; RAP 2002), however, controlled exposure experiments would be necessary to elucidate whether the compounds causing these reproductive irregularities. The above findings emphasize how the population dynamics of an invasive species can be altered in response to an environmental stressor, and show that these demographics can vary even within a small geographical range and while the invasive species is equilibrating with the ecosystem.

Caveats

This study updates our understanding of round goby population dynamics initially presented in Young et al. (2010). Our study further expanded the previous work by adding additional sampling years, sampling at highly contaminated sites, as well as new variables of interest. These results provide a more thorough analysis of the demographics of the round goby population in Hamilton Harbour, and to also address how aspects of the environment (i.e. contaminant load) may affect the demographics of invasive species. We sampled fish using minnow traps that have previously been thought to under-represent round goby population abundance, especially for young-of-the-year fish (compared to seine net or trawling; Johnson et al. 2005). Indeed, we caught few juveniles over the 11-year study. However, given the Harbour's varied substrates with large rocks and boulders, the long-term nature of the study, and the extensive sampling area covered, trapping was the most feasible, consistent and viable method to record changes in adult abundance. Furthermore, we must acknowledge two issues of long-term monitoring that the current work does not address. First, we cannot guarantee that our sampling sites represent separate distinct populations. Due to the pelagic phase of round goby larvae (Hensler and Jude 2007), it is likely that they are not distinct, but genetic relatedness assays would be required to confirm this statement. However, adult round goby are known to be highly philopatric (Marentette et al. 2011; Ray and Corkum 2001), indicating that our results are most attributable to environmental differences between sampling sites of varying contaminant load. Second, we treated our high contamination sampling sites as a uniform stressor on round goby, but we recognize that contaminants are likely to be present at different concentrations and forms at each site. We have attempted to account for this variability in our statistical modeling by allowing individual sites to act as a random effect.

General conclusions

We have characterized the persistence and integration stages of an invasion a highly successful invasive species in the Laurentian Great Lakes, the round goby. Our work has documented how the population characteristics of a well-established invasive species can shift across time as they equilibrate with the non-native ecosystem, and also how an environmental stressor can impact these characteristics, even within a small geographic range. Our results have important implications for understanding the maintenance of round goby population abundance through potential predator–prey interactions. While round goby may have a positive impact on some native species by providing a food source, negative impacts from the mobilization of contaminants to higher trophic levels in the invaded ecosystems underscores the importance for close monitoring and management of round goby in areas of contamination. We will continue to monitor the round goby population in Hamilton Harbour, with specific focus on the declining body size and abundance of round goby in the Harbour, as this will have direct consequences for their interactions with native species. Future work will focus on elucidating the mechanisms behind the increased proportion of males in reproductive condition, higher reproductive tissue investment, and smaller body size at contaminated sites using controlled contaminant exposure studies. Combining this experimental work with our long-term population monitoring data, will allow us to thoroughly understand the mechanisms causing the patterns observed at the population level, both across time and spatial scales.

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Appendix C: Supplementary Table S1, Chapters 3, 4, 5 & 6

Supplementary Table S1

Ethogram for scoring round goby behaviour.

BEHAVIOUR	**DESCRIPTION**
1. Movement & territorial maintenance	
Hop (**H**)	Smooth locomotion on substrate driven by *a single stroke* of the pectoral fins. Forward or sideways movement of distance *less than* one body length.
Swim (**Sw**)	Sustained locomotion in the water column using *multiple strokes* of the pectoral fins. Movement *greater than* one body length
Glass Swimming (**Gs**)	Focal fish is oriented towards the side of the tank and repeatedly swims up and down, nose to the glass
Glass Ram (**Gr**)	Focal fish is oriented towards the side of the tank and approaches surface quickly making contact with glass once followed by a still posture.
Darting (**D**)	A spontaneous, rapid swim along the substrate not directed at anything.
Yawn (**Y**)	Big stretch of the mouth outward, not feeding.
Feed (**Fe**)	Focal fish opens its mouth to take in particles.
Scraping (**Sc**)	Focal fish quickly scrapes its side against substrate.
Digging (**Dg**)	Focal fish picks up object or pieces of substrate, in mouth moves it to another location
Burying (**Bu**)	Focal fish vigorously partially or completely hides its body in the substrate.
2. Aggression *including codes for aggression given by focal and aggression received from opponent*	
Mouth-fight (**MF**)	Mouth of focal fish makes contact with the mouth of another fish. Mouths are interlocked and fish push back and forth.
Shelter-fight **(ShF)**	Both fish are in the shelter and are fighting over this resource. Note: while in the shelter, the fish are partially obscured from the observer and individual aggressive behaviours cannot be differentiated.
Parallel display (**Pd**)	Both focal fish and opponent are ~ 1-8 cm away from each other. Fish wave their spine in an “s-curve” horizontally against the other fish. Can be given singly by focal fish or the opponent fish, or by both fish at the same time.

Bite (**B**) Bitten (**Bn**)	Focal fish rapidly approaches the opponent, and opens and closes its mouth somewhere on the opponent's body.
Puff (**Pf**) Puffed at (**Pf'd)**	Focal fish arches its back, flares out its operculum and lower jaw cavity in direction of another fish.
Ram (**Rm**) Rammed (**Rm'd**)	Focal fish uses head to physically ram against opponent's body, no biting.
Chase (**Ch**) Chased (**Ch'd)**	Focal fish rapidly approaches or lunges at opponent fish and pursues them. No bodily contact. Other fish escapes.
Displace (**Ds**) Displaced (**Ds'd)**	Focal fish approaches opponent, not rapidly. Other fish is forced to move from their initial position. Focal fish does not pursue opponent.
Bark (**Bk**) Barked at (**Bk'd**)	Focal fish holds body rigid while quivering caudal fin and waving pectoral fins. Involves the forceful expulsion of water/air from the buccal cavity.

3. Mirror-specific behaviours

Mirror ram (**Mr**)	Focal fish quickly approaches mirror, aggressively ramming against it using pectoral fins
Mirror swim (**Ms**)	Focal fish orients towards mirror, and swims parallel or vertically, always in contact with the mirror
Parallel inspection (**Pi)**	Focal fish orients parallel to mirror, still, and raises all dorsal fins, exposing the black dorsal spot.
Head up (**Hu**)	Focal fish orients towards mirror, props upper body up on pelvic fin, raises head and flares out operculum

Appendix D: Supplementary information for Chapter 4

Supplementary Materials

Fish Plasma Model equations (Huggett et al. 2003) used to estimate steady-state plasma concentration of fluoxetine in fish in our experiments.

$$LogP_{Blood:Water} = 0.73 * LogK_{ow} - 0.88$$

$$Fish_{SS}PC = EC * P_{Blood:Water}$$

Where $P_{Blood:Water}$ is the partitioning between the aqueous phase and arterial blood. $LogK_{ow}$ is the octanol/water partition coefficient for fluoxetine (Kwon & Armbrust, 2008), $Fish_{SS}PC$ is the fish steady state plasma concentration of the given compound, and EC is the exposure concentration.

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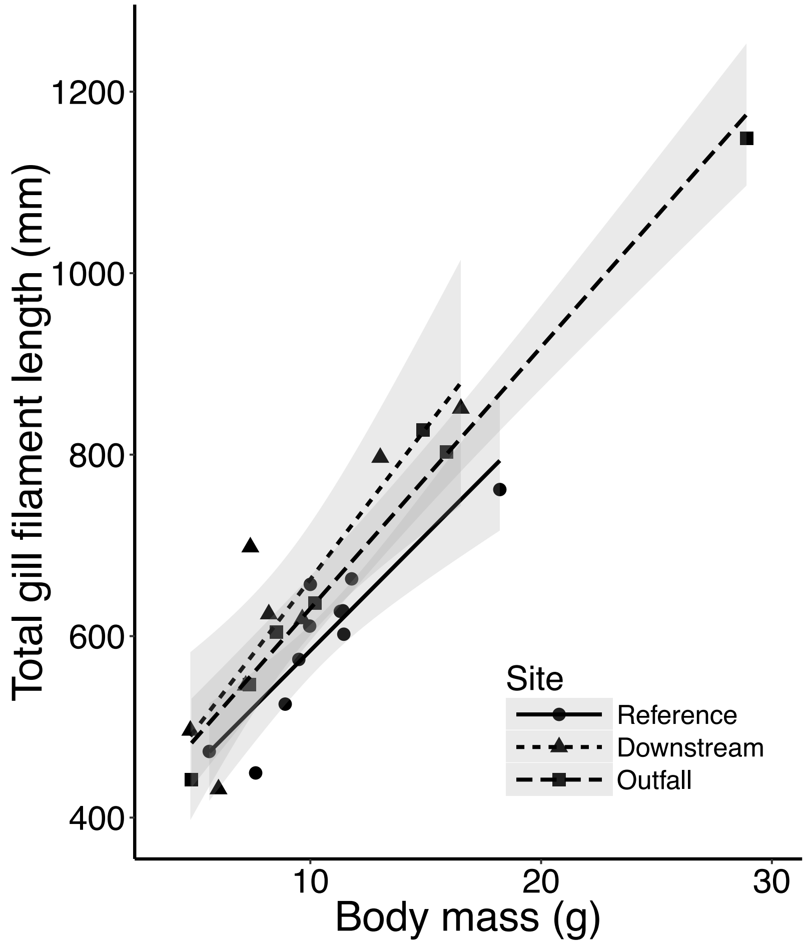
Appendix E: Supplementary information for Chapter 6



Supplementary figure 6.S1

Total gill filament length plotted against body mass, split by exposure site. Graph depicts that fish from the downstream site had longer gill filaments than fish from the reference site.

Supplementary Table 6.S2

Summary of percent survival for round goby from each exposure cage, for each week exposed in the field, split by exposure site.

		Survival (%)		
Site		Week 1	Week 2	Week 3
Reference				
	Cage 1	100.0	91.66	91.33
	Cage 2	93.75	87.50	81.25
	Cage 3	93.33	86.66	80.0
	Cage 4	86.66	86.66	86.66
	Cage 5	60.0	60.0	60.0
Downstream				
	Cage 1	40.0	26.66	26.66
	Cage 2	73.33	73.33	60.0
	Cage 3	92.85	85.71	78.57
	Cage 4	86.66	86.66	86.66
	Cage 5	93.33	86.66	46.66
Outfall				
	Cage 1	20.0	13.33	13.33
	Cage 2	86.66	80.0	66.66
	Cage 3	93.33	66.66	33.33
	Cage 4	93.33	73.33	73.33
	Cage 5	73.33	73.33	60.0
WWTP				
	Cage 1	6.66	6.66	6.66
	Cage 2	86.66	86.66	86.66
	Cage 3	86.66	86.66	0
	Cage 4	53.33	0	0
	Cage 5	0	0	0