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ENDOCRINE DISRUPTION IN ROUND GOBY FROM HAMILTON HARBOUR

ENDOCRINE DISRUPTION IN ROUND GOBY (*NEOGOBIUS MELANOSTOMUS*)

FROM HAMILTON HARBOUR

By

LUCAS A. BOWLEY, B.Sc.(H), B.Ed.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

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ABSTRACT

The occurrence of endocrine disruption in aquatic species is of growing concern in the Great Lakes region. Feminized male white perch (*Morone americana*) and round goby (*Neogobius melanostomus*) have been observed in Hamilton Harbour, a heavily polluted embayment of Lake Ontario. The harbour is impacted by wastewater effluent, containing pharmaceuticals and natural steroid hormones, as well as sediment-bound industrial contaminants including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). This study investigated the severity of endocrine disruption in Hamilton Harbour in an attempt to identify which areas of the harbour are most heavily impacted and thereby, which contaminants are the most potent endocrine disrupting chemicals (EDCs). The round goby, an invasive species found throughout the Great Lakes region, was used as the model species. Three biological endpoints in male fish were observed: intersex (the presence of oocytes in male gonads), feminization of secondary sexual characteristics, and vitellogenin (Vtg) gene upregulation. Vtg is an egg yolk precursor protein normally highly expressed in reproductively active females. Vtg qPCR assays were developed and tested using an *in vivo* lab exposure to estradiol, a known Vtg-inducing compound. Field studies looked for site differences and revealed that there were significant differences among sites in all three biological endpoints. More heavily impacted sites had high signals from each endpoint while moderately impacted sites displayed Vtg upregulation and feminization of secondary sexual characteristics, but not intersex. As expected, reference sites were not impacted by endocrine disruption. Sites influenced primarily by wastewater effluent were moderately impacted, suggesting

that wastewater effluent is not the dominant endocrine disrupting agent in the harbour. Sites that were most heavily impacted were those characterized by high sediment PAH and/or PCB levels. PAHs and PCB appear to be the most potent EDCs in the Hamilton Harbour.

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ABBREVIATIONS

11KT	11-ketotestosterone	OSS	Ottawa Street Slip
AhR	aryl hydrocarbon receptor	P25	Pier 25
BNP	Bruce Peninsula	P27	Pier 27
BWW	Burlington wastewater	PAH	polycyclic aromatic hydrocarbon
CA	cortical alveolar	PCB	polychlorinated biphenyl
CDF	closed disposal facility	PM	parental male
C_t	cycle threshold	PN	perinucleolar
DC	Desjardins Canal	PQ	Presque'ile
E2	17 β -estradiol	Pv	phosvitin
EDC	endocrine disrupting chemicals	SI	Sherman Inlet
ER	estrogen receptor	SL	standard length
ERα	estrogen receptor alpha	SM	sneaker male
GC	Grindstone Creek	SZ	mature spermatozoa
GSI	gonadosomatic index	T	testosterone
HH	Hamilton Harbour	TL	total length
HSD	homologous subdomain	VG	vitellogenic
HSI	hepatosomatic index	Vtg	vitellogenin
LS	LaSalle Marina	VtgR	Vtg receptor
LvH	heavy chain lipovitellin	Vwfd	von Willebrand factor type D
LvL	light chain lipovitellin	WWTP	wastewater treatment plant
NRM	non-reproductive male		

CHAPTER 1:

GENERAL INTRODUCTION

1.1 Endocrine disruption in the Great Lakes

1.1.1 *Background*

Human population growth and industrialization in the Great Lakes region has introduced many novel anthropogenic compounds, that may be endocrine disruptors, into aquatic systems. Many of these compounds have been shown to elicit negative impacts on aquatic species, including impaired reproduction. Major categories of contaminants that have been studied in an endocrine disruption context include pulp mill effluent, industrial waste and pharmaceuticals from wastewater treatment plant discharge. Bleached pulp mill effluent first became of interest in the early 1980s in Sweden where fish toxicity was demonstrated (reviewed in McMaster et al., 2006). Later, studies in Canada showed that both bleached and unbleached mill effluent caused reproductive impacts on aquatic species; these studies led to the regulation of dioxin and furan release into the environment and the implementation of mandatory environmental effects monitoring (McMaster et al., 2006). Despite these advances, monitoring studies provided evidence that paper mill effluents continued to cause negative effects including altered hormone

levels (Shaughnessy et al., 2007), intersex, altered sex ratios and vitellogenesis (Orn et al., 2006) in male fish.

There have been numerous studies describing the effects of industrial wastes that have been discarded into aquatic environments. Polycyclic aromatic hydrocarbons (PAHs) are combustion by-products that have been shown to be carcinogenic to fish species (Bunton, 1996). Polychlorinated biphenols (PCBs), although banned from use in Canada and the US, persist in areas where they have been historically dumped into aquatic environments. PCBs have been shown to bioaccumulate in fish and are related to numerous pathologies (van der Oost et al., 2003). Both PAHs and PCBs have been linked to endocrine disruption endpoints including intersex (Rank, 2009; Stentiford et al., 2003) and vitellogenesis (George et al., 2004) in male fish, although the mechanisms behind these effects are unclear. PAHs and PCBs are ligands for aryl hydrocarbon receptors (AhRs) which have been shown to up-regulate (Mortensen and Arukwe, 2008) and down-regulate (Arukwe et al., 2001) estrogen-responsive genes including estrogen receptor α (ER α) and vitellogenin (Vtg) via AhR/ER cross-talk pathways.

Pharmaceuticals may be potent endocrine disrupting compounds in the aquatic environment due to their ability to elicit physiological responses at low doses. Many pharmaceuticals are released from wastewater treatment plants and are often found in ng/L to μ g/L concentrations in aquatic systems (Halling-Sorensen et al., 1998). Even pharmaceutical compounds that are effectively removed by wastewater treatment, such as acetaminophen, may be detected in the μ g/L range (Kolpin et al., 2004) due to high

human consumption. Since drugs of the same class utilize similar physiological pathways, there may be additive or synergistic effects in aquatic species exposed to wastewater effluent. Natural or synthetic estrogens, such as ethinylestradiol found in birth control and hormone replacement therapies, are a major concern to aquatic species; they have been shown to cause changes in hormone levels (Desbrow et al., 1998), reduced fecundity (Maunder et al., 2007), developmental abnormalities (Fenske et al., 2005), intersex (Sole et al., 2003), vitellogenesis in males (Routledge et al., 1998) and population crashes (Kidd et al., 2007) in aquatic environments. Pharmaceutical contamination is a concern in many areas of the Great Lakes where large human populations generate a constant release of these compounds and their metabolites into the environment.

1.1.2 Hamilton Harbour

Hamilton Harbour is a 2150 hectare body of water that has numerous contaminant inputs as well as a legacy of industrial activity that has left toxic deposits of various compounds in sediments. Fish populations have been impacted by contaminants in Hamilton Harbour; studies have demonstrated feminization of male fish in several species (Kavanagh et al., 2004; Marentette et al., In Press) in the harbour and in Cootes Paradise. Cootes Paradise is an important fish nursery for Lake Ontario that is located at the western end of Hamilton Harbour. This area has been shown to be at high risk from dredging, invasive carp, a loss of aquatic plant-habitat for spawning and developing fish, and endocrine disruption (O'Connor et al., 2003). The harbour is home to four wastewater treatment plants along with 23 combined sewer overflows that release untreated sewage

during high rainfall events (O'Connor et al., 2003). The harbour is surrounded by a 49 400 hectare watershed that is heavily populated and industrialized (O'Connor et al., 2003). Randle Reef, a historic PAH sediment deposit that has been designated as an Area of Concern by the International Joint Commission, is found within Hamilton Harbour (O'Connor et al., 2003). Sediment PAH levels at this site exceed >800 µg/g (Marvin et al., 2000; O'Connor et al., 2003). Estrogenic compounds (Mayer, 2008), PAHs (Slater et al., 2008) and PCBs (Labencki, 2008) have been characterized in Hamilton Harbour sediments and are known to persist there. These compounds pose a potential exposure threat to native fish populations. The harbour has been under a monitoring and remediation effort by the Hamilton Harbour Remedial Action Plan since 1985, but many of the goals of this effort are presently unmet. The harbour is one of the most polluted areas in the Great Lakes and yet very little work has been done to characterize endocrine disruption here or anywhere else in the region. Biological monitoring of aquatic species is requisite to ensuring that native fish populations are able to thrive in dynamically and heavily polluted systems.

1.2 Vitellogenin (Vtg)

1.2.1 Gene expression pathway and gene characteristics

Vtg is an egg yolk precursor lipophosphoprotein that is produced in the liver of sexually mature female fish in a coordinated endocrine cascade involving the brain, ovary,

liver and circulatory system (Finn, 2007). Vtg expression is controlled transcriptionally by estrogen receptors upon binding with estrogenic compounds. Vtg is exported into circulation as homodimeric complexes after being post-translationally glycosylated and phosphorylated in the endoplasmic reticulum and Golgi complex (Finn, 2007). Once in the ovary, Vtg is bound by specific Vtg receptors (VtgR) that are anchored in the oocyte plasma membrane. Bound VtgR are then internalized via clathrin-mediated endocytosis (O'Connor et al., 2003). Endocutes containing Vtg are sorted to early endosomes and acidified by ATP-dependent V-class proton pumps (Wallace and Selman, 1990). Vtg is cleaved into its major yolk protein constituents by cathepsin D after acidification (Carnevali et al., 2006).

Teleosts have a variable number of Vtg genes and each gene can be classified based on the motifs and subdomains present. There are up to twenty Vtg genes in rainbow trout (*Oncorhynchus mykiss*; Trichet et al., 2000), seven Vtg genes in zebrafish (*Danio rerio*; Wang et al., 2005), and only two Vtg genes in Japanese common goby (*Acanthogobius flavimanus*; Ohkubo et al., 2004). Complete vertebrate Vtg genes contain a signal peptide, a heavy chain lipovitellin (LvH), a phosvitin (Pv), a light chain lipovitellin (LvL), and a von Willebrand factor type D domain (Vwfd). Vwfd is cleaved into the beta component, and C-terminal coding region in teleosts (reviewed in Finn, 2007). Teleost Vtg genes have five homologous subdomains (HSDs). The first three HSDs are found in LvH, a conserved motif found in all fish Vtg genes; when present, the last two HSDs are found in LvL (Wang et al., 2005). There are three distinct Vtg gene types. Type I Vtg genes contain all three major motifs (LvH, Pv and LvL) with LvL

lacking HSD IV and V (Wang et al., 2005). Type II Vtg genes have all three motifs and all five HSDs (Wang et al., 2005). Type III Vtg genes lack the Pv motif and HSD IV and V from the LvL motif (Wang et al., 2005).

1.2.2 Vtg as a biomarker of endocrine disruption

Vtg genes can be expressed in male fish when exposed to high levels of estrogenic compounds (reviewed in Finn, 2007; Rotchell and Ostrander, 2003). Since male fish do not normally express Vtg at high levels (Biales et al., 2007a), and because Vtg is responsive to estrogens as well as other compounds (Arukwe et al., 2001; Mortensen and Arukwe, 2008; Orn et al., 2006; Stentiford et al., 2003), it is commonly used as a hallmark of endocrine disruption in many aquatic and terrestrial species. Measuring Vtg levels has been shown to be a sensitive and reliable endpoint and a large body of work exists that characterizes Vtg responses to EDCs in many species and in many different aquatic environments around the world (reviewed in Rotchell and Ostrander, 2003). For example, significant increases in circulating Vtg protein levels have been detected after exposure to 10 ng/L estradiol in Japanese medaka (*Oryzias latipes*; Thompson et al., 2000). Celius et al. (2000) found that 0.1 mg/kg body weight estradiol significantly induced Vtg mRNA expression in rainbow trout (*Oncorhynchus mykiss*).

Vtg induction in male fish can be measured at the mRNA or protein level. In order to measure circulating Vtg protein, immunoassays with specific antibodies are required. Compared to mRNA assays, developing protein-based assays is relatively difficult, expensive and time consuming. Vtg proteins have high inter-species variability and

antibodies typically show poor inter-species cross-reactivity. This often makes it difficult to use protein-based assays in non-model species. Assays to measure Vtg mRNA expression using qPCR can be readily developed for new species. Once sequences for Vtg genes are established, qPCR primers can be easily designed to amplify gene fragments for expression studies (Biales et al., 2007b). Cloning previously uncharacterized Vtg genes in new species is relatively easy due to the many available sequences from other species. Observing multiple Vtg genes in a single species using mRNA-based assays has proven to be useful; distinct Vtg genes are typically expressed at different levels and the inducibility of each gene is variable (Wang et al., 2005). This approach allows researchers to incorporate new species into endocrine disruption studies rather than rely on existing model species that may not be as useful or appropriate for their field sites. Vtg mRNA has been shown to be induced days sooner than Vtg protein following exposure (Biales et al., 2007b), although Vtg protein levels tends to persist much longer than mRNA transcripts after exposure halts (Craft et al., 2004). Measuring mRNA transcripts allows a better understanding of temporal aspect of contamination events and may be useful in characterizing combined sewage overflow or intermittent industrial dumping.

1.3 Intersex and feminization

Intersex and feminization of male fish in wild populations have been correlated with numerous types of EDCs. Intersex, or testis-ova, has been defined as the presence of one or more individual oogenic cells within male gonad tissue, according to published

USEPA guidelines (Ankley et al., 2006). Ideally, gonads are robustly screened by taking multiple serial sections covering the entire gonad. The severity of intersex can be graded, based on the number of oocytes present in one gonad section, as either absent (0), minimal (1 to 2), mild (3 to 5), moderate (6 to 8) or severe (more than 9). Oocytes can be individually staged based on the level of development as either perinucleolar (early-development), cortical alveolar (mid-development) or vitellogenic (late-development), based on histological size and appearance (Ankley et al., 2006).

Less severe forms of feminization may also be assessed through altered morphology of secondary sexual characteristics (Marentette et al., In Press; Van der Kraak et al., 1992). For example, one study showed that spawning white sucker (*Catostomus commersoni*) exposed to bleached kraft mill effluent near Terrace Bay, Ontario had fewer nuptial tubercles compared to a reference site (McMaster et al., 1991). Marentette et al. (In Press) found that male round goby from contaminated sites in Hamilton Harbour had urogenital papillae that were less pointed and more closely resembled female urogenital papillae. Other measures of feminization that have been used to identify endocrine disruption in aquatic environments include alterations in sex ratios (Orn et al., 2006; Xu et al., 2008), gonadosomatic index (GSI; gonad compared to body weight; Li and Wang, 2005; Marentette et al., In Press), hepatosomatic index (HSI; liver compared to body weight; Verslycke et al., 2002), Fulton's condition factor ($K = 100 \times$ mass (g) / total length (cm)³; Danylchuk and Fox, 1996), social and behavioural changes (Vos et al., 2000), and sperm quality (Gross-Sorokin et al., 2006). These endpoints have been used extensively in toxicological and monitoring studies to display endocrine

disruption from pulp mill effluent (McMaster et al., 2006; Orn et al., 2006; Van der Kraak et al., 1992), industrial by-products, such as PAHs (Rank, 2009; Stentiford et al., 2003; van der Oost et al., 2003) and PCBs (Rank, 2009), and pharmaceuticals (Jones et al., 2001; Tyler et al., 1998) in aquatic species.

1.4 Round goby (*Neogobius melanostomus*)

1.4.1 Basic biology and background

The round goby is an invasive species that is currently widely distributed throughout the Great Lakes. The goby was inadvertently introduced from the Black and Caspian seas via ship ballast water (Jude et al., 1992). In North America, round goby consume benthic species; they primarily eat molluscs including zebra mussels, but may also consume worms and other fish (Charlebois et al., 1997). Their reproductive season extends from May to October and individual fish spawn multiple times during that period (Charlebois et al., 1997). Reproductive male round goby exist as either parental or sneaker male morphs that are morphologically, behaviourally and physiologically distinct. Parental males are larger, darkly coloured fish that build a nest for the brood and care for eggs during development (Marentette et al., 2009). Sneaker males are small, lightly coloured fish with a higher gonadosomatic index and a larger papilla for its body size, compared to parental males (Marentette et al., 2009). Sneaker males fertilize eggs in a parental male-built nest and abandons the clutch which is cared for by the resident male.

Sneaker males have higher circulating levels of testosterone, while parental males have higher circulating levels of 11-ketotestosterone (Marentette et al., 2009).

The round goby has numerous characteristics that make it an ideal model species for studying endocrine disruption in the Great Lakes region. Their widespread distribution and large population size allows comprehensive, large scale monitoring without disrupting native fish populations. Male goby, particularly parental male morphs, inhabit small areas ($5 \pm 1.2 \text{ m}^2$) and show high site fidelity (Ray and Corkum, 2001). This trait makes it possible to pinpoint contaminant sources since these fish do not move between sites as other species do. Certain EDCs, like estrogens (Mayer, 2008), PAHs (Slater et al., 2008) and PCBs (Labencki, 2008) are known to bind and persist in Hamilton Harbour sediments. Goby are benthic and may be at higher exposure risk to sediment-bound contaminants than non-benthic species. Catching goby is relatively simple using corn-baited minnow traps deployed overnight, which often yields a high catch. The round goby is a small fish (up to 290 mm total length; Charlebois et al., 1997) that is easily used in lab-based exposure studies under flow-through conditions. The difference in circulating steroids associated with the presence of two distinct reproductive tactics (Oliveira et al., 2001) provides the opportunity to study two unique male physiological systems in the same species. Non-reproductive male goby may also be useful in endocrine disruption studies as they represent a third reproductive status that are distinct from the two reproductive males classes.

1.4.2 Endocrine disruption in round goby

Round goby have been shown to be sensitive to endocrine disruption using various biological endpoints. Marentette et al. (In Press) identified gonadal intersex and feminization of secondary sexual characteristics in male round goby from contaminated sites in Hamilton Harbour. Certain species appear to be sensitive to endocrine disruption in the harbour while others do not. Kavanagh et al. (2004) showed a high prevalence of intersex in harbour-caught white perch (*Morone americana*), but did not find intersex in goldfish (*Carassius auratus*), common carp (*Cyprinus carpio*), gizzard shad (*Dorosoma cepedianum*), brown bullhead (*Ictalurus ameiurus*), pumpkinseed (*Lepomis gibbosus*), or bluegill (*Lepomis macrochirus*), although sample size was small for many of the species screened. Based on the knowledge of basic round goby biology and its history of sensitivity to endocrine disruption, this species was selected as an excellent model for endocrine disruption in Hamilton Harbour and the Great Lakes region.

1.5 Research questions and hypotheses

The goals of this project were to characterize endocrine disruption within Hamilton Harbour and to begin to understand which contaminants are the most disruptive to resident aquatic species. The round goby was used as a model species by evaluating numerous biological endpoints to address these questions. It was predicted that Vtg assays would show high levels of gene upregulation in the harbour, particularly at sites

where endocrine disruption has already been demonstrated in round goby. It was predicted that Vtg expression would correlate with the prevalence of intersex and feminization at the most heavily impacted sites. Sites that were less impacted were expected to show lower levels of intersex, if at all. It was predicted that sites with different contaminant inputs would display different levels of endocrine disruption and provide evidence for which pollutants were most harmful. It was expected that goby collected from sites adjacent to wastewater effluents would show the most severe endocrine disruption in the harbour, indicating that wastewater effluents were the primary source of feminizing contaminants in Hamilton Harbour fish species.

The results are included here, presented in a format for publication, in chapters 2 and 3. Chapter 2 describes the development of qPCR assays to assess the expression of two Vtg genes in the round goby and to establish the goby as a useful model species for endocrine disruption studies in the Great Lakes. The assays were validated by *in vivo* exposure of round goby to estradiol, a natural steroid known to induce Vtg expression in other fish species. Vtg was observed in field-caught goby from Hamilton Harbour to test the ability of the assays to detect differences between variably impacted sites. The assays were also used to observe differences between the three male reproductive classes and female goby. Intersex was analysed histopathologically in three other fish species to evaluate the relative strength of the round goby as a model to predict contamination in the harbour. Finally, circulating steroid hormone levels were evaluated in the three male reproductive classes of round goby to validate the inherent differences in dominant

androgens used by each class. Chapter 2 has been accepted for publication and is in press in the Journal of Environmental Toxicology and Chemistry.

Chapter 3 encompasses a field study of endocrine disruption in Hamilton Harbour using round goby as the model species. Vtg expression, intersex and feminization of secondary sexual characteristics were determined at seven field sites within Hamilton Harbour and two reference sites. Sites were selected based on the contaminant sources found at each site, particularly those with high concentrations of PAHs and wastewater effluent discharge. Differences in severity and prevalence of the three biological endpoints were evaluated in order to discover which sites were most impacted, and infer which contaminants were most harmful to Hamilton Harbour fish populations.

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CHAPTER 2:

CHARACTERIZATION OF VITELLOGENIN GENE EXPRESSION IN ROUND GOBY (*NEOGOBIUS MELANOSTOMUS*) USING A QUANTITATIVE PCR ASSAY

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Abstract

A growing concern over endocrine disruption in aquatic species has prompted the development of molecular assays to monitor environmental impacts. This study describes the development of qPCR assays to characterise the expression of two vitellogenin (Vtg) genes in the invasive round goby (*Neogobius melanostomus*). Fragments from the 18SrRNA (housekeeping gene), Vtg II, and Vtg III genes were cloned and sequenced. qPCR assays were developed to detect hepatic Vtg expression in goby. The assays detected induction of both Vtg genes in non-reproductive males following a two week laboratory exposure to 17 β -estradiol (≥ 1 mg/kg i.p. injection). The assays were applied to goby from Hamilton Harbour, Lake Ontario; including those from sites where feminization and intersex of goby has been documented. Both Vtg genes had significantly higher expression in females compared to males. Male reproductive goby adopt either parental or sneaker tactics; Vtg II expression was higher in sneaker than in parental males but parental and non-reproductive males did not differ from each other. Vtg III expression was significantly higher in sneaker males followed by parental males and non-reproductive males, respectively. Vtg II and III expression in non-reproductive males was elevated in the contaminated site with documented intersex. This assay provides an important tool for the use of an invasive species in monitoring endocrine disruption in the Great Lakes region.

Key words—Gobiidae, Hamilton Harbour, Biomarker, Vitellogenin, Endocrine disruption

Contributions: F.A. completed gene cloning, J.R.M. and S.B. collected field samples and L.A.B. developed the qPCR assays, completed lab experiments and completed the qPCR and intersex analysis under the supervision of J.Y.W.

2.1 Introduction

The concentrations and impacts of endocrine disrupting chemicals (EDCs) from wastewater and industrial effluents on aquatic species have not been adequately characterised in the Great Lakes region of North America. Increases in human populations, pharmaceutical use and industrial activity in the region have the potential of increasing harmful loads of EDCs into receiving waters. Endocrine disruption has been observed in aquatic environments and has been linked to both emerging contaminants, such as pharmaceuticals [1] and estrogenic compounds [2], and more traditional contaminant sources, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) [3-5]. *In vitro* and *in vivo* studies have demonstrated that exposure to EDCs can alter gene transcription [6], induce vitellogenesis in males [7], alter plasma steroid levels [8], and cause developmental impacts [9], intersex [10], reduced or arrested fecundity [11] and multigenerational effects [12] in aquatic species. It is predicted that EDCs could have population-level impacts including population crashes in native fish species [13]. Monitoring biologically relevant exposures is critical to assessing the potential risks of EDCs to populations prior to population level impacts.

One of the most effective and widely used biomarkers to measure estrogenic exposure and effects is the inappropriate expression of the egg yolk precursor lipophosphoprotein vitellogenin (Vtg) in males. Vtg is highly expressed in egg-producing, sexually mature females during the reproductive season, although males may express low levels without significant contaminant exposure [14]. This protein is regulated at the transcriptional level; activation of estrogen receptor alpha (ER α) up-regulates Vtg genes

in liver of oviparous animals [15]. Aryl hydrocarbon receptor (AhR) ligands have been shown to up-regulate [4] and down-regulate [3] estrogen-responsive genes including ER α and Vtg via AhR/ER cross-talk pathways. Expression of high levels of Vtg in males is considered a hallmark of exposure to biologically relevant concentrations of natural or synthetic estrogens [16].

Since Vtg expression is under transcriptional control, it can be measured at the mRNA or protein level; most assays measure circulating Vtg protein since plasma Vtg protein levels tend to persist much longer than transcriptional Vtg levels, particularly after exposure has ceased [17]. An attractive advantage of mRNA-based assays is that they have detected maximal Vtg induction days earlier than protein-based assays [14] and significant induction in exposure studies when protein-based assays failed to do so [17]. Temporal data on intermittent exposure events from sources such as combined sewer overflows may be better characterized with mRNA-based assays, although induction may not be seen unless recent exposure has occurred. Antibodies for Vtg protein typically show poor cross-species reactivity and are only available for a select few model species. Antibody-based assays typically require plasma volumes that would be difficult to obtain from individual small fish, including round goby. Thus, protein-based assays are more limited in the study of many native and/or wild caught fish because these assays are not available for many desirable species. mRNA based assays, such as quantitative PCR (qPCR), can be readily developed for any fish species of interest once the Vtg gene sequences for that species are known.

The use of the round goby (*Neogobius melanostomus*) for the study of contaminant research in the Great Lakes has several advantages; these fish are invasive, benthic, highly territorial and widely distributed throughout the Great Lakes [18]. Catching this species in large numbers is relatively simple and sampling many individuals will not be environmentally detrimental, as it might be for native fish. The round goby have high site fidelity and occupy small territories ($5\pm1.2 \text{ m}^2$) [19], especially parental males that care for their eggs, making them an ideal species for pinpointing point sources of contamination or local environmental differences between sites. Housing this species for lab-based studies requires less room than many other fish species due to their small body size. However, maintaining round goby in a laboratory can be challenging in static tanks and they are better held under flow through conditions. In addition, round goby have several features that are particularly useful in the study of EDCs. First, these fish have multiple alternative reproductive tactics; adult, reproductively mature males are either parental or sneaker male morphs. Parental males are larger in size, darkly coloured, build a nest and care for the brood. Sneaker males are small, lightly coloured, invest in larger gonads and exploit parental male efforts to attract females and care for eggs [20]. Sneaker and parental males primarily use different androgen hormones, providing the opportunity to examine the impact of contaminants on male physiology dominated by either 11-ketotestosterone (parental male) or testosterone (sneaker male) within a single species [20, 21]. Second, this species is sensitive to feminization; round goby have been found with altered secondary sexual characteristics (i.e. urogenital papilla shape) and intersex at selected contaminated sites in Hamilton Harbour [22]. Other available species

that have the advantage of large databases of historical data such as brown bullhead do not appear to be as sensitive to intersex as the round goby. To our knowledge, there are no documented cases of intersex in brown bullhead, even in Cootes Paradise, an area of Hamilton Harbour with very high intersex in white perch [23].

This study was conducted in Hamilton Harbour, a 2150 hectare body of water, on the western most end of Lake Ontario. The Harbour is surrounded on all sides by heavily populated and industrialized land; 46% of the 42 km shoreline is dedicated to heavy industry [24]. The Harbour is nearly cut off from Lake Ontario, except for a narrow canal on its eastern shore (Figure 1) which strongly limits water exchange with the lake. Contaminants released into Hamilton Harbour are likely to remain there since water flow is restricted. Cootes Paradise, a protected wildlife sanctuary at the western tip of Hamilton Harbour, is an important fish nursery for Lake Ontario and has been greatly impacted by dredging, invasive carp, wastewater effluent discharge and untreated sewage from a combined sewer system. Water quality in Cootes Paradise has been significantly improved by a physical barrier to invasive fish species, improvements to wastewater treatment and decreased untreated sewage discharge yet, fish therein continue to display elevated levels of PCBs [24] and intersex [23]. Currently, there are four wastewater treatment plants (WWTP) and 23 combined sewer overflows that discharge into Hamilton Harbour and Cootes Paradise. Potential EDCs from WWTP effluents have been found in sediments in Cootes Paradise and appear to persist in sediment for decades [25]. Potential EDCs from industrial sources such as PAHs [26] and PCBs [27] are known to persist in sediment deposits in the harbour. Hamilton Harbour has been identified as an Area of

Concern (AOC) and is presently home to the second most contaminated PAH deposit in Canada [24].

The current study describes the establishment of a qPCR assay for Vtg gene expression in round goby. The assay was optimized using hepatic mRNA from reproductively active females. The capacity for induction of Vtg in males was determined by laboratory exposures of non-reproductive males to 17β -estradiol. The assay was then used to assess Vtg expression in male and female round goby collected from several sites in Hamilton Harbour. Two Vtg genes were cloned from round goby and gene specific primers were developed to determine if both genes were equally responsive to estrogens. This assay may be widely applicable for determining the presence and impacts of EDC contamination in the Great Lakes region.

2.2 Materials and Methods

2.2.1 17β -estradiol exposure design

Sixty non-reproductive male round goby were collected from LaSalle Marina, Burlington, Ontario between July 15 and August 4, 2009 (LS site, Figure 1). Fish were acclimated to a flow-through system consisting of de-chlorinated tap water in 40 L tanks for a period of at least six days prior to i.p. injection with 17β -estradiol (E2; Sigma-Aldrich, St. Louis, MO, USA) suspended in a coconut oil carrier (Alpha Health Products Ltd, Burnaby, BC, Canada). Coconut oil is a solid below 25°C providing a slow E2

release after bolus injection since the water temperature was kept at ~17°C. Each of the six tanks held ten fish; two fish per treatment group were included in each tank to remove possible tank effects ($n = 12$ fish per treatment). Fin clips were used to identify treatment groups. Fish were randomly assigned to treatment groups; the control group received an injection of coconut oil only, the remaining treatment groups were dosed with 1, 5, 10 or 20 mg/kg E2 suspended in coconut oil. Fish were fed once daily with Nutrafin tropical flake food (Hagen, Montreal, ON, Canada). Artificial light was used to maintain a 14L:10D photoperiod. Two fish died in the 5 mg/kg E2 treatment group leaving ten fish in this group; there was no mortality in any other treatment group. After two weeks of exposure, fish were sacrificed and tissues collected (see Tissue and plasma sampling below). The average (\pm SD) fish mass and total length was 15.3 (\pm 8.18) g and 10.3 (\pm 1.59) cm, respectively.

2.2.2 Field sampling

Non-reproductive and reproductive (parental and sneaker) male and gravid and non-gravid female round goby were collected between April 25 and September 12, 2007. Field sites were located in Hamilton Harbour, on the north-west tip of Lake Ontario. The field sampling sites included two cleaner sites at Grindstone Creek (GC; 43° 17' 21" N, 79° 53' 13" W) and LaSalle Park Marina (LS; 43° 18' 1" N, 79° 50' 47" W), a site presumed to be contaminated at Pier 27 (P27; 43° 17' 3" N, 79° 47' 33" W) and a highly contaminated site at Sherman Inlet (SI; 43° 17' 3" N, 79° 47' 33" W; Figure 1). The cleaner sites were not near any known sources of contamination and sediments contain low levels of

contaminants [28]. The Sherman Inlet site was adjacent to Randle Reef, a site highly contaminated with PAHs from historical coal tar deposits and a site of intermittent combined sewer overflow discharge [24]. The Pier 27 site was adjacent to a closed disposal facility (CDF) that received contaminated sediments from dredging activity [24]. High levels of PCBs have been documented near Pier 27 [28]; its close proximity to the CDF and two WWTP effluent sources and the presence of feminization in round goby collected at Pier 27 [22] strongly suggested this was a contaminated site.

Goby were caught in minnow traps baited with kernel corn and left overnight. Goby were processed on site or brought back to the lab and processed within two days, as described below (see Tissue and plasma sampling section). Male and female goby were identified based on external secondary sexual characteristics and visual inspection of the gonads upon dissection. Female and male goby have a broad, U-shaped or triangular urogenital papilla, respectively. Gravid and non-gravid females have traditionally been defined as those fish with either a gonadosomatic index (GSI) above or below 8%, respectively [29]. Reproductive males were defined as having a GSI over 1% and a developed papilla [20]. Parental male morphs were clearly identified in the field based on physical characteristics including a developed papilla, black body colouration, a large body, and swollen cheeks. Sneaker males were identified by a small body, developed papilla and large gonads. Non-reproductive males ranged widely in size but lacked a developed papilla and gonads [20, 22]. Eggs and seminal fluid were typically visible upon dissection in animals above the GSI cut-offs.

2.2.3 Tissue and plasma sampling

Gobies were placed in an ice bath prior to caudal puncture with a 26.5 gauge needle and a 1 ml syringe to collect blood samples. When this method failed to produce large enough volumes of blood, caudal severance followed by plasma collection with 10 µl coagulation capillary tubes was used. Immediately after blood collection the fish were sacrificed by spinal severance using a scalpel blade or scissors. Blood was stored on ice until it was centrifuged at 14500 rpm for 10 min at 4°C. Plasma was removed and stored at -80°C until extraction for determining plasma hormone concentrations. Liver samples were removed, flash frozen in 2 ml cryogenic vials in liquid nitrogen and stored at -80°C.

2.2.4 Steroid hormone quantification

Plasma samples were extracted by vortexing three times for 30 s in diethyl ether. The ether layer was removed between each vortex, transferred to a new vial and allowed to evaporate prior to reconstitution in reaction buffer to the desired concentration. The extraction recovery, based on the addition of a known amount of hormone into the samples prior to extraction, was $94.7 \pm 3.3\%$ ($n=4$), $88.0 \pm 3.5\%$ ($n=4$) and $90.5 \pm 10.8\%$ ($n=4$) for E2, T and 11KT, respectively. Samples were assayed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI, USA). All standards and samples were run in duplicate. Standard curves of 6.6 to 4000 pg/ml, 3.9 to 500 pg/ml and 0.78 to 100 pg/ml with an average lower detection level (80% binding limit) of 26.79 ± 8.7 pg/ml ($n=5$), 7.36 ± 1.9 pg/ml ($n=4$) and 2.26 ± 0.7 pg/ml ($n=3$) were used for E2, T and 11KT, respectively. The intra-assay

coefficients of variation (calculated from sample duplicates) was $8.10 \pm 5.46\%$ ($n=52$), $8.11 \pm 5.85\%$ ($n=73$) and $8.83 \pm 5.54\%$ ($n=68$) for E2, T and 11KT, respectively. The inter-assay coefficient of variation at 50% binding was 18.32% ($n=5$), 17.80% ($n=4$) and 15.30% ($n=3$) for E2, T and 11KT, respectively. In some cases, low plasma sample volume precluded determination of steroid hormone concentrations.

2.2.5 Total RNA isolation and cDNA synthesis

Total RNA was extracted from liver samples using the TRIzol[®] Reagent protocol (Invitrogen, Carlsbad, CA, USA) using ≤ 100 mg of liver tissue in 1 ml of reagent. Tissues were homogenized in TRIzol[®] for 10 to 30 s using an OMNI GLH homogenizer (OMNI International, Marietta, GA, USA) and the extraction followed the manufacturer's protocol. The total RNA was reconstituted in molecular biology grade water, quantified spectrophotometrically, and absorbance at 260 and 280 nm was used as a measure of relative purity and quantity. Samples showed 260:280 nm ratios of 1.86 or greater. The total RNA samples were analyzed for structural integrity using agarose gel electrophoresis. Samples were diluted to 400 ng/ μ l with molecular biology-grade water. cDNA was made using random hexamer primers and M-MLV reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

2.2.6 Cloning and sequencing of round goby genes

Using pools of liver from reproductively mature gravid females as a total RNA source, Vtg II, Vtg III and 18SrRNA genes were cloned and sequenced for the

development of a qPCR assay. Based on known Vtg sequences in the closely related Japanese common goby and pufferfish, the only other percomorpha species with a complete genome or exhaustive approaches for Vtg gene identification, we expected to find multiple Vtg genes in round goby. Both pufferfish and Japanese common goby contain two Vtgs, type II and III [30], and these genes, along with 18SrRNA, were targeted with gene specific degenerate primers (Table 1). For each gene, a multiple sequence alignment of available fish sequences (Supplemental table 1) was made using Clustal X [31] and used to add degeneracy to primers (Table 1) designed in Primer3 [32] based on Japanese common goby (Vtg II and III) or previously described for fathead minnow (18SrRNA) [33]. PCR amplification of target genes was completed with the Platinum taq polymerase kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Amplified products were gel purified using the Wizard SV gel and PCR clean-up kit, ligated into the pGEM-T easy vector and transformed into JM109 bacterial cells (Promega, Madison, WI, USA). Four colonies per amplicon were grown in LB broth and those colonies with a confirmed insert were plasmid purified using the QIAprep Spin Miniprep kit (Qiagen, Maryland, USA) and bi-directionally sequenced using T7 and SP6 primers in the MOBIX lab (McMaster University, Hamilton, ON, Canada). Sequences were assessed for read quality and consensus sequences were used to create primers for qPCR.

2.2.7 Quantitative PCR

All qPCR primers were designed in Primer3 [32], straddled an intron-exon boundary and had an amplicon size of approximately 100 bp (Table I). Standard curves for two Vtg genes (Vtg II and Vtg III) and one housekeeping gene (18SrRNA) were made using cDNA from a pool of five reproductively active females using 10-fold serial dilutions (1 µg to 100 pg). Reaction parameters included assay efficiencies between 90 to 105%, R² values of 0.98 or better, similar cycle threshold (C_t) values for replicate wells (less than 0.5 C_t difference) and the production of a single peak in the melt-curve analyses to ensure single product synthesis. All qPCR reactions were done on a Stratagene Mx3000TM thermocycler (La Jolla, CA, USA) using the Platinum SYBR Green qPCR kit with ROX (Invitrogen, Carlsbad, CA, USA). cDNA template, made from 1 µg total RNA, was used in each reaction. The reactions were carried out as follows: 2 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 50°C (60°C for Vtg III) and 30 s at 72°C. The melt curve analyses were done by terminating the reaction at 95°C then stepping up one degree every 30 s from 50°C (60°C for Vtg III) to 95°C. Expression levels of Vtg II and III for individual fish were normalized using 18SrRNA expression levels and fold changes were calculated based on the expression levels in the laboratory exposure control group.

2.2.8 Gonad histology

Gonad samples were collected from 30 reproductively mature common carp (*Cyprinus carpio*), pumpkinseed (*Lepomis gibbosus*), and brown bullhead (*Ameiurus nebulosus*), respectively, between August 27 and November 5, 2009 from Cootes

Paradise ($43^{\circ} 16' 46''$ N, $79^{\circ} 53' 36''$ W; Figure 1). Fish were caught by electrofishing or live trap. The average (\pm SD) fish mass and total length was 3.20 (\pm 0.69) kg and 56.0 (\pm 4.28) cm for carp, 42.43 (\pm 15.24) g and 11.6 (\pm 1.12) cm for pumpkinseed, and 517.68 (\pm 92.45) g and 33.9 (\pm 2.46) cm for bullhead. Entire gonads were taken for pumpkinseed and bullhead. A small piece, approximately 8 cm^2 , was removed from the caudal tip of each carp gonad. Gonads were stored in formaldehyde for 1 week, rinsed in 50% ethanol for 20 min and stored in 70% ethanol. Tissues were embedded in paraplast (McCormick, St. Louis, MO, USA) and sectioned at 5 μm . Ten serial sections were taken from each tissue and mounted on superfrost slides (Fisher Scientific, Toronto, ON, Canada). Slides were stained with hematoxylin and eosin (Richard-Allen Scientific, Kalamazoo, MI, USA). Sections were scanned using a Nikon Eclips^c TE2000-S light microscope at 100 X magnification and images were captured using a Nikon DXM1200F digital camera.

2.2.9 Statistics

Statistical analyses were performed using SigmaPlot 11.0 (Systat Software Inc, Point Richmond, CA, USA). Data were log-transformed and statistical differences were determined using one-way ANOVAs, except Vtg II and III expression in field-caught goby which were assessed using a two-way ANOVA on field sites and reproductive classes. Data from sneaker males were omitted from the two-way ANOVA as samples were unavailable for this reproductive class from one field site (GC). To determine whether sneaker male Vtg levels were different across field sites, a one-way ANOVA was used. Following one or two-way ANOVAs, significant differences were determined using

a Holm-Sidak post-hoc test. Data which were non-normal after log-transformation (Vtg III expression from exposure, GSI for males and females) were tested using Kruskal-Wallis analysis of variance on ranks followed by a Dunn's post-hoc test. Sneaker male data were omitted from the statistical test for 11KT due to low sample size. A Spearman rank order correlation test was performed to evaluate the relationship between plasma E2 and Vtg mRNA levels. The significance level for all statistical tests was $P \leq 0.05$.

2.3 Results

2.3.1 Development of a qPCR assay for Vtg expression in round goby

Two vitellogenin (Vtg) genes were specifically targeted based on data from a complete genome (pufferfish) and DNA library screening (Japanese common goby) in the most closely related species with available Vtg sequences. The Japanese common goby [30], a closely related gobiidae, and the pufferfish [7], a fellow percomorpha with a complete genome, were known to have two vitellogenin (Vtg) genes, one type II and one type III Vtg gene per species. Our cloning strategy targeted Vtg II and III genes using liver from reproductively mature, gravid female goby. Approximately 1900, 1650 and 720 bp were amplified from Vtg II (GenBank accession #HM238184), Vtg III (GenBank accession #HM238185) and 18SrRNA (GenBank accession #HM238183) genes from round goby. The 18SrRNA sequence was 89 to 97% identical to other fish sequences; Vtg

II and III sequences were 19 to 70% and 57 to 78% similar to other fish Vtg genes, respectively.

qPCR assays were optimized for all genes; products were tested by melt-curve analysis to confirm the amplification of no more than a single product in each reaction well. Single peaks were generated for every sample with a melting point of 77.3, 83.8 and 82.3°C for Vtg II, Vtg III and 18SrRNA, respectively. Amplification of all genes was seen in all lab control animals and most field samples. Negative controls (no cDNA template) did not produce amplification products indicating that non-target amplification or primer dimerization was not an issue for quantification.

2.3.2 Exposure of non-reproductive male round goby to 17 β -estradiol

Non-reproductive males were collected from a cleaner site (LaSalle Marina) and maintained in the laboratory for a two-week, controlled E2 exposure study. Average plasma levels of E2 increased across treatment groups. Control fish had a mean (\pm SD) of 0.38 (\pm 0.24) ng/ml E2 while E2-exposed fish ranged from 55 to 1290 (\pm 67 to 1531) ng/ml in the 1 and 20 mg/kg groups, respectively (data not shown). Mean E2 levels were significantly higher in fish from all treatment groups compared to control fish. Fish E2 levels in the 5, 10 and 20 mg/kg groups were significantly higher than the 1 mg/kg group ($P \leq 0.05$) but did not vary from each other. The average concentrations of plasma E2 in the males (from all treatment groups) were much higher than levels observed in reproductively active female goby; the average plasma E2 concentration in pooled and non-pooled gravid female goby plasma was 7.92 \pm 5.62 ng/ml ($n=9$; data not shown).

Expression of Vtg II and Vtg III (normalized to 18SrRNA) in all treatment groups were significantly higher than controls, but treatment groups were not statistically different from each other ($P \leq 0.05$, data not shown). A linear trend in dose-response was seen when Vtg II and III expression levels were compared to measured plasma E2 levels (Figure 2 and 3). Plasma E2 levels were significantly correlated with Vtg II ($R = 0.574$; Figure 2) and Vtg III ($R = 0.416$; Figure 3) mRNA levels. Vtg II and Vtg III mRNA levels were significantly correlated with each other ($R = 0.761$; data not shown). The levels of Vtg II and Vtg III expression in E2 exposed male fish were similar to levels seen in field collected females (Figure 4 and 5). Based on normalized cycle threshold values, Vtg III expression levels were approximately 18X higher than Vtg II levels overall (data not shown). Vtg II expression appears to be more sensitive to induction by E2; Vtg II expression increased approximately 10X more than that seen with Vtg III induction (1238% increase versus 100% increase at the highest dose of E2; Figure 2 and 3).

2.3.3 *Vtg expression in field-caught fish*

Females were identified as gravid or non-gravid based on clear differences in GSI ($P \leq 0.05$) yet, gravid and non-gravid females did not differ significantly in body mass or total length (Table 2). As expected, sneaker males were significantly smaller than both parental and non-reproductive males in both length and weight, had a developed papilla and a significantly higher GSI (Table 2; $P \leq 0.05$). Non-reproductive males were not significantly different from parental males in length or weight and had a significantly lower GSI values than both parental and sneaker males (Table 2; $P \leq 0.05$). The number

of fish collected varied across sites for each reproductive class and no sneaker males were available from GC (Table 2).

Vtg II and Vtg III expression in gravid and non-gravid females were not significantly different, but were significantly higher than all male expression levels (Figure 4 and 5). Vtg II expression was not different between non-reproductive males and parental males, but sneaker males had higher Vtg II expression levels than the other two male reproductive classes (Figure 4). Non-reproductive males had the lowest Vtg III expression, parental males intermediate expression levels and sneaker males the highest expression (Figure 5). Both Vtg II and Vtg III levels were significantly higher in non-reproductive males from Sherman Inlet compared to non-reproductive males from the other sites ($P \leq 0.05$). There were no site differences amongst other reproductive classes tested. Vtg III had about 18X higher overall expression than Vtg II in both our field and laboratory exposed fish (data not shown).

2.3.4 Plasma steroid hormone concentration in field-caught fish

Average plasma concentrations of testosterone (T), 11-ketotestosterone (11KT), and 17β -estradiol (E2) were compared between the three male reproductive classes, averaged over all field sites. Sneaker males had a significantly higher concentration of T compared to the other two male reproductive classes ($P \leq 0.05$; Figure 6). Although there were only three samples from sneaker males, a significant difference was detected due to the large difference in plasma T levels. The lowest measured value for sneaker male plasma was 29.79 ng/ml while the highest value for parental males was 4.15 ng/ml.

Parental males had a significantly higher concentration of 11KT compared to non-reproductive males (sneaker males omitted from analysis due to small sample size; Figure 6). There were no significant differences in E2 concentrations between the three male reproductive classes (Figure 6).

2.3.5 Gonad histology

Intersex has been characterized for the round goby used in this study by Marentette et al. [22]. No incidence of intersex were found in any pumpkinseed, brown bullhead or carp screened in this study.

2.4 Discussion

We recommend the use of the round goby as a very suitable model species for studying endocrine disruption in the Great Lakes region. In Hamilton Harbour, this species has demonstrated intersex [22] while other species including goldfish (*Carassius auratus*), gizzard shad (*Dorosoma cepedianum*), bluegill (*Lepomis macrochirus*) [23], common carp, pumpkinseed, and brown bullhead [this study and 23] have not. This lack of intersex in several species is in stark contrast to the high intersex (50-80%) documented in white perch from the same location [23]. Thus, many alternative species do not appear as sensitive to feminization and intersex as round goby. With such a small home range and high site fidelity, round goby offer a significant advantage over more mobile species for determining sources of EDCs into the aquatic environment. These fish

are very easy to capture in large numbers with minimal effort. The round goby offers an invasive, benthic, widely distributed species with the added advantages of having multiple reproductive tactics driven by unique androgens and a prominent secondary sexual characteristic (urogenital papilla) that has been shown to be sensitive to endocrine disruption [22].

Fish populations in the Great Lakes are declining [34] and, at some sites, show signs of endocrine disruption [22, 23]. Though the compounds responsible for endocrine disruption in the lakes may be as diverse as the species that inhabit them, little work has been done to identify which anthropogenic factors are responsible and which areas are at the greatest risk. If more information was available on the nature of biological effects and EDC sources, remediation and prevention efforts could be better guided. One major category of pollutants is natural and synthetic estrogens; these compounds have been well documented as endocrine disruptors [2, 9-13]. The induction of Vtg transcription and translation after estrogen receptor (ER) activation has been well characterized making Vtg expression in male fish a useful biomarker of EDCs [7, 8, 14-16, 35-37].

The ability to monitor endocrine disruption is currently limited to a small number of biomarker assays that are typically only available for common model species. Quantifying Vtg expression has proven to be a reliable and sensitive approach to demonstrate endocrine disruption mediated through the ER [36]. Such assays are generally less expensive and require less time to develop than protein-based immunoassays, providing an opportunity to expand the number of species available for

use in biological monitoring. This approach seems particularly relevant in a comparative context since species appear to vary greatly in their sensitivity to estrogen insults. A fifty-fold increase in sensitivity to a phytoestrogen (the isoflavone genistein), between rainbow trout (*Oncorhynchus mykiss*) and Siberian sturgeon (*Acipenser baeri*) [38] suggests high interspecies variability in sensitivity to estrogenic compounds. Transcriptional assays have measured Vtg responses after 24 h exposures to environmentally relevant concentrations of E2 [14] indicating that qPCR assays may provide better data on temporal aspects of EDC contamination associated with transient and intermittent events such as combined sewer overflow.

Fish species have a variable number of Vtg genes; seven Vtg genes are present in the zebrafish (*Danio rerio*) genome [7] and up to twenty Vtg genes have been identified in rainbow trout (*Oncorhynchus mykiss*) [39]. Vitellogenin genes can be classified based on the motifs and subdomains present; Vtg genes may contain a signal peptide, heavy chain lipovitellin (LvH), phosvitin (Pv), light chain lipovitellin (LvL), beta component, and C-terminal coding region motif and five homologous subdomains (HSDs). The first three HSDs are found in LvH, a conserved motif found in all fish Vtg genes; when present, the last two HSDs are found in LvL [7]. There are three distinct Vtg gene types. Type I Vtg genes contain all three major motifs (LvH, Pv and LvL) with LvL lacking HSD IV and V [7]. Type II Vtg genes have all three motifs and all five HSDs [7]. Type III Vtg genes lack the Pv motif and HSD IV and V from the LvL motif [7].

The diversity of Vtg genes among species and a lack of knowledge of how sensitive each gene is to estrogens made it prudent to target multiple Vtg genes for this

study. Round goby were assumed to have at least type II and III Vtg genes, based on the genes found during DNA library screening for Vtg genes in the Japanese common goby, a close relative [30]. The possibility of other Vtg genes in round goby cannot be ruled out as our approach was not exhaustive, nor are there genome data for this species. The cloned round goby Vtg II and III sequences were part of the LvH motif and shared low homology with each other; only four regions of high identity (>93%) were present, totalling <3% of the total sequence coverage (~1650 bp; data not shown). Primers for each gene were required since amplifying large fragments on both genes would be difficult, if not impossible, with the same primer set. Full-length gene sequences were not obtained for either gene as this was not necessary for the development of a qPCR assay.

Initially, a preliminary waterborne study was conducted in an attempt to mimic likely field exposure to estrogens. The round goby do not fair well in static holding conditions at high densities. As a result, significant induction of Vtg expression was difficult to confirm due to small sample sizes. Based on our preliminary data, we exposed larger numbers of round goby to E2 in a flow-through tank system and using a coconut oil ip injection for slow release estrogen delivery which has been previously used in Vtg induction studies [40-43]. The coconut oil carrier provided significant E2 exposure over a two week period and the increase in plasma E2 concentrations was approximately proportionate to the doses given (data not shown). Higher concentrations of E2 were observed in lab exposed males than those found in reproductive females. Lab exposure to E2 showed that Vtg II and III mRNA expression levels were inducible. Both Vtg II and III mRNA expression levels correlated with plasma E2 levels (Figure 2 and 3) and with

each other, demonstrating that these assays are able to detect increased activation of the ER pathway. After 48 h of exposure, Davis et al. [40] observed 100 to 10 000-fold induction of three Vtg genes in male tilapia (*Oreochromis mossambicus*) liver after injecting 5 mg/kg E2 via a coconut oil carrier. The lowest dose (1 mg/kg) in this study was below that used in tilapia [40] yet produced a similar fold induction (data not shown). Lomax et al. [42] injected English sole (*Pleuronectes vetulus*) with 1 and 5 mg/kg E2 and observed approximately 450 and 600 fold increases in plasma Vtg protein levels, respectively. In this study the same treatment doses produced a 250 and 1200 fold increase in mRNA Vtg II expression, respectively. Waterborne studies using rainbow trout (*Oncorhynchus mykiss*) found that exposed fish with average measured plasma E2 levels of approximately 10 ng/ml had 100 fold induction of plasma Vtg protein levels [44]. Similar fold induction of Vtg II mRNA was observed in this study for fish with plasma E2 levels near 10 ng/ml (Figure 2).

Vtg II and III were not expressed at the same level, nor were they induced to the same degree in treated versus control fish. Vtg III expression was higher than Vtg II expression overall in round goby. In contrast, hepatic expression of type III Vtg genes was lower than expression of type II Vtg genes in zebrafish [7]. Zebrafish, which have seven Vtg genes, likely have many more Vtg genes than round goby; the difference in gene copy number may account for these differences in relative expression. After exposure of male goby to E2, overall induction of Vtg II expression (above control fish Vtg II levels) was about 10X higher than induction of Vtg III expression (above control fish Vtg III levels; Figure 2 and 3). Similar to the round goby, zebrafish Vtg II genes were

more inducible than Vtg III genes with E2 exposure [7]. Clearly, the differences in expression and inducibility of Vtg genes, and the biological relevance of these differences, will require further study.

Both gravid and non-gravid female round goby showed high expression of Vtg II and III genes (Figure 4 and 5). Round goby spawn multiple times during an extended reproductive season (May to October) [45]. Considering that gravid and non-gravid females were not significantly different in either length or weight (Table 2), both groups were likely reproductively active. The non-gravid females may have recently spawned resulting in a lower GSI, rather than being immature. The fact that Vtg levels were relatively constant between gravid and non-gravid females and that round goby spawn continually during their long reproductive season [45] suggest that Vtg mRNA expression levels may not fluctuate during the reproductive season of this species. Further studies validating these points would provide a reasonable basis for using these assays to monitor anti-estrogenic effects in female fish.

Male round goby had significantly lower expression of both Vtg genes compared to females, yet the assays were sensitive enough to detect differences between male reproductive classes. Sneaker males had significantly higher Vtg II levels than the other two male reproductive classes and there were differences between all three male classes in Vtg III expression, although it is currently unclear why this might occur. The difference in Vtg levels between male reproductive classes may be due to the type and amount of circulating steroid hormones; higher levels of 11KT have been reported in parental male goby [20]. We also found that parental males had higher levels of 11KT

and that sneaker males use higher levels of T. There did not appear to be differences in circulating E2 levels between male reproductive classes (Figure 6). Sample sizes for steroid analyses were small, particularly in SM, and the samples sizes were not equal between the field sites for either NRM or PM. As such, an examination of the potential differences in steroid hormone concentrations between sites was difficult. With these samples, we did not detect a statistical difference in mean steroid hormone concentrations across the field sites in NRM or PM. However, the possibility of site differences in steroid hormone concentrations could not be robustly tested and must be revisited with a more complete dataset, particularly by increasing sample sizes for SM and ensuring equal sample numbers across the field sites. This study demonstrates for the first time that there are differences in Vtg expression between male reproductive classes in round goby. Our findings suggest that these assays are sufficiently sensitive to determine differences in expression between reproductive classes and that the use of different male morphs may provide unique information regarding EDC exposure and effects.

Both Vtg II and III assays detected a site difference in non-reproductive males showing higher levels of Vtg expression in fish caught at Sherman Inlet. Sherman Inlet is near Randle Reef, a large sediment deposit of PAHs, that receives intermittent discharge from combined sewer overflow. There are limited data characterizing the mechanism of estrogenic effects of PAHs and PCBs in aquatic species, and most of these compounds have not been shown to be estrogenic. Yet, several studies have reported an increase in vitellogenesis after PAH and PCB exposures [3-5]. Since PAHs are such a dominant contaminant at Sherman Inlet and intersex has been reported at this site in round goby

[22], it cannot be discounted that PAHs are a possible culprit for endocrine disruption. Interestingly, elevated Vtg expression was not seen at Pier 27, a site in Hamilton Harbour where PCB exposure is expected and where feminization of secondary sexual characteristics (urogenital papilla), but no gonadal intersex, has been observed [22]. The lack of significant differences in Vtg II and III expression across some field sites may be attributed to lower sample numbers for some sites. For example, there were no sneaker males collected from one clean site (GC) and GC had lower numbers of fish for most reproductive classes (Table 2).

One potential issue with using Vtg as an endpoint is the variability of expression in response to EDCs. It has been noted that even genetically identical fishes can display large variability in response to the same dose of an ER agonist under identical holding conditions [35]. Prior exposure may be a critical factor in the responsiveness of an individual animal to estrogens. Fish appear to be more sensitive to estrogens and produce much higher levels of Vtg during subsequent exposures compared to animals that have never been exposed [15, 17]. When animals have been previously exposed, long-term exposures to relatively low estrogen concentrations can elicit much larger responses in Vtg gene transcription [15]. In addition, variability between fish may be accounted for by the kinetics of transcription and clearance of mRNA. Plasma E2 levels showed high variability suggesting that uptake and metabolism of E2 could have also been a contributing factor to the variability of Vtg expression. Fish exposed in the lab to E2 were collected in 2009, two years after the field samples for this study were collected. Hence the variability observed between lab control group fish and field-caught fish from cleaner

sites may have been a result of year to year differences in field sites. Increasing sample sizes and expanding data sets with multi-year studies would help to clarify this issue.

Measurement of hepatic Vtg gene expression by qPCR appears to be a sensitive assay, capable of detecting differences between lab exposed and control fish, major reproductive classes and across field sites with and without intersex. The overall expression of Vtg II was lower than Vtg III, and Vtg II was more inducible than Vtg III, but both assays were able to detect significant differences in Vtg expression in lab and field samples. We suggest that these are suitable assays for measurement of Vtg gene expression in the field. The round goby appears to be a useful sentinel species for determining the presence and impacts of EDC pollution in the Great Lakes. These fish are widely distributed, easily caught, and have been shown to be sensitive to several important endpoints including intersex, abnormal secondary sex characteristics [22] and, now with this study, Vtg induction by an ER agonist.

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2.6 Tables

Table 1. Degenerate and qPCR primer sequences for vitellogenin II, vitellogenin III, and 18SrRNA genes in round goby. IUPAC degenerate bases are bolded for degenerate primers used in cloning genes from round goby. All sequences are listed 5' to 3'.

Primer		Primer sequence	Tm ^a	Size ^b	Amplicon length ^c
qPCR primers					
Vtg II	F ^d	ACATGCTTGAGCCATCTAGTGATA	50	24	90
	R ^e	GCAAGACTGGTCCATAGTTTCTT		24	
Vtg III	F	GCAGCTGTGCAGGCCATGAGA	60	21	96
	R	AGCCTCCAGCTCCGGTTCA		20	
18SrRNA	F	CCTGAATACCGCAGCTAGGA	50	20	124
	R	ACCTCTAGCGGCACAATACG		20	
Degenerate primers					
Vtg II	F	HHB GAG TAC ART GRH RTB TGG CC	50	23	1886
	R	AND GTN GCA GCR TCR TTG AT		20	
Vtg III	F	TBW CCT WYG GCT CYC TGG TG	50	20	1658
	R	GWA TCC VAG GAA RTR GTA CAG		21	
18SrRNA	F	GCG GCG ACG WYT CWT TCG AAT GTC	54	24	719
	R	CAT BGT TTA RGG TCG GAA CT		20	

^a Tm = melting temperature.

^b Size = primer length, in base pairs.

^c Amplicon length = PCR product length, in base pairs.

^d F = Forward.

^e R = Reverse.

Table 2. Morphometrics of field-caught round goby. Mean values (\pm SD) of all sites combined are given for round goby of different reproductive classes caught in Hamilton Harbour in 2007.

Class ^a	Length (cm)	Weight (g)	GSI ^b	Sample number per site ^c				
				GC	LS	P27	SI	Total
Females^d								
NGF	7.44 (A) ± 1.09	5.44 (A) ± 2.23	4.62 (A) ± 2.37	5	15	11	19	50
GF	7.41 (A) ± 1.21	6.01 (A) ± 3.05	13.87 (B) ± 4.70	6	7	9	7	29
Males^e								
NRM	9.16 (C) ± 1.71	10.99 (C) ± 6.16	0.29 (C) ± 0.79	9	10	11	7	37
PM	10.06 (C) ± 2.24	14.97 (C) ± 11.34	2.32 (D) ± 0.76	3	12	14	12	41
SM	6.79 (D) ± 1.09	4.18 (D) ± 2.24	6.59 (E) ± 11.43	0	9	12	18	39

^a NGF = non-gravid female; GF = gravid female; NRM = non-reproductive male; PM = parental male; SM = sneaker male.

^b GSI = gonadosomatic index.

^c GC = Grindstone Creek; LS = LaSalle Marina; P27 = Pier 27; SI = Sherman Inlet.

^d One-way ANOVAs comparing NGF and GF for differences in length, weight or GSI. The same letter indicates means are not statistically different at $P \leq 0.05$.

^e One-way ANOVAs comparing NRM, PM and SM for differences in length, weight or GSI. The same letter indicates means are not statistically different at $P \leq 0.05$.

Supplementary Table 1. Accession numbers from fish gene sequences used to design primers for vitellogenin II, vitellogenin III and 18SrRNA genes.

Gene	Species	Common name	Genbank Accession Number
Vtg II	<i>Oryzias latipes</i> *	Medaka	AB064320
	<i>Oryzias latipes</i> *	Medaka	AB074891
	<i>Cyprinus carpio</i>	Common carp	AB086796
	<i>Oncorhynchus mykiss</i>	Rainbow trout	X92804
	<i>Acanthogobius flavimanus</i>	Japanese common goby	AB088473
	<i>Fundulus heteroclitus</i> *	Mummichog	U07055
	<i>Fundulus heteroclitus</i> *	Mummichog	U70826
	<i>Melanogrammus aeglefinus</i> *	Haddock	AF284035
	<i>Melanogrammus aeglefinus</i> *	Haddock	AF284034
	<i>Oreochromis aureus</i>	Blue tilapia	AF017250
	<i>Takifugu rubripes</i>	Fugu	SINFRUT00000179796
Vtg III	<i>Acanthogobius flavimanus</i>	Japanese common goby	AB088474
	<i>Pagrus major</i>	Red seabream	AB181840
	<i>Gambusia affinis</i>	Mosquitofish	AB181837
	<i>Takifugu rubripes</i>	Fugu	SINFRUT00000131263
18SrRNA	<i>Pimephales promelas</i>	Fathead minnow	AY855349
	<i>Cyprinus carpio</i>	Common carp	AF133089
	<i>Danio rerio</i>	Zebrafish	BX296557
	<i>Channallabes sanghaensis</i>	Anguilliform catfish	AJ876387

* indicates multiple gene sequences used from the same species.

2.7 Figures

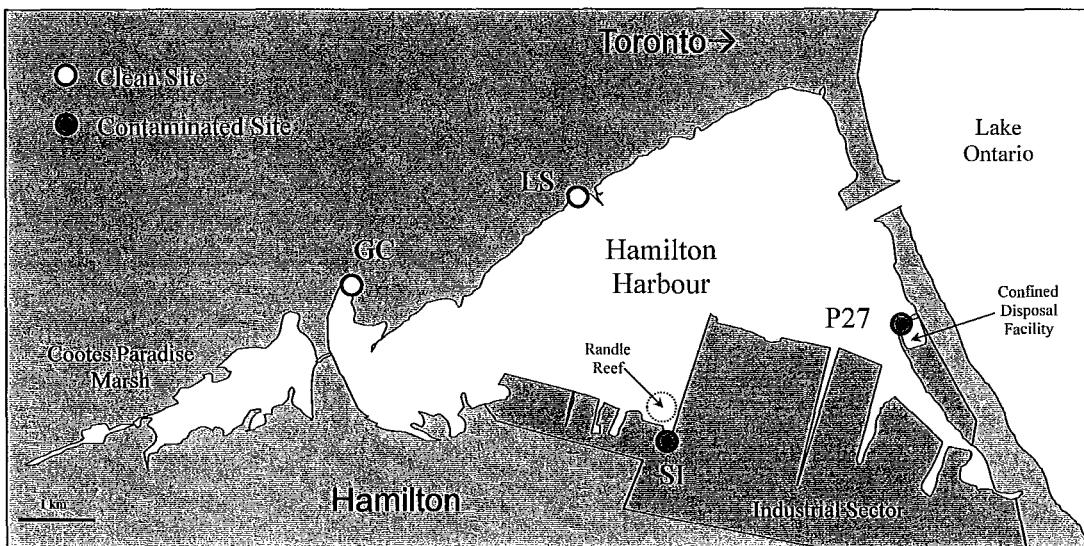


Figure 1. Field sites for round goby collections in Hamilton Harbour [22]. Cleaner sites are indicated by open circles and contaminated sites are indicated by closed circles. GC = Grindstone Creek, LS = LaSalle Marina, P27 = Pier 27, SI = Sherman Inlet.

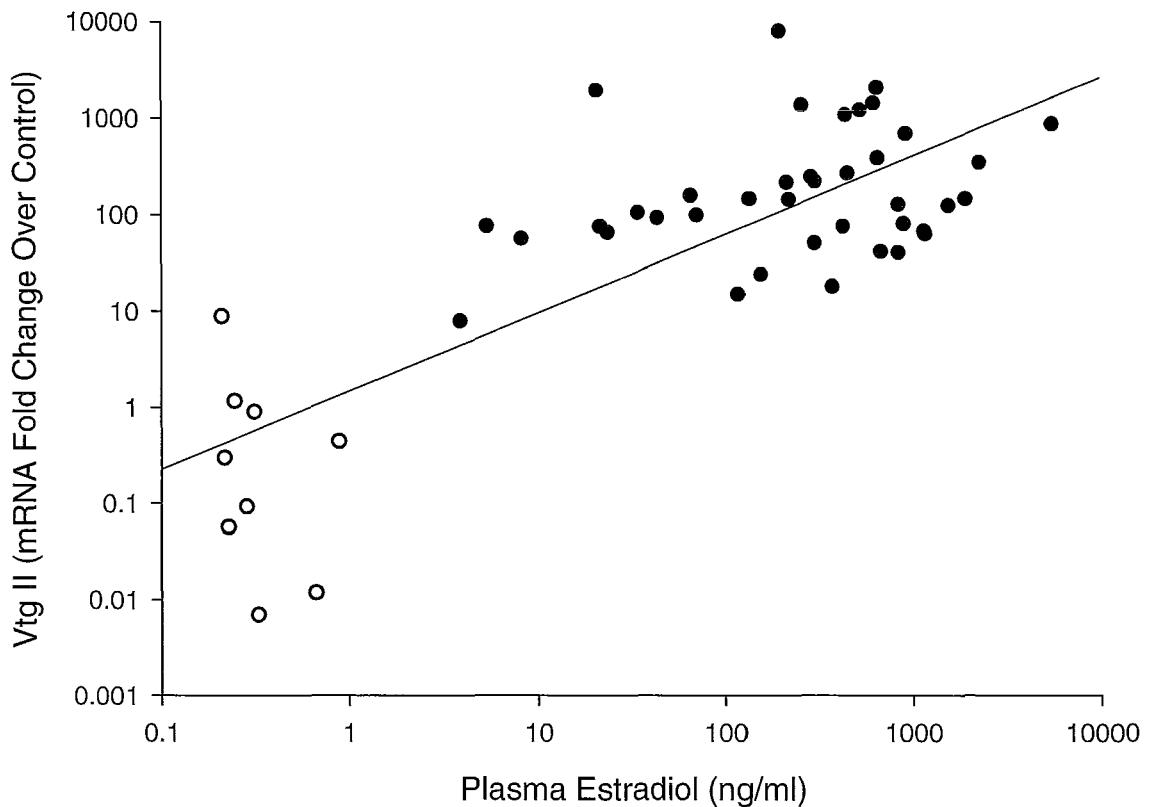


Figure 2. Plasma 17 β -estradiol and hepatic Vtg II mRNA levels in non-reproductive male round goby after two weeks injection with 17 β -estradiol in a coconut oil carrier.
Gene expression was normalized for the expression of 18SrRNA housekeeping genes. Data is shown as the fold change relative to control (coconut oil carrier injection only) animals. Open and closed circles represent control and treated fish, respectively. R = 0.574; Spearman rank order correlation test.

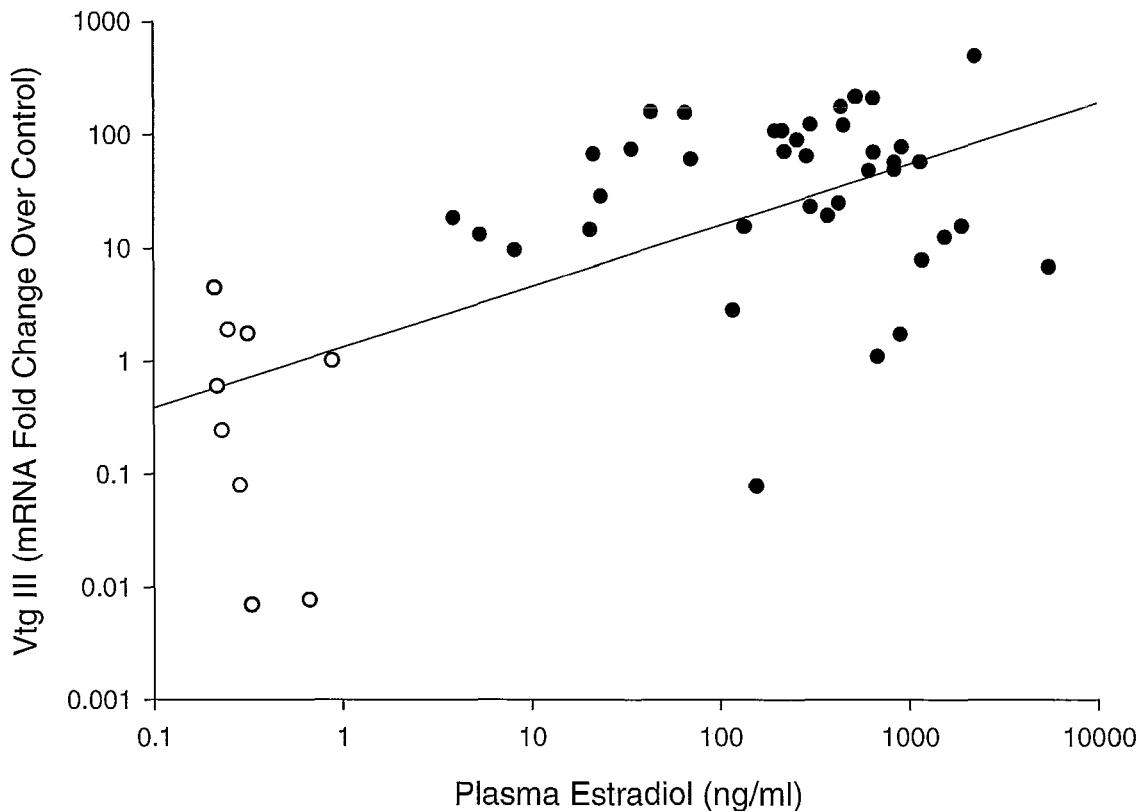


Figure 3. Plasma 17β -estradiol and hepatic Vtg III mRNA levels in non-reproductive male round goby after two weeks injection with 17β -estradiol in a coconut oil carrier.
Gene expression was normalized for the expression of 18SrRNA housekeeping genes. Data is shown as the fold change relative to control (coconut oil carrier injection only) animals. Open and closed circles represent control and treated fish, respectively. $R = 0.416$; Spearman rank order correlation test.

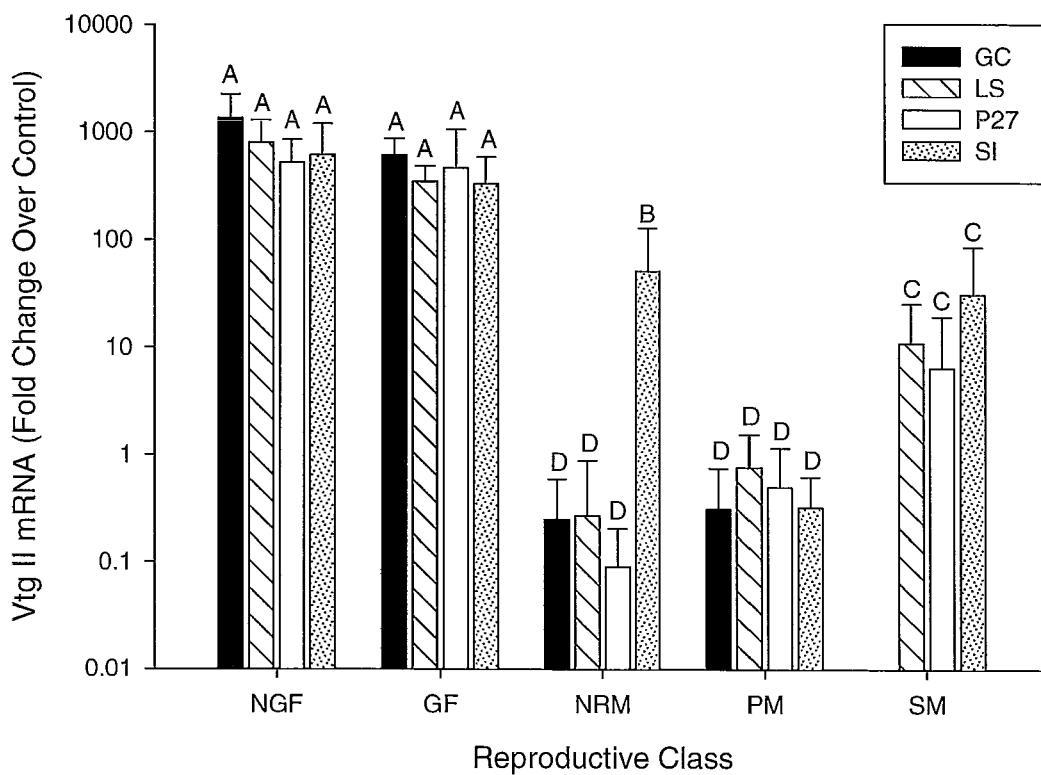


Figure 4. Vitellogenin II gene expression levels in round goby liver collected from Hamilton Harbour. Gene expression was normalized by 18SrRNA expression and is shown as fold change from expression in the lab control group (i.e. non-reproductive males collected from LS and maintained under laboratory conditions). Error bars are standard deviations from group means. Letters indicate statistical differences ($P \leq 0.05$) in a two-way ANOVA; Holm-Sidak post-hoc test. GC = Grindstone Creek, LS = LaSalle Marina, P27 = Pier 27, SI = Sherman Inlet, NGF = non-gravid female, GF = gravid female, NRM = non-reproductive male, PM = parental male, SM = sneaker male.

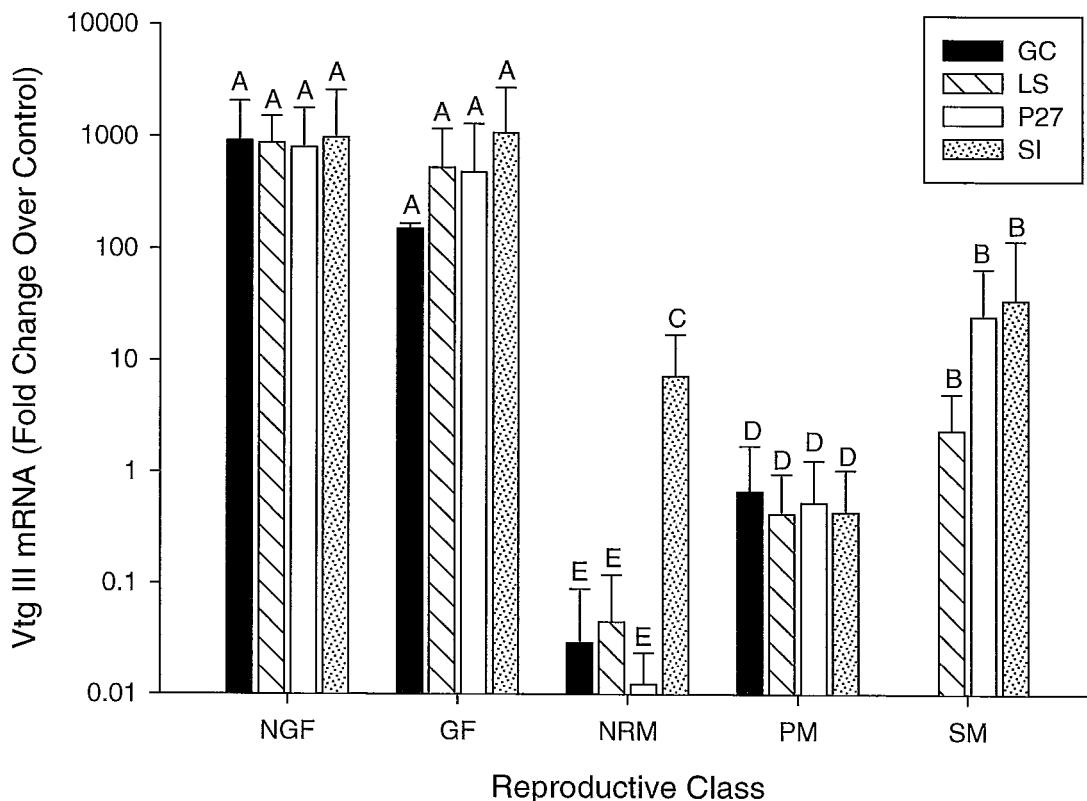


Figure 5. Vitellogenin III gene expression levels in round goby liver collected from Hamilton Harbour. Gene expression was normalized by 18SrRNA expression and is shown as fold change from expression in the lab control group (i.e. non-reproductive males collected from LS and maintained under laboratory conditions). Error bars are standard deviations from group means. Letters indicate statistical differences ($P \leq 0.05$) in a two-way ANOVA; Holm-Sidak post-hoc test. GC = Grindstone Creek, LS = LaSalle Marina, P27 = Pier 27, SI = Sherman Inlet, NGF = non-gravid female, GF = gravid female, NRM = non-reproductive male, PM = parental male, SM = sneaker male.

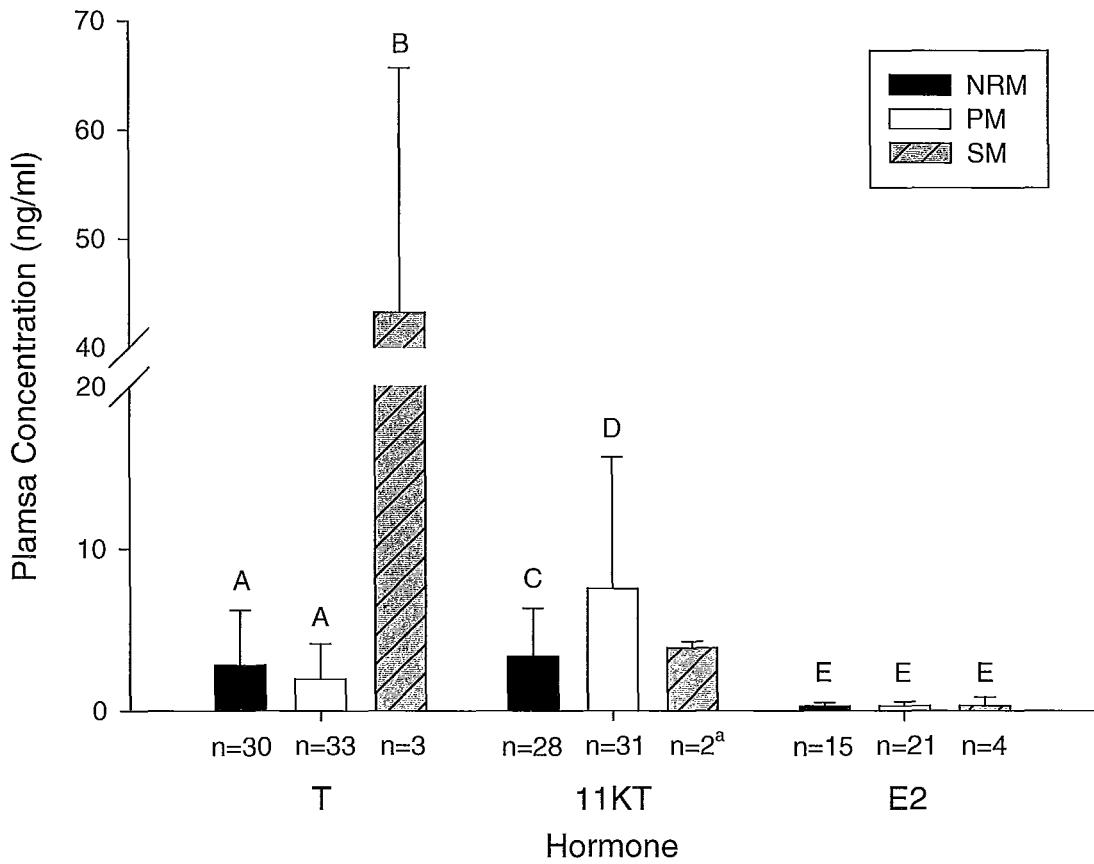


Figure 6. Hormone concentrations in round goby plasma collected from Hamilton Harbour. Error bars are standard deviations from group means. Letters indicate statistical differences ($P \leq 0.05$) in a one-way ANOVA; Holm-Sidak post-hoc test. T = testosterone, 11KT = 11-ketotestosterone, E2 = 17β -estradiol, NRM = non-reproductive male, PM = parental male, SM = sneaker male. ^a SM data were omitted from the statistical test for 11KT due to low sample size.

CHAPTER 3:

**FEMINIZATION OF MALE ROUND GOBY (*NEOGOBius MELANOSTOMUS*)
FROM HAMILTON HARBOUR, LAKE ONTARIO**

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Abstract

Feminization of white perch and round goby has been previously identified at selected sites in Hamilton Harbour, Lake Ontario. The sources of contaminants to Hamilton Harbour are complex and are dominated by steel mill industry and wastewater effluent discharges. Several sites in the harbour have high sediment polycyclic aromatic hydrocarbon (PAH) contamination. Using multiple biological endpoints, we have examined feminization of round goby at seven sites in Hamilton Harbour and two clean sites, one from Lake Ontario and one from Lake Huron. Intersex, identified by the presence of oocytes in male gonads, feminization of the sexually dimorphic urogenital papilla, and hepatic expression of vitellogenin, an egg yolk precursor protein normally expressed in spawning females but not in males, were used to assess endocrine disruption. Vitellogenin gene expression is an early and sensitive endpoint that has been widely used to characterize endocrine disruption in male fish. Endocrine disruption was most severe at sites impacted by industrial contaminants including PAHs and/or polychlorinated biphenyls (PCBs). Sites near wastewater effluent were impacted by endocrine disruption with less severity. Reference sites outside the harbour and one cleaner site in the harbour showed no signs of feminization. This study demonstrates that feminization of round goby (*Neogobius melanostomus*) is widespread in Hamilton Harbour and suggests that PAHs and/or PCBs may be responsible.

Contributions: M.A.Y. and L.A.B. collected field samples and L.A.B. completed analysis of all samples under the supervision of J.Y.W.

3.1 Introduction

Endocrine disruption in aquatic species is a major concern in areas where pollution has been shown to affect viability and reproduction in fish. Hamilton Harbour is an embayment of Lake Ontario that has been heavily impacted by contaminants and fish within this area show signs of endocrine disruption. Intersex, or testis-ova, is identified by the presence of oocytes in male gonads. Intersex has been reported in Hamilton Harbour white perch (Kavanagh et al., 2004) and round goby (Marentette et al., In Press), but not other species of fish such as brown bullhead (*Ameiurus nebulosus*), common carp (*Cyprinus carpio*), or pumpkinseed (*Lepomis gibbosus*; Bowley et al., In Press; Kavanagh et al., 2004). Feminization of the urogenital papilla, a secondary sexual characteristic, has been observed in round goby (Marentette et al., In Press). Induced expression of vitellogenin, an estrogen responsive gene, have been reported in both white perch (Kavanagh et al., 2004) and round goby (Bowley et al., In Press) from Hamilton Harbour. Endocrine disruption in aquatic species has been linked to effluent exposure from both sewage (reviewed in Tyler et al., 1998) and industrial (Stentiford et al., 2003) sources. Yet, the contaminants responsible for these effects in Hamilton Harbour are unclear because of the complex contaminant sources within the harbour. Wastewater effluent has been suggested as the source of endocrine disrupting compounds (EDCs) because the white perch with intersex were downstream of wastewater effluent discharge (Kavanagh et al., 2004) and wastewater effluent often contains known EDC compounds including both natural and synthetic estrogens (Tyler et al., 1998). Since the previously observed feminized round goby were located at sites with significant polycyclic aromatic

hydrocarbon (PAH) and/or polychlorinated biphenyl (PCB) contamination (Bowley et al., In Press; Marentette et al., In Press), these remain possible causative agents.

Hamilton Harbour is a 2150 hectare body of water surrounded by a densely populated region; 46% of the 42 km shoreline is dedicated to heavy industry (O'Connor et al., 2003). There is minimal water exchange with Lake Ontario through a canal on the eastern shore (Figure 1). The harbour has a 49 400 hectare watershed and receives effluent from four sewage treatment plants and 23 combined sewer overflow sites. Hamilton Harbour currently houses the second most contaminated PAH deposit site in Canada as a result of historic coke oven discharge and has been designated as an Area of Concern by the International Joint Commission (Marvin et al., 2000; O'Connor et al., 2003). PCBs are found throughout harbour sediments, particularly in Windermere arm, an area near heavy industrialization (O'Connor et al., 2003). The current study reports several biological endpoints of EDCs, including intersex, in the round goby (*Neogobius melanostomus*). Discerning the distribution and potential contaminant sources responsible for feminization, particularly intersex, in fish found in Hamilton Harbour is critical to protecting native fish populations from EDCs. This is particularly true since Cootes Paradise, located at the western tip of Hamilton Harbour, is an important fish nursery for Lake Ontario.

Anthropogenic inputs into aquatic environments, including sewage (Tyler et al., 1998) and industrial (Stentiford et al., 2003) effluents, have been linked to intersex and the upregulation of vitellogenin (Vtg) expression in males. Vtg is a transcriptionally controlled phospholipoprotein that is normally synthesised by females during egg

development (Denslow et al., 2001). Vtg mRNA expression and consequently, Vtg protein synthesis, is activated in female and male fish when an agonist such as 17 α -ethynylestradiol binds to the estrogen receptor alpha (ER α ; Denslow et al., 2001). The compounds found in receiving waters that contribute to these endpoints may include pharmaceuticals like 17 α -ethynylestradiol (Hirai et al., 2006), or organic compounds such as PAHs (Stentiford et al., 2003) or PCBs (Arukwe et al., 2001; Mortensen and Arukwe, 2008). Most PAH and PCB compounds have not been shown to be estrogenic and yet, several studies have reported increases in Vtg expression upon exposure to certain PAHs and PCBs (Arukwe et al., 2001; George et al., 2004; Mortensen and Arukwe, 2008). PAHs and PCBs that are aryl hydrocarbon receptor (AhR) ligands have been shown to induce estrogen response elements such as vitellogenin genes in the absence of circulating estrogen (Pocar et al., 2005).

Although it is difficult to predict the outcome of extended exposure to mixtures of endocrine disrupting chemicals (EDCs) on fish populations in dynamic systems, reproductive failure and population crashes are possible. Dosing an experimental lake with 5 to 6 ng/L of 17 α -ethynylestradiol led to induction of Vtg and intersex in male fathead minnow (*Pimephales promelas*), as well as the near eradication of the species from the lake (Kidd et al., 2007). Evaluating which areas are at greatest risk of endocrine disruption is an important step in protecting native fish populations as it may guide efforts for remediation and policy development.

The round goby is an ideal model species for the Great Lakes region. Goby are invasive, benthic, highly territorial and widely distributed throughout the region (Jude et

al., 1992). These fish are easily caught in large numbers with minnow traps deployed from shore, allowing high sample numbers without disturbing native fish populations. As many potential EDCs, such as estrogens (Mayer, 2008) PAHs (Marvin et al., 2000; Slater et al., 2008) and PCBs (Labencki, 2008), are found in Hamilton Harbour sediments, round goby may be at greater risk for sediment-bound exposure and therefore a more sensitive species for biomonitoring. Certainly, male goby are sensitive to feminization. Round goby from contaminated sites in Hamilton Harbour had increased Vtg mRNA expression (Bowley et al., In Press), altered secondary sexual characteristics (feminized urogenital papilla) and intersex (Marentette et al., In Press). These fish have high site fidelity and small territories ($5\pm1.2\text{m}^2$; Ray and Corkum, 2001) making this species useful for isolating contamination signatures from different areas.

Mature male goby use parental or sneaker male reproductive tactics; parental males are larger, display dark colouration, and build a nest and care for the brood (Marentette et al., 2009). Sneaker males are small, lightly coloured, have larger gonads relative to body size (gonadosomatic index; GSI) and exploit parental males by fertilizing eggs in the parental male's brood. Parental males use 11-ketotestosterone, while sneaker males use testosterone, as their primary circulating androgen hormone (Bowley et al., In Press; Marentette et al., 2009). The use of different androgens has been correlated with controlling mating tactics (Oliveira et al., 2001). Non-reproductive male goby are a third male morph that can also be utilized, providing the opportunity to observe endocrine disruption in multiple, physiologically distinct reproductive classes within a single species.

This study measured vitellogenin gene expression, intersex and feminization in the round goby from seven sites in Hamilton Harbour and two reference sites. Sites were chosen to include those dominated by wastewater or industrial effluent to distinguish between these possible sources of EDCs. Due to the small home range and high site fidelity of this species, it is highly likely that endocrine disruption at any given site is caused by the major contaminant source at that location. We describe the results of this study in Hamilton Harbour, Ontario, Canada to assess the extent of endocrine disruption in Hamilton Harbour round goby and to isolate which contaminant sources are the most likely causative endocrine disrupting agent.

3.2 Materials and Methods

3.2.1 Field sites

Non-reproductive and reproductive (parental and sneaker) male round goby were collected between May 8 and September 1, 2009 at nine sites; seven sites in Hamilton Harbour and two reference sites. Hamilton Harbour is located on the north-west tip of Lake Ontario and has historical coke oven deposits and ongoing industrial and wastewater effluent inputs. LaSalle Park Marina (LS; 43°17'57" N, 79°50'42" W) is a cleaner site in the harbour that was not near known sources of contamination. Sediments at this site have been tested for PAHs, PCBs and metals (Zeman, 2008), but not for other potential pollutants such as pesticides or pharmaceuticals. Three sites were primarily impacted by wastewater effluent; Desjardins Canal (DC; 43°16'44" N, 79°53'36" W), Pier 25 (P25;

43°16'7" N, 79°46'52" W), and Burlington wastewater (BWW; 43°18'29" N, 79°48'13" W), a site that was adjacent to a discharge pipe from the Burlington wastewater treatment plant. Pier 25 was in Windermere arm where sediment PCB levels were high (Zeman, 2008). Pier 27 (P27; 43°17'3" N, 79°47'31" W), had a closed disposal facility (CDF) that received contaminated sediments from dredging activity (O'Connor et al., 2003). High levels of PCBs have been documented near Pier 27 in Windermere arm (Zeman, 2008); the close proximity to the CDF and two WWTP effluent sources and the presence of feminized urogenital papilla in round goby collected at Pier 27 (Marentette et al., In Press) strongly suggested that this was a contaminated site. The Ottawa Street Slip site (OSS; 43°16'14" N, 79°48'30" W), received effluent from an active steel mill and sediments were known to contain high levels of PAHs (O'Connor et al., 2003). The site at Sherman Inlet (SI; 43°16'12" N, 79°50'1" W) was adjacent to Randle Reef, a site highly contaminated with PAHs from historical coal tar deposits (Marvin et al., 2000; O'Connor et al., 2003). Both OSS and SI received intermittent combined sewer overflow discharge. The two reference sites were located outside the harbour at the Bruce Peninsula National Park on Georgian Bay, Lake Huron (BNP; 45°15'26" N, 81°40'29" W) and Presque'ile Provincial Park on the north east tip of Lake Ontario (PQ; 44°0'15" N, 77°41'49" W; Figure 1).

3.2.2 Field sampling

Non-reproductive and reproductive male fish were collected at each site. Male round gobies have alternative reproductive tactics and both the sneaker and parental male

morphs were collected. With the exception of P25 (n=4 for all classes), parental males from PQ (n=7) and DC (n=5) and sneaker males from DC (n=2), there were at least nine animals collected from each site, for each reproductive class. Goby were caught in minnow traps baited with kernel corn and left overnight. Goby were processed on site as described (see *Tissue sampling*, below). Male goby were identified based on external secondary sexual characteristics and visual inspection of the gonads upon dissection. Female and male goby have a broad, U-shaped or triangular urogenital papilla, respectively. Reproductive males were defined as having a GSI over 1% and a developed papilla (Marentette et al., 2009). Parental male morphs were clearly identified in the field based on physical characteristics including a developed papilla, black body colouration, a large body, and swollen cheeks. Sneaker males were identified by a small body, developed papilla and large gonads. Non-reproductive males ranged widely in size but lacked a developed papilla and gonads. Seminal fluid was typically visible upon dissection in fish identified as either sneaker or parental males by the criteria described above.

3.2.3 Tissue sampling

Goby were sacrificed by spinal severance using a scalpel blade or scissors. The mass of each fish, liver and gonads (including accessory glands) were measured to 0.01 g and 0.001 g, respectively. The standard and total length of the fish were measured to 0.1 cm. The length and width at the base of the urogenital papilla were measured with image analysis software (AxioVision 29A, 4.6.3; Zeiss, Toronto, ON, Canada) to 0.01 mm.

Liver samples were removed, flash frozen in liquid nitrogen and stored at -80°C. Testes lobes were randomly selected (right or left), stored in formaldehyde for 1 week, rinsed in 50% ethanol for 20 min and finally stored in 70% ethanol.

3.2.4 Quantitative PCR

Total RNA was extracted from liver samples using the TRIzol® Reagent protocol (Invitrogen, Carlsbad, CA, USA) using ≤100 mg of liver tissue in 1 ml of reagent. Tissues were homogenized in TRIzol® for 10 to 30 s using an OMNI GLH homogenizer (OMNI International, Marietta, GA, USA) and the extraction followed the manufacturer's protocol. The total RNA was reconstituted in molecular biology grade water, quantified spectrophotometrically, and absorbance at 260 and 280 nm was used as a measure of relative purity and quantity. Samples showed 260:280 nm ratios of ≥1.90. The total RNA samples were analyzed for structural integrity using agarose gel electrophoresis. Samples were diluted to 400 ng/μl with molecular biology grade water. cDNA was made using random hexamer primers and M-MLV reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

Hepatic expression of two Vtg genes (Vtg II and Vtg III) and one housekeeping gene (18SrRNA) were determined with qPCR as previously described (Bowley et al., In Press). Primers straddled an intron-exon boundary and had an amplicon size of approximately 100 bp for each gene. Briefly, qPCR was completed on a Stratagene Mx3000™ thermocycler (La Jolla, CA, USA) using the Platinum SYBR Green qPCR kit with ROX (Invitrogen, Carlsbad, CA, USA). cDNA template, made from 1 μg total RNA,

was used in each reaction. Expression levels of Vtg II and III for individual fish were normalized using 18SrRNA expression levels and fold changes were calculated based on the expression levels in non-reproductive males collected from LaSalle Park (LS) in 2009, housed in the laboratory and used as a control group in previously published research (Bowley et al., In Press).

3.2.5 Gonad histology

Gonads were embedded in paraplast (McCormick, St. Louis, MO, USA) and sectioned at 5 µm. Ten serial sections were taken from each tissue and mounted on superfrost slides (Fisher Scientific, Toronto, ON, Canada). Slides were stained with hematoxylin and eosin (Richard-Allen Scientific, Kalamazoo, MI, USA), using standard histology procedures. Sections were scanned using a Nikon Eclipse TE2000-S light microscope at 100 X magnification and images were captured using a Nikon DXM1200F digital camera. Severity of intersex was characterised according to Ankley et al. (2006).

3.2.6 Statistics

Statistical analyses were performed using SigmaPlot 11.0 (Systat Software Inc, Point Richmond, CA, USA). All data were log-transformed and assessed using a two-way ANOVA on field sites and reproductive classes. Significant differences were determined using a Holm-Sidak post-hoc test. The significance level for all statistical tests was $P \leq 0.05$.

3.3 Results

3.3.1 Morphometrics

Our goal was to collect 30 fish per site (10 for each non-reproductive and reproductive class); P25 ($n=4$ for all classes combined), PQ ($n=7$ parental males) and DC ($n=2$ sneaker males; $n=5$ parental males) had incomplete collections (Table 1). The weight and length of fish were significantly different between the three reproductive classes. Parental males were the largest followed by non-reproductive males and sneaker males (Table 1; $P \leq 0.05$). GSI and HSI were significantly different between all three reproductive classes. Sneaker males had the highest GSI value followed by parental males and non-reproductive males (Table 1; $P \leq 0.05$). HSI values were highest in non-reproductive males followed by parental males and sneaker males (Table 1; $P \leq 0.05$). HSI and GSI were negatively correlated with each other ($R = -0.543$; data not shown) over all samples collected.

There were significant differences in morphometrics among the sites. Fulton's condition factor ($K = \text{weight (g)} / \text{length (cm)}^3$; Danylchuk and Fox, 1996) was applied to all field caught goby as an overall indicator of health in individual fish (data not shown). PQ, a reference site, had very few parental and sneaker males per catch effort and those that were caught had significantly lower K values ($P \leq 0.05$, data not shown) than parental and sneaker males from all other field sites except DC, where K values were also low. Sneaker males from PQ were smaller overall but appeared to be healthy while parental males from this site were severely emaciated. GSI values were significantly

lower in sneaker males from PQ than in sneaker males from all other sites except for DC. Parental males from OSS had significantly lower K values than parental males from SI, despite the close proximity of these sites and similar contaminant inputs. Unlike SI, OSS received effluent from an active steel mill, had higher water temperature than all other sites sampled and the surface of the water was typically covered in an opaque white film.

Feminization of the urogenital papilla was determined by the ratio of the width at the base to the length. Healthy female papillae are short, broad and square-shaped (ratio ~ 0.8; Marentette et al., In Press) while male papillae are long, thin, and triangular in shape (ratio ~ 0.5; Marentette et al., In Press). Male papillae with higher width to length ratios were considered feminized. Non-reproductive male papilla ratios from DC and P27 were significantly higher than papilla ratios from either reference site (Figure 2). Sneaker male papilla ratios from all sites inside the harbour, except DC (n=2 for this site), were significantly higher than those from one reference site (PQ) but not the other (BNP; Figure 3). The papilla ratio from parental males did not differ significantly among sites.

3.3.2 Vitellogenin gene expression

Sneaker males had significantly higher Vtg II and III expression levels than parental or non-reproductive males at each site except for Vtg III expression at PQ (Figure 4 and 5). Parental and non-reproductive male Vtg II and III expression levels were similar at nearly all sites. Vtg III had about 13 times higher expression than Vtg II overall, based on normalized cycle threshold values (data not shown).

Vtg II and III expression was significantly higher in all three reproductive classes at SI compared to reference sites ($P \leq 0.05$; Figure 4 and 5). Other sites in the harbour, including OSS, P27, BWW and DC, had significantly elevated Vtg II and III expression levels, but none were consistently as high as SI. In the Vtg II assay, sneaker males from SI had the highest expression levels. Expression was highest in sneaker males from BWW in the Vtg III assay. Vtg levels were typically lowest in all classes from the two reference sites and LS, a cleaner site in the harbour. Parental male Vtg levels were very low at OSS, despite the fact that the other two classes had elevated Vtg expression levels at this site (Figure 4 and 5).

3.3.3 Gonad histology

Of nine field sites sampled, five sites (DC, OSS, P25, P27, SI; Table 2) had round goby with intersex (Figure 6 and 7). The prevalence of intersex was 25% at P25, but only four samples were screened due to low catch numbers. The small sample size at P25 makes it difficult to predict the actual prevalence of intersex at this site. No intersex was found at either control site (BNP and PQ), the cleaner Hamilton Harbour site (LS), nor at the site immediately adjacent to a wastewater effluent discharge (BWW). The prevalence of intersex ranged from 3 to 25% across sites (Table 3) and the highest incidence was found at SI. The stage of oocyte development in male round goby gonads ranged from perinucleolar (early-development; Figure 6) to vitellogenic (late-development; Figure 7), although only one animal from one site (SI) had vitellogenic oocytes. All other individuals had either perinucleolar and/or cortical alveolar oocytes. Two individuals had

more than one stage of oocyte; the perinucleolar oocytes were the most prevalent in these individuals. The severity of testis-ova (number per section) ranged from grade 1 (least-severe) to grade 4 (most-severe; Ankley et al., 2006; Table 2); grade 4 testis-ova were found at SI, OSS and DC. Spermatozoa were present in all reproductively active male round goby that had testis-ova (Figure 6 and 7). Spermatogonia were present in all non-reproductive male round goby that had testis-ova.

3.4 Discussion

Intersex, feminization of secondary sexual characteristics and elevated Vtg expression in fish species had been previously demonstrated in Hamilton Harbour (Bowley et al., In Press; Kavanagh et al., 2004; Marentette et al., In Press), yet little is known about which anthropogenic factors are responsible for these effects. At least two species in the harbour have intersex, white perch in Cootes Paradise (Kavanagh et al., 2004) and round goby within Hamilton Harbour (present study; Marentette et al., In Press). Several other common species have been screened for intersex without a single case identified (Bowley et al., In Press; Kavanagh et al., 2004). The harbour is impacted by numerous pollutants including four wastewater treatment plants, 23 combined sewage overflows and various types of historic and current industrial contaminants including heavy metals and organic compounds like PAHs and PCBs (O'Connor et al., 2003). This makes it difficult to identify which compounds, individually or in combination, are the most dominant causes of endocrine disruption in native fish populations. Biomarkers such

as Vtg mRNA expression in male fish are relatively sensitive compared to the presence of intersex, which is indicative of more severe impacts. Evaluating multiple endpoints at sites with different contaminant inputs is helpful to assess which sites in the harbour are of greatest concern and to identify which compounds or contaminant sources are likely involved.

Morphological measures provided basic information about the goby collected across our field sites and provided clues about the environmental conditions at each site. As expected, sneaker males were smaller in size than the other two reproductive classes and had the highest GSI and lowest HSI values (Table 1). Low HSI values in sneaker males are likely a trade-off to their large gonadal investment. In contrast, non-reproductive males had a very low GSI but the highest HSI of all reproductive classes. Energy and mass investment in gonads is typically at the expense of liver investment during pre-spawning and spawning periods in fish (Huntingford et al., 2001). Since HSI and GSI were negatively correlated, it follows that round goby sacrifice liver weight to compensate for larger gonads, particularly in sneaker males where small body size has added constraints to gonadal versus hepatic investment.

Parental males from PQ appeared to be very unhealthy and had low Fulton's body condition factor values. Sneaker males from this site were small compared to other sites, but appeared healthy. These differences may simply be related to food availability, but since fish from PQ were different from other sites data should be interpreted with caution. Condition factor was also significantly lower at the OSS site. The water at OSS is typically warmer than the rest of the harbour due to steel mill effluents (Cheng, 2009).

Temperature affects the dissolved oxygen content of water and metabolism in poikilotherms (Buentello et al., 2000). It is likely that high temperature and low dissolved oxygen were important factors in arresting growth at this site and these factors cannot be discounted when considering the morphological condition of round goby from OSS.

Vtg genes are up-regulated after activation of the ER receptor by a ligand in male and female fish (Denslow et al., 2001). The use of Vtg as an endpoint for exposure to natural and synthetic estrogens has been well characterized (Biales et al., 2007; Denslow et al., 2001; Rotchell and Ostrander, 2003; Scott et al., 2006; Wang et al., 2005). All three male round goby classes expressed Vtg, although the level of expression was quite different even at the same site. Sneaker males almost always had significantly higher Vtg II and III expression levels than parental or non-reproductive males (Figure 4 and 5). Bowley et al. (In Press) found significantly higher levels of Vtg mRNA expression levels in sneaker male compared to non-reproductive and parental males, although the reason for this is unknown. Sneaker males have higher plasma levels of testosterone while parental males have higher plasma levels of 11-ketotestosterone, yet estradiol levels were not different between reproductive male morphs (Bowley et al., In Press; Marentette et al., 2009). This apparent difference in circulating steroid androgen hormone may be related to Vtg expression, but a more robust study of circulating estradiol levels in fish with upregulated Vtg expression is required to clarify this point. It has been previously shown that parental males had significantly higher plasma 11-ketotestosterone levels than non-reproductive males, but no difference in plasma testosterone (Bowley et al., In Press; Marentette et al., In Press). Vtg expression levels are similar between parental and non-

reproductive males, supporting the notion that androgen levels, particularly testosterone and not 11-ketotestosterone, are an important factor to the induction of Vtg expression in male round goby liver. Since testosterone is the precursor to estradiol and Vtg genes are estrogen responsive, the lack of difference in plasma estradiol levels across reproductive male morphs is contradictory. We have yet to determine if there are differences in steroid levels in fish caught at different sites in Hamilton Harbour or compared to our reference sites.

Vtg II and III expression levels were highly variable in field collected fish in this study. It is not uncommon for transcriptional Vtg assays to show high inter (Latonnelle et al., 2002) and intra (Celsius et al., 2000) -species variability in controlled lab exposure studies. Even genetically identical fish have shown high variability after exposure to an ER agonist (Celsius et al., 2000). Prior exposure to estrogens has been shown to make individual fish much more responsive to subsequent exposures in Vtg-based studies (Craft et al., 2004; Denslow et al., 2001). High variability is expected in field studies where exposure to potential EDCs is undefined and uncontrolled. Considering that combined sewage overflow discharge may have occurred at several of our sites, particularly DC, SI and OSS, intermittent exposure to additional estrogenic compounds may have occurred during sampling periods at these sites and contributed to variability.

Vtg II and III were not expressed at the same level in this study. Vtg III levels were about 13 times higher than Vtg II. In a controlled estradiol exposure, Bowley et al. (In Press) found that Vtg III expression was about 18 times higher than Vtg II, but Vtg II was about 10 times more inducible over control animals in non-reproductive male round

goby. Zebrafish have been shown to have higher expression levels of type II Vtg genes compared to type III (Wang et al., 2005). This difference may be accounted for by the fact that zebrafish have seven Vtg genes (Wang et al., 2005), while the round goby likely has fewer Vtg genes. Without more information on the nature of round goby Vtg genes, it is not apparent why they are expressed and induced at different levels.

Round goby collected from our two reference sites outside the harbour and from LaSalle marina, a cleaner site in the harbour, were not as impacted by endocrine disruption, if at all. Intersex was not observed at any of these sites. Vtg II and III levels were relatively low except for in parental males from PQ. Parental males from this site appeared to be very unhealthy and this may be related to the higher Vtg levels seen in these fish. LaSalle is not near any industrial or wastewater contaminant sources and fish from this site were expected to be less impacted than at any other site in the harbour. Vtg mRNA expression levels at LaSalle are higher than at BNP, but are not significantly different. Sneaker male papilla ratios were significantly higher at LS than at PQ, but this may be related to the small size of sneaker males from PQ. A lack of significant endocrine disruption at this site suggests that EDCs in Hamilton Harbour were localized and probably sediment bound rather than distributed in solution throughout the harbour from a single point source.

Lower levels of endocrine disruption were observed at DC and BWW, two of our wastewater effluent impacted sites. Sneaker males at both sites had elevated Vtg mRNA expression levels. Feminization of the papilla was found in non-reproductive males at DC, and one male had intersex at this site. No intersex was found in BWW goby despite a

comprehensive screening of 29 males. Feminization of the papilla was found in sneaker males from BWW. These sites, especially BWW where sampling was immediately adjacent to the effluent source, were impacted by wastewater effluent from the Dundas and Burlington wastewater treatment plants, respectively (O'Connor et al., 2003). It was predicted that goby from BWW would show high levels of intersex and Vtg expression in all reproductive classes if wastewater was a significant source of EDCs in the harbour. As this was not the case, our data suggests that wastewater effluents may not be the major contributor to endocrine disruption in Hamilton Harbour round goby. Goby from sites where other contaminant classes and sources dominate showed a higher prevalence and more severe forms of endocrine disruption.

Round goby from P27 had intersex and high levels of Vtg II and III expression. Feminization of the papilla was seen in non-reproductive and sneaker males from this site. Feminization of secondary sexual characteristics had been reported at this site, but not intersex (Marentette et al., In Press) or elevated Vtg levels (Bowley et al., In Press). P27 is about three kilometres from two wastewater effluent sources, but its close proximity to Windermere arm, an area of high PCB sediment contamination, and a confined disposal facility where contaminated sediment dredged from the harbour has been dumped (O'Connor et al., 2003; Zeman, 2008), would strongly suggest that wastewater effluent sources are not the dominant contaminants at P27. Since only four goby were collected from P25 and Vtg levels were not tested, it was difficult to robustly characterise endocrine disruption at this site. Intersex was discovered in one male, but goby are in too low an abundance at this site to use this as a major site for study. P25 is located in

Windermere arm and is close to the Woodward wastewater treatment plant effluent source. Since goby from BWW did not have intersex, it is unlikely that intersex in fish from P25 was caused by wastewater effluent contaminants, although it may have been a factor. This site, like P27, is heavily polluted with sediment-bound industrial pollution (O'Connor et al., 2003).

Round goby from OSS were among the most impacted in this study. OSS had elevated levels of Vtg II and III expression as well as intersex. Sneaker males had feminized urogenital papillae at this site. Interestingly, Vtg levels were very low in parental males from this site. Both OSS and SI receive combined sewage overflow and have high levels of sediment PAH contamination (O'Connor et al., 2003). OSS received effluent from an active steel mill and the water quality was characterized by high temperatures and low light penetration. Parental males from this site had low weight to length ratios compared to non-reproductive and sneaker males which may be related to low Vtg levels observed there.

Endocrine disruption was most severe in all reproductive classes at SI. The Vtg II and III assays showed significant elevation in expression at SI in all reproductive classes (Figure 4 and 5). This is consistent with previous work on round goby from the harbour (Bowley et al., In Press). Goby at this site had the highest prevalence and the most advanced stages of intersex. 10% of all male fish screened had intersex (n=39). Marentette et al. (In Press) found 13% intersex at the same site (n=31). Sneaker male papillae were feminized at this site in this study and in Marentette et al. (In Press). SI was the most heavily impacted site in this study. The influence of PAHs on endocrine

disruption is highly likely since PAHs were the major contaminant at SI (Marvin et al., 2000; O'Connor et al., 2003). PAHs and PCBs are known to induce vitellogenesis (Arukwe et al., 2001; George et al., 2004; Mortensen and Arukwe, 2008), although the mechanism behind this effect is unclear. It is likely regulated by aryl hydrocarbon receptor (AhR) ligands via AhR/ER cross-talk pathways. This site received effluent from a combined sewage overflow, but wastewater effluents do not appear to be a major EDC in the harbour since sites like BWW and DC were less impacted.

Intersex was observed in round goby at five of the nine field sites. As expected, sites where intersex was observed also had higher Vtg expression and feminization of the urogenital papilla. SI showed a high level of intersex and the most advanced oocyte development; one sample from SI, but no other site, had vitellogenic oocytes (Table 2 and Figure 7). Of the eight fish that had testis-ova, five were sneaker males, two were non-reproductive males and only one was a parental male. Interestingly, sneaker males had higher Vtg expression levels overall suggesting that sneaker males are more vulnerable to feminization. We speculate that the apparent increase in sensitivity to feminization in sneaker males is related to higher circulating levels of testosterone, their primary androgen. The only site that had a higher Vtg signal but no intersex was BWW. Our screening for intersex was comprehensive; we had reasonable numbers of fish at this site and for each individual fish we had multiple sections of the entire gonad for identification of oocytes. A lack of intersex at BWW indicates that wastewater effluents may not be as influential as other contaminants at causing endocrine disruption in Hamilton Harbour. Our study shows intersex in round goby for the first time at DC, P25, P27 and OSS,

suggesting that intersex in round goby is more broadly found in Hamilton Harbour than previously thought (Marentette et al., In Press).

The round goby is a useful model species for endocrine disruption studies as several endpoints can be readily used to assess contaminated sites. The Vtg II and III assays employed in this study were able to show significant site differences with relatively low sample numbers in several reproductive classes. Non-reproductive and sneaker males showed similar site differences in Vtg expression; parental males appear to be the least informative for identifying site differences with Vtg expression. All male reproductive classes displayed intersex, but sneaker males were most affected, suggesting that sneaker males are more sensitive to feminization than the other two male classes. This study has shown high Vtg expression, feminization of secondary sexual characteristics and intersex at several sites in Hamilton Harbour; feminization of round goby appears to be broadly distributed within the harbour. Sites with the highest prevalence of intersex and the highest Vtg levels were most impacted by industrial contaminants; although intermittent combined sewage overflow or low level wastewater effluent inputs are concurrently found at several of these sites. This data suggests that PAHs and/or PCBs are the most likely contaminants responsible for endocrine disruption in round goby from Hamilton Harbour.

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3.6 Tables

Table 1. Morphometrics of field-caught round goby. Mean values ($\pm SD$) from each site are given for round goby of different reproductive classes caught in Hamilton Harbour in 2009.

Category ^b	Site ^a								
	BNP	PQ	LS	DC	BWW	P27	OSS	SI	All Sites ^c
TL (cm)									
NRM	6.7 ± 1.2	7.7 ± 1.3	10.3 ± 2.4	10.7 ± 1.8	9.7 ± 1.7	8.5 ± 1.3	7.5 ± 1.3	6.0 ± 1.0	8.5 (A) ± 2.1
PM	11.0 ± 1.4	9.0 ± 1.4	10.9 ± 1.6	10.4 ± 0.8	10.7 ± 2.1	10.2 ± 1.5	9.6 ± 1.1	8.3 ± 1.1	10.0 (B) ± 1.7
SM	8.2 ± 1.0	6.0 ± 0.4	7.0 ± 1.1	7.8 ± 2.9	6.8 ± 1.3	6.7 ± 0.8	7.6 ± 0.9	6.0 ± 0.9	6.9 (C) ± 1.2
Weight (g)									
NRM	3.98 ± 2.27	6.62 ± 3.30	20.24 ± 12.83	19.43 ± 7.50	15.27 ± 9.23	9.77 ± 6.07	6.12 ± 3.27	3.25 ± 1.40	10.82 (A) ± 9.06
PM	20.31 ± 8.00	7.57 ± 3.49	22.57 ± 9.58	12.92 ± 1.67	21.20 ± 13.08	17.30 ± 8.23	12.88 ± 4.30	10.26 ± 5.35	15.63 (B) ± 9.06
SM	8.54 ± 3.31	2.78 ± 0.41	5.79 ± 2.84	7.64 ± 7.12	5.21 ± 3.05	4.74 ± 2.01	6.89 ± 2.47	3.50 ± 1.70	5.64 (C) ± 3.03

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Category ^b	BNP	PQ	LS	DC	BWW	P27	OSS	SI	All Sites ^c
NRM	0.4	0.1	0.1	0.1	0.1	0.2	0.2	0.6	0.2 (A)
	±0.4	±0.1	±0.0	±0.0	±0.1	±0.2	±0.2	±0.5	±0.3
PM	3.1	1.7	2.1	1.0	2.7	2.2	2.6	3.4	2.5 (B)
	±1.0	±0.8	±0.9	±0.9	±1.1	±0.7	±0.9	±3.1	±1.6
SM	10.1	1.2	7.0	4.5	7.1	6.2	6.1	5.0	6.0 (C)
	±4.6	±0.5	±6.5	±1.1	±3.7	±1.2	±1.9	±2.0	±4.0
HSI									
NRM	2.6	2.7	4.8	4.1	3.4	3.3	3.4	3.2	3.4 (A)
	±0.8	±0.3	±1.3	±0.7	±1.6	±0.8	±1.1	±0.9	±1.2
PM	2.0	1.3	3.9	2.3	4.1	2.4	3.1	3.1	2.8 (B)
	±0.5	±0.4	±1.4	±0.5	±1.5	±0.7	±0.7	±0.6	±1.2
SM	1.2	1.7	1.7	1.5	1.8	1.4	2.0	2.2	1.7 (C)
	±0.4	±0.6	±0.8	±0.6	±0.8	±0.5	±0.9	±0.7	±0.7
n									
NRM	9	12	10	12	10	14	10	9	86
PM	11	7	11	5	9	10	10	12	75
SM	11	10	10	2	10	11	10	17	81

^a BNP = Bruce Peninsula, PQ = Presque'ile, LS = LaSalle Marina, DC = Desjardins Canal, BWW = Burlington Wastewater, P27 = Pier 27, OSS = Ottawa Street Slip, SI = Sherman Inlet.

^b TL = total length, NRM = non-reproductive male, PM = parental male, SM = sneaker male, GSI = gonadosomatic index, HSI = hepatosomatic index.

^c Two-way ANOVAs comparing NRM, PM and SM in each category. Different letters indicate means are statistically different at $P \leq 0.05$.

Table 2. Prevalence of gonadal intersex in round goby from Hamilton Harbour and reference sites.

Site	n	Incidence	Prevalence (%)
Bruce Peninsula	31	0	0
Presque'ile	29	0	0
LaSalle Marina	38	0	0
Desjardins Canal	21	1	5
Burlington Wastewater	29	0	0
Pier 25	4	1	25
Pier 27	35	1	3
Ottawa Street Slip	31	1	3
Sherman Inlet	39	4	10

Table 3. Characterization and severity of intersex in Hamilton Harbour round goby.

Site ^a	Class ^b	Grade	Oocyte stage ^c
DC	NRM	4	PN
P25	SM	1	CA
P27	PM	2	CA
OSS	SM	4	PN, CA
SI	SM	4	PN, CA, VG
SI	SM	2	CA
SI	SM	1	CA
SI	NRM	3	PN

^a DC = Desjardins Canal, OSS = Ottawa Street Slip, P25 = Pier 25, P27 = Pier 27, SI = Sherman Inlet.

^b NRM = non-reproductive male, PM = parental male, SM = sneaker male.

^c PN = perinucleolar, CA = cortical alveolar, VG = vitellogenic.

3.7 Figures

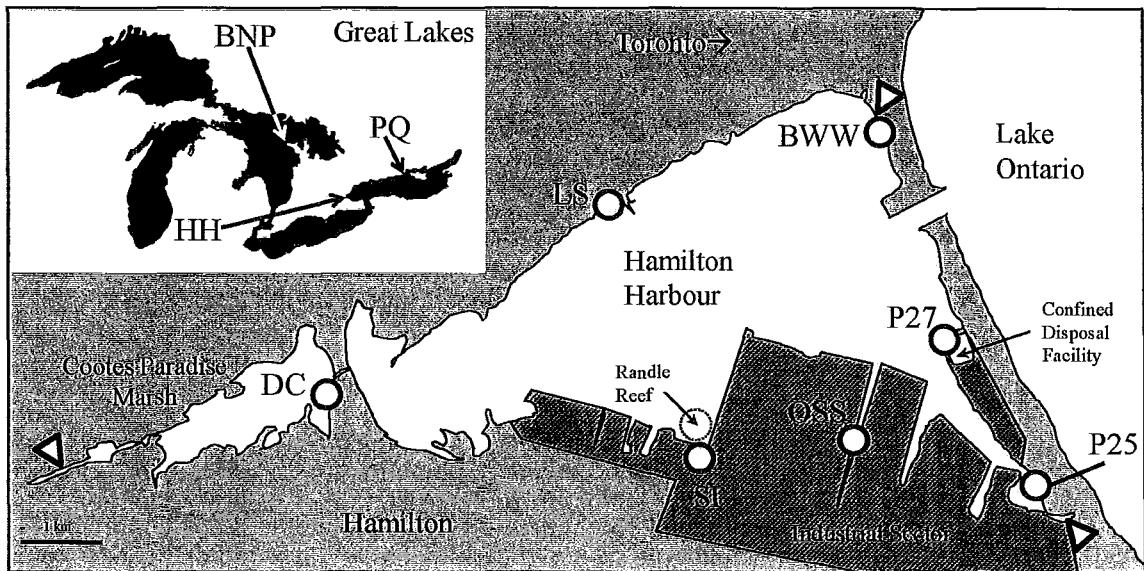


Figure 1. Location of field sites for assessment of feminization in round goby. Collection sites are indicated by bold, open circles. Wastewater effluent sources are indicated by open triangles. Randall Reef, the site of very high sediment associated PAHs, is shown with a circle and arrow. Inset shows the location of the two reference sites and Hamilton Harbour within the Great Lakes region. BNP = Bruce Peninsula, PQ = Presque'ile, LS = LaSalle Marina, DC = Desjardins Canal, BWW = Burlington Wastewater, P27 = Pier 27, OSS = Ottawa Street Slip, SI = Sherman Inlet, HH = Hamilton Harbour.

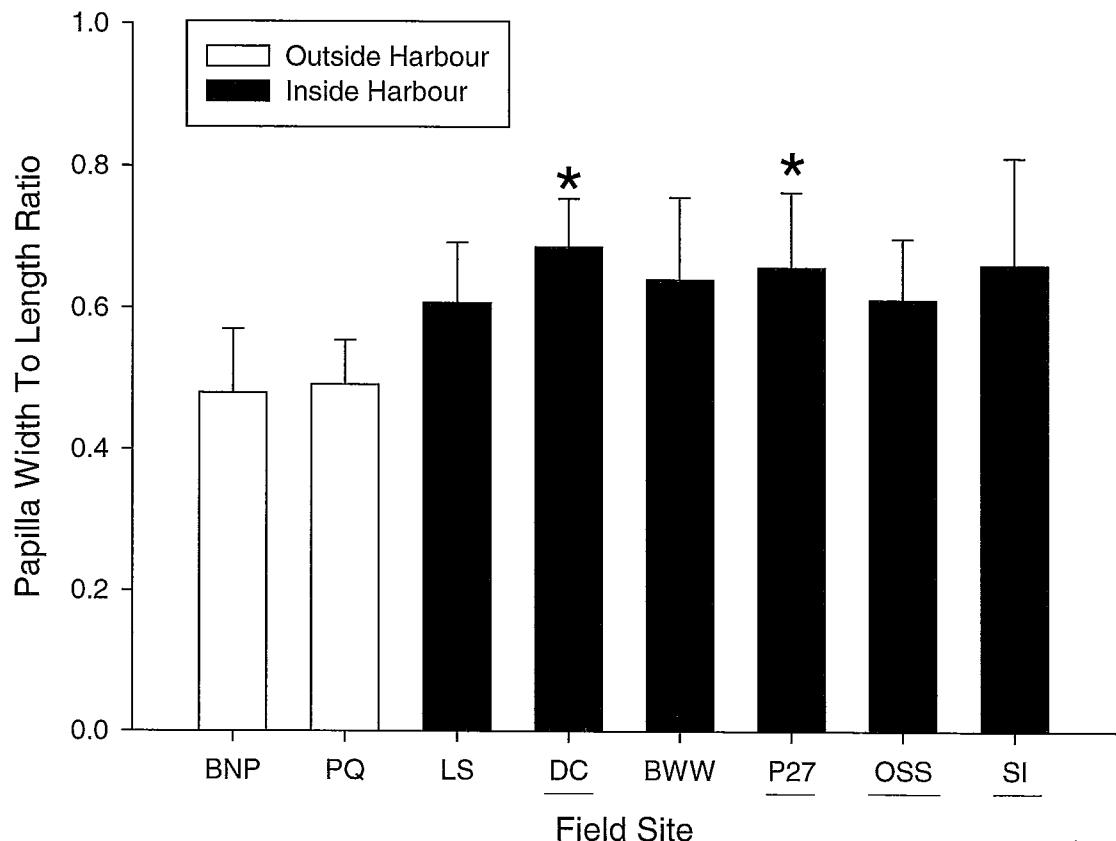


Figure 2. Feminization of non-reproductive male urogenital papilla at sites inside and outside Hamilton Harbour. Field sites with intersex are underlined for reference. Asterisks indicate significantly different from both clean sites outside the harbour ($P \leq 0.05$) in a two-way ANOVA; Holm-Sidak post-hoc test. BNP = Bruce Peninsula, PQ = Presque'ile, LS = LaSalle Marina, DC = Desjardins Canal, BWW = Burlington Wastewater, P27 = Pier 27, OSS = Ottawa Street Slip, SI = Sherman Inlet.

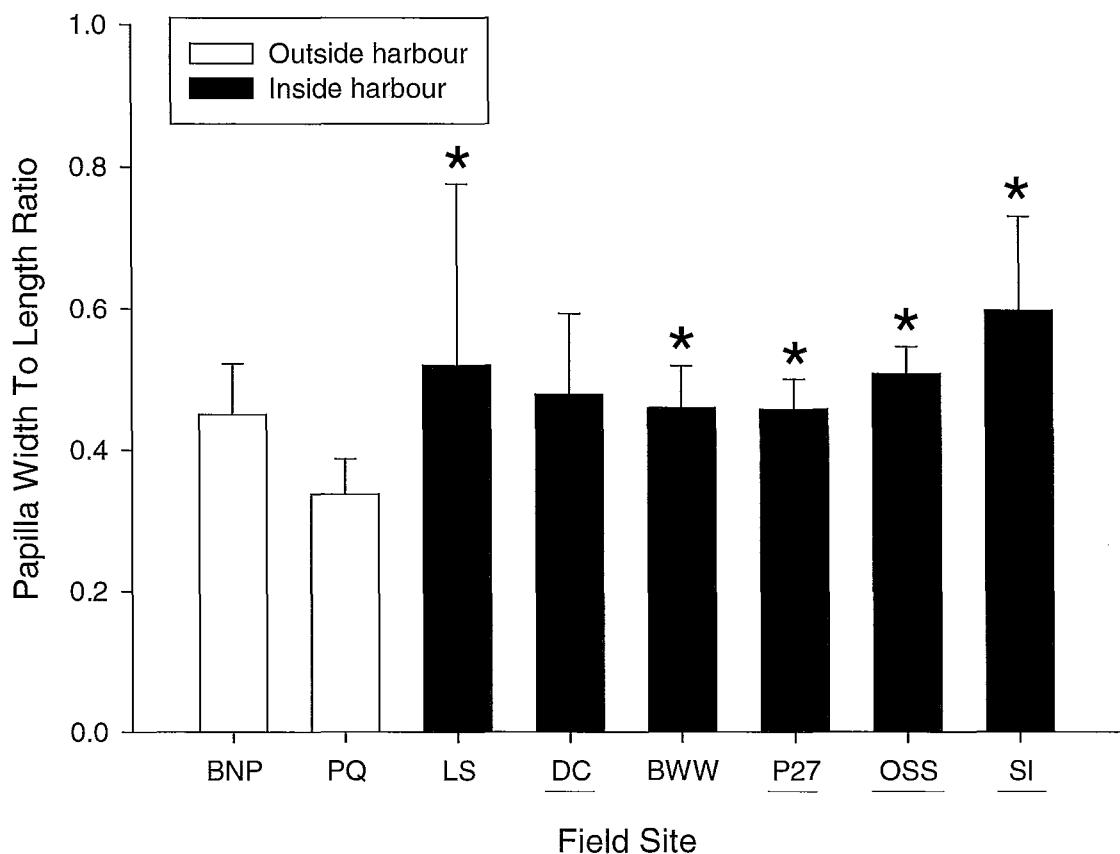


Figure 3. Feminization of sneaker male urogenital papilla at sites inside and outside Hamilton Harbour. Field sites with intersex are underlined for reference. Asterisks indicate significantly different from one clean site outside the harbour (PQ; $P \leq 0.05$) in a two-way ANOVA; Holm-Sidak post-hoc test. BNP = Bruce Peninsula, PQ = Presque'ile, LS = LaSalle Marina, DC = Desjardins Canal, BWW = Burlington Wastewater, P27 = Pier 27, OSS = Ottawa Street Slip, SI = Sherman Inlet.

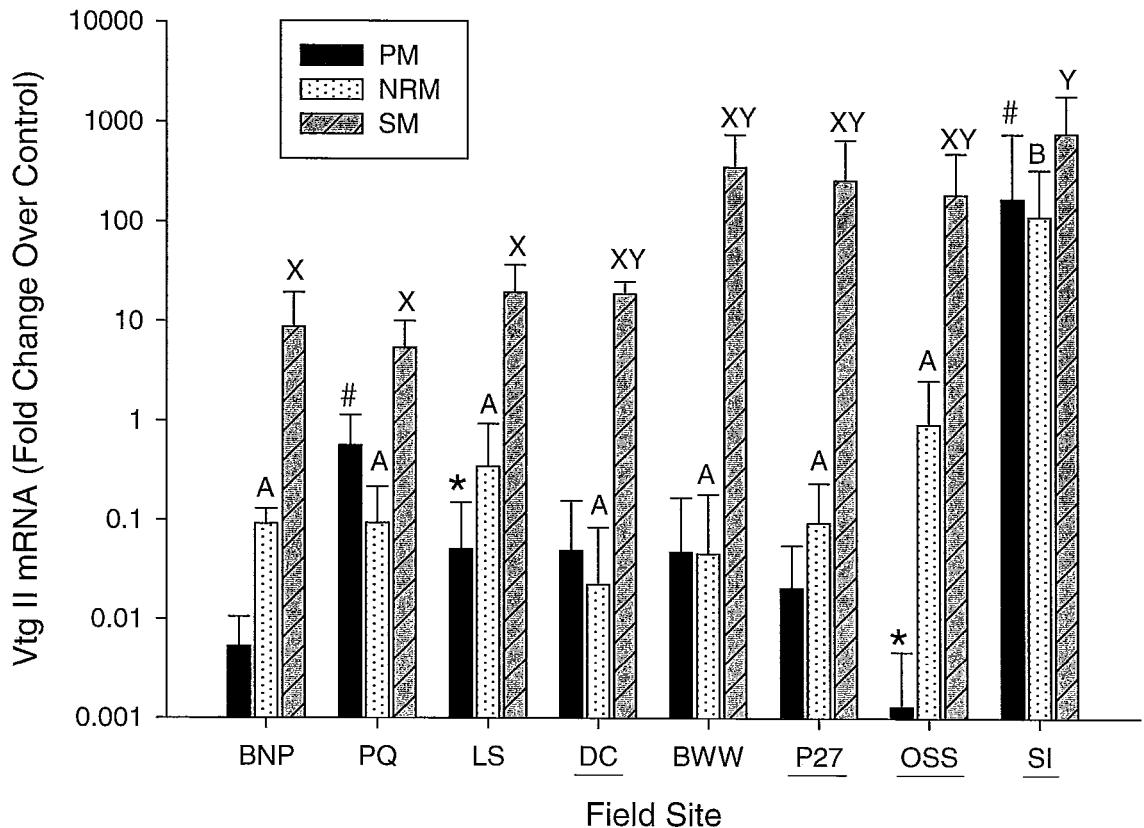


Figure 4. Hepatic vitellogenin II mRNA expression levels in round goby. Gene expression was normalized by 18SrRNA expression and is shown as fold change. The fold change is based on the expression of non-reproductive male fish held in the lab during a previous study (Bowley, In Press). Error bars are standard deviations from group means. Sites with intersex are underlined for reference. Letters indicate statistical differences between sites, within reproductive classes; * = different from PQ; # = different from OSS ($P \leq 0.05$) in a two-way ANOVA; Holm-Sidak post-hoc test. BNP = Bruce Peninsula, PQ = Presque'ile, LS = LaSalle Marina, DC = Desjardins Canal, BWW = Burlington Wastewater, P27 = Pier 27, OSS = Ottawa Street Slip, SI = Sherman Inlet, NRM = non-reproductive male, PM = parental male, SM = sneaker male.

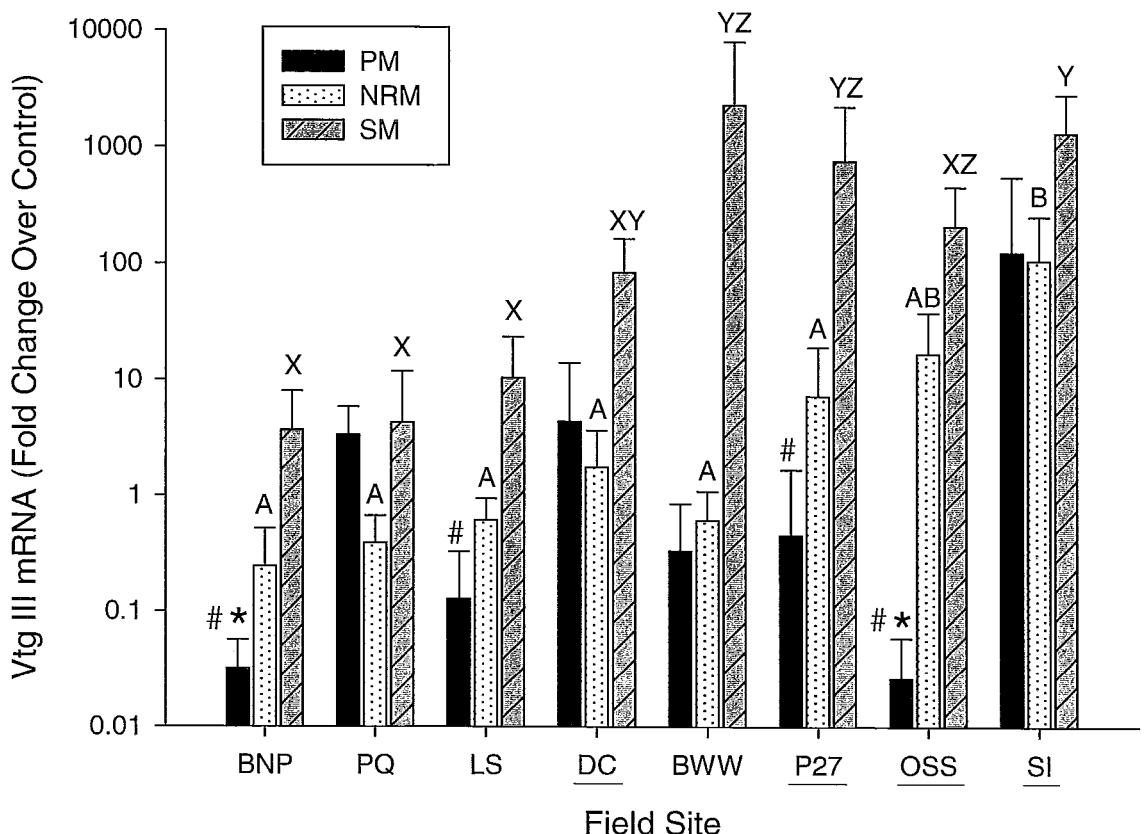


Figure 5. Hepatic vitellogenin III mRNA expression levels in round goby. Gene expression was normalized by 18SrRNA expression and is shown as fold change. The fold change is based on the expression of non-reproductive male fish held in the lab during a previous study (Bowley, In Press). Error bars are standard deviations from group means. Sites with intersex are underlined for reference. Letters indicate statistical differences between sites, within reproductive classes; * = different from PQ; # = different from SI ($P \leq 0.05$) in a two-way ANOVA; Holm-Sidak post-hoc test. BNP = Bruce Peninsula, PQ = Presque'ile, LS = LaSalle Marina, DC = Desjardins Canal, BWW = Burlington Wastewater, P27 = Pier 27, OSS = Ottawa Street Slip, SI = Sherman Inlet, NRM = non-reproductive male, PM = parental male, SM = sneaker male.

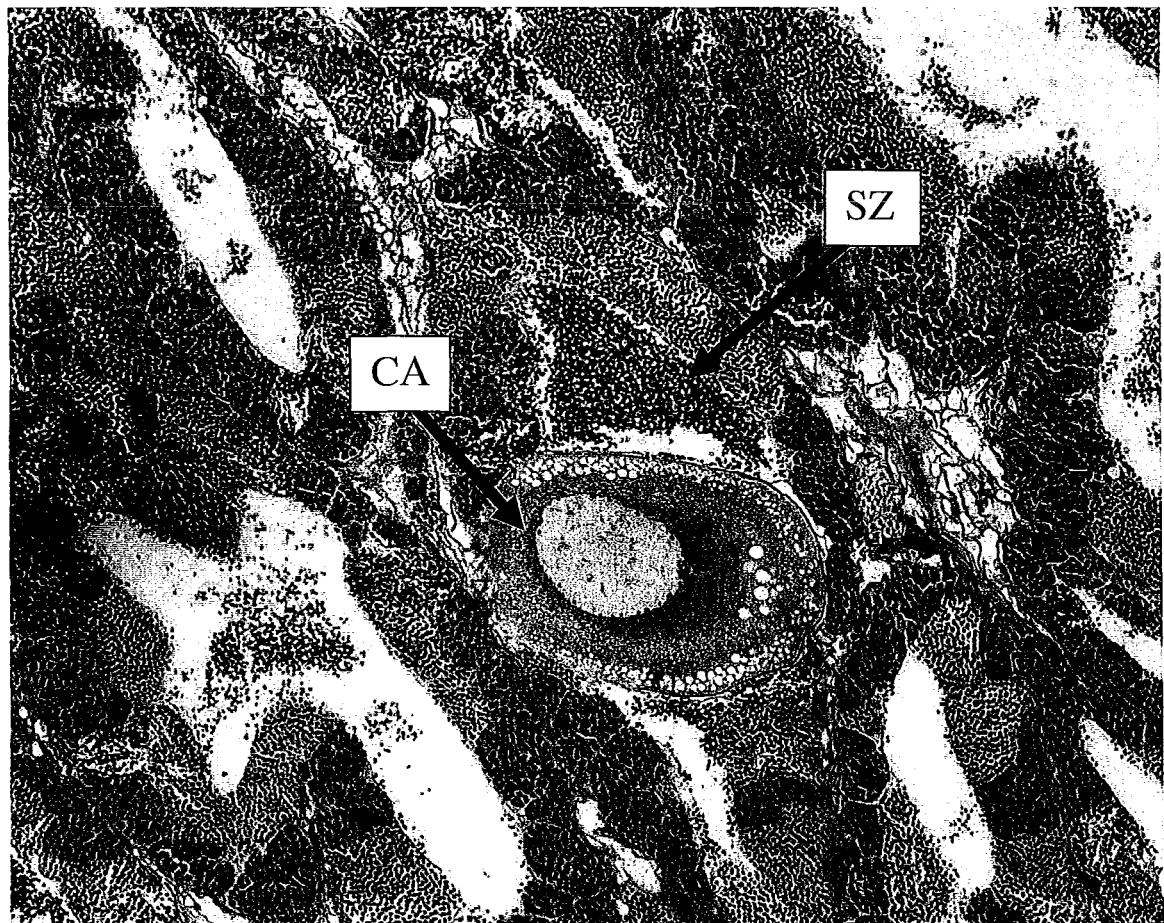


Figure 6. Grade one testis-ova in round goby. Testis is from a mature sneaker male from Sherman Inlet. Sperm cells can be seen at all stages of development. A single oocyte was present in the section. SZ = mature spermatozoa, CA = cortical alveolar stage oocyte. Magnification 200x.

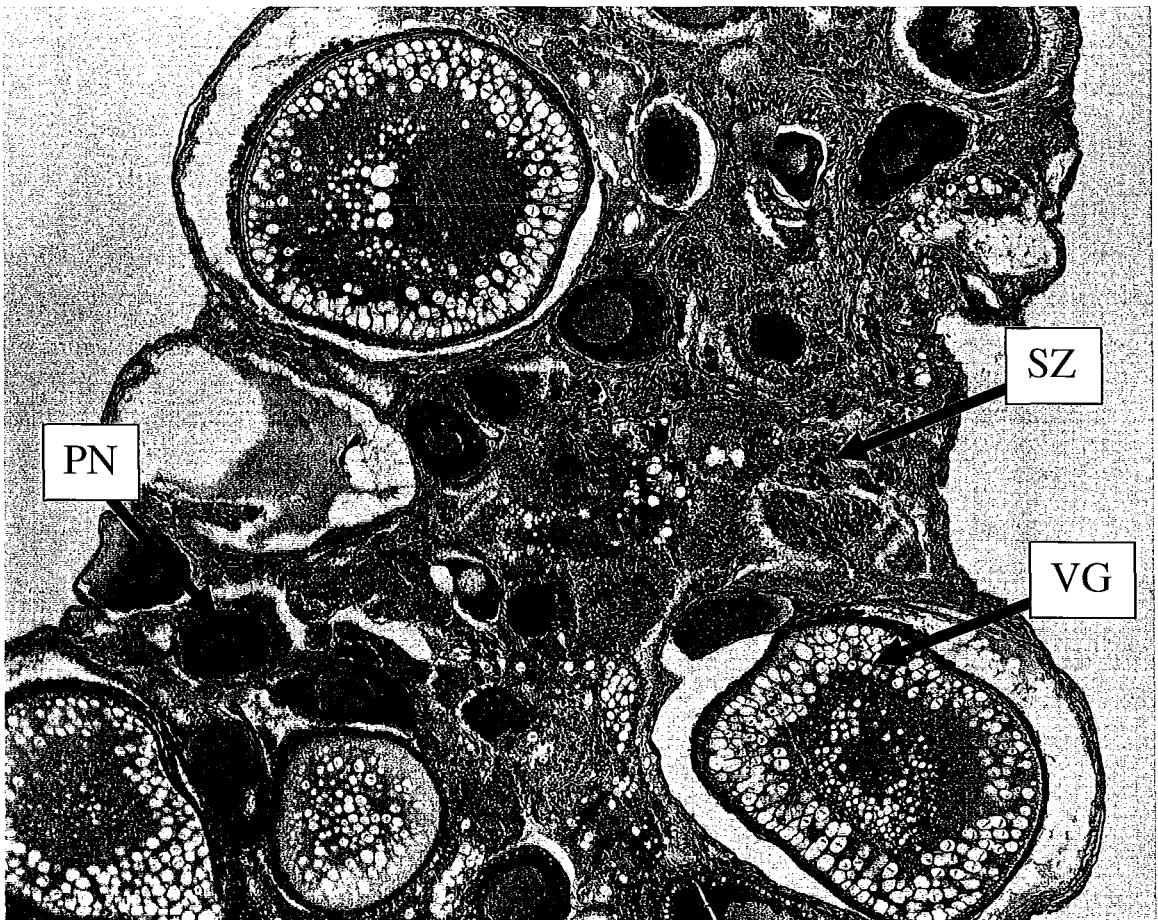


Figure 7. Grade four testis-ova in round goby. Testis is from a mature sneaker male from Sherman Inlet. Sperm cells can be seen at all stages of development, but are sparse. Over 70 oocytes were observed in the section and dominated the gonad area. SZ = mature spermatozoa, PN = perinucleolar stage oocyte, VG = vitellogenic stage oocyte. Magnification 100x.

CHAPTER 4:

GENERAL DISCUSSION

4.1 Endocrine disruption in the Great Lakes

Native fish species in the Great Lakes region are under enormous pressure from commercial fisheries, pollution, invasive species and changing ecosystem dynamics (Mills et al., 2003). Scientific investigation evaluating endocrine disruption from certain forms of pollution, such as pulp mill effluent (McMaster et al., 2006; Orn et al., 2006; Shaughnessy et al., 2007; Van der Kraak et al., 1992), has been comprehensively conducted; wastewater effluent and industrial forms of pollution have not been adequately characterized for many regions in the Great Lakes, including Hamilton Harbour. Hamilton Harbour is a designated Area of Concern due to heavy pollution from past and present industrial activity (O'Connor et al., 2003). It is also impacted by four wastewater treatment plant effluent sources. Despite there being several reports of intersex in numerous fish species (Kavanagh et al., 2004; Marentette et al., In Press and Chapter 3), and declines in native fish populations (Mills et al., 2003), there has never been a comprehensive study evaluating differences in site-specific impacts in order to discover which compounds are causing endocrine disruption in the harbour. It has been assumed that intersex was caused by wastewater effluent, as there were significant inputs

of wastewater effluent but not industrial effluent, into Cootes Paradise, the region of Hamilton Harbour where very high rates of intersex were seen in white perch (Kavanagh et al., 2004). Yet, intersex in round goby was first identified at Sherman Inlet, an area adjacent to the highest sediment PAH contaminant site in the harbour (Marentette et al., In Press). Thus, endocrine disruption by industrial contaminants could not be completely ruled out and it was unknown whether intersex was influenced more heavily by wastewater effluent or industrial contaminants.

4.2 Biological endpoints and the round goby as a model species

In order to evaluate which pollution sources were most critical in disturbing fish populations, an appropriate model species was selected and several biological endpoints were established. Numerous species have been screened and showed no signs of intersex in Hamilton Harbour including goldfish (*Carassius auratus*), gizzard shad (*Dorosoma cepedianum*), bluegill (*Lepomis macrochirus*; Kavanagh et al., 2004), common carp (*Cyprinus carpio*), pumpkinseed (*Lepomis gibbosus*), and brown bullhead (*Ameiurus nebulosus*; Kavanagh et al., 2004 and Chapter 2). The samples sizes in the original survey of intersex in Hamilton Harbour were quite small (Kavanagh et al., 2004). Intersex in brown bullhead, common carp and pumpkinseed have been examined more thoroughly (Chapter 2) because these species are particularly widespread and common in the Great Lakes and are very good model species. Due to a lack of intersex in these species, an alternative model was chosen in the round goby (*Neogobius melanostomus*). The goby is

ideal for characterising site-specific impacts due to its high site fidelity and small territory. Based on scuba follows, its home range is only approximately 5 m^2 (Ray and Corkum, 2001). They are benthic, making them good targets for exposure to sediment bound endocrine disrupting chemicals (EDCs). They are easy to catch in high numbers and due to their small size (Charlebois et al., 1997), round goby offer the potential for larger scale experimental exposures that would be impossible in other common model species. The goby has been shown to be sensitive to intersex and feminization (Marentette et al., In Press and Chapter 3) and can now be used to evaluate vitellogenin (Vtg) levels in male fish (Chapter 2 and 3) as an indicator of endocrine disruption.

The three male reproductive classes of round goby use different types and levels of primary circulating androgens. Sneaker males use testosterone, parental males use 11-ketotestosterone and non-reproductive males have both androgens in low levels (Marentette et al., 2009 and Chapter 2). This provides an opportunity to study two physiologically distinct reproductive classes and a non-reproductive class within a single species. Our work has shown that there are differences in sensitivity to endocrine disruption between the three reproductive classes. Sneaker males are the most sensitive to intersex and show significant increases in Vtg expression at contaminated sites. Sneaker males also had consistently higher expression levels of both Vtg genes overall. In contrast, non-reproductive and parental males showed a low prevalence of intersex, but non-reproductive males had significant increases in Vtg expression at contaminated sites. Parental males were less useful in demonstrating endocrine disruption, but may still be

valuable for studies of circulating hormone levels since they have high levels of 11-ketotestosterone (Marentette et al., 2009; Oliveira et al., 2001 and Chapter 2).

Previous to this study, assays to measure Vtg gene expression in the round goby did not exist. Using Vtg expression as an endpoint in Hamilton Harbour was limited to existing model species such as carp or rainbow trout (*Oncorhynchus mykiss*). These species were not conducive to this research for several important reasons. First, intersex carp have not been found in Hamilton Harbour. Second, the large size of both fish at reproductive maturity makes them difficult to maintain in the laboratory for experiments. Third, both carp and trout have a much larger home range which would preclude their use in assessing specific contaminant sources based on their location at capture. Rainbow trout use Cootes Paradise as a fish nursery but this species is not found at all the sites we specifically targeted in this study.

In chapter two, we developed assays to measure Vtg II and III expression in round goby. These assays were validated by a lab study exposing goby to 17 β -estradiol (E2) *in vivo*, demonstrating an induction of Vtg gene expression in response to an estrogen receptor (ER) agonist. The degree of upregulation in Vtg II and III genes in our lab-based exposure study was comparable to that seen in similar studies using other species (Davis et al., 2008; Folmar et al., 2001; Lomax et al., 1998; Pinto et al., 2006). There are multiple Vtg genes in many fish species and there is variability in both overall expression and inducibility between genes (Trichet et al., 2000; Wang et al., 2005) in a given species. Wang et al. (2005) found that type II Vtg genes in zebrafish (*Danio rerio*) liver had higher expression than type III genes. Round goby Vtg III (a type III gene) had higher

overall expression than Vtg II (Chapter 2). Both our study (Chapter 2) and the study by Wang et al. (2005) found that type II Vtg genes were more inducible upon exposure to an ER agonist. Differences in type II and III Vtg gene expression between round goby and zebrafish may be due to the fact that zebrafish have seven Vtg genes (Wang et al., 2005), although the total number of Vtg genes in round goby has not yet been determined.

We have shown that the round goby has at least two Vtg genes, one type II and one type III Vtg gene. Type II Vtg genes have three motifs (heavy chain lipovitellin (LvH), phosvitin (Pv) and light chain lipovitellin (LvL), and five homologous subdomains (HSDs; Wang et al., 2005). Type III Vtg genes lack the Pv motif and HSD IV and V from the LvL motif (Wang et al., 2005). Both Vtg II and III gene sequences that were cloned in round goby were part of the heavy chain lipovitellin (LvH) motif; yet, there were only four regions of high identity (>93%) present, covering <3% of the total sequence alignment between cloned Vtg II and III genes in round goby. It is unlikely that the round goby has many more than two Vtg genes, if any, since its close relative, the sand goby (*Acanthogobius flavimanus*), has only two Vtg genes (Ohkubo et al., 2004). Further study on the differences in expression and inducibility of Vtg genes, and the biological relevance of these differences, will require further study in the round goby.

4.3 Site differences and endocrine disruption in Hamilton Harbour

This study has shown which sites in Hamilton Harbour are the most heavily impacted by measuring multiple biological endpoints in the round goby. In chapter two,

we demonstrated that there was significantly higher Vtg II and III expression in non-reproductive males at Sherman Inlet (SI) compared to three other sites in the harbour. This site is heavily impacted by sediment PAH contamination (Marvin et al., 2000; O'Connor et al., 2003). In chapter three, field sampling was expanded to include more sites and higher sample numbers to allow a more robust evaluation of feminization of goby in the harbour. Intersex, secondary sexual characteristics, and Vtg expression were evaluated at each site, providing progressively more severe endpoints for endocrine disruption. There were site differences in the biological endpoints that clarify which sites were most impacted. This information allows us to predict which types of contamination are the most harmful to aquatic species based on known pollution inputs at each site.

LaSalle Marina (LS) is a less contaminated site in Hamilton Harbour. To our knowledge, endocrine disruption has never been demonstrated in round goby at this site. With no proximal contaminant inputs from either industrial or wastewater sources, male fish sampled from this site were expected to show low levels of Vtg expression, no feminization of urogenital papillae and no intersex. Vtg mRNA expression levels appeared to be slightly higher than at the two reference sites outside the harbour, but were not significantly different, as expected. A lack of intersex, some feminization of secondary sexual characteristics, and low levels of Vtg expression at this site in round goby suggest that native fish populations that are being impacted by EDCs, such as the white perch (Kavanagh et al., 2004), are being exposed to pollutants locally at contaminated sites rather than by waterborne EDCs moving throughout the harbour. Since round goby are territorial and have a small home range, endocrine disruption in fish from

LS would only occur if EDCs in the harbour were mobile or if contaminants existed at this site.

Desjardins Canal (DC) and Burlington wastewater (BWW) are sites impacted by wastewater effluent from the Dundas and Burlington wastewater treatment plants, respectively (O'Connor et al., 2003). Sampling was done much closer to the effluent source at BWW than at DC. Elevation in Vtg expression was apparent at BWW, but less so at DC. Feminization of urogenital papillae was observed at both sites, but was higher at DC. Intersex was found in one male goby at DC but no intersex was found at BWW despite adequate sample sizes and a robust screening of gonads. The lack of intersex at BWW suggests that wastewater effluents are not the major contributor to endocrine disruption in Hamilton Harbour round goby, especially when considering the proximity of sampling to the effluent source. Elevated Vtg levels at BWW are likely due to wastewater effluent, but this endpoint is a more sensitive measure of endocrine disruption. There are often low levels of natural and synthetic estrogens in wastewater effluent (Kidd et al., 2007; Kolpin et al., 2004; Larsson et al., 1999; Nichols et al., 1999) which could account for elevated Vtg levels at these sites. Increases in Vtg expression is often seen after low level chronic exposures to EDCs, while intersex is not (Rotchell and Ostrander, 2003).

Pier 27 (P27) is near Windermere arm, an area with high sediment PAH and PCB contamination, and adjacent to a confined disposal facility for contaminated, dredged sediment from the harbour (O'Connor et al., 2003). Intersex and feminization of secondary sexual characteristics was found at this site and Vtg expression levels were significantly elevated, indicating a high degree of endocrine disruption in round goby.

Feminization of secondary sexual characteristics has been previously reported in goby from this site (Marentette et al., In Press), but this is the first report of intersex and elevated Vtg mRNA expression levels. P27 is located between two wastewater treatment effluent sources from the Woodward and the Burlington plants, but the distance to each effluent source is about three km. Intersex was found in one of four male fish sampled from Pier 25 (P25), which is much closer to the Woodward treatment plant than P27, but Vtg levels were not tested due to low sample numbers. P25 is located in Windermere arm and industrial waste cannot be discounted as a major EDC at this site. The lack of intersex at BWW suggests that intersex at P27 and P25 is primarily due to PAHs and/or PCBs, and not wastewater effluent.

Ottawa Street Slip (OSS) receives effluent from a combined sewage overflow and an active steel mill and has high levels of PAH contamination (O'Connor et al., 2003). Vtg expression levels were elevated at this site and feminization of secondary sexual characteristics and intersex was present. The combined sewage overflow, a transient source of effluent, is likely not the major contributor to endocrine disruption at this site in round goby since there was no intersex in fish sampled from BWW where wastewater effluent is constantly released. The current and historic industrial activity at OSS has lead to significant PAH contamination; the Hamilton Harbour Remediation Action Plan has included this site, along with Windermere arm and Randle Reef, in a \$46 million remediation effort (O'Connor et al., 2003). PAHs are the probable, major EDC at OSS.

The most impacted site for intersex, secondary sexual characteristics and induction of Vtg in gobies was Sherman Inlet (SI). Of all male goby sampled at SI (n=39),

10% had intersex. Marentette et al. (In Press) found a similar prevalence of intersex at SI (13%; n=13). SI was the only site screened where more than one animal had testis-ova, and was the only site where vitellogenic oocytes were found in male gonads. Vtg II and III levels were significantly higher at SI than at both reference sites outside the harbour, and one less contaminated site (LS), within the harbour. Even though SI has intermittent sewage overflow during high rainfall events, the dominant contaminant source is Randle Reef which contains high levels of PAHs (>800 µg/g PAH; Marvin et al., 2000; O'Connor et al., 2003). Since the round goby is benthic, it seems likely that resident fish at SI had significant PAH exposure. During our field sampling, and in another study, it was noticed that many goby from SI had damaged pectoral fin peduncles (Marentette et al., In Press). The goby uses this fin to move itself across sediment and it is highly likely that damage to this feature is due to sediment contamination. This evidence strongly suggests that goby at SI were exposed to high levels of PAHs and that these compounds are likely the most influential in causing endocrine disruption at this site.

4.4 Revisiting research questions and hypotheses

This study has helped to characterize endocrine disruption in Hamilton Harbour and has provided useful assays that will allow the use of the round goby in future studies in the Great Lakes region. Initially, it was predicted that round goby from wastewater effluent impacted sites would have the highest levels of Vtg mRNA expression levels, the most severe feminization of urogenital papillae and intersex since wastewater effluents

contain both synthetic and natural estrogens from human sources. These compounds are known to induce vitellogenesis and intersex in male fish (Kausch et al., 2008; Kidd et al., 2007; Lai et al., 2002; Larsson et al., 1999) and have been shown to be the major EDC in several studies (Rotchell and Ostrander, 2003; Tyler et al., 1998). Our study has shown that sites influenced primarily by wastewater effluent are not as impacted as sites with high levels of sediment PAH and PCB contamination. Endocrine disruption in Hamilton Harbour appears to be heavily influenced by past and present industrial activity. Remediation efforts to clean up contaminated sediment and to prevent contaminant loading are vital to ensuring that native fish populations have a future in the harbour.

4.5 Future directions

The information generated from this study should be augmented by future investigations on how industrial pollution is impacting fish species in areas like Hamilton Harbour. Other studies have identified PAHs and PCBs as EDCs, yet the mechanism of action is unclear; aryl hydrocarbon receptor ligands (including PAHs and PCBs) have been shown to have estrogenic effects in fish (Mortensen and Arukwe, 2008), but the ability to demonstrate a mechanistic explanation for endocrine disruption is necessary. Lab-based studies exposing round goby to harbour sediment or purified PAHs and PCBs would provide better evidence for industrial contaminants as EDCs. Testing contaminated sediment for estrogenic effects in assays such as the yeast estrogenic screen (YES) assay, which measures ER binding (Schultis and Metzger, 2004), can help to verify if the

mechanism of action is mediated directly through the ER or by another pathway. Hormone levels in round goby from this study should be evaluated in order to establish a link between Vtg expression, intersex and circulating steroid hormones. Sneaker males have much higher levels of testosterone, compared to parental or non-reproductive males, and appear to be the most sensitive to feminization. The concentrations of plasma circulating steroids in goby from different field sites and the possible toxicological role of hormone pathways in feminization of round goby requires significant attention.

The assays developed in this study and the data collected on round goby from inside and outside Hamilton Harbour will be useful to future research in the Great Lakes region. Understanding endocrine disruption in aquatic species, in a North American context, is a relatively young discipline that has many questions yet to be answered. Many native fish species are currently under pressure by multiple anthropogenic factors that threaten to affect native fish species at the population level. Understanding the dynamic and complex interactions between EDCs that humans continue to release into aquatic ecosystems will become the foundation for remediation and preservation of important native aquatic species.

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APPENDIX

Table 1. Levels of circulating steroid hormones in reproductively active females collected from field sites inside and outside Hamilton Harbour. 50P = Fifty Point Conservation Area, GC = Grindstone Creek, LS = LaSalle Marina, P27 = Pier 27, SI = Sherman Inlet.

Year	Site	Hormone	Mean concentration (ng/ml)	Standard deviation	n
2008	50P	11KT	4.02	4.52	3
2007	GC	11KT	1.31	0.52	2
2007	LS	11KT	6.56	7.14	6
2007	P27	11KT	0.60	0.75	4
2007	SI	11KT	3.88	0.42	2
2008	50P	T	12.68	8.91	3
2007	GC	T	18.15	3.28	2
2007	SI	T	3.28	0.68	2
2008	50P	E2	4.30	3.39	3
2007	GC	E2	14.11	na	1
2007	LS	E2	13.48	11.05	2
2007	P27	E2	2.74	na	1

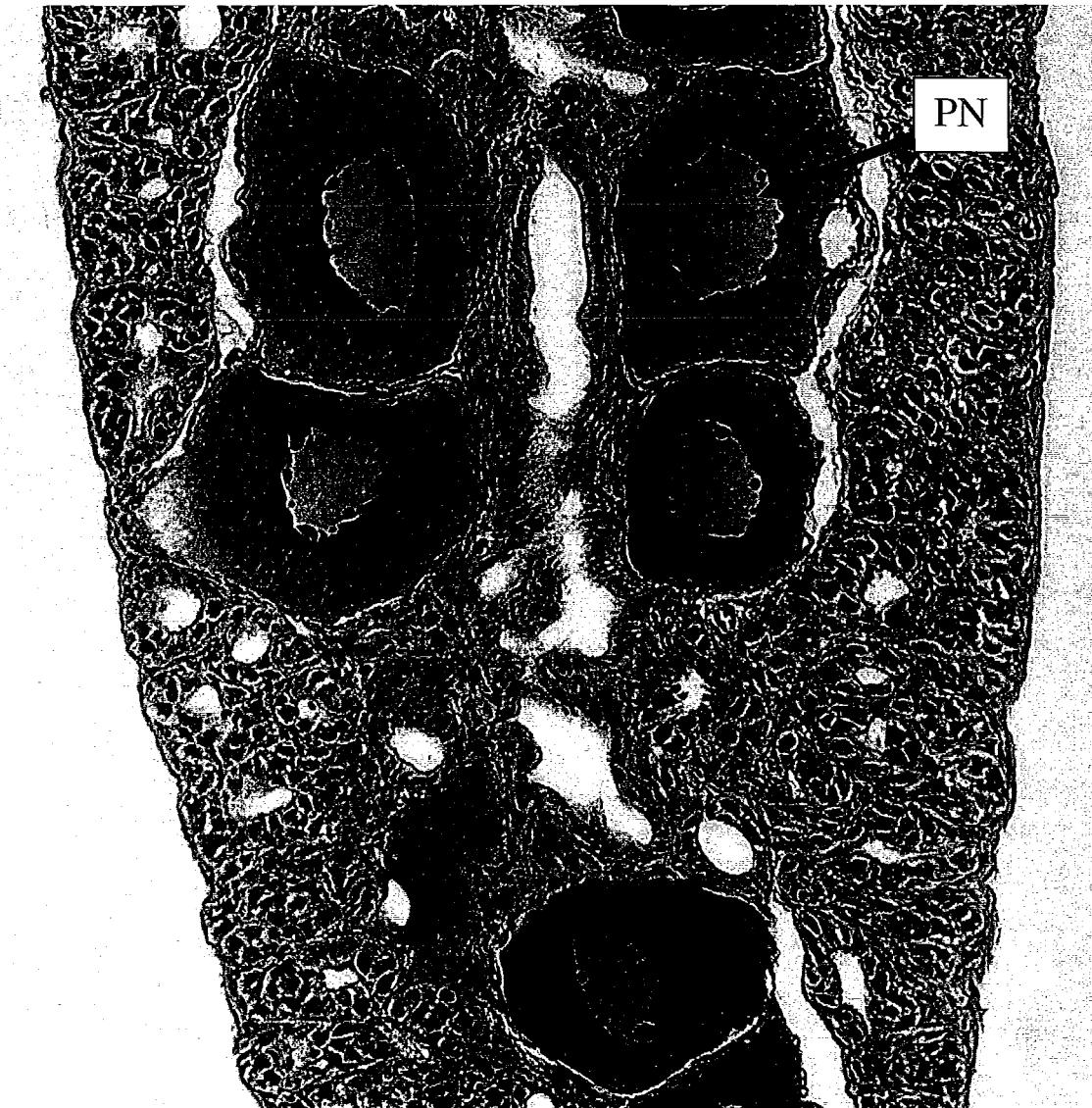


Figure 1. Grade four testis-ova in round goby from Desjardins Canal. Testis is from a non-reproductive male. Sperm cells can be seen at early stages of development. PN = perinucleolar stage oocyte. Magnification 200x.

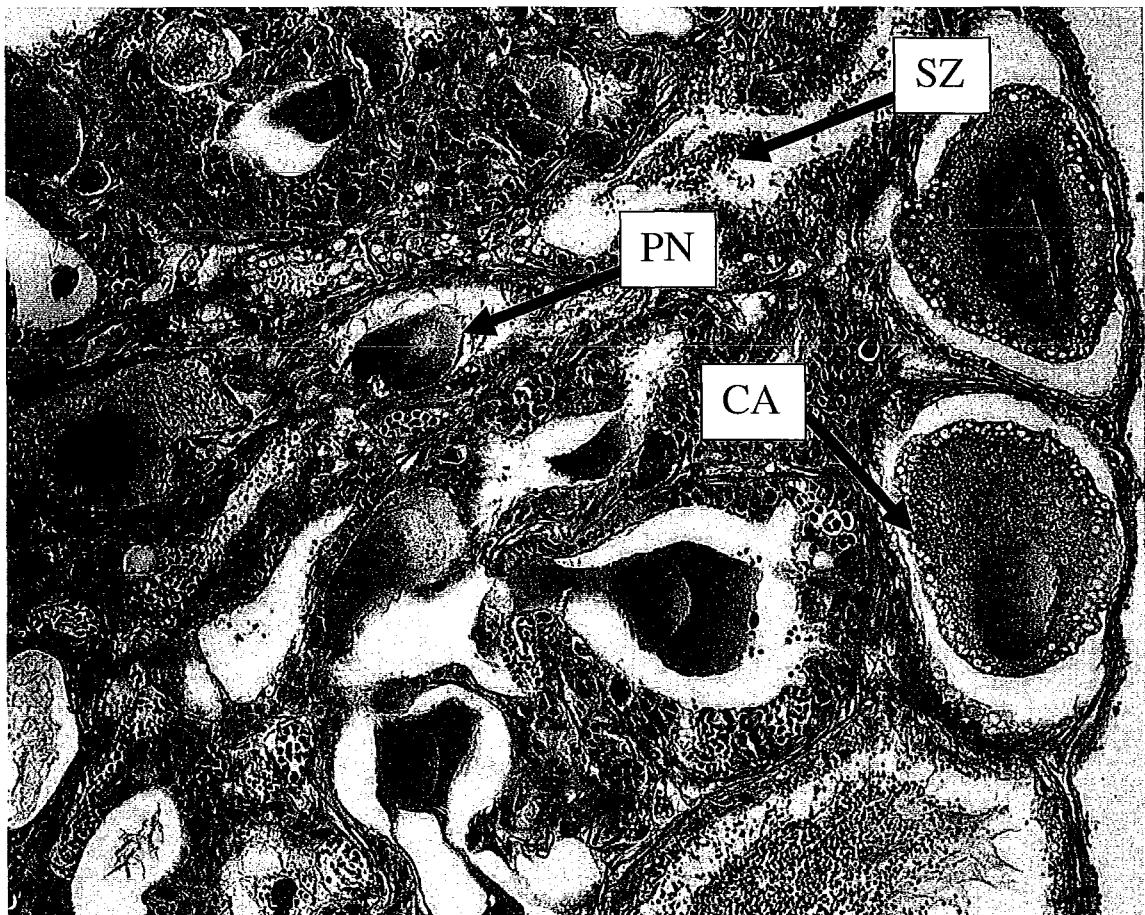


Figure 2. Grade four testis-ova in round goby from Ottawa Street Slip. Testis is from a mature sneaker male. Sperm cells can be seen at all stages of development. PN = perinucleolar stage oocyte, SZ = mature spermatozoa, CA = cortical alveolar stage oocyte. Magnification 200x.

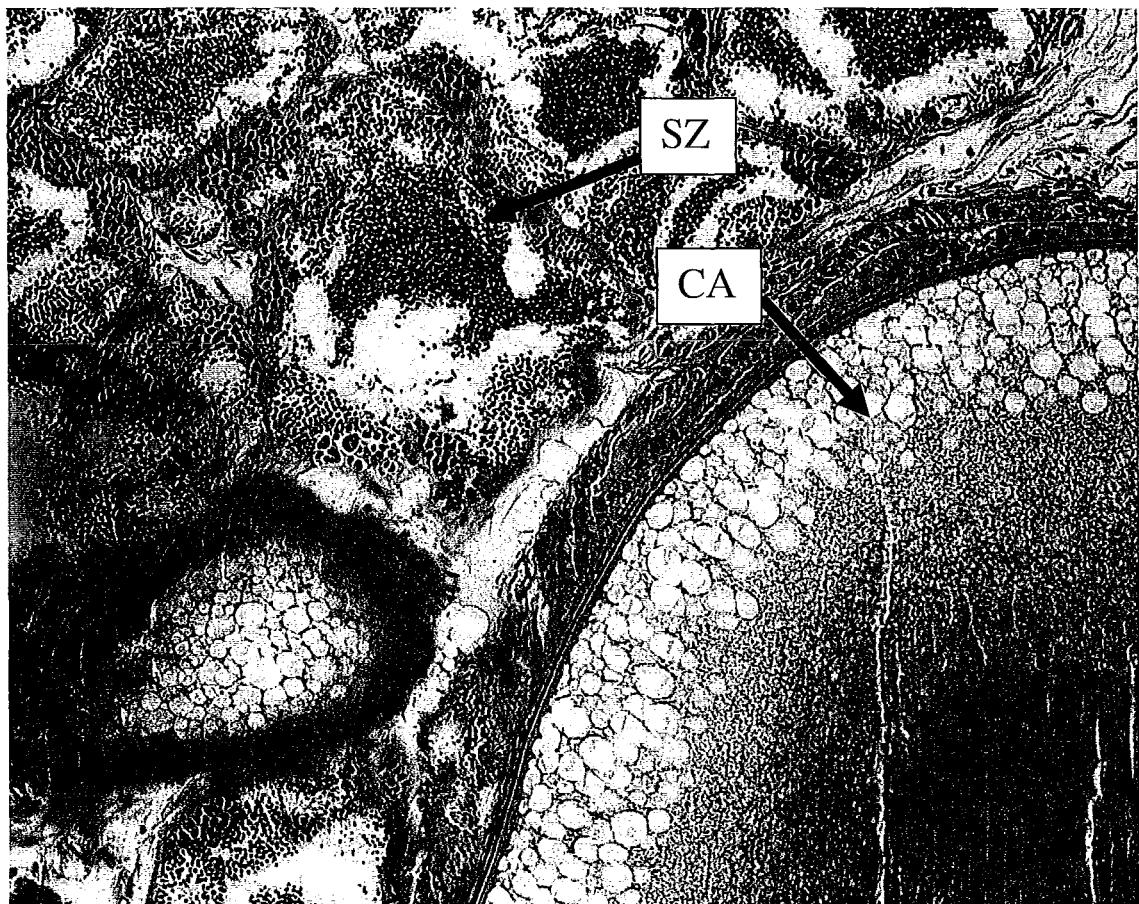


Figure 3. Grade two testis-ova in round goby from Pier 27. Testis is from a mature parental male. Sperm cells can be seen at all stages of development. SZ = mature spermatozoa, CA = cortical alveolar stage oocyte. Magnification 200x.