12-2016

Gonadal intersex in teleosts: Mechanisms, molecular biomarkers and diagnostic assays

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GONADAL INTERSEX IN TELEOSTS: MECHANISMS, MOLECULAR BIOMARKERS AND DIAGNOSTIC ASSAYS

by

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A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Forestry and Natural Resources

West Lafayette, Indiana

December 2016

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For my parents, Mohamed El-Mehrezy and Manal, my wife, Amira, and my kids, Celine and Yassin.

ACKNOWLEDGMENTS

I would like to start by thanking my wonderful wife, Amira, and my great parents, Mohamed El-Mehrezy and Manal. They have provided tremendous support and encouragement throughout my graduate career, I could not have made it through without them. I would also like to thank and acknowledge by advisor, Dr. Maria Sepúlveda, for her support, patience and guidance during my time here at Purdue. I would also like to thank the rest of my committee, Drs. Cecon Mahapatra, GuangJun Zhang, and Jennifer Freeman for giving me the opportunity to work with and learn from great scientists and great people. This research could not have been done without the help and advice of many people whom I would like to thank, including: Daragh Deegan, Dr. Jiejun Gao, Monica Hensley, Samuel Guffey, Dr. Azadeh Hatef, Jenny Zenobio, Dr. Shuai Chen, Paulina Moraga, Tatumn Vernon, Grace Weisenbach, Jennifer Serafin, Amy Godfrey and others. Last, but not least, I would like to thank my beautiful children, Celine and Yassin, for being a source of joy for me every day after a long tiring day of work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Author: Abdel-moneim Mohamed, Ahmed, M. E. Ph.D.
Institution: Purdue University
Degree Received: December 2016
Title: Gonadal Intersex in Teleosts: Mechanisms, Molecular Biomarkers and Diagnostic Assays.
Major Professor: Maria S. Sepúlveda.

Natural and synthetic estrogenic and androgenic compounds are continuously released into aquatic ecosystems. Exposure of teleost fishes to these contaminants can negatively impact sex differentiation and reproductive output. Specifically, development of gonadal intersex in gonochoristic (fixed sex) fish species has been studied extensively in relation to exposure to this class of compounds. The main objectives of this dissertation were to: 1) conduct field and laboratory studies to investigate the molecular signaling pathways behind the development of gonadal intersex; and 2) establish molecular biomarkers and assays for testing the ability of environmental pollutants to develop this condition using a battery of molecular, cellular and organ-level tools.

First, we conducted a literature review to summarize all available articles reporting gonadal intersex in wild populations of gonochoristic fish (Chapter 1). We also included the limited information available on this topic in amphibians. We analyzed studies from across the globe, identified families and species with reported cases of gonadal intersex, and highlighted the contaminants often linked with this condition. In addition, we discussed the current knowledge of molecular signaling pathways behind the development of gonadal intersex and summarized molecular biomarkers tested and others that require further investigation. We then conducted a field study investigating the prevalence of testicular oocytes (TOs), the most prevalent form of gonadal intersex, in a sentinel freshwater fish species, the smallmouth bass *Micropterus dolomieu*, inhabiting

the St. Joseph River and its tributaries in northern Indiana (Chapter 2). This constitutes the first study of this nature. Sites on this river were previously identified as having medium to high intersex induction potential based on contaminant quantification and estrogen equivalence estimations. We reported prevalence and severity of gonadal intersex reaching 100% in some sites, and significant decreases in prevalence and increases in severity of TOs occurrence after the spawning season. We evaluated changes in the transcription levels of several genes involved in sex differentiation and gonadal development. Significantly higher levels of vitellogenin (v*tg*) transcripts were found in livers of males with TOs, but only when sampled in the spawning season. Further, we quantified contaminant levels in surface water to identify possible correlations between contamination levels and the observed prevalence of gonadal intersex. Multiple sites had detectable levels of endocrine disruptors, but no correlations with the prevalence or severity of TOs was recorded.

In order to develop molecular biomarkers and assays that test contaminants' ability to develop gonadal intersex, short-term laboratory exposures were performed using Japanese medaka *Oryzias latipes,* a fish model with a well-understood sex determination mechanism and high sensitivity to exogenous hormone exposure (Chapter 3). First, we identified a gene, ovary structure protein 1 (*osp1*), with strong female-specific expression during gonadal differentiation and observed a significant downregulation in its expression in females following short-term (10 d) exposure to a potent synthetic androgen, 17β-trenbolone. Importantly, this decrease in *osp1* expression was correlated with changes in ovarian phenotype, namely ovarian intersex, later in life. We decided to further utilize this promising molecular biomarker by incorporating it in a visual *in vivo* reporter assay for rapid detection of contaminants with estrogenic/androgenic potential (Chapter 4). For this purpose, we built a pOSP1-AcGFP (promoterOSP1-*Aequorea coerulescens* green florescence protein) Japanese medaka transgenic line with *osp1* promoter region driving the expression of a reporter protein, AcGFP. After establishing this line, we tested its use in an *in vivo* visual reporter system for identifying estrogenic contaminants. Significant upregulation in fluorescence intensity was recorded in 30 d post hatch females following a 24 h exposure to 500 ng/L of a synthetic potent estrogen, ethinyl estradiol.

Overall, our results support earlier findings suggesting that gonadal intersex is highly prevalent in impacted rivers across the US and that smallmouth bass are highly sensitive to developing this condition. We also conclude that hepatic and plasma VTG are promising biomarkers for diagnosing gonadal intersex, but only in males sampled during the spawning season. Our findings also support the hypothesis that molecular biomarkers, such as *osp1*, are sensitive tools that can be used for early detection of the effects of contaminants with estrogenic and androgenic activity on fish and are ideal endpoints in wide-scale contaminant screening assays.

CHAPTER 1. INTERSEX IN FISHES AND AMPHIBIANS: POPULATION IMPLICATIONS, PREVALENCE, MECHANISMS AND MOLECULAR BIOMARKERS

Reproduced from:

Abdel-moneim, A.; Coulter, D. P.; Mahapatra, C. T.; Sepúlveda, M. S., Intersex in fishes and amphibians: population implications, prevalence, mechanisms and molecular biomarkers. J. Appl. Toxicol. 2015, 35, (11), 1228-1240.

1.1 Abstract

Intersex is defined as the abnormal presence of both testicular and ovarian cells in gonads of gonochoristic animals. Its occurrence is widespread and reports on its presence in the gonads of vertebrates continues to increase. In this review, we use standardized terminology to summarize the current knowledge of intersex in gonochoristic fishes and amphibians. We describe the different indices that have been used to assess the severity of intersex and synthesize reports discussing the prevalence of intersex in relation to different types of pollutants. In addition, we evaluate the geographic distribution and chronology of the reported cases of intersex in fishes and amphibians, their pathological descriptions and severity and discuss species sensitivities. We also summarize molecular biomarkers that have been tested for early detection of intersex in wild populations and highlight additional biomarkers that target molecular pathways involved in gonadal development that require further investigation for use in the diagnosis of intersex. Finally, we discuss the needs for future research in this field.

1.2 Introduction

Over the past decades, an increase in awareness has developed regarding the adverse effects of endocrine-disrupting chemicals (EDCs) on aquatic organisms (Damstra *et al.*, 2002; Diamanti-Kandarakis *et al.*, 2009). Numerous studies in natural populations of gonochoristic (fixed sex) fish and amphibians (e.g., Jobling *et al.*, 1998; Hinck *et al.*, 2006; Murphy *et al.*, 2006; Amberg *et al.*, 2010) have reported a gonadal abnormality

known as intersex; the development of incomplete or complete gonads of a gender opposite to that of an organism's genotype (Yamamoto, 1969). Intersex can occur at different levels of severity, ranging from a few cells to large masses of gonadal tissue of the opposite sex. At the functional level, low severities of gonadal intersex are generally not associated with impairments in reproductive function; however, as severity increases, adverse reproductive effects are likely in fish (Jobling *et al.*, 2002; Harris *et al.*, 2011). No comparative studies, though, have been conducted in amphibians.

Despite the widely reported cases of gonadal intersex in wild fish and amphibian populations, few studies have directly connected intersex in wild populations to adverse reproductive effects (Jobling *et al.*, 2002; Harris *et al.*, 2011). However, evidence suggests that chronic exposures to EDCs and the resulting gonadal intersex can have dramatic population-level consequences. Kidd *et al.* (2007) demonstrated this by exposing fathead minnow (*Pimephales promelas*) populations to ethinyl estradiol (EE_2; 4–6 ng l^{-1}) in experimental lakes for 7 years. Chronic exposure caused males to develop intersex gonads, altered oogenesis in females, and ultimately led to a population collapse owing to almost a complete lack of reproduction. In contrast, studies investigating other fishes such as pearl dace (*Margariscus margarita*) and lake trout (*Salvelinus namaycush*) did not observe detrimental population-level effects of EE_2 exposure, despite observing gonadal intersex in wild fish (Palace *et al.*, 2006; Werner *et al.*, 2006). Overall, existing literature in this area suggests that the broader implications of intersex on wild populations of fish are still unclear, with no comparable data available for amphibians.

Understanding the ecological consequences of intersex has become increasingly important as EDCs are an ever-present component of aquatic environments. Although much research has been focused on examining the causes and consequences of intersex in aquatic organisms, it is difficult to synthesize general patterns from such a diverse group of studies. This is in part owing to the inconsistent terminology that is oftentimes used among studies to refer to different forms of intersex (Hecker *et al.*, 2006). For example, the term ovotestis has been used to refer to different degrees of gonadal intersex, from the presence of a few oocytes in testicular tissue (Vethaak *et al.*, 2002), to the co-occurrence of large clusters of oocytes with scattered testicular tissue (Getsfrid *et al.*, 2004). Additionally, many studies examining intersex in wild populations of fish and

amphibians often focus on a single species from a single geographic region. Summarizing studies across taxa from around the world will help focus future research on the causes and consequences of EDC exposure to aquatic organisms. Furthermore, future studies will probably involve the use of molecular biomarkers for an assessment of abnormal gonadal development in wild populations.

In this review, we summarize the available literature reporting gonadal intersex in wild populations of gonochoristic fish and amphibians. Intersex in birds and reptiles was not a focus for this review article, yet it was notable that very few studies have examined how intersex in these groups is related to EDC exposure (Guillette *et al.*, 1994; Hart, 1998). In order to assess results from a diversity of studies, we use standardized terminology to describe different forms of intersex, and synthesize reports regarding the frequency of intersex occurrences and the different indices used to assess its severity. In addition, we analyze studies from across the globe to identify families and species with reported cases of gonadal intersex and highlight the EDCs that are oftentimes attributed to intersex development. Finally, we summarize the molecular biomarkers of gonadal intersex that have been identified and describe potential biomarkers that should be further investigated to improve the noninvasive early detection of intersex.

1.3 Paper selection and terminology

This review is a summarization of studies examining intersex in wild populations of gonochoristic fish and amphibians and we only provide some examples of laboratory studies to help clarify specific points. Readers should refer to the peer-reviewed literature including recent reviews that have discussed laboratory data related to intersex in vertebrates (Mills and Chichester, 2005; Solomon *et al.*, 2008; Leet *et al.*, 2011). Among the field studies examining intersex in wild populations, we omitted studies that did not indicate the sites from which samples were collected, nor did we describe studies that only reported feminization or masculinization in animals based on secondary sex characteristics and not gonadal morphology.

We use standardized terminology developed by Hecker *et al.* (2006) to classify intersex as either testicular oocytes (TOs), ovotestis, or mixed gonadal tissue (Figure 1.1).

TO are testes containing oocytes that have an intact nucleus, nucleoli, and a surrounding squamous epithelial layer (Figure 1.1A; Coady *et al.*, 2004, 2005). The term ovotestis describes the histological presence of scattered testicular tissue or spermatogenic nests within a mature ovary (Figure 1.1B; Getsfrid *et al.*, 2004; Körner *et al.*, 2005). Finally, we use the term mixed gonadal tissue to refer to the gross morphological occurrence of testicular and ovarian tissue masses (Figure 1.1C; Kinnison *et al.*, 2000).

1.4 Geographic distribution and chronology of intersex research

We identified 114 manuscripts describing intersex in wild populations of gonochoristic fish and amphibians (Supplementary Tables 1.1 and 1.2), with the majority of papers (97 manuscripts) examining fish. Most of the fish intersex studies examined teleost species (92 manuscripts) and only five examined other fish groups, particularly sturgeons (family Acipenseridae). The geographical distribution of the reported cases and prevalence of intersex in the manuscripts we reviewed show a distinct pattern (Figure 1.2). Intersex studies were by far more commonly reported from North America and Europe than any other continent; moreover the United States, Canada and the United Kingdom were the most represented countries. This biased distribution is likely not attributable to a higher incidence of intersex in these regions, but rather a reflection of a larger number of researchers studying this phenomenon in these countries.

The majority of the manuscripts (84%) were published after 1999 (Figure 1.3), which followed the passing of both the Food Quality Protection Act (FQPA) and the amendments to the Safe Drinking Water Act (SDWA) by the federal government of the USA in 1996. These acts emphasized the dangers of exposure to EDCs and required the US Environmental Protection Agency (USEPA) to develop programs for screening and testing of chemicals for possible endocrine disrupting effects. The interest in this area of research has also coincided with the adoption of the European Union (EU) Water Framework Directive (WFD) in 2000 and the registration of the Endocrine Disruptor Screening Program (EDSP) by the USEPA in 1999 in response to the recommendations of the FQPA and SDWA (EC, 2000; US-EPA, 2011). In addition to the increased awareness, we believe that several other factors, including the widespread exposure of

fish and wildlife to EDCs and increased monitoring efforts, have also increased research interest in this area.

1.5 Types and severity of intersex lesions

Among the reviewed studies, TOs was the most commonly reported form of gonadal intersex in both fish (120 reported cases) and amphibians (17 reported cases; Figure 1.4). Of these, only 41% quantified the severity of the observed lesions by counting the number and distribution of oocytes from replicate gonadal histological sections (e.g., Jobling *et al.*, 1998, 2006; van Aerle *et al.*, 2001; Anderson *et al.*, 2003; Faller *et al.*, 2003; Blazer *et al.*, 2007; Tanna *et al.*, 2013). The indices or ranking systems developed by Jobling *et al.* (1998) and Blazer *et al.* (2007) were the most commonly adopted for quantifying intersex severity. In brief, the ranking system developed by Jobling *et al.* (1998) examines six gonadal sections from each fish and considers the presence of developing eggs (oocytes) or ovarian cavities as signs of intersex. Each section examined is given a score on a numerical scale from 0 to 7. A score of zero is assigned to sections with no signs of intersex, whereas scores of 1, 2 and 3 are assigned to testicular sections where ovarian cavities and oocytes are observed either infrequently, occasionally or frequently, respectively. A scoring of 4 is given to testicular sections lacking sperm ducts, harbor obvious ovarian cavities and numerous interspersed oocytes, and scores of 5, 6 and 7 are assigned to sections with percentages of ovarian tissues that are either < 50%, > 50% or 100%, respectively. Blazer *et al.* (2007) developed an intersex severity index that assigns severity ratings to gonads after scanning five gonadal sections for TOs while focusing on the central zone of the sections examined. For each testicular section examined, the most severe score observed in a given microscopic field with 10X objective (4 mm^2 of tissue) is assigned to the whole section and the average of all section scores used to calculate severity. This ranking system has a scale of 1 to 4, with a score of 1 given to testes with single oocytes focally distributed within each microscopic field. Testes with a diffuse distribution of oocytes harboring more than one oocyte per field of view with no physical association in between are assigned a score of 2. Higher scores, (3; cluster distribution) and (4; zonal

distribution), are given to testes with < 5 or > 5 closely associated oocytes per field of view, respectively.

In the studies we reviewed, mixed gonadal tissue (sometimes referred to as macroscopic hermaphroditism) was the second most commonly reported intersex lesion with 19 reported cases in fish and 7 in amphibians (Figure 1.4). This condition was mostly reported as isolated cases recorded while collecting organisms for other research purposes. Finally, ovotestis was the least recorded lesion, with only 11 reported cases in fish and none in amphibians (Figure 1.4). Several controlled studies have suggested that this type of intersex is a result of androgenic or anti-estrogenic exposure (Koger *et al.*, 2000; Cevasco *et al.*, 2008), although no correlations have been established in wild populations. In support of this, we recently documented the development of ovotestis in female Japanese medaka exposed to 5 ng L^{-1} of the potent synthetic androgen 17β-trenbolone during sex differentiation (Abdel-moneim *et al.*, 2015).

1.6 Intersex in fish

Among the reviewed manuscripts, a total of 20 fish families and 54 species had reported cases of gonadal intersex (Figure 1.4 and Supplementary Table 1.1). Cyprinidae and Centrarchidae were the most commonly reported families with 26% and 18% of the reported intersex cases in fish, respectively. At the species level, smallmouth bass *Micropterus dolomieu* was the most commonly reported species with intersex (10% of the reported cases in fish), followed by roach *Rutilus rutilus* (8%) and largemouth bass *Micropterus salmoides* (7%). The only type of intersex reported from these species was TOs. In contrast, mixed gonadal tissue was predominantly observed in members of the Salmonidae family (12 out of 18 cases) with only two recorded cases in Cyprinidae and Centrarchidae. An interesting finding from these studies is the apparent enhanced sensitivity of some fish species and families to develop intersex. For instance, Hinck *et al.* (2009) studied the prevalence of intersex in 16 freshwater fish species inhabiting nine rivers across the US. Intersex was detected only in four species: channel catfish *Ictalurus punctatus*, common carp *Cyprinus carpio*, largemouth bass and smallmouth bass. This finding suggest that some fish species may be more likely to develop intersex than others.

We believe these differences in susceptibility are primarily attributed to the variation in the sex differentiation mechanisms among species. The molecular and cellular processes that allow an undifferentiated gonad to develop into a testis or ovary, referred to as sex differentiation, are genetically and environmentally influenced. In different species, instabilities in intrinsic factors including behavior, or extrinsic factors such as temperature or exposure to EDCs, can dramatically influence the process of gonadal differentiation (Devlin and Nagahama, 2002; Leet *et al.*, 2011). For instance, life-long exposure in the laboratory to 5 ng L^{-1} of EE_2 in zebrafish (*Danio rerio*) caused a 56% reduction in fecundity with a complete failure of fertilization (Nash *et al.*, 2004). These observations were attributed to the absence of functional testes in males and the development of either undifferentiated or intersex gonads. Exposure of European sea bass *Dicentrarchus labrax* juveniles to high temperature induced masculinization of females as a result of alterations in the DNA methylation-mediated control of aromatase gene expression (Navarro-Martín *et al.*, 2011). It is important to consider that several other factors might be influencing the observed differences in the prevalence of intersex among species, particularly the sampling seasons and ages of the sampled fish. Therefore, standardizing sample sizes and the timing of sample collection in relation to the spawning season of each species is necessary when sampling multiple species (Barrett and Munkittrick, 2010).

1.7 EDC concentrations and intersex in wild fish: little evidence of causality

In this review, we identified 97 scientific manuscripts on gonadal intersex in wild populations of fish (Supplementary Table 1.1). The majority of these studies (84%) focused on freshwater fish inhabiting streams and rivers, whereas only 16% investigated estuarine or marine species (De Metrio *et al.*, 2003; Ferreira *et al.*, 2004; Bizarro *et al.*, 2014). This bias may be a result of observations of intersex in fish inhabiting rivers and streams that are more directly associated with specific point-source pollution that is discharged into these systems close to the sampling sites. This is evident in the majority of the fish studies reviewed (88% of the 97 manuscripts reporting intersex in fish), where intersex has been studied in relation to point-sources including urban discharges (mostly

sewage wastewater treatment effluents) and industrial discharges (Figure 1.5), whereas the remaining cases (12%) were reported from 'reference sites'. These reference sites were primarily located in relatively pristine or underdeveloped areas where no known sources of contaminants were observed. The status of 'reference', however, is questionable in 55% of the studies reviewed, as these studies either did not report any contaminant data to support their assertion of a 'reference site', or the data they presented was inadequate for monitoring endocrine-active compounds from surface water.

Similarly, a certain degree of ambiguity was noted in a number of studies linking gonadal intersex to particular pollution sources. Studies (32% of fish studies linking intersex to pollution) reported only qualitative information on potential sources of contamination at the sampling sites, without actually quantifying chemical concentrations in water or fish tissues (e.g., Viganò *et al.*, 2001; Woodling *et al.*, 2006; Douxfils *et al.*, 2007). Others measured the concentrations of several potential contaminants in water, soil or tissue samples collected from the sites only at the time of sampling but found few to no significant correlations between measured contaminant concentrations and the prevalence of intersex (Reeder *et al.*, 1998, 2005; Bjerregaard *et al.*, 2006). Using data presented in multiple studies we reviewed, we analyzed the relationship between intersex prevalence from multiple fish families and contaminant data for a number of chemicals for which these data were available using weighted regressions (weighted by sample size). We observed one positive relationship between the concentration of a xenobiotic, diclofenac; a non-steroidal anti-inflammatory drug with endocrine-disrupting potential (Hong *et al.*, 2007), and the prevalence of intersex (measured as the percentage of fish sampled with intersex lesions; R^2= 39, P = 0.05, n= 10 studies), and one marginally significant negative relationship between dichlorodiphenyldichloroethylene (DDE), a metabolite of dichlorodiphenyltrichloroethane (DDT) concentration and the prevalence of intersex (R^2= 60, P = 0.07, n= 6 studies). We did not, however, observe a significant relationship between the prevalence of intersex and concentration of atrazine (R^2= 20, P= 0.38, n= 6 studies), an estrogenic contaminant. Other contaminants, including carbamazepine, DDT, ibuprofen and venlafaxine, were also not related to the prevalence of intersex. These findings illustrate the challenges in attempting to establish relationships between EDC concentrations and the prevalence of intersex in natural

systems. Specifically, small sample sizes and the timing of sample collection are problematic in establishing these relationships in the field. For example, many studies attempting to examine these relationships relate EDC concentrations in water samples collected at the same time that fish were sampled for examinations of intersex. However, it is well known that concentrations of EDCs can rapidly change through time (Rocha *et al.*, 2014). Therefore, studying the relationship between EDC concentrations when fish are undergoing gonadal development or identifying long-term fluctuations (week or months) that occur before sampling would be more meaningful than assessing chemical concentrations at the time of sample collection. Gonadal intersex is primarily induced upon the exposure of juvenile fish to exogenous hormones during the critical period of sexual differentiation, and, to a lesser extent, upon the exposure of reproductively active adult fishes to EDCs for weeks or months (Gimeno *et al.*, 1998; Gray *et al.*, 1999; Gronen *et al.*, 1999; Depiereux *et al.*, 2014; Abdel-moneim *et al.*, 2015). Therefore, field studies attempting to relate ambient concentrations of EDCs to the prevalence of intersex should measure EDC concentrations over a longer time to avoid the problems associated with relating intersex to EDC concentrations at a single point in time. Furthermore, increasing sample sizes, examining multiple study sites, and determining the preferred type of sample collected for measuring EDC concentrations (e.g., surface water versus sediment) will help elucidate these relationships in natural ecosystems. These issues are especially important to consider when working with species with large home ranges. Field sampling protocols are needed that consider all of these factors and guide researchers in relation to where, when, and how many individuals to collect during sampling to evaluate relationships between EDC exposure and intersex.

1.8 Intersex in Amphibians

Despite the fact that amphibian populations are declining worldwide, primarily as a result of high mortality and reproductive failure (Hayes *et al.*, 2010), limited effort has been made to examine the prevalence of intersex in wild amphibians and its effect on the reproductive success of this group (Supplementary Table 1.2). We recorded five families and 12 species with reported cases of gonadal intersex (Figure 1.4 and Supplementary

Table 1.2). Ranidae was the most widely reported family with gonadal intersex (50% of the reported intersex cases), with Northern leopard frogs *Lithobates pipiens* the most commonly reported species (17% of the reported cases), followed by green frogs *Lithobates clamitans* (13%). The majority (68%) of the studies examined reported the prevalence of intersex in amphibians sampled from agricultural landscapes (e.g., Hayes *et al.*, 2003; Smith *et al.*, 2005; McCoy *et al.*, 2008; McDaniel *et al.*, 2008), as atrazine and other pesticides applied to these landscapes have been linked to amphibian intersex in laboratory studies (Hayes *et al.*, 2002; Solomon *et al.*, 2008). For example, atrazine (≥ 0.1 ppb) induced hermaphroditism among African clawed frogs *Xenopus laevis* and leopard frogs when exposed throughout their larval developmental period (Hayes *et al.*, 2003, 2002). A small percentage of these studies (13%), however, found a significant increase in the prevalence of intersex in animals sampled from agricultural sites as compared with reference sites, with significant correlations between the increase in intersex or effects on sexual differentiation and the exposure to these contaminants (Reeder *et al.*, 1998; Hayes *et al.*, 2003). Indeed, several studies have failed to find a relationship between the prevalence of intersex and agricultural activities. For instance, Papoulias *et al.* (2013) recorded ovarian dysgenesis, female-biased sex ratios and higher rates of testicular oocytes in Plains leopard Frog *Lithobates blairi* in Nebraska's Rainwater Basin when compared with a reference site. However, this study found no relationship between exposure to pesticides (atrazine, metolachlor or glyphosate) and the prevalence of intersex. In some frog species, such as the green frog, intersex has been reported in males sampled from agricultural landscapes (6%), but much more so from males collected from urban/suburban landscapes (21%; Skelly *et al.* 2010). It is unclear whether the same causes are operating to induce intersex in both landscape types, but sewage wastewater effluents that are known to contain potent xenoestrogens (Sumpter and Johnson, 2005; Ying *et al.*, 2009) provide a major contribution of contaminants in urban settings. Owing to the few number of studies reporting ambient EDC concentrations in relation to intersex prevalence in amphibians, we were unable to explore these potential relationships. Future studies should simultaneously collect and examine intersex in both fish and amphibians to provide a more comprehensive examination of the effects of EDCs on a variety of aquatic organisms.

1.9 Existence of a basal rate of gonadal intersex

In teleost fishes, researchers have speculated on the existence of a "basal rate" of intersex among different fish populations. These speculations were primarily derived from the observation of background levels of gonadal intersex among populations inhabiting reference or non-polluted sites (Jobling *et al.*, 1998; van Aerle *et al.*, 2001; Hecker *et al.*, 2002; Hinfray *et al.*, 2010; Supplementary Table 1.1) and the occurrence of gonadal intersex incidences in control groups used in laboratory studies (Grim *et al.*, 2007). Similarly, the existence of a basal rate of gonadal intersex has been speculated in amphibians (Cheng, 1929; Christensen, 1929; Smith *et al.*, 2005; Murphy *et al.*, 2006; Orton *et al.*, 2006). Moreover, genetic and ontology studies have revealed that amphibians, particularly frogs, are highly plastic during their sexual development and can easily undergo gonadal changes in response to environmental conditions, including temperature fluctuations (Wallace *et al.*, 1999). Whether intersex develops and at what rates it occurs in fish and amphian populations from reference sites remains unknown.

A factor that might be influencing the observed basal rates of intersex is water temperature. A growing body of evidence suggests that temperature changes originating from natural and human activities can have long-lasting impacts on the gonadal development of aquatic organisms extending up to complete sex reversal (Goto-Kazeto *et al.*, 2006; Selim *et al.*, 2009; Navarro-Martín *et al.*, 2011; Coulter *et al.*, 2015). Despite the different sex determination mechanisms aquatic organisms possess (e.g., gonadal (GSD) and temperature (TSD) sex determination), they all undergo a sex differentiating pathway in which a bipotential gonad develops towards either ovary or testis (Wallace *et al.*, 1999; Devlin and Nagahama, 2002). The methylation levels of cytochromeP450 19a (*cyp19a*) promoter region, a key gene in the sex differentiation process of non-mammalian vertebrates, were found to be sex-specific and highly influenced by changes in temperature during early life stages (Navarro-Martín *et al.*, 2011). Exposure to high temperature during the period of sex differentiation increases the methylation levels of *cyp19a* promoter, causing signs of masculinization as a result of the down-regulation of the expression of this gene. Moreover, elevated water temperature and high EDC concentrations have recently been shown to have an additive effect on skewing sex ratios in zebrafish (Brown *et al.*, 2015). Although this area of research is still in its infancy, it

might direct attention to several other factors influencing the development of gonadal intersex among wild populations of fish and amphibians.

1.10 Molecular biomarkers of gonadal intersex in wild populations

Despite the fact that gonadal intersex in wild populations of fish and amphibians has been associated with adverse effects on reproduction, little progress has been made concerning the development of suitable non-invasive molecular biomarkers for this condition (Jobling *et al.*, 2002; Williams *et al.*, 2009; Zhao and Hu, 2012). Establishing these (non-invasive) molecular biomarkers will provide more sensitive, faster and more cost effective tools that prevents sacrificing animals, thus allowing for increased sample sizes and the better ability of detection. Although some promising biomarkers have been identified for use in intersex detection, more research is needed to understand fully the pathways involved with the development of intersex and to identify further molecular biomarkers that can be used.

The traditional identification of gonadal intersex in the field studies we reviewed was primarily performed using histopathological observations of the gonads (94% of manuscripts reviewed) and, to a lesser extent, from gross lesions during necropsy. Macroscopic observation of gonadal intersex can rarely be used because it requires a severe degree of gonadal intersex that is often not encountered. Histopathological determination of gonadal intersex has well-established guidelines (Johnson *et al.*, 2009), but also involves several drawbacks. Generally, histopathological analysis results in low sensitivity of detecting intersex owing to the limited observation area arising from the limited sampling of selected tissue sections and the high costs required for sample processing (Zhao *et al.*, 2014). Additionally, histopathological observation of gonadal intersex is often performed on mature individuals or individuals approaching sexual maturity to allow for the complete differentiation of the gonad. Whereas, intersex might develop following exposure to EDCs during the sexual differentiation period, thus identifying relationships between the lesions observed in adults and contaminant levels identified at the time of gonadal sampling can be misleading. In contrast, quantifying changes occurring at the molecular level, that can be measured in a minimally invasive

way (e.g., blood, plasma or body swab), during the period of sex differentiation can allow for the prediction of phenotypic changes (i.e., intersex) occurring at later stages (Koger *et al.*, 2000; Abdel-moneim *et al.*, 2015) while being directly linked to the observed contamination levels. Thus, the use of molecular biomarkers to identify intersex would complement histopathological techniques by circumventing some of the drawbacks of this traditional approach.

The molecular biomarkers ultimately used for identifying the presence of intersex should principally be the products of genes associated with sex differentiation or gonadal development or maintenance in order to provide a sensitive and specific response to the development of gonadal intersex. A number of genes have been tested or adopted as biomarkers of intersex in wild fish populations (Table 1.1), whereas none was tested among amphibians. However, the success of these biomarkers in identifying intersex in fish compared to histopathological examination varies. Among the genes tested in the studies we reviewed, *doublesex and mab-3 related transcription factor 1 (dmrt1)*; a gene that encodes a transcription factor which plays a critical role in male sex determination and differentiation through controlling the male germ cell proliferation and testicular development (Kobayashi *et al.*, 2004), showed similar transcription levels in intersex male, normal males and female shovelnose sturgeon (Amberg *et al.*, 2010). In contrast, the same genes in rainbow darters *Etheostoma caeruleum* were only expressed in males despite variability in contaminant levels and intersex prevalence among sites (Bahamonde *et al.*, 2014). *Forkhead transcription factor (foxl2),* an essential gene in the differentiation of the granulosa cell and ovarian maintenance (Schmidt *et al.*, 2004), showed a sexually dimorphic expression pattern in both shovelnose sturgeon and rainbow darter, suggesting its significance as a biomarker for sex determination. However, its transcription levels did not differ among sites harboring different contaminant levels nor did it show significant differences between normal and intersex males. The ratio between expression levels of *foxl2* and *dmrt1* provided a promising tool to identify intersex, however further research is needed to validate this method (Amberg *et al.*, 2010; Bahamonde *et al.*, 2014). In thicklip gray mullet *Chelon labrosus*, the transcriptional levels of 5S rRNA and its associated proteins involved in oocyte differentiation and maturation (e.g., *42Sp43* and *tfIIIA*) were orders of magnitude higher in females than

males. Intersex mullets collected from a highly polluted site had expression levels between both sexes or comparable to females (Diaz de Cerio *et al.*, 2012). Finally, transcription levels of vitellogenins (*vtg*), the egg yolk precursors in fish, have been correlated to intersex in one lake study (Kidd *et al.*, 2007) with no correlation in wild populations (Amberg *et al.*, 2010; Bahamonde *et al.*, 2014; Bizarro *et al.*, 2014).

Overall, the available data suggest that adopting molecular biomarkers to identify intersex is a promising tool and that adoption of a single gene as a biomarker may be insufficient for identifying intersex. More research is needed in this area, including the testing of different biomarkers developed under laboratory exposures to test their suitability in field studies. It is also important that larger sample sizes be examined to overcome the high variability that can occur in gene transcription levels in wild animals.

1.11 Candidate biomarkers associated with sex differentiation and development

Sex differentiation in egg-laying vertebrates is a hormone-dependent process that involves complex interactions among a large network of genes (Baron *et al.*, 2005; Figure 1.6). Among these genes, *cyp19a* plays an important role directing sexual differentiation toward a particular sex in the majority of non-mammalian vertebrates (Guiguen *et al.*, 2010). The expression levels of this gene can be altered through positive and negative feedbacks originating from other genes involved in this process. These genes are well-conserved between different species with a characteristic expression during the sexual differentiation process (Baron *et al.*, 2005; Leet *et al.*, 2011) regardless of the sex-determination mechanism (Alfaqih *et al.*, 2009). Exposure to exogenous hormones and endocrine disruptors throughout sex differentiation can alter the expression of these genes (Figure 1.6), causing alterations in the sex differentiation process that ultimately leads to reproductive abnormalities, including intersex. Monitoring the alterations in expression levels of individual or multiple genes involved in the sex differentiation process can be a sensitive approach for diagnosing gonadal intersex in wild populations of fish and amphibians.

Recent research articles and reviews have described the genes involved in the sex differentiation process, how expression is regulated and how expression is altered upon exposure to EDCs (Leet *et al.*, 2011; Bahamonde *et al.*, 2013). Quantifying the

expression levels of those genes among individuals belonging to wild populations of fish and amphibians is necessary to estimate the ability of those genes to be used as molecular biomarkers of gonadal intersex. A promising candidate gene is ovarian structure protein 1 (*osp1*); a molecular biomarker of gonadal intersex that has been recently identified in Japanese medaka (Zhao and Hu, 2012). The expression levels of *osp1* show a sexual dimorphic pattern with changes in its expression upon exposure to endocrine disruptors that are related to the development of gonadal intersex in both males and females (Zhao and Hu, 2012; Abdel-moneim *et al.*, 2015). Strong correlations have also been found between the severity of male gonadal intersex and the alterations in expression of this gene upon exposure to EE_2 (Zhao *et al.*, 2014). Although the function of the protein encoding this gene is still unknown, the identification of regulatory protein binding sites that influence the expression of genes involved in sex differentiation in the promoter region of *osp1* supports its role in sex differentiation and explains its sensitivity to hormone exposure (Abdel-moneim *et al.*, 2015). The identification of this biomarker in wild populations can provide a more sensitive tool for detection of gonadal intersex.

1.12 Recommendations for future research

The studies we summarized in this review article provide valuable information concerning the prevalence and severity of gonadal intersex in different aquatic species from around the globe. The majority of these studies, however, were unable to establish causal relationships to ambient concentrations of EDCs due to limitations in sample sizes, study site selection, and sampling protocols. Several considerations should be made in future studies when attempting to relate intersex observations with contaminant exposure in the field. One consideration is that the same type of effluent can contain different chemicals and concentrations, thus summarizing findings from multiple studies can sometimes be misleading. For instance, pulp mill effluent has been related to feminization in fathead minnows (Parrott *et al.*, 2003), but has also been linked to masculinization in other species (Larsson *et al.*, 2000). Another consideration is that the contaminants leading to certain intersex conditions might not always be released from the source closest to the sampling site. Other point or non-point sources of contamination might be present several miles upstream of a sampling site. Thus, careful sample site

selection is an important consideration. An additional issue is that gonadal intersex does not always result from exposure to a single contaminant. Evidence suggests that mixtures of several chemicals with estrogenic or androgenic activity can have synergistic effects causing this condition to develop (Brian *et al.*, 2005; Vajda *et al.*, 2008; Leet *et al.*, 2015). Therefore, the quantification of a wide array of chemicals with endocrine disrupting potential and the adoption of methods that accurately quantify the estrogenicity or androgenicity levels among those mixtures is necessary to make well-informed decisions concerning the contamination status of a particular site (Drewes *et al.*, 2005; Vajda *et al.*, 2008). Moreover, the development of intersex can not only result from exposure to certain types and concentrations of contaminants during the period of sexual differentiation in juveniles, but can also occur in mature adults (Gimeno *et al.*, 1998; Gray *et al.*, 1999; Gronen *et al.*, 1999). Thus, quantifying chemical concentrations from water samples collected at the same time as adult organisms can provide insufficient information concerning the contaminants present at earlier developmental periods. It is also important to consider that several contaminants present in water bodies have a seasonal pattern of release, which is particularly evident with contaminants in agricultural runoff. Seasonal patterns of release suggest that individuals living in confined areas might be exposed to different contaminants and concentrations during different seasons of the year. One aspect we recently highlighted concerning the latter point, is that use of grab samples alone in chemical measurements is inadequate for capturing the presence of compounds, and that long-term passive sampling approaches are preferable for capturing contaminant spikes (Zenobio *et al.*, 2015). Temperature fluctuations resulting from natural or man-made sources might be an influencing factor that should carefully be considered while interpreting the causes of the observed incidences of gonadal intersex among aquatic organisms. Finally, the development and use of suitable molecular biomarkers for gonadal intersex will provide sensitive tools for identification of this condition, while reducing the time of sampling and costs.

Acknowledgements: We wish to thank S. Guffey for his assistance in reviewing earlier drafts of this manuscript. We also thank John Wiley & Sons Limited and Taylor & Francis publishers for permitting the use of Figure 1.1B and 1.1C in this manuscript,

respectively. We thank the Department of Forestry and Natural Resources at Purdue University, the Cultural Affairs and Mission Sector at the Egyptian Ministry of Higher Education, the Ohio River Ecological Research Program (ORERP) and the Electric Power Research Institute (EPRI) for providing funding for this study.

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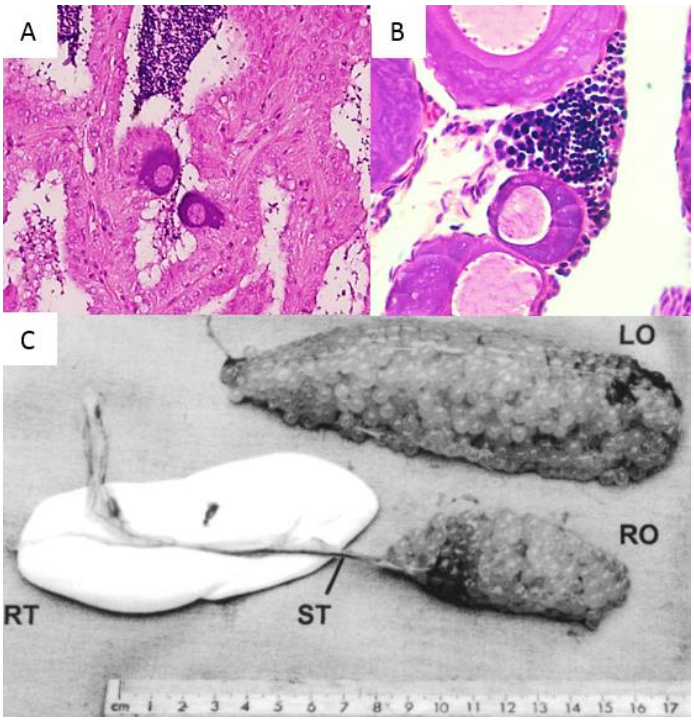

Figure 1.1: Representative microphotographs of different gonadal lesions in intersex individuals; A) testicular oocytes in smallmouth bass (*Micropterus dolomieu*) (200X), B) ovotestis in brown trout (*Salmo trutta fario*) (200X) (Körner *et al.*, 2005), and C) mixed gonadal tissue in chinook salmon (*Oncorhynchus tshawytscha*) showing anterior/posterior separation in right gonad (Kinnison *et al.*, 2000). LO = left ovary; RO = right ovary; RT = right testis; and ST = supporting tissue.

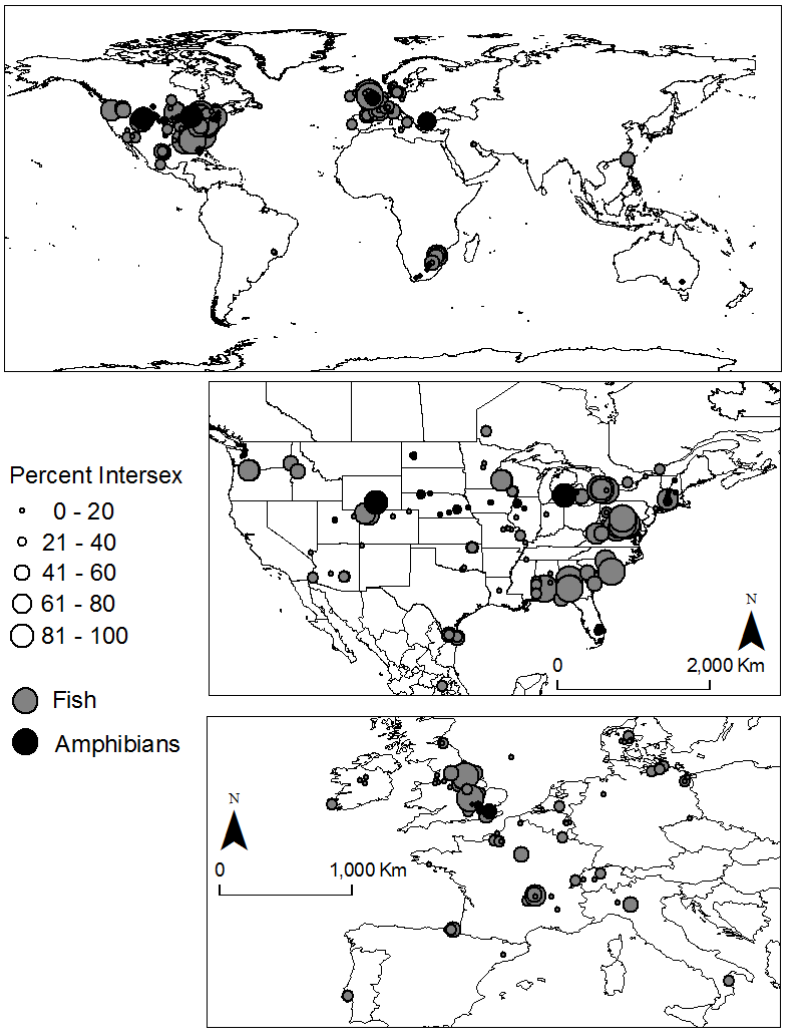

Figure 1.2: Geographical distribution and prevalence of gonadal intersex reported in wild populations of fish and amphibians.

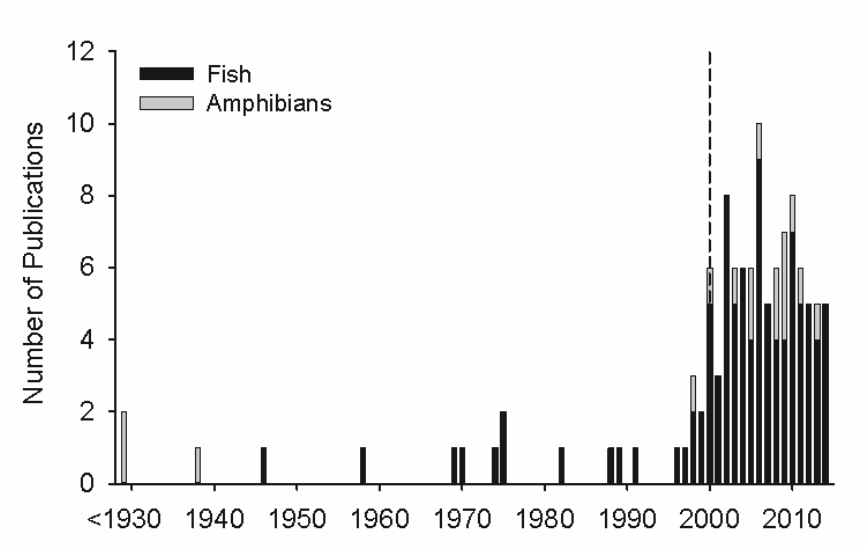

Figure 1.3: The number of scientific publications examining gonadal intersex in wild populations of fish and amphibians through time. The dashed vertical line represents the boundary between research published before and after the year 1999.

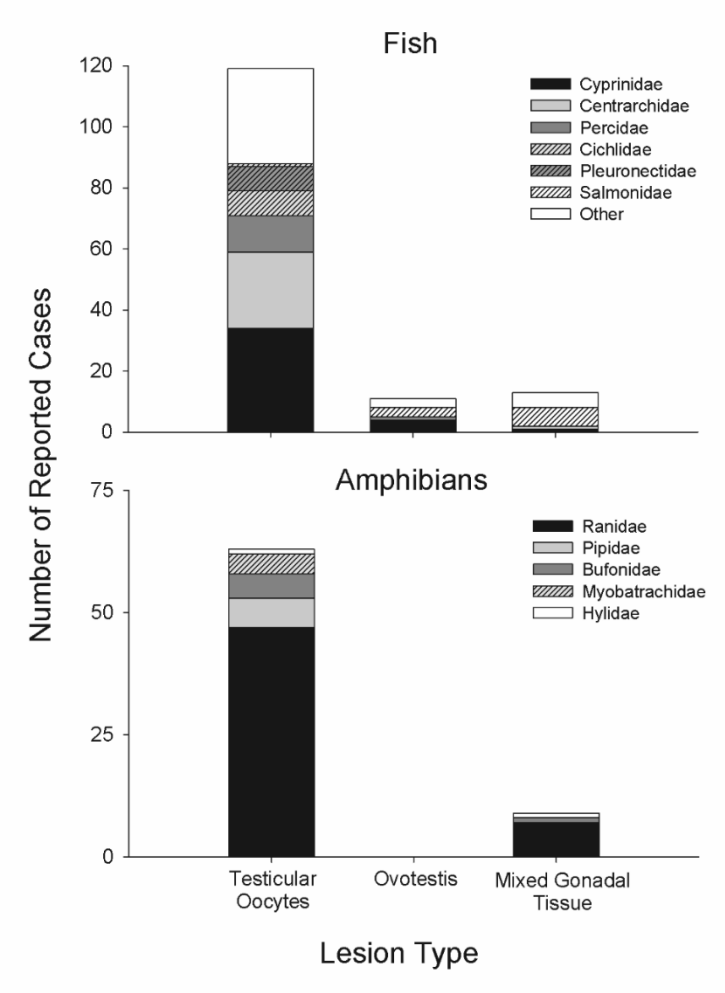

Figure 1.4: The number of reported cases of gonadal intersex for fish and amphibians, showing the major lesions and affected families described in the scientific publications.

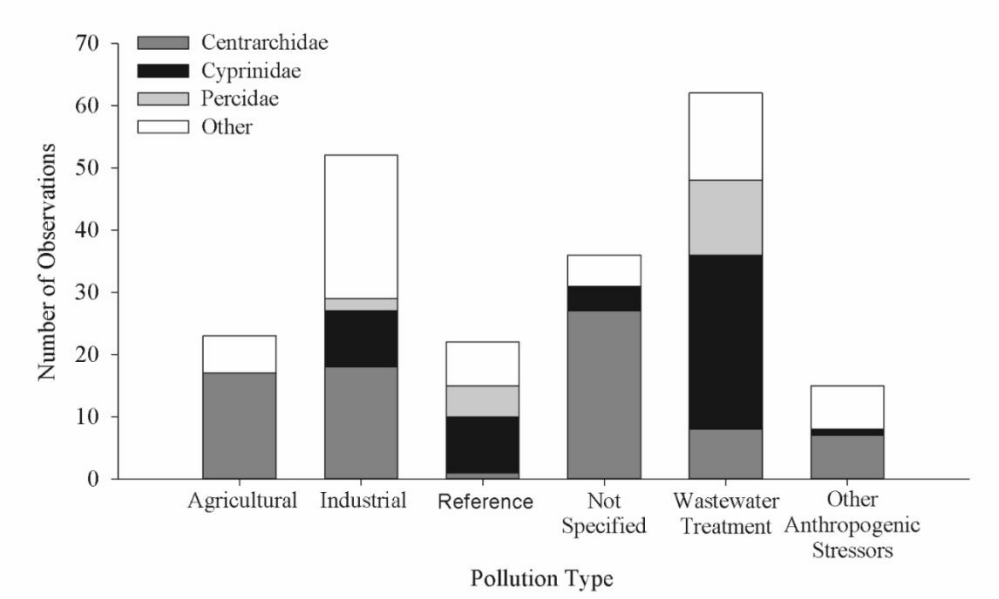

Figure 1.5: Observed cases of gonadal intersex in wild fish populations classified by the type of contaminant associated with the sampling sites as reported in the scientific publications reviewed. “Reference” refers to sites with no direct pollution discharges identified.

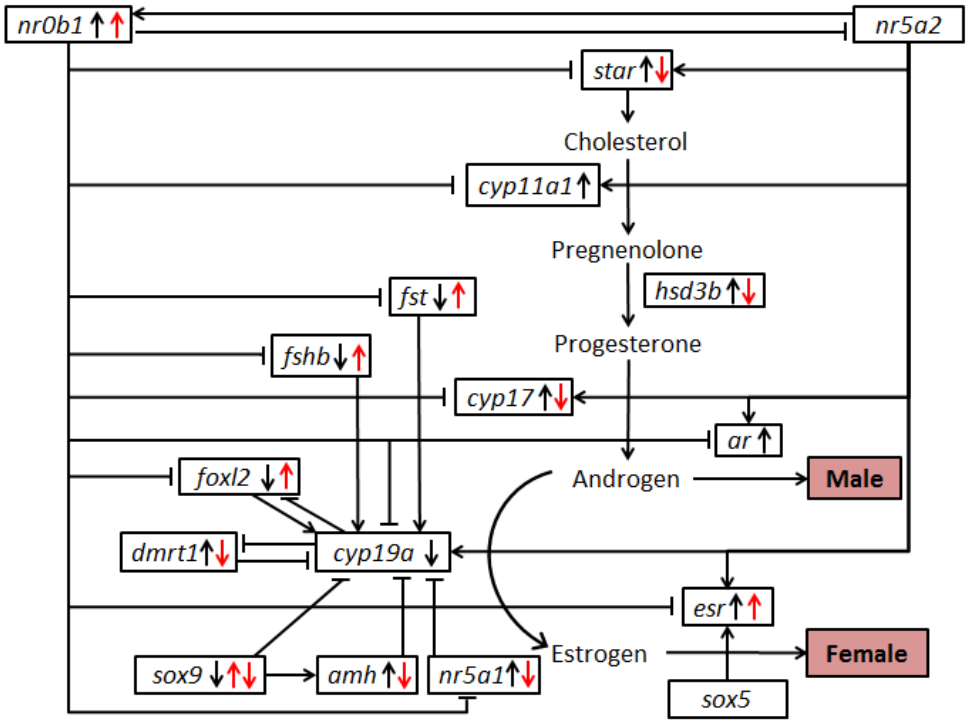

Figure 1.6: A representation of several genes involved in the sex differentiation process of fish and amphibians in relation to exposure to endocrine disrupting compounds. Some reported effects of exposure to exogenous androgens or aromatase inhibitors: black arrow (↑), exogenous estrogens: red arrow (↑). Complete gene names are cytochromeP450 19a, 11a1 and 17 (*cyp19a, cyp11a1* and *cyp17*), doublesex and mab-3 related transcription factor 1 (*dmrt1*), anti-Müllerian hormone (*amh*), sry-related HMG box-9 and 5 (*sox9 and* sox5), forkhead transcription factor (*foxl2*), estrogen receptors (*esr*), androgen receptor (*ar*), steroidogenic acute regulatory protein (*star*), dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X-chromosome, gene 1 (*nr0b1* or *dax1*), 3b-hydroxysteroid dehydrogenase (*hsd3b*), nuclear receptor subfamily 5, group A, member 1 and 2 (*nr5a1* and *nr5a2*), follistatin (*fst*) and follicle-stimulating hormone, β subunit (*fshb*).

Table 1.1: Genes tested as potential molecular biomarkers of intersex in wild populations of fish, species and tissue tested, and the reported response.

Gene	**Species**	**Tissue**	**Reference**	**Transcription levels**	**Additional comments**
P43 5S RNA-binding protein *(42Sp43)*	*Chelon labrosus* (Thicklip gray mullet)	Gonad	Diaz de Cerio *et al.* (2012)	Females and intersex > males	
5S rRNA	*Chelon labrosus* (Thicklip gray mullet)	Gonad	Diaz de Cerio *et al.* (2012)	Females > intersex > male	
Androgen receptor *(ar)*	*Etheostoma caeruleum* (Rainbow darter)	Gonad	Bahamonde *et al.* (2014)	Sites with different intersex % did not show differences in *ar* levels, except the closest site to WWTP sites (males = females).	Levels of *ar* in males and intersex were not compared.
CytochromeP450 11a *(cyp11a)*	*Etheostoma caeruleum* (Rainbow darter)	Gonad	Bahamonde *et al.* (2014)	Males > females at a reference (10% intersex) and a WWTP's (70% intersex) sites. Males = females at WWTP's site (70% intersex) closest to the sources.	Levels of *cyp11a* in males and intersex were not compared.
CytochromeP450 19a1 *(cyp19a1)*	*Chelon labrosus* (Thicklip gray mullet)	Gonad	Puy-Azurmendi *et al.* (2013)	Similar levels among all groups and sites (suggesting exposure to estrogenic active compounds)	
CytochromeP450 19a1b *(cyp19a1b)*	*Chelon labrosus* (Thicklip gray mullet)	Brain	Bizarro *et al.* (2014)	Trend to intersex > males, no significant differences due to high variability within groups.	Levels were positively correlated with pesticides and total EDCs load in water.

Gene	Species	Tissue	Reference	Transcription levels	Additional comments
CytochromeP450 19a2 *(cyp19a2)*	*Chelon labrosus* (Thicklip gray mullet)	Brain	Puy-Azurmendi *et al.* (2013)	Similar levels among all groups and sites (suggesting exposure to estrogenic active compounds)	
Doublesex and mab-3 related transcription factor (*dmrt1)*	*Scaphirhynchus platorynchus* (Shovelnose sturgeon)	Gonad	Amberg *et al.* (2010)	Similar levels among all groups, high variability within groups limits its use in identifying intersex.	*foxl2/dmrt1* transcript abundance, is a useful tool for identifying intersex
	Etheostoma caeruleum (Rainbow darter)	Gonad	Bahamonde *et al.* (2014)	Only in males, no differences among sites (different intersex %). No comparison between normal and intersex individuals was done.	
Estrogen receptor alpha (*esr1)*	*Chelon labrosus* (Thicklip gray mullet)	Gonad, Liver & Brain	Puy-Azurmendi *et al.* (2013)	Transcribed similarly in all groups	
	Etheostoma caeruleum (Rainbow darter)	Gonad	Bahamonde *et al.* (2014)	Females > males, with no differences between sites (different intersex %).	Levels of *esr1* in males and intersex were not compared.
Estrogen receptor beta (*esr2)*	*Etheostoma caeruleum* (Rainbow darter)	Gonad	Bahamonde *et al.* (2014)	Females = males, with no differences between sites (different intersex %).	Levels of *esr2* in males and intersex were not compared.
Exportin 5 (*exp5*)	*Chelon labrosus* (Thicklip gray mullet)	Gonad	Diaz de Cerio *et al.* (2012)	Similar transcription levels among all groups	
Exportin 6 (*exp6*)	*Chelon labrosus* (Thicklip gray mullet)	Gonad	Diaz de Cerio *et al.* (2012)	Females > males and intersex	

Gene	Species	Tissue	Reference	Transcription levels	Additional comments
Forkhead transcription factor (*foxl2)*	*Scaphirhynchus platorynchus* (Shovelnose sturgeon)	Gonad	Amberg *et al.* (2010)	Females > males and intersex.	*foxl2/dmrt1* transcript abundance, is a useful tool for identifying intersex
	Etheostoma caeruleum (Rainbow darter)	Gonad	Bahamonde *et al.* (2014)	Females > males, with no change in expression between sites (different intersex %).	Levels of *exp6* in males and intersex were not compared.
Importin α1 (*impα1*)	*Chelon labrosus* (Thicklip gray mullet)	Gonad	Diaz de Cerio *et al.* (2012)	Females and intersex > males	
Importin α2 (*impα2*)	*Chelon labrosus* (Thicklip gray mullet)	Gonad	Diaz de Cerio *et al.* (2012)	Females and intersex > males	
Importin β2 (*impβ2*)	*Chelon labrosus* (Thicklip gray mullet)	Gonad	Diaz de Cerio *et al.* (2012)	Similar transcription levels among all groups	
Piwi like protein 1 (*piwil1*)	*Chelon labrosus* (Thicklip gray mullet)	Gonad	Diaz de Cerio *et al.* (2012)	Testis > intersex > ovary	In July (Similar transcription levels among all groups).
Piwi like protein 2 (*piwil2*)	*Chelon labrosus* (Thicklip gray mullet)	Gonad	Diaz de Cerio *et al.* (2012)	Similar transcription levels among all groups.	In July (females > intersex > males).
retinoid X receptor (*rxr*)	Chelon labrosus (Thicklip gray mullet)	Gonad, Liver & Brain	Puy-Azurmendi *et al.* (2013)	Similar transcription levels among all groups.	
Sry-related HMG box-9 (*sox9)*	*Etheostoma caeruleum* (Rainbow darter)	Gonad	Bahamonde *et al.* (2014)	Females > males during recrudescence at reference site (10% intersex). Females = males at contaminated sites (70% intersex).	Levels of *sox9* in males and intersex were not compared.

Gene	Species	Tissue	Reference	Transcription levels	Additional comments
Transcription factorIIIA (*tfIIIA*)	*Chelon labrosus* (Thicklip gray mullet)	Gonad	Diaz de Cerio *et al.* (2012)	Females and intersex > males	
Vitellogenin *(vtg)*	*Scaphirhynchus platorynchus* (Shovelnose sturgeon)	Liver	Amberg *et al.* (2010)	Similar transcription levels among all groups.	No significant differences due to high variability within groups.
	Chelon labrosus (Thicklip gray mullet)	Liver	Bizarro *et al.* (2014)	Up-regulation of *vtg* levels at contaminated sites. Intersex and males at the same site show same *vtg* levels.	Positive correlation between *vtg* levels and pesticide concentrations in water.
	Etheostoma caeruleum (Rainbow darter)	Liver	Bahamonde *et al.* (2014)	Up-regulation of *vtg* levels in males at site closest to the effluent outfall (70% intersex), whereas comparable levels between far-field site (70% intersex) and reference site (10% intersex).	Females exposed to the MWWEs showed a decrease in *vtg* levels.
Vitellogenin alpha (*vtga)*	*Chelon labrosus* (Thicklip gray mullet)	Liver	Puy-Azurmendi *et al.* (2013)	Transcribed similarly in all groups.	Positive correlation between *vtga* and VTG protein levels

Abbreviations: WWTP, wastewater treatment plant.

1.14 Supplementary information

Supplementary Table 1.1. Reported cases of gonadal intersex in fish. The table reports the family and species affected, country and location of sampling, sampling date, reported sources of contamination at the sampling sites, samples used for chemical analysis and its results, type of gonadal intersex reported and prevalence and severity of gonadal intersex recorded.

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
Acipenseridae	*Acipenser oxyrinchus* (Atlantic sturgeon)	Van Eenennaam and Doroshov (1998)	USA	Hudson River and Bight	1992-1995				TO	1.86% (161 m)	
	Acipenser transmontanus (White sturgeon)	Chapman *et al.* (1996)	USA	San Francisco Bay, CA (area between Golden Gate Bridge, Angel Island, and San Francisco Bay Bridge)	1983-1986				TO	0.12% (855)	
	Scaphirhynchus albus (Pallid sturgeon)	Harshbarger *et al.* (2000)	USA	Mississippi River (south of Saint louis)		Organochlorines (Chlordane, PCBs and DDTs)	Chlordane (2960 in fillets and 1926 in roe), PCBs (5810 in roe) and DDE (780 in roe)	T	TO	29% (7 m)	
	Scaphirhynchus platorynchus (Shovelnose sturgeon)	Koch *et al.* (2006)	USA	Mississippi River (south of Saint louis)	2003	Organochlorines (OCPs and PCBs)	PCBs (6469.4 in gonads, 3953.2 in brain and 7171.2 in fillets) and OCPs (1370.9 in gonad, 838.8 in brain and 1361.8 in fillets)	T (gonads, brain, fillets)	TO, MGT	10.4% (48 m)	
		Amberg *et al.* (2010)	USA	Wabasha River (RM 300, near Lafayette, IN)	2008				TO	7.5% of the non-females (61, 52 non-female fish)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
Balitoridae	*Barbatula barbatula* (Stoneloach)	Douxfils *et al.* (2007)	Belgium	Vesdre River (upstream and downstream Goffontaine and Wegnez WWTPs)	2004-2005	WWTP effluents			TO	11% (18 downstream Goffontaine WWTP)	
Catostomidae	*Catostomus commersoni* (White sucker)	Sikstrom *et al.* (1975)	Canada	Athabasca River	1974				MGT	one case	
		Woodling *et al.* (2006)	USA	Boulder Creek (downstream of the city of Boulder WWTP, and South Platte River downstream of the Denver Metropolitan Sewage District WWTP)	2002	WWTP effluents			TO, OT	10.3% (39) at downstream Boulder WWTP and 20% (20) at downstream Denver Metro WWTP, South Platte	Percentage of each type of gonadal tissue
		Vajda *et al.* (2008)	USA	Boulder Creek (200m downstream city of Boulder WWTP, CO)	2003-2004	WWTP effluents (17B-estradiol (E2), 17a-ethynylestradiol, alkylphenols, and bisphenolA)	Total estrogen equivalence (up to 0.031) and 4-nonylphenolmonoethoxycarboxylate (up to 120)	W	TO, OT	18–22% (64)	Severity estimated following Jobling *et al.* 1998
Centrarchidae	*Micropterus cataractae* (Shoal bass)	Ingram *et al.* (2011)	USA	Flint River (above lake Blackshear (Hw 96), between	2010	WWTP effluents			TO	Total: 77% (61 m), Hw96: 79% (14 m),	Severity estimated following

Family	Species	Reference Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
			Blackshear Dam and Lake Worth (Hw 32, Warwick), below albany dam to 64 RK downstream (Albany, Plant Mitchell, Norman's Ferry))						Warwick: 67% (9 m), Hw32: 81% (16 m), Norman F: 100% (3 m), Plant M: 71% (14 m), Albany: 80% (5m)	Blazer *et al.* (2007)
	Micropterus dolomieu (Smallmouth bass)	Schmitt *et al.* (2002) USA	Mississippi River Basin (Allegheny River at Natrona (Station 67), Wisconsin River at woodman (Station 72), Mississippi River at Little Falls (74), Mississippi River at Lake city (Station 111))	1995-1996	Agricultural, urban and industrial discharges (a dioxin-like contaminant and non-accumulative organic contaminants)	TCDD: site 67 (0.028), site 72 (0.021), site 74 (0.003), site 111 (0.008). tPCBs: site 67 (1100), site 72 (250), site 74 (50), site 111 (1000). dieldrine: site 67 (15), site 72 (5), site 74 (5), site 111 (5). tDDT: site 67 (300), site 111 (100). Hg: site 67 (100), site 72 (130), site 74 (200), site 111 (90)	T (whole fish)	TO	Total range: 14%-73%, Station 67: 20% (5 m), Station 72: 25% (4 m), Station 74: 14% (7 m), and Station 111: 73% (11 m)	
		Anderson *et al.* (2003) USA	Kalamazoo River (downstream the city of Kalamazoo, MI)	1995	Agricultural, paper mills and WWTP effluents (PCBs)	tPCB (2190 in liver and 1010 in fillet)	T (liver, fillet)	TO	Mild (8 m and 7 f)	Scored from 0 to 3 depending on lesion severity
		Anderson *et al.* (2003) USA	Kalamazoo River (upstream, near the city of Ceresco, MI)	1995	No pollution	tPCB (260 in Liver and 90 in fillet)	T (liver, fillet)	TO	Mild (8 m and 7 f)	Scored from 0 to 3 depending on lesion severity

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
		Baldigo *et al.* (2006)	USA	Feeder Dam (RK 323)	1998	No pollution	Hg (470)	T	TO	35%	
		Baldigo *et al.* (2006)	USA	Hudson River (Waterford (RK 249) and Poughkeepsie (RK 122))	1998	PCBs, Hg and estrogenic contaminants	Hg (280)	T	TO	25-50% at middle and lower reaches of the basin	
		Hinck *et al.* (2006)	USA	Snake River at Lewiston, ID and Columbia River at Warrendale, OR	1997-1998	Urban and industrial effluents (Hg, PCBs, and TCDD)	In Lewiston: As (140), Cd (30), Hg (170), Pb (50), Se (340), Zn (17100), DDE (110), PCB (350). In Warrendale: As (400), Cd (30), Hg (190), Pb (60), Se (500), Zn (12400), DDE (180), PCB (380).	T (whole fish)	TO	Snake River: 42% (7), Columbia River: 67% (3)	
		Blazer *et al.*(2007)	USA	Potomac River (South Branch), Greenbrier River, Tygart River, Gauley River, Elk River, Greenbrier River, Shenandoah River	2003-2005	Anthropogenic stressors			TO	Overall: 14-100%, South branch of Potomac River: 80-100% (15 m), Greenbrier River: 22-75% (30 m), Tygart River: 14% (7 m), Gauley River: 17% (6 m), Elk River: 36% (11 m), Shenandoah River (south fork: 80% (10 m),	Scored from 0-4 based on the distribution of oocytes within the testes.

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
										North fork and mainstem: 100% (8 m, 13 m))	
		Hinck *et al.* (2007)	USA	Colorado River and its large tributaries (site 311: Yamba River near Lay, CO)	2003	Range of contaminant sources, including mining, agricultural, and urban areas (Selenium, Hg and OCs)	Hg (250), Se (950), DDE (2)	T (whole fish)	TO	Station 311: 70% (10)	mild to sever (NO criteria specified)
		Hinck *et al.* (2009)	USA	Colorado, Columbia and Mississippi River basins (Mississippi River at Lake City, MN (site 111), Yampa River at Lay, CO (site 311), Salmon River at Riggins, ID (site 42), Columbia River at Warrendale, OR (site 502), Allegheny River at Natrona, PA (site 67), Wisconsin River at Woodman,	1995-2004	Mercury ,DDE, DDD, TCDD, PCBs, trans-nonachlor, dieldrin, cis-nonachlor, cis-chlordane, and DDT.		T (whole fish)	TO	Overall: 33% (70), Site 111: 73% (11), Site 311: 70% (10), Site 42: 43% (7), Site 502: 67% (3), Site 67: 25% (4), Site 72: 25% (4), Site 74: 14% (7)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
				WI (site 72), Mississippi River at Little Falls, MN (site 74))							
		Iwanowicz *et al.* (2009)	USA	Potomic River Basin (upstream and downstream conococheague and monocacy WWTP)	2005	WWTPs effluent (PCBs, PAHs, Endosulfan, metolachlor, atrazine)	tPCBs (0.066 upstream and 0.215 downstream of Conococheague, and 410 downstream of Monocacy), tPAHs (3.9 upstream and 16.7 downstream of Monocacy, 4.1 Conococheague), tDDT (0.094 upstream and 0.144 downstream of Conococheague and 0.655 downstream of Monocacy), metolachlor (0.730 upstream and 1.115 downstream of Conococheague, and 12 upstream and 10.7 downstream of Monocacy), atrazine (90 upstream and 46.9 downstream of Conococheague, and 88 upstream and 44 downstream of Monocacy)	W (passive samplers)	TO	82-100% (upstream (20) and downstream (20) conococheague, and upstream (20) and downstream (20) monocacy WWTP)	Severity estimated following Blazer *et al.* (2007)
		Blazer *et al.*(2012)	USA	Gauley River	2006-2007	no pollution	Total estrogenicity using Yeast estrogen screen (<LOD)	W	TO	11% (17)	Severity estimated following Blazer *et al.* (2007)
		Blazer *et al.*(2012)	USA	Shenandoah River, South Branch of Potomac River	2006-2007	Animal feeding opertions, agricultual	Total estrogenicity using Yeast estrogen screen: Shenandoah (South Branch (0.0036), main stem	W	TO	54.5-100% depending on site and season,	Severity estimated following

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
				and Conococheague Creek		and WWTP effluents	(0.0023), North fork (0.0041), South fork (0.005)), Conococheague Creek (0.0091)			South Branch Springfield (74-82%), Shenandoah River (100%), Conococheague Creek (upper 100%, lower 88%) (20/site, 10 m/site)	Blazer *et al.* (2007)
		Kolpin *et al.* (2013)	USA	Gauley River	2006-2007	no pollution	Atrazine (0.007 in discrete water, 260 ng/sampler in passive water and 100 in sediment), dieldrine (0.009 in discrete water, 0.46 ng/sampler in passive water and 71 in sediment), galaxolide (0.5 in discrete water, 63 ng/sampler in passive water and 50 in sediment), sitosterol (2 in discrete water and 500 in sediment), sigmastinol (2 in discrete water, 510 ng/sampler in passive water and 500 in sediment), estrone (0.004 in discrete water, 1 ng/sampler in passive water and 1 in sediment), androstenedione (0.008 in discrete water, 2 ng/sampler in passive	W, S	TO	11% (17)	Severity estimated following Blazer *et al.* (2007)

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
							water), 17B-estradiol (0.004 in discrete water, 1 ng/sampler in passive water and 1 in sediment)				
		Kolpin *et al.* (2013)	USA	Shenandoah River, South Branch of Potomac River and Conococheague Creek	2006-2007	Animal feeding opertions, agricultual and WWTP effluents	Atrazine (0.007 in discrete water, 260 ng/sampler in passive water and 100 in sediment), dieldrine (0.009 in discrete water, 0.46 ng/sampler in passive water and 71 in sediment), galaxolide (0.5 in discrete water, 63 ng/sampler in passive water and 50 in sediment), sitosterol (2 in discrete water and 500 in sediment), sigmastinol (2 in discrete water, 510 ng/sampler in passive water and 500 in sediment), estrone (0.004 in discrete water, 1 ng/sampler in passive water and 1 in sediment), androstenedione (0.008 in discrete water, 2 ng/sampler in passive water), 17B-estradiol (0.004 in discrete water, 1 ng/sampler in passive water and 1 in sediment)	W, S	TO	54.5-100% depending on site and season, South Branch Springfield: 74-82%, Shenandoah River: 100%, Conococheague Creek: upper 100%, lower 88% (20/site, 10 m/site)	Severity estimated following Blazer *et al.* (2007)

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
	Huro salmoides (Largemouth black bass)	James (1946)	USA	Ridge Lake, IL	1942				MGT	not specified (393)	
		James (1946)	USA	Fork Lake, IL	1942				TO		
	Micropterus salmoides (Largemouth bass)	Schmitt *et al.* (2002)	USA	Mississippi River Basin (Illinois River at Beardstown, IL (station 26), Virdigris River at Oolagah, OK (station 78), Red River at Alexandria, LA (station 81), Red River at Lale Texoma, TX/OK (station 82), Missouri River at Hermann, MO (station 83), Wolf River at LaGrange, TN (station 213))	1995-1996	Agricultural, urban and industrial discharges (a dioxin-like contaminant and non-accumulative organic contaminants)	TCDD: Site 26 (0.005), Site 78 (0.024), Site 81 (0.002), Site 82 (0.0005), Site 83 (0.001), Site 213 (0.003). tPCB: Site 26 (550), Site 78 (50), Site 81 (100), Site 82 (50), Site 83 (100), Site 213 (50). Dieldrine: Site 26 (75), Site 78 (5), Site 81 (5), Site 82 (5), Site 83 (40), Site 213 (5). tDDT: Site 81 (400), Site 213 (200). Hg: Site 26 (100), Site 78 (70), Site 81 (220), Site 82 (150), Site 83 (150), Site 213 (280)	T (whole fish)	TO	Overall: 7.7%-28.6%, Stations 26: 14% (7 m), Station 78: 29% (7 m), Station 81: 12.5% (8 m), Station 82: 8% (13 m), Station 83: 17% (6 m), Station 213: 14% (7 m)	
		Schmitt *et al.* (2005)	USA and Mexico	lower Rio Grande River basin (Below Falcon Dam, TX (site 513), Mission, TX (site 16), Brownsville, TX (site 512))	1997-1998	Agriculture and energy extraction contaminants (OCs, Se, Hg, As)	DDE: Site 16 (380), Site 512 (90), Site 513 (150). Hg: Site 16 (200), Site 512 (110), Site 513 (120). Pb: Site 16 (470), Site 512 (40), Site 513 (80).	T (whole fish)	TO	35% of sampled males (29 m)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
		Baldigo *et al.* (2006)	USA	Hudson River (Catskill (CAT, RK 180) and Albany/Troy (AL, RK 246))	1998	PCBs, Hg and other contaminants	In sediment: DDE (AT (4.1) and CAT (3.9)), DDD (AT and CAT (1.8)), DDT (AT (0.4) and CAT (1.9)), aroclor (AT (150-520) and CAT (130-150)), tPCBs(AT (720) and CAT (305)), tPAHs (AT (3732) and CAT (2652)), tOC except PCBs (AT (185) and CAT (152)), In Tissue: Hg (AT (640) and CAT (280-500))	S, T	TO	20-40% (N/A)	
		Hinck *et al.* (2007)	USA	Colorado River and its large tributaries (Colorado River near Imperial Dam, AZ (site 322), Gila River near Hayden, AZ (site 323))	2003	Range of contaminant sources, including mining, agricultural, and urban areas (Selenium, Hg and OCs)	In Site 322: Hg (40), Se (2670), DDE (36). In Site 323: Hg (100), Se (530), DDE (16)	T (whole fish)	TO	Station 322: 40% (10), Station 323: 40% (10)	
		Hinck *et al.* (2008)	USA	Mobile River (MRB) (M1, M2, M4), Apalachicola River (ARB) (A1, A2, A3), Savannah River (SRB) (S1, S2, S3), Pee Dee River (PRB) (P1, P2, P3)	2004	Industrial and agricultural effluents (Hg and PCBs)	Hg (MRB (530), ARB (370), SRB (460), PRB (460)), PCB (MRB (683), ARB (448), SRB (125), PRB (311))	T (whole fish)	TO	Overall: 42%, Mobile River: M1 25% (8), M2 10% (10), M4 25% (8), Apalachicola River: A1 30% (10), A2 30% (10), A3 60% (10),	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
										Savannah River: S1 43% (7), S2 50% (4), S3 50% (10), Pee Dee River: P1 67% (3), P2 27% (11), P3 9% (11)	
		Hinck *et al.* (2009)	USA	Apalachicola, Colorado, Mobile, Mississippi, Pee Dee, Rio Grande, Savannah Rivers	1995-2004	Mercury ,DDE, DDD, TCDD, PCBs, trans-nonachlor, dieldrin, cis-nonachlor, cis-chlordane, and DDT.		T (whole fish)	TO	Overall: 18% (390), Site 338: 91% (11), Site 336: 67% (3), Site 332: 60% (10), Site 334: 50% (4), Site 333 43% (7), Site 330: 30% (10), Site 331: 30% (10), Site 326: 25% (8), Site329: 25% (8), Site 327: 10% (10), Site 512: 50% (8), Site 513: 44% (8), Site 322:	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
										40% (N/A), Site 323: 40% (5), Site 78: 22% (8), Site 213: 14% (7), Site 83: 11% (6), Site 26: 10% (10), Site 81: 14% (7), Site 82: 8% (13)	
		Iwanowicz *et al.* (2009)	USA	Potomic River basin (15Km upstream and downstream Blue Plains WWTP)	2005	WWTPs effluent (PCBs, PAHs, endosulfan, metolachlor, atrazine)	tPCBs (2.55), tPAHs (21.8), tDDT (0.355), metolachlor (1.85), atrazine (30.9)	W (passive samplers)	TO	23% (20, 13 m)	Severity estimated following Blazer *et al.* (2007)
		Ingram *et al.* (2011)	USA	Flint River	2010	WWTP effluents			TO	100% (3 m)	Severity estimated following Blazer *et al.* (2007)
		Kellock *et al.* (2014)	USA	Impoundment hapitats across Georgia, without direct municipal or agricultural wastewater inputs (Hatchary bond at Ben Hill, Private bonds at Wilkes and Hancock,	2010-2011	No direct municipal or agricultural wastewater (p-nonylphenol)			TO	Overall: 48% (0 to 82% across impoundments), (155 m, 8-22 m/site)	Severity estimated following Blazer *et al.* (2007)

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
				Paradise Puplic fishing area at Berrien, Dodge Puplic fishing area at dodge, Health and Antioch lakes at Floyd, Walter F George at Clay)							
		Yonkos *et al.* (2014)	USA	Surface waters on the Delmarva Peninsula	2005-2009	Poultry production and agricultural contaminants			TO	In 2005-2007: 17% (12 m), in 2008-2009: 57% (95 m)	Severity estimated following Blazer *et al.* (2007)
Characidae	*Astyanax fasciatus* (Lambari - Mexican tetra)	Prado *et al.* (2011)	Brazil	Furnas reservoir, Grande River (Boa Esperanca, Guape)	2006-2007	Untreated agricultural, industrial and municipal discharges			TO	0-29% (1265)	Focal and multifocal
		Prado *et al.* (2014)	Brazil	Furnas reservoir, Grande River (Boa Esperanca, Guape)	2010-2011	Untreated municipal, agricultural and industrial effluent	Total phosphorous: BE (44-62), G (68-180), and total estrogen: BE (0.131), G (0.121)	W	TO	Boa Esperança: 10% (13 m), and Guapé: 16% (14 m)	Focal and multifocal
Cichlidae	*Oreochromis* spp. (Tilapia)	Sun and Tsai (2009)	Taiwan	Era-Jiin River (6 km upstream of the River mouth (EJ-1), 10.2 km upstream the River mouth (EJ-2))	1994	Heavy metals and PCBs (Hg, Pb, Cr, Cu, PCBs, PCDDs, TCDDs)			TO	50% (6 m)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
	Oreochromis mossambicus (Mozambique tilapia)	Marchand *et al.* (2008)	South Africa	Luvuvhu River (Albasini dam, Limpopo Province)		No pollution (low DDT contamination)	In river water samples (DDT, DDE and DDD (< 0.01)), and in runoff water (DDT, DDE and DDD (0.1))	W	TO	Reaction pattern index values (Irp) = 3 (6)	
		Marchand *et al.* (2008)	South Africa	Luvuvhu River (Xikundu weir, Limpopo Province)		DDT	In river water samples (DDT, DDE and DDD (< 0.01)), and in runoff water (DDT, DDE and DDD (0.12-0.27))	W	TO	Reaction pattern index values (Irp) = 2.8 (13)	
		Barnhoorn *et al.* (2010)	South Africa	Luvuvhu River (Albasini dam (AD), Limpopo Province)		No pollution (low DDT contamination)	In Water: DDE and DDD (1), DDT (0.3), dieldrin (2).	W, S	TO	AD: 48% (39 m)	
		Barnhoorn *et al.* (2010)	South Africa	Luvuvhu River (Nandoni Dam (ND) and Xikundu Weir (XW), Limpopo Province)		DDT	In fat: DDT (948 at ND, 5889 at XW), DDD(860 at ND, 847 at XW), DDE (1764 at ND and 7609 at XW), Lindane (48 at ND and 18 at XW), dieldrin (14 at XW), endosulfan I (42 at ND), PCB153 (119 at ND). In Water: DDE (0.3-1.1 at ND and 0.4-1 at XW), DDT (0.12 at ND), dieldrin (2.4 at ND and 4 at XW). In sediment: DDT (1.4 at ND and 4.1 at XW), DDE (3.7 at ND and 13 at XW)	T (fat), W, S	TO	ND: 63% (30 m), and XW: 58% (50 m)	
		Marchand *et al.* (2010)	South Africa	Luvuvhu River (Nandoni Dam (ND) and Xikundu Weir	2007-2008	DDT	DDE (<LOD-0.3 at ND and <LOD-0.6 at XW), DDT (<LOD-0.12 at ND and XW)	W	TO	ND: 60% (10), XW: 59% (34)	

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				(XW), Limpopo Province)							
		Marchand *et al.* (2010)	South Africa	Luvuvhu River (Albasini dam, Limpopo Province)	2007-2008	No pollution (low DDT contamination)	DDE (<LOD-0.95), DDD (<LOD-0.5), DDT (<LOD-0.3)	W	TO	55% (20)	
	Apollonia melanostoma (Round goby)	Marentette *et al.* (2010)	Canada	Hamilton Harbour, Lake Ontario (Sherman inlet)	2006-2008	Agricultural,WWTP effluents, and steel mills (Cu, Cd, PAHs)	Cd (70), Cu (45000) and Ni (300)	T (liver, gill, gut)	TO	12.9% (31 m)	
Clariidae	*Clarias gariepinus* (Sharptooth catfish)	Yalçin *et al.* (2002)	Turkey	Asi River		Agricultural, industrial and WWTP effluents			TO	0.42% (720)	
		Barnhoorn *et al.* (2004)	South Africa	Rietvlei Nature Reserve (Marais Dam and Rietvlei Dam)		Agricultural, industrial and WWTP effluents (p-nonylphenol)	In MD: p-nonylphenol (6360 in water, 4 in sediment, 2-48 in serum). In RVD: p-nonylphenol (113 in sediment)	W, S, T (serum)	TO	20% (100)	
		Kruger *et al.* (2013)	South Africa	Rietvlei Nature Reserve (Marais Dam and Rietvlei Dam)		Agricultural, industrial and WWTP effluents	DDT (56.34-660.1), DDE (< OD-28.43), lindane (33.74-145.61), aldrine (< OD- 44.62), PCB153 (15-67.78), p-nonylphenol (98.24-1440.51)	T (mesentric fat)	TO, MGT	MD: 35% (52), and RVD: 22% (45)	
Cyprinidae	*Abramis brama* (Bream)	Slooff and Klootwijk-Vandijk (1982)	Netherland	Waal, Rhine and Lek Rivers, and Lake Brassem	1979-1980				MGT	0.09% (5533)	

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		Hecker *et al.* (2002)	Germany	Balksee (lake)	1999-2000	No pollution			TO	0.5% (N/A)	Severity estimated following Jobling *et al.* (1998)
		Hecker *et al.* (2002)	Germany	Elbe River (Schmilka, Haseldorf, Meissen, Barby, Magdeburg, Hohenwarte, Zollenspeiker, Koehlbrand, Geesthacht)	1999-2001	Complex mixture of chemicals (including PCBs, PAHs, p-nonylphenol, DDX, HCH, BPA, Zn)	PCBs (30-137), PAHs (1320-5050), DDX (29-391)	W, S	TO	Schmilka: 4%, Haseldorf: 3%, Meissen: 6%, Barby: 2%, Magdeburg: 5%, Hohenwarte: 5%, Zollenspeiker: 5.5%, and Koehlbrand: 6% (N/A)	Severity estimated following Jobling *et al.* (1998)
		Vethaak *et al.* (2002)	Netherland	Dommel River	1999	WWTP effluents			TO	37% (~ 23 m)	
	Barbus plebejus (Barbel)	Viganò *et al.* (2001)	Italy	Po River (15-18Km upstream and downstream from the inlet of Lambro River)	1999	Industrial and urban contaminants (ammonia, trace metals, surfactants, pesticides, PAH, and PCB)			TO, OT	50% (16)	Severity estimated by looking at the percent of each gonadal tissue

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
		Viganò *et al.* (2006)	Italy	Po River (15-18Km upstream and downstream from the inlet of Lambro River)		Industrial and urban contaminants (natural steroids (E1 and E3) and xenoestrogens (p-nonylphenol and tOP))	In bile: tOP (130-520), OP (<LOD-20), p-nonylphenol (2240-32790), BPA (<LOD-380). In water: OP (0.01), p-nonylphenol (0.011), BPA (0.302), tOP (0.019). In Sediment: p-nonylphenol (120), tOP (6.09), E3 (22.5). In macroinvertebrates: p-nonylphenol (95.66), tOP (49.81), E3 (45.2)	W, S, T (bile), macroinvertebrates	OT	Downstream: 30% (10)	Severity estimated by identifying the number of cysts at three random fields on three sections
	Cyprinus carpio (Common carp)	Solé *et al.* (2002)	Spain	Anoia River (A1: 5Km upstream a WWTP, A2: 23Km downstream the WWTP and A3: 27Km downstream the WWTP)	2000	WWTP effluents (p-nonylphenol)			TO	41% (17 m)	
		Solé *et al.* (2003)	Spain	Anoia River (23Km downstream the WWTP)	2000	WWTP effluents (p-nonylphenol)			TO	19% (31)	
		Baldigo *et al.* (2006)	USA	Hudson River (Catskill (RK180))	1998	PCBs, Hg and other contaminants	In sediment: DDE (3.9), DDD (1.8), DDT (1.9), aroclor (25-150), tPCBs (305), tPAHs (2652), tOC except PCBs (152), In Tissue: Hg (90-180)	S, T	TO	10% (N/A)	
		Hinck *et al.* (2007)	USA	Colorado River at Willow	2003	Mining, agricultural, and urban	Hg (20), Se (1630) and DDE (105)	T (whole fish)	OT	0.8% (132)	Mild to sever

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				Beach, AZ (site 320)		contaminants (Se, Hg and OCs)					
		Hinck *et al.* (2009)	USA	Colorado River at Willow Beach, AZ (site 320)	1995-2004	Mining, agricultural, and urban contaminants (Me, ,DDE, DDD, TCDD, PCBs, trans-nonachlor, dieldrin, cis-nonachlor, cis-chlordane, and DDT)		T (whole fish)	OT	0.1% (798 f)	
	Gobio gobio (Gudgeon)	Minier *et al.* (2000)	France	Seine River (Poses)	1998	WWTP effluents			TO	33% (3)	Severity estimated following Jobling *et al.* (1998)
		Van Aerle *et al.* (2001)	United Kingdom	Longton Park Lake	1997	No pollution			TO	6% (50)	Severity estimated by looking at the mean number of oocytes/section
		Van Aerle *et al.* (2001)	United Kingdom	Aire River (Silsden Bridge (upstream WWTP),	1995-1998	WWTP effluents			TO	Lakeside Fisheries: 15.1% (66), Silsden	Mean no. of oocytes/section

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
				Thwaite Weir (13 Km downstream Esholt WWTP), Knostrop (Swillington Bridge, downstream Knostrop WWTP), Lea River (Harpenden (downstream of East Hyde WWTP), and Lakeside Fisheries (Midlands)						Bridge: 13.3% (15), Thwaite Wier: 14.28% (44), Knostrop: 12.24% (49), Harpenden: 6% (83)	
		Faller *et al.* (2003)	Switzerland,	Suhre River (upstream and downstream of the Surental WWTP (9.5 Km downstream the lake outlet))	2000	WWTP effluents, Pesticides and Atmospheric deposition			TO	Overall: 22% (125 m), Suhre: upstream 23.3% and downstream 24.5%, Ron River: 14%	Scored into three groups based on the mean no. of oocytes/section
		Douxfils *et al.* (2007)	Belgium	Vesdre River (upstream and downstream Goffontaine and Wegnez WWTP)	2004-2005	WWTP effluents			TO	Wegnez upstream site: 5% (20), Goffontaine upstream site: 20% (15)	
		Sanchez *et al.* (2011)	France	Dore River (Site A (downstream a WWTP), Site	2008-2009	Industrial (Pharmaceutical factory			TO	Site A: 5-8% (32), Site B: 55-	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
				B (downstream urban and industrial discharges), Site C (dilution of site B))		producing steroid compounds) and urban contamination				80% (30), and Site C: 44-56% (30)	
	Hypophthalmichthys molitrix (Silver carp)	Papoulias *et al.* (2006)	USA	Missouri River (between RM 132 (near mouth of the Osage River) and 202 (near mouth of the Lamine River))	2003-2005				TO	8% (38 m)	
	Hypophthalmichthys nobilis (Bighead carp)	Papoulias *et al.* (2006)	USA	Missouri River (between RM 132 (near mouth of the Osage River) and 202 (near mouth of the Lamine River))	2003-2005				TO	7% (28 m)	
	Leuciscus cephalus (European chub)	Hinfray *et al.* (2010)	France	Drôme River (Saillans)	2006	No pollution			TO	13% (15 m)	Number of oocytes/slide
		Hinfray *et al.* (2010)	France	Rhône River (Givors)	2006	Urban and Industrial effluents (aromatase inhibitors)	42%-76% inhibition in *in vitro* aromatase activity	S	TO	Summer: 9% (11 m), and fall: 7% (15 m)	Number of oocytes/slide
	Notropis hudsonius (Spottail shiner)	Aravindakshan *et al.* (2004)	Canada	St. Lawrence River (Île Dorval, Îlet Vert (4km downstream	1999-2002	WWTP effluents			TO	Ile Dorval: 15% (13), Ilet Vert: 31% (13), and Ile	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
				WWTP) and Île Beauregard (35km downstream WWTP))						Beauregard: 27% (11)	
		Aravindakshan *et al.* (2004)	Canada	St. Lawrence River (upstream WWTP at Îles de la Paix)	1999-2002	No pollution			TO	2.6% (38)	
	Rhinicthys cataractae (longnose dace)	Tanna *et al.* (2013)	Canada	Grand River (DK1, DK3)	2010	WWTP effluents	DK1: Ibuprofen (0.4), venlafaxine (0.275), diclofenac (0.125), carbazepine (0.08). DK3: Venlafaxine and ibuprofen (0.2), diclofenac (0.15), ibuprofen (zero), remaining tested chemicals (~0.05).	W	TO	DK1: 60% (N/A) and DK3: 80% (N/A)	Severity estimated using a method developed (RETO)
	Rutilus rutilus (Roach)	Jobling *et al.* (1998)	United Kingdom	Lakes and canals in British Isles	1995-1996	No pollution			TO	4-18.1% (60-100 fish/site)	Number of oocytes/slide
		Jobling *et al.* (1998)	United Kingdom	15km upstream and downstream of WWTP in Wreake/Eye, Ouse, Lea, Arun, Nene, Trent, Rea and Aire Rivers	1995-1996	WWTP effluents			TO	Downstream: 16% (Wreake/Eye)-100% (Nene and Aire), and upstream: 11.7% (Lea) - 44.4% (Nene) (60-100/site)	Number of oocytes/slide
		Minier *et al.* (2000)	France	Blesle (Eu), Bethune (Neufchatel-en-	1998	WWTP effluents			TO	Neufchatel-en-Bray: 21% (14 m), Eu: 9% (23	Severity estimated following

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
				Bray), Seine (Poses) Rivers						m), Poses: 25% (4 m)	Jobling *et al.* (1998)
		Nolan *et al.* (2001)	United Kingdom	Random collection from Rivers, lakes and dtreams in British Isles					TO	150 cases (N/A)	Focal or multifocal
		Jobling *et al.* (2002)	UK	Royal Canal, Ireland, Grantham Canal, Leicestershire	1999-2000	No pollution			TO	Royal Canal: 9% (43), and Grantham Canal: 10% (30)	Number of oocytes/slide
		Jobling *et al.* (2002)	UK	Nene (Northamptonshire) and Aire (Yorkshire) Rivers	1999-2000	WWTP effluents			TO	100% (206)	Number of oocytes/slide
		Bjerregaard *et al.* (2006)	Denmark	Aarhus Brook, Egaa, and Kristrup Landkanal streams in Aarhus County, Jutland	1999	WWTP effluents			TO	Egaa: 12% (104 m), Aarhus: 7% (14 m), and Kristrup: 36% (50 m)	Severity estimated following a modified version of Jobling *et al.* (1998)
		Bjerregaard *et al.* (2006)	Denmark	Almind and Ravn Lakes	1999	No pollution			TO	Almind: 5% (21 m), and Ravn: 5% (37)	Severity estimated following a modified version of Jobling *et al.* (1998)

Family	Species	Reference Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
		Jobling *et al.* (2006) UK	1km upstream and downstream of the WWTP in 39 Rivers throughout the United Kingdom (Flit, Hiz, Ivel, Churnet, Erewash, Eye, Don, Trib of Dearne, Foss, Wey, Stanford, Ray, Foudry Brook, Sincil Dyke, Witham, Louth Canal, Nene, Brain, Blackwater, Colne, Gipping, Little Ouse, R. Cam, Whittlesey Dyke, Anker, Anker, Erewash, Avon, Bow, Calder, Calder, Grand Union Canal, Great Stour, Great Stour, Ray Brook, Trib of Lee, Blackwater, Loddon, Mole, Bourne, Langford Brook, Kennet, Medway, Hiz,	2002-2003	WWTP effluents			TO	Atreas of high-risk: 31.25% (128 m), medium-risk: 22.24% (409 m), low-risk sites: 9.1% (55 m)	Severity estimated using a numerical scale from 0 to 7

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
				Severn, Wey, Itchen)							
		Maltret-Geraudie *et al.*(2008)	France	Seine River		Urban and Industrial effluents			TO	14% (110)	
		Harris *et al.* (2011)	UK	River Bourne (downstream of Chertsey WWTP) and River Arun (downstream of Horsham WWTP)	2006-2008				TO	Bourne: 39% (38 m), Arun: 41% (76 m)	Severity estimated following Jobling *et al.* (2006)

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
		McGee *et al.* (2012)	Ireland	Brosna River (downstream Mullingar WWTP), Inny River(downstream Ballymahon WWTP)and Suck River (downstream Ballinasloe WWTP)	2007	WWTP effluents			TO	Brosna: 14.3% (7), Inny: 27.3% (11) and Suck: 10% (10)	
	Squalius cephalus (Chub)	Minier *et al.* (2000)	France	Bethune River (Isambertheville)	1998	WWTP effluents			TO	3.6% (28 m)	Severity estimated following Jobling *et al.* (1998)
		Randak *et al.* (2009)	Czech Republic	Elbe River (Downstream Usti nad Labem site)	2004	Industrial effluents (PCB, DDT, HCH, HCB, OCs)	HCHs (53), DDTs (6480), HCB (521), octachlorostyrene (221), Hg (260), alkylphenol (2.7)	T (muscle)	TO	50% (4)	
Esocidae	*Esox lucius* (Northern pike)	Vine *et al.* (2005)	United Kingdom	Colne and Blackwater Rivers, Chesterfield Canal (less than 5Km downstream WWTP)	2001-2003	WWTP effluents (Estrogenic contamination)	Estrogenic activity (significantly higher downstream than upstream)	T (bile)	TO	Chesterfield: 33.3% (18), Colne: 25% (4), Blackwater 25% (4)	Scored based on no. of intersex clumbs/section
		Vine *et al.* (2005)	United Kingdom	Gara, River Hull, Moretons Leam, River Trent (upstream WWTP)	2001-2003	No pollution (upstream WWTP)	Estrogenic activity (significantly higher downstream than upstream)	T (bile)	TO, OT	Hull: 4.3% (23), Gara: 50% (2), Moretons: 33.3% (3),	Scored based on no. of intersex clumbs/section

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
										Trent: 57% (7)	
Gasterosteidae	*Gasterosteus aculeatus* (Three-spined stickleback)	Gercken and Sordyl (2002)	Germany	Uecker and Randow Rivers (Neu Sulstorf and Moraas)	1999-2000	WWTP effluent			TO	Neu Sulstorf: 12.5% (16), and Moraas: 4.8% (21)	Scored from mild to sever
		Pettersson *et al.* (2007)	Sweden	near Askő	2001-2003	No pollution		T (bile)	TO	0.91% (110)	
Ictaluridae	*Ictalurus punctatus* (Channel catfish)	Hinck *et al.* (2007)	USA	Colorado River (Oyrary NWR, UT, Hogback Diversion, NM, and Phoenix, AZ)	2003	Mining, agricultural, and urban contaminants (Selenium, Hg and OCs)	Ouray (Hg (150), Se (1430), DDE (12)), Hogback (Hg (100), Se (1500), DDE (17)), Phoenix (Hg (40), Se (1690), DDE (310))	T (whole fish)	TO	7% (42 m)	Scored from mild to sever
		Hinck *et al.* (2009)	USA	Colorado River (Oyrary NWR, UT, Hogback Diversion, NM, and Phoenix, AZ)	1995-2004	Mercury ,DDE, DDD, TCDD, PCBs, trans-nonachlor, dieldrin, cis-nonachlor, cis-chlordane, and DDT.		T (whole fish)	TO	7% (42 m)	
Moronidae	*Morone americana* (White perch)	Kavanagh *et al.* (2004)	Canada	Cootes Paradise region, Hamilton Harbour, Lake Ontario	1998	Domestic and industrial effluents	Cootes Paradise: p-nonylphenol (1000-5000) and octylphenol (10-600)	S	TO	50% (16)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
		Kavanagh *et al.* (2004)	Canada	Cootes Paradise region in Hamilton Harbour, Bay of Quinte and Lake St. Clair, Ontario	1999-2000	Domestic and industrial effluents	Cootes Paradise: p-nonylphenol (1000-5000) and octylphenol (10-600)	S	TO	Bay of Quinte: 22% (37)– 44% (16), Lake St. Clair: 45% (11), and Cootes Paradise: 83% (12)	
Mugilidae	*Chelon labrosus* (Thicklip gray mullet)	Diaz de Cerio *et al.* (2012)	France & Spain (Basque Countries)	Bay of Biscay (Bilbao, Plentzia, Urdaibai, Ondarroa and Pasaia)	2009-2011	Endocrine disruptor compounds (Pasaia)			TO	N/A (30/sampling)	
		Puy-Azurmendi *et al.* (2013)	Spain	Estuary of Urdaibai Biosphere Reserve (Gernika)	2007, 2008	WWTP effluents (phthalate and organotins, OPs, p-nonylphenol)	In sediment: total Ots (3-37), total Aps (91.93-193.7), total phthalates (687.99-8871.15). In Bile: p-nonylphenol (17781-44896), OP (126-599)	S, T (bile)	TO	< 33% (N/A)	
		Puy-Azurmendi *et al.* (2013)	Spain	Abra estuary	2007, 2008	Heavy industrial pressure (phthalate and organotins, OPs, p-nonylphenol)	In sediment: total Ots (2178-12460), total Aps (<LOD-257.22), total phthalates (2019.58-3885.62). In Bile: p-nonylphenol (3083-7393), OP (66-765)	S, T (bile)	TO	1 case (N/A)	
		Bizarro *et al.* (2014)	Spain	Basque coast (Bay of Biscay) (Deba, Gernika, Pasaia)	2012	Urban and industrial effluents (Phthalates	In water: Gernika (HCH (<LOD-0.321), chlorpyrifos (1.213), DEHP (0.641)), Pasaia	W, T (bile)	TO	Deba: 20%, Gernika: 23.5%, and Pasaia: 50%	Severity estimated following

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
						and pesticides (most abundant), followed by alkylphenols, musks, bisphenol-A and estrogenic hormones)	(DEHP (0.806)). In Bile: Deba (BBP (694-879)), Gernika(HCH (<LOD-2202.9), DDT (1096-2972), BBP (1639.3-1966), DEHP (<LOD-1966)), Pasaia (HCH (<LOD-1769), DDT (1100-1560), BBP (900.6-1388.2))			(12-20 m/site)	Jobling *et al.* (1998)
	Mugil cephalus (Grey mullet)	Ferreira *et al.* (2004)	Portugal	Douro estuary	2001-2002	WWTP and industrial effluents (PCBs and tDDT)	tPCB: in liver (190.3 - 686.4) and muscle (310.7-344.4 ppb DW), tDDT: in liver (39.2 -137.8) and muscle (63.1- 69.4)	T (muscle, liver)	TO	21% (53 m)	
		Aoki *et al.* (2010)	Korea and Japan	Ansan, Tongyeong, and Busan in Korea and Omuta in Japan	2003-2005	Enviromental estrogens			TO	Ansan: 11.1% (18), Tongyeong: 15.8% (19), Busan: 7.4% (27), Omuta: 7.7% (52)	
	Mullus barbatus (Red mullet)	Martin-Skilton *et al.* (2006)	NW Mediterranean	French coast "Cortiou"	2001-2002	Urban and industrial contaminants (alkylphenols, PCBs, organochlorinated compounds)	p-nonylphenol (28310) and octylphenol (250)	T (bile)	TO	Cortiou: 6.7% (15)	
Pangasiidae	*Pangasius nasutus*	Rodriguez *et al.* (2012)	Indonesia	Indragiri River (Riau provence,					MGT	1 case (N/A)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
	(Pangasiid catfish)			Central Sumatra)							
		Bernet *et al.* (2004)	Swizerland	Lake Thun	2000-2003				MGT	1.1% (818) and 10 additional specimes collected by fishermen	
Percidae	*Etheostoma blennioides* (Greenside darter)	Tetreault *et al.* (2011)	Canada	Grand River (Kiwanis, Mannheim)	2007-2009	No pollution			TO	Kiwanis: 20% (N/A), and Mannheim: 10% (N/A)	
		Tetreault *et al.* (2011)	Canada	Grand River, (Downstream Waterloo WWTP, downstream Kitchner WWTP)	2007-2009	WWTP Effluent, domestic and industrial wastes			TO	Downstream Waterloo WWTP: 33% (N/A), and downstream Kitchner WWTP: 60% (N/A)	
	Etheostoma caeruleum (Rainbow darter)	Tetreault *et al.* (2011)	Canada	Grand River (Kiwanis, Mannheim 2, Horse Ranch)	2007-2009	No pollution			TO	Kiwanis: 9% (N/A), Mannheim 2: 33% (N/A), and Horse Ranch: 50% (N/A)	
		Tetreault *et al.* (2011)	Canada	Grand River (Downstream Waterloo WWTP, downstream	2007-2009	WWTP Effluent, domestic and industrial wastes			TO	Downstream Waterloo WWTP: 33% (N/A), downstream Kitchner	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
				Kitchner WWTP, Blair)						WWTP: 60-75% (N/A), and Blair: 100% (N/A)	
		Tanna *et al.* (2013)	Canada	Grand River (Ref1, Ref2, Ref3, Ref4)	2010	no pollution (upstream effluent)	Atrazine, venlafaxine carbamazepie, diclofenac (~ 0.025)	W	TO	Ref1: 20% (25 m), Ref2: 18% (17 m), Ref3: 20% (20m), Ref4: 9% (22m)	Severity estimated using a method developed (RETO)
		Tanna *et al.* (2013)	Canada	Grand River (DW1, UK1, UK2, DK1, DK2, DK3, DK4, DK5, DG1)	2010	WWTP effluent	DW1: venlafaxine (0.35), diclofenac and ibuprofen (0.2), carbamazepine (0.1), atrazine and fluoxetine (0.025).UK1 and UK2: all chemicals tested (<0.1). DK1: Ibuprofen (0.4), venlafaxine (0.275), diclofenac (0.125), carbazepine (0.08). DK2: Venlafaxine and ibuprofen (0.275), diclofenac (0.15), remaining tested chemicals (~0.075). DK3 and DK4: Venlafaxine and ibuprofen (0.2), diclofenac (0.15), ibuprofen (zero), remaining tested chemicals (~0.05). DK5: Venlafaxine (0.15), remaining tested chemicals (< 0.1). DG1: Venlafaxine (0.65), carbamazepine (0.35), diclofenac (0.15) and remaining tested chemicals (< 0.05)	W	TO	DW1: 33% (19 m), UK1: 40% (19 m), UK2: 50% (17 m), DK1: 85% (19 m), DK2: 100% (6 m), DK3: 75% (30 m), DK4: 25% (21 m), DK5: 45% (20 m), DG1:35% (19 m)	Severity estimated using a method developed (RETO)

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
		Bahamonde *et al.* (2014)	Canada	Grand River (upstream of the city of Waterloo and Kitchener)	2010	no pollution			TO	10% (10)	
		Bahamonde *et al.* (2014)	Canada	Grand River (downstream of the city of Waterloo and Kitchener)	2010	WWTP effluent			TO	70% (21)	
	Etheostoma nigrum (Johnny darter)	Tanna *et al.* (2013)	Canada	Grand River (DK3)	2010	WWTP effluent	DK3: Venlafaxine and ibuprofen (0.2), diclofenac (0.15), others (0.05) and ibuprofen (<LOD)	W	TO	40% (N/A)	Severity estimated using a method developed (RETO)
	Perca fluviatilis (Eurasian perch)	Gercken and Sordyl (2002)	Germany	Uecker and Randow Rivers	1999-2000	WWTP effluent & Reference sites			TO	Uecker (Rochow): 33.3% (3), and Randow 16.7% (6)	Scored from mild to sever
	Sander vitreus vitreus (Walleye)	Pollock *et al.* (2010)	Canada	Wabigoon River (5km downstream of the pulp mill effluent discharge, Dryden limm)	2002	Pulp mill effluents, WWTP effluent, hypoxia			TO, OT	TO: 50% (14 m), and OT: 28% (32 f)	
		Miller *et al.* (2012),	USA	Upper Mississippi River (Lake Bemidji (RK 2063), Upstream of Grand Rapids (RK 1934), Grand Rapids (RK 1903),	2007-2009	Pulp mill effluents and EDCs			TO	<1% (377)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
				Aitkin (RK 1706), Brainerd (above and below dam, RK 1622 and 1617), Little Falls (RK 1553), Sartell (RK 1495), St. Cloud (RK 1485), Monticello (RK 1445), St. Paul (RK 1345), Red Wing (RK 1275), Lake city (RK 1241))							
Pleuronectidae	*Limanda limanda* (Common dap)	Stentiford and Feist (2005)		Northern Dogger Bank, North Sea	2003				TO	14.3% (14 m)	
	Pseudorhombus arsius (large-toothed flounder)	Stentiford *et al.* (2014)	Kuwait	Kuwait Bay (El-Doha, Ras Al-Ajoza, between Al-Bedaa and Ras Al-Ardh)		Oil and industrial contamination			TO	2.5% (40)	
	Platicthys flesus (Flounder)	Allen *et al.* (1999a)	UK	Mersey and Tyne Rivers	1997	Industrial effluents (OCs based on previous studies)			TO	Mersey River: 9.2% (65m), and Tyne River: 7.5% (65m)	
		Allen *et al.* (1999b)	UK	Mersey estuary	1996-1997	Industrial and domestic effluents			TO	16.67% (30 m)	
		Simpson *et al.* (2000)	UK	Mersey and Dee estuaries		Heavy metals, PCBs,			TO	Mersey estuary: 4 cases (410	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
						DDT, other chlorinated hydrocarbons and PAHs				in both esturaies, ~76 males in Mersey)	
		Stentiford *et al.* (2003)	UK	Mersey and Tees estuaries	2000	Anthropogenic contamination (PAHs)			TO	Tees: 7.7% (13), and Mersey: 8.3% (12)	
		Kleinkauf *et al.* (2004)	UK	Mersey and Dee estuaries	1997-2000	Urban, industrial and agricultural effluents			TO	Mersey estuary: 0.5% (4 cases)	
	Pleuronectes yokohamae (Marbled flounder)	Hashimoto *et al.* (2000)	Japan	Tokyo Bay	1997-1998	Industrial and domestic effluents (APEs and nonylphenol)			TO	15% (20 m)	
Salmonidae	*Coregonus clupeaformis* (Lake whitefish)	Chen (1969)	Canada	Hogan's Pond (St. John's, Newfoundland)	1965-1966				MGT	0.38% (261)	
		Porter and Corey (1974)	Canada	South bay, Lake Huron	1970				MGT	1 case (N/A)	
		Mikaelian *et al.* (2002)	Canada	St. Lawrence River (Saint-Nicolas, 10km upstream of Quebic city)	1996	WWTP effluents (aroclor, PCB congeners, other organochlorinated	As (< 400), Cd (230), Cr (130), Cu (30440), Hg (41), Pb (130), Zn (27500), aroclor (1752), DDTs (128.2), dieldrin (2.6), endrin (6.6)	T (liver, ovary, muscles)	MGT, OT	MGT: 1.2% (497),OT: 11.7% (223 f)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
						compounds (OCs) and some trace metals (e.g., Cd, Cu, Pb)					
	Coregonus lavaretus (common whitefish)	Scott (1975)	Scotland	Loch Lomond	over 15 years				MGT	1 case (10000)	
		Brown and Scott (1988)	Scotland	Loch Lomond	1986				MGT	1 case (N/A)	
	Coregonus hoyi (bloater)	Edsall (1970)	USA	Lake Michigan (7.5 miles NW of Frankfort)	1955				MGT	1 case (N/A)	
	Oncorhynchus clarki lewisi (westslope cutthroat trout)	Benson (1958)	USA	Yellowstone Lake, WY	1957				MGT	1 case (N/A)	
	Oncorhynchus kisutch (Coho salmon)	Kinnison *et al.* (2000)	Chile	Fish farm near Puerto Montt	1996				MGT	1 case (3000)	
	Oncorhynchus tshawytscha (Chinook salmon)	Kinnison *et al.* (2000)	New Zealand	Glenariffe Hatchery	1997				MGT	1 case (2660)	
	Salvelinus alpinus (Arctic char)	Fraser (1997)	Scotland	Loch Rannoch					MGT	1 case (N/A)	
	Salmo trutta fario (Brown trout)	O'Farrell *et al.* (1989)	Irland	Hatchery operations at Crumlin, Connemara	1987				MGT	1 case (N/A)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
		Körner *et al.* (2005)	Switzerland	Liechtensteiner Binnenkanal and Venoge Rivers (one site near the source of the River and two sites downstream)	2002-2003	WWTP effluents and areas with no effluent load			OT	Liechtensteiner Binnenkanal: 20% (64 f), Venoge: 26.7% (57 f)	
		Körner *et al.* (2007)	Switzerland	Liechtensteiner Binnenkanal and Venoge Rivers (one site near the source of the River and two sites downstream)	2002-2003	WWTP effluents and areas with no effluent load			OT	Liechtensteiner Binnenkanal: 20% (64 f), Venoge: 26.7% (57 f)	
	Thymallus thymallus (Grayling)	Blachuta *et al.* (1991)	Poland	Nysa Klodzka River (left tributary of the Odra River, SW Poland)	1989				MGT	1 case (N/A)	
Soleidae	*Synaptura orientalis* (Oriental sole)	Stentiford *et al.* (2014)	Kuwait	Kuwait Bay (El-Doha, Ras Al-Ajoza, between Al-Bedaa and Ras Al-Ardh)		Oil and industrial contaminants			TO	1.67% (60)	
Xiphiidae	*Xiphias gladius* (Mediterranean swordfish)	De Metrio *et al.* (2003)	Italy & Spain (Mediterranean sea)	Gulf of Taranto (North Ionian Sea) & the Western Mediterranean (Spanish seas)	2000-2001				TO	North ionian sea: 27% (121), and western mediterranean: 17% (41)	
Zoarcidae	*Zoarces viviparus* (Viviparous	Gercken and	Germany	Coastal waters in in Mecklenburg-	1999-2000	WWTP effluents &			TO	Wismar: 25% (24), Salzhaff:	Scored from mild to sever

Family	Species	Reference Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
	blenny, European eelpout)	Sordyl (2002)	Western Pomerania (Wismar, Salzhaff, Rostock)		Reference sites				28% (18), Rostock: 24% (17)	
		Stentiford *et al.* (2003) UK	Tyne estuary	2000	Anthropogenic contamination (PAHs)			TO	25% (~16 m)	

Abbreviations: RM, river mile; RK, river kilometer; WWTP, wastewater treatment plant; LOD, limit of detection; W, water; T, tissue; S, sediment; TO, testicular oocyte; OT, ovotestis; MGT, mixed gonadal tissue.

Supplementary Table 1.2: Reported cases of gonadal intersex in amphibian. The table reports the family and species affected, country and location of sampling, sampling date, reported sources of contamination at the sampling sites, samples used for chemical analysis and its results, type of gonadal intersex reported and prevalence and severity of gonadal intersex recorded.

Family	**Species**	**Reference**	**Country**	**Location and sampling sites**	**Sampling date**	**Pollution reported**	**Chemical concentrations measured (ppb)**	**Sample used for chemical analysis**	**Lesion recorded**	**Incidence of Intersex (n)**	**Intersex severity assessment**
Hylidae	*Acris crepitans* (Northern cricket Frogs)	Reeder *et al.* (1998)	USA	Ponds and agricultural areas in Illinois	1993-1995	PCBs, PCDFs and atrazine	Atrazine (<LOD-3 ppb in W, <LOD-70 ppb in S), metolachlor (1-2 ppb in W, <LOD-40ppb in S), chlorpyrifos (<LOD-3100 ppb in W), Lead (<LOD-11500 ppb in W, <LOD-13000 ppb in S)	W, S	TO, MGT	2.6% (341)	
		Beasley *et al.* (2005)	USA	Ponds in northern and southern Illinois				W, S	TO, MGT	2.6% (341)	
Bufonidae	*Bufo bufo* (Common toad)	Dönmez *et al.* (2009)	Turkey	Gelibolu (Çanakkale-Turkey)	2006-2007				MGT	67.9% (28)	
	Bufo marinus (Cane toad)	McCoy *et al.* (2008)	USA	South Florida: Lake Worth (LW), Lake Wellington (WT) (no agriculture), Homestead (HS) (34% agricultural land), Canal Point (CP) (51%) and Belle Glade (BG) (97%)		Pesticides (glyphosate and atrazine)			TO	5% at HS, 30% at CP and 40% at BG (20/site)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
Myobatrachidae	*Limnodynastes fletcher* (Long-thumbed Frog)	Hyne *et al.* (2009)	Australia	Northern region of Coleambally irrigation area (NSW),	2005-2006	Herbicides and insecticides (fipronil, endosulfan, chlorpyrifos, thiobencarb, molinate, metolachlor, diuron, clomazone, atrazine)	In rice only field: fipronil (0.0025 ppb), endosulfan (0.001 ppb), chlorpyrifos (2.09 ppb), thiobencarb (204 ppb), molinate (96.2 ppb), metolachlor (0.08 ppb), diuron (0.19 ppb), clomazone (3.53 ppb), atrazine (0.26 ppb). In rice and corn fields: fipronil (0.0016 ppb), endosulfan (0.007 ppb), chlorpyrifos (0.72 ppb), thiobencarb (185 ppb), molinate (6.9 ppb), metolachlor (0.39 ppb), diuron (0.11 ppb), clomazone (0.37 ppb), atrazine (0.66 ppb)	W (passive samplers)	TO	6.3% (41, 16 m)	
		Spolyarich *et al.* (2011)	Australia	Rice bays of Coleambally Irrigation Area (CIA), New South Wales	2005-2007	Pesticides (atrazine and metolachlor)	Atrazine (0.11 ± 0.03 ppb), metolachlor (0.01 ± 0.01ppb), thiobencarb (49.81 ± 30.71ppb), molinate (66.13 ± 46.13ppb), chlorpyrifos (0.90 ± 0.15 ppb)	W (passive samplers)	TO	0.7% (138)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
	Limnodynastes tasmaniensis (Spotted grass frog)	Hyne *et al.* (2009)	Australia	Southern region of Coleambally irrigation area (NSW)	2005-2006	Herbicides and insecticides (fipronil, endosulfan, chlorpyrifos, thiobencarb, molinate, metolachlor, diuron, clomazone, atrazine)	In rice only field: fipronil (0.0025 ppb), fndosulfan (0.001 ppb), chlorpyrifos (2.09 ppb), thiobencarb (204 ppb), molinate (96.2 ppb), metolachlor (0.08 ppb), diuron (0.19 ppb), clomazone (3.53 ppb), atrazine (0.26 ppb). In rice and corn fields: fipronil (0.0016 ppb), endosulfan (0.007 ppb), chlorpyrifos (0.72 ppb), thiobencarb (185 ppb), molinate (6.9 ppb), metolachlor (0.39 ppb), diuron (0.11 ppb), clomazone (0.37 ppb), atrazine (0.66 ppb)	W (passive samplers)	TO	4.2% (54, 24m)	
		Spolyarich *et al.* (2011)	Australia	Rice bays of Coleambally Irrigation Area (CIA), New South Wales	2005-2007	Pesticides (atrazine and metolachlor)	Atrazine (0.11 ± 0.03 ppb), metolachlor (0.01 ± 0.01ppb), thiobencarb (49.81 ± 30.71ppb), molinate (66.13 ± 46.13ppb), chlorpyrifos (0.90 ± 0.15 ppb)	W (passive samplers)	TO	0.8% (120)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
Ranidae	*Lithobates blairi* (Plains leopard Frog)	Papoulias *et al.* (2013)	USA	South-central Nebraska's Rainwater Basin Management District (Killdeer, McMurtrey, Morger, Atlanta, Valentine)	2007-2009	Pesticides (atrazine, metolachlor and glyphosate) and nutrient runoff	Atrazine: Killdeer (0.41-49.1ppb), McMurtrey (<LOD-47.85ppb), Morger (0.09-0.99ppb), Atlanta (0.26-3.23ppb), Glyphosate: Killdeer (<LOD-1.15ppb), McMurtrey (<LOD-10.7ppb), Morger (0.09-0.11ppb), Atlanta (<0.15ppb), Metolachlor: Killdeer (<LOD-0.52ppb), Morger (<0.07-0.29ppb)	W	TO	Killdeer 1: 12% (50, 14 m), Killdeer 2: 3% (39, 21 m), Killdeer 3: 0% (10, 4 m), McMurtrey: 7% (50, 13 m), Morger: 2% (50, 13m), Atlanta: 10% (50, 19 m), Valentine: 7% (44, 13 m)	
	Lithobates catesbeianus (American bullfrogs)	Sower *et al.* (2000)	USA	Southern New Hampshire	1998				MGT	6.9% (29)	
	Lithobates sylvaticus (Wood frog)	Cheng (1929)	USA	Pond of White Woods in Ann Arbor, Michigan	1928				MGT	1 case	
	Lithobates catesbeiana (American bullfrogs)	Moore and Byers (1938)	USA	Stillwater by Frank Briscoe of Tulsa, Oklahoma	1937				MGT	1 case	
		Murphy *et al.* (2006)	USA	South-central Michigan (Kalamazoo (KZ), Greater Lansing area (GLA), and Lapeer (LPR))	2002-2003	Atrazine	Atrazine: at agricultural areas (< 2 ppb, Ag5 in LBR reached 250 ppb in 2002), at non-agricultural sites (< 0.23 ppb), As: <12 ppb, and Zn exceeded	W	TO	In 2002, KZ Ag2 7.7% (13) and LPR Ag5 50% (2)	Number of TO/animal, severity determined using Dumont (1972)

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
							20ppb in three samples.				classificati on system
	Lithobates clamitans (Green frogs)	Murphy *et al.* (2006)	USA	South-central Michigan (Kalamazoo (KZ), Greater Lansing area (GLA), and Lapeer (LPR))	2002-2003	Atrazine	Atrazine: at agricultural areas (< 2 ppb, Ag5 in LBR reached 250 ppb in 2002), at non-agricultural sites (< 0.23 ppb), As: <12 ppb, and Zn exceeded 20ppb in three samples.	W	MGT	MGT: less than 5% per site. TOs: 0-18%/site at non-agricultural and 0-28%/site at agricultural sites, no significant difference.	Number of TO/animal, severity determined using Dumont (1972) classificati on system
		McDaniel *et al.* (2008)	Canada	Farm ponds and agricultural drains within Thames River watershed, Southwestern Ontario	2003–2005	Pesticides and nutrient inputs (atrazine, metolachlor and nitrate)	Atrazine: Chatham (0.035-0.4 ppb), London (<LOD-0.078 ppb), agricultural reference (0.043 ppb), non-agricultural reference (<LOD). Metolachlor: Chatham (0.073-0.55 ppb), London (<LOD -0.18 ppb), agricultural reference (<LOD -0.05 ppb), non-agricultural reference (<LOD).	W	TO	11% at reference sites and 10% at agricultural sites	Developed a severity index of 4 levels

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
		Skelly *et al.* (2010)	USA	Wetlands of different landscape within Connecticut River watershed	2005				TO	Overall 13% (233m, 20/site), undeveloped landscapes (0%), agricultural (5%), suburban (22%), urban (16%)	
	Lithobates pipiens (Northern Leopard Frogs)	Christensen (1929)	USA	West Lake Okoboji and Iowa city, Iowa	1928-1929				MGT	3 cases	
		Hayes *et al.* (2003)	USA	All localities between 39°N and 43°N latitude (Cache county (Utah), Carbon county (Wyoming), Cherry and York counties (Nebraska) and Polk and Clinton counties (Iowa))	2001	Atrazine	Atrazine: Juab (111°52.23W, 39°46.63N) ND, Cache (111°50.14W, 39°43.40N) 0.2 ppb, Carbon (107°03.28W, 41°51.68N) 0.2 ppb, Cherry (101°42.89W, 42°41.67N) 0.3 ppb, York (97°22.38W, 40°55.88N) 0.8 ppb, Polk (93°27.39W, 41°48.11N) 6.7 ppb, Polk (93°25.50W, 41°47.47N) NA, Clinton (90°21.28W, 41°44.46N) 0.5 ppb	W	TO	Juab 0%, Cache 10%, Carbon 90%, Cherry 40%, York 30%, Polk 18%, Polk 20%, Clinton 22% (800, 100/site)	

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
		Murphy *et al.* (2006)	USA	South-central Michigan (Kalamazoo (KZ), Greater Lansing area (GLA), and Lapeer (LPR))	2002-2003	Atrazine	Atrazine: at agricultural areas (< 2 ppb, Ag5 in LBR reached 250 ppb in 2002), at non-agricultural sites (< 0.23 ppb), As: <12 ppb, and Zn exceeded 20ppb in three samples.	W	TO	In 2002, NA2 33.3% (12) and Ag6 81.5% of the juveniles (27) in GLA	Number of TO/animal, severity determined using Dumont (1972) classification system
		McDaniel *et al.* (2008)	Canada	Farm ponds and agricultural drains within Thames River watershed, Southwestern Ontario	2003–2005	Pesticides and nutrient inputs (atrazine, metolachlor and nitrate)	Atrazine: Chatham (0.035-0.4 ppb), London (<LOD -0.078 ppb), agricultural reference (0.043 ppb), non-agricultural reference (<LOD). Metolachlor: Chatham (0.073-0.55 ppb), London (<LOD -0.18 ppb), agricultural reference (<LOD -0.05 ppb), non-agricultural reference (<LOD).	W	TO	Chatham/Kent 45% (169m), London 25% (20m), agri. reference sites 25% (22m) and non-agri ref sites 5% (44m)	
Pipidae	*Xenopus laevis* (African clawed frogs)	Smith *et al.* (2005)	South Africa	Maize-growing areas in Viljoenskroon area (Free State Province) and non-maize-growing areas north of Potchefstroom (North West Province)	2002	Agricultural chemicals used in maize production (atrazine and terbuthylazine)			TO	NMGR 3% (115, 55 m), MGR 2% (92, 44 m)	Severity determined using Dumont (1972) classification system

Family	Species	Reference	Country	Location and sampling sites	Sampling date	Pollution reported	Chemical concentrations measured (ppb)	Sample used for chemical analysis	Lesion recorded	Incidence of Intersex (n)	Intersex severity assessment
		Du Preez *et al.* (2009)	South Africa	Site A: Lewis Gay Dam, Cape Point, Site B: Klapmuts, Bellville, Site C: Jonkershoek, Stellenbosch, Site D: Jonkershoek Hatchery, Stellenbosch, Site E: Jaques Well, Laingsburg, Site F: Ko ka Tsjara, Beaufort West, Site G: Sophiasdal, Redersburg, Site I: Taggart Farm, Potchefstroom		Atrazine	Site I: atrazine (0.1 ppb), simazine (0.4 ppb), and terbuthylazine (0.3 ppb), remaining sites: <LOD	W	TO	Site E: 14%, Site F: 8%, Site G: 2-8%, Site I: 8-10% (up to 50/site)	Total number of TOs/individual

Abbreviations: WWTP, wastewater treatment plant; LOD, limit of detection; W, water; S, sediment; TO, testicular oocyte; MGT, mixed gonadal tissue.

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CHAPTER 2. GONADAL INTERSEX IN SMALLMOUTH BASS *MICROPTERUS DOLOMIEU* FROM NORTHERN INDIANA: PREVALENCE, SEVERITY, MOLECULAR BIOMARKERS AND POSSIBLE LINKS TO LEVELS OF ENDOCRINE DISRUPTING CHEMICALS

2.1 Abstract

Over the past decade, studies have shown that exposure to endocrine disrupting chemicals (EDCs) can cause gonadal intersex in fish. Smallmouth bass (*Micropterus dolomieu*) males appear to be highly susceptible to developing this condition in the form of testicular oocytes (TOs) as observed in various areas across the U.S. The main objective of this study was to quantify the prevalence and severity of TOs in smallmouth bass sampled from the St. Joseph River in northern Indiana and develop biomarkers for the diagnosis of this condition. Prevalence and severity of TOs reached maximum levels in some sites, while showing significant decreases in prevalence and increases in severity after the spawning season. We examined the relationship between the presence of TOs and expression of gonadal and liver genes involved in sex differentiation and reproductive functions (*esr1, esr2, foxl2, fshr, cyp19a, star, lhr* and *vtg*). We found that vitellogenin (v*tg*) transcript levels were significantly higher in the liver of males with TOs, but only when sampled in the spawning season. Further, we identified positive correlations between plasma VTG levels and *vtg* transcript levels, suggesting its use as a non-destructive biomarker of intersex in this species. Finally, we quantified the levels of 43 contaminants in surface water at representative sites using passive sampling to look for contamination with possible links to the observed prevalence of intersex. No detectable levels of steroids were recorded at any of the sampling sites, but levels of other endocrine disruptors were found. Our findings suggest that the observed prevalence levels of TOs might be the result of exposures to unforeseen endocrine disruptors.

2.2 Introduction

Gonadal intersex in gonochoristic (fixed-sex) fish species is an abnormal condition that has been reported in wild populations across the globe (Abdel-moneim *et*

al., 2015a). Development of oocytes within testicular tissues of male fish, generally referred to as testicular oocytes (TOs), is the most prevalent form of gonadal intersex (Hecker *et al.*, 2006; Abdel-moneim *et al.*, 2015a). Studies have speculated on the existence of a basal rate of TOs among different gonochoristic fish species (Jobling *et al.*, 1998; van Aerle *et al.*, 2001; Hecker *et al.*, 2002; Hinfray *et al.*, 2010) ; however, increases in its prevalence over the past few years have been primarily associated with exposure to EDCs (Jobling *et al.*, 1998; Pait and Nelson, 2003; Blazer *et al.*, 2007). These compounds enter aquatic ecosystems from various point and non-point sources, predominantly wastewater treatment plants and runoff from land-applied with animal manure (Kusk *et al.*, 2011; Ciparis *et al.*, 2012; Kortenkamp, 2012; Leet *et al.*, 2012). In addition, prevalence and severity of TOs is season-dependent, with highest values recorded immediately prior to spawning (Blazer *et al.*, 2007), likely because of an enhanced sensitivity to hormonal exposure during the period of gonadal recrudescence (Okutsu *et al.*, 2006).

Numerous studies have attempted to establish relationships between TOs and exposure to EDCs; however the majority of these studies have failed to find strong cause-effect relationships (Reeder *et al.*, 2005; Bjerregaard *et al.*, 2006; Woodling *et al.*, 2006; Douxfils *et al.*, 2007). In fact, only a handful of studies have reported strong relationships between the prevalence of TOs and specific contaminants, such as steroids and atrazine (Iwanowicz *et al.*, 2009; Kolpin *et al.*, 2013). However, these findings were based on field observations so they do not necessarily imply causation. Despite the debate behind the etiology of TO development, researchers have been able to withdraw a number of conclusions and recommendations from these field studies including the use of passive sampling approaches over grab sampling for assessing contaminant concentrations in surface waters. Passive sampling approaches, such as Polar Organic Chemical Integrative Samplers (POCIS), can better represent aqueous contaminant exposures to biota in streams receiving fluxes of chemicals and can increase detection of more rare contaminants compared to conventional sampling techniques (Alvarez *et al.*, 2005; Zenobio *et al.*, 2015; Van Metre *et al.*, 2016). These samplers accumulate ultra-trace levels of chemicals over their deployment periods, resulting in masses of sequestered chemicals much greater than what is generally recovered using grab

sampling approaches (Alvarez *et al.*, 2007). Another important conclusion from these studies is the need for more studies investigating the prevalence of TOs in relation to contaminant concentrations over large spatial scales, which is particularly important when working with species with large home ranges (Abdel-moneim *et al.*, 2015a).

Currently, TOs can only be diagnosed after sacrificing the fish for histological analyses of the testes. Plasma vitellogenin (VTG), the egg yolk female-specific precursor, has been the most promising candidate tested as a potential minimally invasive biomarker of intersex in wild fish populations (Abdel-moneim *et al.*, 2015a). In female fish, *vtg* is synthesized in the liver and transported systemically to the developing oocytes (Bahamonde *et al.*, 2013). Since males do not express *vtg* under normal conditions and vitellogenesis is a process stimulated by exposure to endogenous or exogenous estrogens (Anderson *et al.*, 1996), changes in the expression levels of this gene and its protein in male fish have been widely used as indicators of estrogenic exposure (Sumpter and Jobling, 1995; Jobling *et al.*, 1998; Rotchell and Ostrander, 2003; Maltret-Geraudie *et al.*, 2008; Matozzo *et al.*, 2008). However, the expression of VTG in plasma and its mRNA in liver have been predictive of TOs only in a few studies (Jobling *et al.*, 2006; Kidd *et al.*, 2007; Amberg *et al.*, 2010; Bahamonde *et al.*, 2014; Bizarro *et al.*, 2014).

Our objectives were several-fold. First, we investigated the prevalence and severity of TOs in smallmouth bass sampled from multiple sites along the St. Joseph River, northern Indiana over multiple years and seasons. This waterbody has previously been identified as having medium to high estrogenic and intersex induction potential, due to the existence of levels of EDCs and compounds with potential for adverse biological effects (Baldwin *et al.*, 2016). Next, we examined the relationship between candidate molecular biomarkers and presence of TOs for the development of biomarkers for this condition. Changes in hepatic expression of *vtg* in males with TOs was compared to the changes observed at the plasma protein level to validate our findings and identify the sensitivity of each approach. Finally, we quantified the levels of 43 contaminants including chemicals with known endocrine disrupting potential through the use of POCIS in a subset of sites where fish had been collected along the St. Joseph River and its tributaries to investigate possible links between TO prevalence/severity and contaminant

levels. This study constitutes the first to report TOs in smallmouth bass inhabiting freshwater systems in Indiana.

2.3 Material and Methods

2.3.1 Sampling sites

We sampled the St. Joseph River for smallmouth bass from Bristol, Elkhart and South Bend, northern Indiana (Figure 2.1). Smallmouth bass were sampled over six consecutive years (2010-2015) targeting both their spawning (spring) and post-spawning (summer) seasons (Table 2.1). This watershed receives runoff from both urban and agricultural discharges, primarily from wastewater treatment plants and surface-runoff from agricultural land (Baldwin *et al.*, 2016). The location of our sampling sites was determined using methods similarly described by Young *et al.* (2014). Briefly, flow lines and watershed boundaries from the first version of the National Hydrography Dataset Plus (NHDPlus) hydrologic framework (US-EPA, 2006) were used to locate stream outflow points on twelve digit Hydrologic Unit Code (HUC 12) watersheds. The ratio of urban, agriculture, and forest (Table 2.2) from the National Land Cover Dataset (2006) along with the locations of wastewater treatment plants from the Environmental Protection Agency's (EPA) National Pollutant Discharge Elimination System (NPDES) permits were examined to determine the relative impact of humans and animals on water quality. Finally, water flow information in the NHDPlus hydrography lines were used to determine if a sampling point would be affected by input from upstream watersheds. A distance of ~10 river miles was selected to separate between the different sampling sites along St. Joseph River given our knowledge that smallmouth bass inhabiting this river show limited movement (average radius of 2.65 miles) (Foy, 2004). In addition, the selected sampling sites along St. Joseph River were separated by dams, namely Elkhart Dam (ED), Twin Branch Dam (TBD), Central Park Dam (CPD) and South Bend Dam (SBD) (Figure 2.1), which further prevents fish migration from site to site.

2.3.2 Fish tissue sample collection

Smallmouth bass were captured by boat electroshocking. Fish were euthanized using MS-222 (300 mg/L), weighed, measured (total length), examined for gross lesions, and bled from the caudal vein. Blood (~1 mL) was placed in heparinized vials for the collection of plasma, which was stored after separation at –80 ºC until protein analyses. Fulton's condition factor (Nash *et al.*, 2006) ($K = W\ (g)/L^3\ (mm)\ x100{,}000$) was calculated to estimate the health status of fish captured from different sites (see Table 2.1). Gonads were weighed to calculate the gonadosomatic index (GSI, %) following the formula gonad weight in g/body weight in g x100 (see Table 2.1). Liver and gonad samples were flash-frozen in liquid nitrogen and stored at –80 ºC for gene expression analyses. A portion of each testes was fixed in 10% neutral buffered formalin for histological analysis as described below.

2.3.3 Histological analysis of gonads

Microtomy and standard hematoxylin and eosin staining were performed at Purdue Histology & Phenotyping Laboratory. For each sample, five sections (5 µm each), covering cross sections of the whole testes with 200 µm intervals in between, were examined at a magnification of 200X to identify the presence of TOs. The prevalence of TOs in each sample was recorded and the severity of the condition determined based on the ranking system developed by Blazer *et al.* (2007) with some modifications. In brief, each gonad was assigned a rank on a scale of 1 to 4 (see Supplementary Figure 2.1 for microphotographs exemplifying severity scales) after scanning five histological sections for TOs while focusing on the central zone of the examined section. The most severe score observed in a given microscopic field at 200X was assigned to the whole section and the highest rank recorded in all sections assigned to the whole gonad and used as the fish's TO severity index (Green *et al.*, 2014).

2.3.4 Passive-water sampling

POCIS were purchased from Environmental Sampling Technologies, (St. Joseph, MO, USA) and deployed at six stations (4 "Urban" and 2 "Rural") in the St. Joseph River watershed to detect and characterize concentrations of pesticides and estrogenic

contaminants. Deployment sites for the POCIS (see Figure 2.1) were selected using the same approach described in section 2.3.1. Urban and Rural sites were selected based on their proximity to wastewater treatment plants/combined sewer outputs and registered combined animal feeding operations (CAFOs), respectively. In 2014, POCIS were deployed for 28 d from July 25 to August 22. In 2015, POCIS were deployed for 49 d from June 12 to July 31. A total of three POCIS were deployed at each station and extracts of all three POCIS were composited as one sample. At each sampling location, one POCIS was placed in ambient conditions throughout the POCIS deployment period and their retrieval was used as a field blank.

2.3.5 Contaminant quantification

POCIS were stored at -18°C until extraction. The POCIS were pooled by location with 3 POCIS membranes combined during extraction except for the field blank, which was a single POCIS. A solvent blank was also performed for each set of extractions (one set in 2014 and one set in 2015). POCIS were gently rinsed with ultrapure water (NANOpure® DIamond™ Analytical, Barnstead Thermolyne, Dubuque, IA, USA), and the POCIS holders were unscrewed over clean aluminum foil. A plug of glass wool was inserted at the bottom of a chromatography column, and a funnel was placed on top. The whole system was then rinsed with methanol. The POCIS membranes were separated from the holders over the funnel, and the inside was rinsed with methanol so that the SX-3Ambersorb/Isolute ENV+ phase was poured into the column. After the 3 membranes were in the column the funnel was rinsed with methanol. Extraction was performed using 50 mL of a mixture of methylene chloride:methanol:toluene 8:1:1, which was introduced in the column and eluted at a flow rate of 1.5-3 mL/min. The final extract contained the initial methanol used to rinse the phase and the 50 mL extraction solvent. This extract was evaporated under gentle nitrogen flow. After only a few mL were left, extracts were filtered through a glass pipet containing methanol-wet GF6 filter, and through another pipet containing sodium sulfate to dry water traces. All the rinses during these steps were performed using methanol. The extract was then blown down to <1 mL and transferred to an injection vial through a 0.2 µm polypropylene syringe filter and blown down to dryness. The extract residue was then re-suspended in 0.5 mL methanol

containing an internal standard mix at about 100 ppb for each compound. Along with the second set of extractions, an additional laboratory spike was performed: methanol (20 mL) was introduced into the column along with solution aliquots of 20 µL containing all analytes at about 6 ppm except the pesticides and another of 10 µL of a pesticide mix at 20-40 ppm.

Extracts were analyzed using an AB Sciex TripleTOF 5600+ equipped with a DuoSpray Ion Source and Shimadzu LC system (CBM20A controller, LC30AD gradient pumps, SIL30AC autosampler, and CTO30A oven). A Phenomenex Kinetex™ 100 × 2.1 mm, 5 µm EVO C18 column was used for the chromatography. Three different analyses were performed on each extract as detailed in Supplementary Tables 2.1 and 2.2. Internal calibration was performed using standards of analytes dissolved in 100% methanol, each containing the same amount of internal standards as the samples. Concentrations measured in the field blank POCIS were subtracted from the concentrations measured in the POCIS samples (Supplementary Table 2.3).

2.3.6 Gene expression analysis

Differential gene expression between males without TOs, males with TOs, and females, was quantified using real-time quantitative polymerase chain reaction (qPCR). Total RNA was extracted from testes and liver using QIAzol (Qiagen, Valencia, CA) following vendors' specifications with some modifications. In brief, 50 µg of tissue was homogenized in 1 mL of QIAzol, incubated at room temperature for 5 min, then 200 µL of chloroform was added to the homogenate, vortexed and centrifuged for 15 min. The aqueous layer was again vortexed with 200 µL of chloroform and centrifuged for 5 min, then the newly formed aqueous layer was mixed with an equal volume of isopropanol, vortexed thoroughly and centrifuged for 20 min. The pellet was washed multiple times with 75% ethanol with 5 min centrifugations, then air-dried and dissolved in 50 µL of nuclease-free water. All centrifugations were performed at room temperature in a benchtop centrifuge at a speed of 16,100 g. RNA concentrations, 260/280 and 260/230 ratios, were quantified using a Nanodrop 2000c spectrophotometer (ThermoFishers Scientific, Waltham, MA). RNA concentrations ranged between 0.3 and 1 µg/µL, whereas both 260/280 and 260/230 ratios were always between 1.9 and 2.1, which

indicates the absence of protein and chemical contamination (Eldh *et al.*, 2012). The deoxyribonuclease (DNase) treatment, complementary DNA (cDNA) synthesis, qPCR reaction and its data acquisition and analysis were performed as described by Abdel-moneim *et al.* (2015b).

Primer sequences (Supplementary Table 2.4) for the reference gene, *β-actin*, and estrogen receptor alfa (*esr1*) were designed from partial coding sequence published in the National Center for Biotechnology Information (NCBI) website (Genbank accession numbers reported in Supplementary Table 2.4), whereas vitellogenin (*vtg*) primer sequences were adopted from Biales *et al.* (2007). Primer sets from closely related species (largemouth bass *Micropterus salmoides* and sea bass *Centropristis striata*) targeting conserved regions of the additional target genes (estrogen receptor beta *esr2*; steroidogenic acute regulatory protein *star;* forkhead transcription factor *foxl2*; luteinizing hormone receptor *lhr*; and follicle stimulating hormone receptor *fshr*) were used to develop primer sequences. Amplified sequences of appropriate size were gel purified using the QIAquick gel extraction kit (QIAgen) and ligated to pMD-20 t-vector (TaKaRa, Mountain View, CA), then cloned into One Shot TOP10 Chemically Competent *E. coli* (ThermoFishers Scientific), according to the manufacturer's instructions. After propagation, plasmids were extracted from the cloned cells using QIAprep Spin Miniprep Kit (QIAgen) and submitted to the Purdue genomic core facility for low throughput sequencing on an ABI sequencer. Sequences were then blasted against the NCBI database to identify their homology to the target genes in other species and if high sequence homology (> 95%) was found, sequences were submitted to the NCBI Genbank (Genbank accession numbers reported in Supplementary Table 2.4). Primers were designed according to the sequence read and their efficiency were tested (see Supplementary Table 2.4). All primers were purchased from Integrated DNA.

2.3.7 Western blotting of plasma VTG

Western blots were performed to identify differences in plasma VTG levels between smallmouth bass males with and without TOs as described in Abdel-moneim *et al.* (2015b), with some modifications. Plasma from spawning females (n = 5) with high VTG levels were also run as a positive control. In brief, protein concentration was

determined using the Thermo-Scientific bicinchoninic acid (BCA) Protein Assay Kit and equal sample volumes (100 µg/sample) were used. Samples were loaded in a 10% SDS–PAGE gel and then transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). Blots were first incubated with the primary polyclonal antibody, anti-VTG (1:1000, Biosense, Bergen, Norway) and then incubated with near-infrared dye (IRDye) 680 secondary antibody (1:10000; Li-Cor) after washing with phosphate-buffered saline plus 0.1% Tween 20 (PBST) buffer. The developed bands were viewed using Odyssey infrared imager (Li-Cor).

2.3.8 Statistical analyses

All statistical analyses were performed using SPSS 22.0. Unless otherwise noted, all data are presented as mean ± standard error (SE). First, data were tested for normality using the Shapiro–Wilks test. Response variables showing normal distributions were statistically analyzed using independent sample t tests or one-way ANOVA. Mann–Whitney U and Kruskal-Wallis tests were used to analyze response variables with non-normal distribution. Chi square and Fisher's Exact tests were used to compare the prevalence and severity of TOs across sites, seasons and years. Spearman rank order correlations were used to identify relationships between different variables. A value of α=0.05 was chosen to detect statistically significant differences.

2.4 Results

2.4.1 Sample collections

A total of 246 smallmouth bass males were collected from eight sampling locations along the St. Joseph River and its tributaries over a period of six consecutive years (2010-2015) (Table 2.1). An average of 40 (33-69) fish were sampled every year targeting the spawning (spring, n = 100) and post-spawning (summer, n = 145) seasons. In 2014 and 2015, fish were sampled only during the summer and spring, respectively. Overall, bass weighed 294 ± 11 g and 290 ± 14 g, measured 288 ± 4 cm and 282 ± 4 cm, and had a K of 1.2 ± 0.01 and 1.2 ± 0.01 in spring and summer, respectively. GSI was significantly lower ($p < 0.001$) among males sampled in summer (0.2 ± 0.02 %)

compared to spring (0.8 ± 0.05 %). Detailed morphometric data for each sample collection are described in Table 2.1.

2.4.2 Prevalence and severity of TOs

The overall prevalence of TOs was 71.7%, however, it ranged greatly from 0 to 100% (Figure 2.2). The prevalence for severity scores one, two, three and four were 25.7%, 18.9%, 22.9%, and 32.6%, respectively (Supplementary Figure 2.1 and Table 2.1). Prevalence and severity of TOs did not differ across sites and there were no differences between the prevalence of TOs between the most upstream (Bristol) and the most downstream (Downstream South Bend Dam) sites (Figure 2.2). The prevalence of TOs was highest in 2011 ($p = 0.001$) and 2014 ($p = 0.002$) and the severity was lowest in 2014 (only when compared to 2013, $p = 0.003$ and 2015, $p < 0.001$). Interestingly, the prevalence of TOs was lowest ($p < 0.001$) and the severity highest ($p = 0.002$) in males sampled in the summer.

2.4.3 Differential expression of biomarker candidates

Seven candidate genes involved in sexual differentiation and gonadal development and functions in fish were tested to assess their suitability for use as biomarkers of TOs. Gene expression analysis was performed on males (and a small set of females) sampled during the spawning (spring) and post-spawning (summer) seasons. Transcripts of all seven genes were detected regardless of the sampling season or the presence or absence of TOs (Supplementary Figure 2.2). Significant changes in gene expression was observed only for *vtg* with an up-regulation in livers from males with TOs, but only when sampled during spring ($p = 0.038$) (Supplementary Figure 2.2 and Figure 3A). No significant correlation was detected between *vtg* expression levels and severity of TOs; however, the trend was positive in the spring ($n = 6$, $r = 0.68$, $p = 0.138$) compared to the summer ($n = 5$, $r = -0.32$, $p = 0.604$). As expected, liver *vtg* expression in females was significantly higher compared to males with (spring: $p = 0.037$, summer: $p = 0.004$) or without (spring: $p = 0.016$, summer: $p = 0.004$) TOs and highest in spring ($p = 0.017$) compared to summer (Figure 3A).

2.4.4 VTG plasma protein levels in smallmouth bass

To determine VTG plasma protein levels and its correlation to hepatic *vtg* expression levels, Western blotting was performed using matching plasma samples from the same individuals. The presence and intensity of the VTG bands recorded using Western blotting from plasma of males with TOs corresponded well with *vtg* transcripts levels in the livers of the same individuals (Figure 3B). The intersex male with the highest hepatic *vtg* transcripts levels had the highest levels of plasma VTG using Western blotting, followed by another intersex male which came second in its *vtg* transcript levels and also had detectable levels of plasma VTG but of lower intensity (Figure 3B). The remaining males that were positive to TOs with lower transcript levels of *vtg* in liver had no detectable levels of plasma VTG.

2.4.5 Contaminant concentrations

Estimated contaminant masses (ng/POCIS) recovered from POCIS at each deployment site are presented in Table 2.2. Of the 43 contaminants analyzed, 20 were pesticides, 7 were steroids, and the remaining were miscellaneous contaminants including pharmaceuticals and personal care products (PPCPs) and industrial chemicals. Twenty-two contaminants had detectable levels at no less than one of the POCIS deployment sites in 2014 and 2015. No steroid levels were detected. Other known EDCs, such as the bisphenol family of compounds were always detected below the limit of quantification (LOQs). Perfluorinated compounds, such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), were detected in all sites and years. All four parabens analyzed (methyl, ethyl, propyl, and benzyl) had levels well above LOQs at several POCIS deployment sites, especially in 2015, but their occurrence was not site specific. Atrazine and metabolites were detected at all deployment sites and years, their concentrations were the highest among the different contaminants quantified. Additional herbicides (simazine, metolachlor, prometon ametryn, bromacil, and monuron) and fungicides (azoxystrobin and propicanazole) were also consistently observed across sites. PFOS was consistently detected at higher concentrations at “urban” sites, whereas PFOA and triclosan have shown higher concentrations in most of the POCIS deployed at urban sites.

2.5 Discussion

In this study, we present the first cases of TOs in male smallmouth bass inhabiting the St. Joseph River watershed in northern Indiana. Further, we examined diagnostic biomarkers for this condition and report a significant positive relationship between the presence of TOs and hepatic *vtg* transcripts from the same individuals, but only when sampled during the spring. Hepatic *vtg* transcript expression was more sensitive for detecting TOs compared to VTG plasma protein levels. Finally, we identified the ratios of different land covers in this watershed and selected representative sampling sites to quantify the levels of pesticides and other organic contaminants, comprising chemicals with endocrine disrupting potential. Our findings showed high levels of pesticide contamination in this watershed, including pesticides with endocrine disrupting potential such as atrazine, and detectible levels of other contaminants with endocrine disrupting potential, such as PFOA, PFOS and triclosan. Overall, this study helped identify impacts on the reproductive development of smallmouth bass inhabiting St. Joseph River and its tributaries, in the form of high prevalence and severity levels of TOs, and pointed out multiple contaminants with possible links to these observed biological changes.

2.5.1 Prevalence and severity of TOs

The overall prevalence of TOs in smallmouth bass males sampled from the St. Joseph River and its tributaries between 2010 to 2015 was 71.7% (n = 243). This value is close to the overall prevalence reported in a comprehensive analysis of intersex levels in smallmouth bass inhabiting the Northeast region of the US from 2008 to 2010 (85%, n = 118) (Iwanowicz *et al.,* 2016). However, these prevalence values are more than double compared to a country-wide survey conducted between 1995 and 2004 that reported a prevalence of only 33% (n = 70) in smallmouth bass inhabiting eight US River Basins (Hinck *et al.,* 2009). Numerous other reports have identified TOs occurrences among gonochoristic fish populations inhabiting freshwater systems across the US (Abdel-moneim *et al.,* 2015a). The majority of these reports followed the passing of the amendments to the Safe Drinking Water Act (SDWA) in 1996 (Abdel-moneim *et al.,* 2015a), but whether these numerous reports were due to increased monitoring efforts or exposures to xenoestrogens is still debatable. The suggested existence of a basal rate of

TO development among feral smallmouth bass populations might only explain part of these observed changes. This is owing to the facts that xenoestrogens and other EDCs are known to induce gonadal intersex among different fish species (Kidd *et al.,* 2007; Abdel-Moneim *et al.,* 2015a) and that frequency of detecting levels of gonadal intersex among fish inhabiting natural pristine environments is usually very low (Bahamonde *et al.,* 2013; Abdel-moneim *et al.,* 2015a). No gonadal intersex was observed among 470 roach, a species with widely reported cases of gonadal intersex across Europe, sampled from a reference site (Normandy, France) with no known anthropogenic influences and no estrogenic or mutagenic activity in its sediments (Geraudie *et al.,* 2010). Studies reporting high levels of basal rate of TOs rarely tackle fish movement from and to their study sites and in many cases do not comprehensively study contaminant exposure at these sites (Bahamonde *et al.,* 2013; Abdel-moneim *et al.,* 2015a). These observations suggest that only a limited number of aquatic habitats are genuinely pristine and that high levels of TOs occurrence are usually associated with anthropogenic land uses and contaminant discharges (Blazer *et al.,* 2007; Blazer *et al.,* 2012; Kolpin *et al.,* 2013; Abdel-moneim *et al.,* 2015a).

At the level of sample collections, we recorded a wide array of TOs prevalence values among smallmouth bass sampled from different sites along St. Joseph River and its tributaries. The TOs prevalence values ranged between 0% and 100%, but the majority of sites showed high TO prevalence levels. Despite the fact that the majority of our sampling sites were located more than 10 river miles apart and are separated by dams, which limits the ability of fish to migrate upstream, TO prevalence levels did not show any significant differences between the sampling points along St. Joseph River path neither did it show any patterns of increase or decrease. In agreement with our findings, several studies reported a wide array of TOs prevalence among smallmouth bass sampled from different sites (Blazer *et al.,* 2007, 2014). Others, recorded only high ranges of TOs prevalences among smallmouth bass males sampled from different sites, ranging between 60%-100% (Iwanowicz *et al.,* 2016). Similarly, the percentage of male smallmouth bass with TOs reported in other studies was not consistently higher at downstream sites (Blazer *et al.,* 2014; Iwanowicz *et al.,* 2016). In this study, the severity of TO occurrence did not show significant differences between any of the neighboring sampling sites or

between the most upstream and most downstream sites, unlike other studies who recorded tendencies for the TOs severity to increase at downstream sites (Blazer *et al.,* 2014). Our findings suggest that the stretch of St. Joseph River passing through Indiana harbors smallmouth bass populations with adverse effects in their gonadal development. The high TO prevalence levels observed came in agreement with the findings of an earlier study reporting organic contaminant levels in water samples collected from St. Joseph River at Niles, MI (~ 14 mile downstream our sampling sites) that made them conclude that this watershed has medium to high intersex induction potential (Baldwin *et al.,* 2016).

Season was an important driver for the observed differences in the prevalence and severity of TOs. Significantly higher prevalence and lower severity cases of TOs were found during the spawning season (spring). A previous study with smallmouth bass also reported a higher prevalence of TOs at spawning, but severity was also higher (Blazer *et al.,* 2007). It is important to mention that the latter study included samples with no TOs (as zeros) in the estimation of severity, whereas we only included males with TOs in the severity estimates. Our findings suggest that reproductive condition influences the prevalence and severity of TO occurrence. A possible explanation for this, is that during spawning, gonads are highly sensitive to hormonal cues. During this period of gonadal recrudescence, the population of undifferentiated spermatogonial stem cells which will give rise to spermatozoa, is high (Okutsu *et al.,* 2006). Exposure to exogenous steroids or other EDCs during this period, might induce testicular germ cell differentiation into oocytes (Okutsu *et al.,* 2006). However, the signaling mechanisms and molecular pathways behind the development, continuance, and regression of these TOs remains unknown. On the contrary, after spawning, fish undergo a period of gonadal regression with limited sensitivity to steroid exposure, to prepare for germ cell proliferation and gonadal growth in the next spawning season (Liney *et al.,* 2005).

2.5.2 Molecular biomarkers of intersex

We quantified changes in the expression levels of several target genes in males with and without TOs to identify potential molecular biomarkers for this condition. Among the different genes tested, *vtg* was the only promising candidate. Only a few

studies have explored the relationship between TO development and changes in *vtg* transcript or protein plasma levels (van Aerle *et al.*, 2001; Kidd *et al.*, 2007; Amberg *et al.*, 2010; Bahamonde *et al.*, 2014; Bizarro *et al.*, 2014). In agreement with our findings, Kidd *et al.* (2007) reported positive correlations between increases in hepatic *vtg* expression and plasma protein levels and presence of TOs following exposure of fathead minnows (*Pimephales promelas*) to a synthetic estrogen in a whole lake experiment. Others, however, have failed to establish such correlations among wild fish populations (van Aerle *et al.*, 2001; Amberg *et al.*, 2010; Bahamonde *et al.*, 2014; Bizarro *et al.*, 2014). For instance, Van Aerle *et al.* (2001) found no relationship between presence of TOs and plasma VTG levels in gudgeon (*Gobio gobio*). A possible explanation for the failure of many studies in establishing significant relationships between TOs and *vtg*/VTG is the seasonal variability observed in the sensitivity of this biomarker. In the present study, males with TOs also had increased hepatic *vtg* levels, but only when sampled during the spawning season. Sensitivity of males to xenoestrogens is likely enhanced during this period of gonadal recrudescence, resulting in increases in *vtg* synthesis (Liney *et al.*, 2005). Another possible explanation to this lack of correspondence between cellular changes and *vtg*/VTG levels is that molecular endpoints such as *vtg* would respond swiftly to the changes in contaminant concentration by up or down regulating their expression levels, whereas changes at the cellular and organ levels of biological organization as TOs might take longer time to develop or regress (Hemmer *et al.*, 2002; Abdel-moneim *et al.*, 2015a; Blanchfield *et al.*, 2015). The severity of TO occurrence among males sampled herein yielded non-significant relationships with *vtg* expression levels in both seasons tested. Earlier studies who have investigated possible relationships between the severity of TO occurrence and *vtg* expression levels reported weak but significant correlations in bass (Iwanowicz *et al.*, 2016). However, the relationship reported between VTG levels and intersex severity was positive among smallmouth bass (n = 118) and negative among largemouth bass (n = 291) (Iwanowicz *et al.*, 2016), which provides limited explanation to the significance of this relationship.

VTG plasma protein levels showed patterns similar to changes in hepatic *vtg* transcript levels. Other studies have also reported significant positive correlations between hepatic *vtg* and plasma VTG levels (Kidd *et al.*, 2007; Puy-Azurmendi *et al.*,

2013). However, hepatic *vtg* levels was a more sensitive marker for detecting TOs (based on histology results) compared to plasma VTG since Western blotting was only able to detect VTG in two males with TOs, whereas an up-regulation in *vtg* transcript levels was detected in all males positive to TOs collected in spring (n = 6). Only one out of four male samples negative to TOs and collected in spring, had a detectable up-regulation in *vtg* transcript levels. This observation might be explained by the false negative ratio expected to be encountered during the histological examination of gonadal samples using only five histological sections (Blazer *et al.,* 2007).

2.5.3 Contaminant levels in St. Joseph River and its tributaries in northern Indiana

The presence of TOs among male gonochoristic fishes has been used as an indicator of estrogenic exposures (Iwanowicz *et al.,* 2016). However, we detected no sex steroids at any of the deployment sites using POCIS. Other studies who have witnessed similar prevalence levels of TOs have reported estrogenic concentrations above the probable no effects concentration of 0.73 ng/L (Iwanowicz *et al.,* 2016). However, it is important to note that a different detection method was adopted in the latter study, namely bioluminescent yeast reporter assay, which allows for detection of overall estrogenic bioactivity in samples tested. This method can detect estrogenic bioactivity originating from contaminants not targeted in a conventional chemical analysis or from other contaminants whose estrogenic activity has not been identified yet.

In the chemical analysis performed herein, pesticides were the common contaminant detected and at the highest levels. Studies have established significant positive correlations between the prevalence and severity of TOs occurrence and land use metrics (Blazer *et al.*, 2007, 2012). A number of studies have noted stronger correlations between prevalence and severity of TOs and intensity of agricultural practices, more so than contribution by wastewater treatments plants (Blazer *et al.,* 2007, 2014; Iwanowicz *et al.,* 2009; Ciparis *et al.*, 2012). Others reported associations between the prevalence and severity of TOs in other species and urban-land use (Tanna *et al.,* 2013). Atrazine, was the most prominent agricultural contaminant detected in our POCIS samplers. This widely used herbicide was also detected at high levels (80 ng/L - 330 ng/L) in grab samples collected from the St. Joseph River, especially during summer months (Baldwin

et al., 2016). One study reported significant positive correlations between the prevalence of TOs in smallmouth bass and atrazine levels (Kolpin *et al.,* 2013). Atrazine is a well-studied endocrine disruptor that induces a wide array of adverse effects on the reproduction of aquatic organisms (Van der Kraak *et al.,* 2014; Richter *et al.,* 2016; Wirbisky *et al.,* 2016), however, a weak body of evidence suggests any role atrazine might have in inducing TO development in fish (Van der Kraak *et al.,* 2014). Simazine and metolachlor were next in the order of detected levels of herbicides in both sampling years. The order of herbicide levels detected came in agreement with the application rates of these herbicides in Indiana during our sampling years, atrazine (>64.23 lbs/mile^2), simazine (>3.93 lbs/mile^2), and metolachlor (0-24 lbs/mile^2), according to United States Geological Survey (USGS) National Water-Quality Assessment (NAWQA) program (http://water.usgs.gov/nawqa). Simazine and metolachlor are both contaminants that have shown endocrine disrupting effects (Tran *et al.,* 1996; Lemaire *et al.,* 2006; Fan *et al.,* 2007), but no information is available regarding their gonadal intersex induction potential in fish.

Perfluorinated compounds assessed in this study, PFOS and PFOA, have shown higher levels at urban deployment sites over rural sites in both sampling years. These urban sites receive sewage treatment plant effluent and inputs from sewer overflows in addition to inputs from agriculture and animal feeding activities. Perfluorinated compounds, reported at higher rates in these sites, are widely used for many industrial purposes and consumer-related applications. Several members of this group (including PFOS and PFOA) have shown endocrine disrupting potential (Liu *et al.,* 2007; Wei *et al.,* 2007; Du *et al.,* 2009). Similarly, triclosan, a synthetic broad-spectrum antibacterial agent, was quantified at higher levels in urban deployment sites. Triclosan, PFOS and PFOA have all shown to possess estrogenic activity (Henry and Fair, 2013), suggesting that each of these compounds could provide a source of xenoestrogens to fish inhabiting aquatic environments.

2.5.4 Conclusions

In conclusion, the present study demonstrates impacts on the reproductive development of a sentinel freshwater fish species, smallmouth bass, inhabiting the St.

Joseph River and its tributaries in Indiana and identifies contaminates with possible links to the observed biological changes. We report for the first time, cases of TO in smallmouth bass from this watershed. We also quantified changes in gene expression of several targets in males with TOs in order to identify potential molecular biomarkers for this condition. Hepatic *vtg* was positively correlated with the prevalence of TO, but only during the spawning season. Our comparison of *vtg* transcript levels in the livers of intersex males to its plasma protein levels yielded positive correlation. Higher sensitivity of *vtg* transcript levels in detecting prevalence levels of TOs was observed. We quantified pesticide and organic contamination levels in this river and its tributaries through passive and found high levels of pesticide contamination in this watershed. No detectable steroid levels were recorded, but levels other contaminates with endocrine disrupting potential were found, providing possible links to the observed prevalence levels of TOs. We believe that studies adopting these approaches may help elucidate exposure-effect relationships in natural ecosystems. Our findings support the claimed existence of multiple contaminants with endocrine disrupting potential in this watershed, many of whom possess estrogenic activity and can be linked solely or in mixture to the observed prevalence and severity levels of TOs in male smallmouth bass inhabiting this watershed.

Acknowledgements: We wish to thank Sam Guffey, Cecon Mahapatra, Gary Hoover and Shuai Chen for helping in several aspects of this project. This work was partially funded by the Department of Forestry and Natural Resources at Purdue University and the Cultural Affairs and Mission Sector at the Egyptian Ministry of Higher Education as a form of an assistantship for A.A.M.

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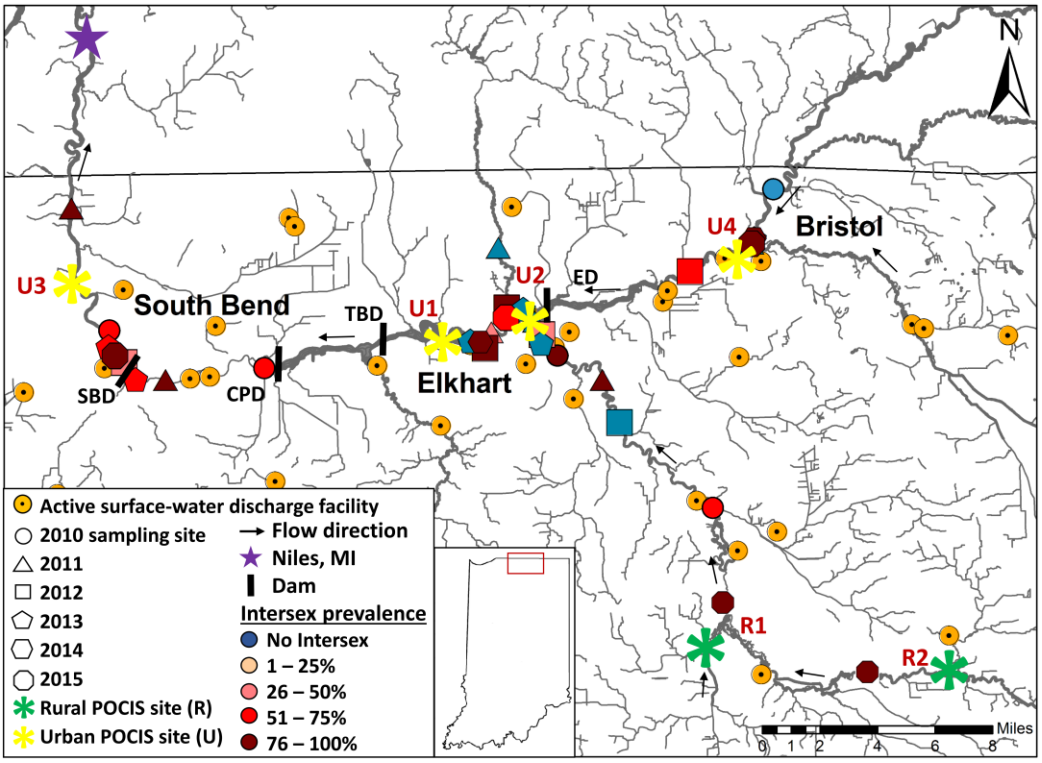


Figure 2.1: Map of northern Indiana showing smallmouth bass sampling sites (2010-2015), POCIS deployment sites, active surface-water discharge facilities and dams along St. Joseph River and its tributaries, and the prevalence of gonadal intersex (testicular oocytes, TOs) recorded over years. The dams along St. Joseph River are Elkhart Dam (ED), Twin Branch Dam (TBD), Central Park Dam (CPD) and South Bend Dam (SBD).

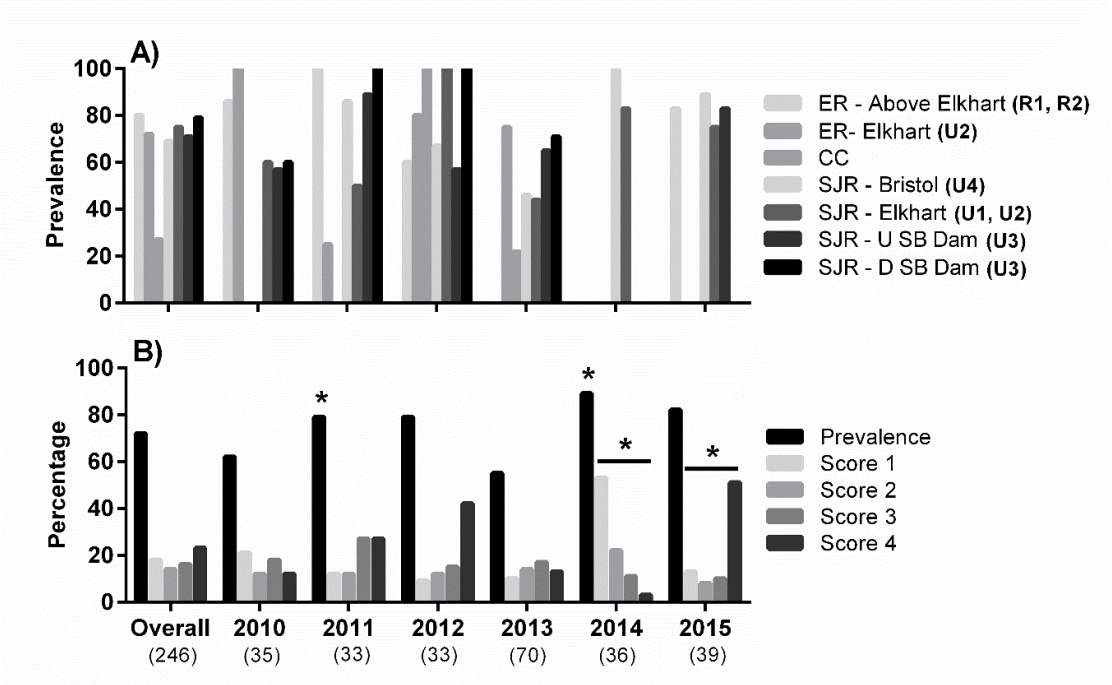


Figure 2.2: A) Prevalence of testicular oocytes (TOs) in male smallmouth bass sampled from different sites along the St. Joseph River watershed, St. Joseph River (SJR), Elkhart River (ER) and Christiana Creek (CC), between 2010 and 2015 (n, number of males sampled). In legend, symbols between parentheses refer to the closest POCIS deployment site, Urban (U1, U2, U3, and U4) and Rural (R1, R2). B) Changes in prevalence and severity of TOs over time. One oocyte per view (score 1), less than 5 oocytes per view (score 2), less than 5 oocytes per view with associations in between (score 3) and five or more oocytes per view with associations in between (score 4). Significant differences between years are denoted by asterisks (*).

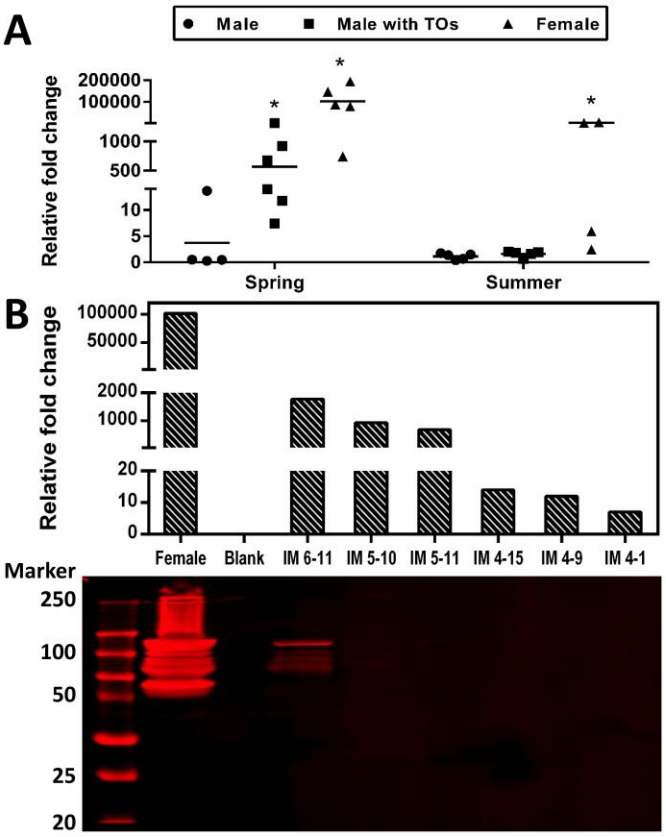


Figure 2.3: A) Scatter plot showing relative fold change in vitellogenin messenger RNA (*vtg*) expression in livers of smallmouth bass males (with and without testicular oocytes, TOs) and spawning females (at least 4 fish examined per condition). B) Relative hepatic *vtg* expression in smallmouth bass males with TOs and C) its corresponding levels of plasma VTG. The fold-change of transcript expression is relative to the expression level of the housekeeping gene (*β-actin*). Plasma samples from spawning females (mean gonadosomatic index= 4.2%) are shown for comparison. Significant differences in hepatic *vtg* expression from males without TOs are denoted by asterisks (*).

Table 2.1: Prevalence and severity of testicular oocytes (TOs) in male smallmouth bass sampled from the St. Joseph River and its tributaries, by sampling date and stream. Data on condition factor (K) and gonadosomatic index (GSI) are also presented.

	Stream	Sampling Site	Sampling Date(s)	Season	GSI (%)	Fulton's Condition Factor	Intersex (n) Prevalence	Severity*	Coordinates
2010	Elkhart River	Above Elkhart	5/28-6/7	Spring		1.2 (0.06)	100% (4)	1 (1), 3 (2), 4 (1)	85°50'34.317"W 41°35'33.013"N
			6/30-8/16	Summer		1.2 (0.09)	67% (3)	0 (1), 1 (1), 4 (1)	
		Elkhart	6/8	Spring		1.1 (0.00)	100% (2)	2 (1), 3 (1)	85°56'45.099"W 41°40'13.076"N
			7/1-7/16	Summer		1.1 (0.13)	100% (3)	1 (2), 3 (1)	
	St. Joseph River	Bristol	6/11	Spring		1.3 (0.00)	0% (1)	0 (1)	85°47'57.203"W 41°45'10.286"N
			6/29-7/9	Summer		1.3 (0.00)	0% (4)	0 (4)	
		Elkhart	6/10	Spring		1.2 (0.02)	100% (2)	1 (1), 2(1)	85°58'49.822"W 41°41'25.084"N
			7/21	Summer		1.2 (0.02)	33% (3)	0 (2), 1 (1)	
		Upstream South Bend Dam	6/16	Spring		1.3 (0.07)	50% (2)	0 (1), 2 (1)	86°8'34.351"W 41°39'56.538"N
			7/23-7/27	Summer		1.4 (0.07)	60% (6)	0 (2), 3 (2), 4 (2)	
		Downstream South Bend Dam	6/24-8/2	Summer		1.2 (0.05)	60% (5)	0 (2), 1 (1), 2 (1), 4 (1)	86°14'48.421"W 41°41'7.148"N
2011	Christiana Creek	Elkhart	7/27	Summer		1.2	0% (1)	0 (1)	85°59'6.301"W 41°43'32.132"N

	Stream	Sampling Site	Sampling Date(s)	Season	GSI (%)	Fulton's Condition Factor	Intersex (n) Prevalence	Severity*	Coordinates
	Elkhart River	Above Elkhart	6/10	Spring		1.1	100% (1)	3 (1)	85°54'58.817"W 41°39'29.664"N
			7/15	Summer		1.1	100% (1)	1 (1)	
		Elkhart	6/13	Spring		1.2	100% (1)	2 (1)	85°57'40.514"W 41°41'17.001"N
			7/18-7/19	Summer		1.1 (0.03)	0% (3)	0 (3)	
	St. Joseph River	Bristol	5/27-6/6	Spring		1.3 (0.01)	100% (6)	1 (1), 2(1), 3 (3), 4 (1)	85°48'52.527"W 41°43'33.894"N
			7/28	Summer		1.2	0% (1)	0 (1)	
		Elkhart	7/14	Summer		1.1 (0.17)	50% (2)	0 (1), 4 (1)	85°59'25.875"W 41°40'58.635"N
		Upstream South Bend Dam	6/15	Spring		1.3 (0.01)	100% (2)	1 (1), 3 (1)	86°12'35.69"W 41°39'38.16"N
			7/21-8/12	Summer		1.2 (0.09)	86% (7)	0 (1), 3 (2), 4 (4)	
		Downstream South Bend Dam	6/16-6/21	Spring		1.3 (0.08)	100% (4)	2 (1), 3 (1), 4 (2)	86°16'19.432"W 41°44'51.416"N
			7/8-7/31	Summer	0.3 (0.03, 9)	1.3 (0.03)	67% (12)	0 (4), 1 (2), 2 (1), 3 (3), 4 (2)	
2012	Christiana Creek	Elkhart	5/31	Spring		1.1	100% (1)	4 (1)	85°58'47.494"W 41°41'42.656"N
	Elkhart River	Above Elkhart	5/25	Spring		1.3 (0.04)	100% (3)	1 (1), 3 (1), 4 (1)	85°54'18.229"W 41°38'11.995"N
			7/11	Summer		1.3 (0.01)	0% (2)	0 (2)	

	Stream	Sampling Site	Sampling Date(s)	Season	GSI (%)	Fulton's Condition Factor	Intersex (n) Prevalence	Severity*	Coordinates
		Elkhart	5/30	Spring		1.3 (0.02)	100% (3)	3 (1), 4 (2)	85°57'23.523"W 41°40'53.796"N
			7/10	Summer		1.4 (0.03)	50% (2)	0 (1), 4 (1)	
	St. Joseph River	Bristol	6/22-7/30	Summer		1.2 (0.07)	67% (3)	0 (1), 2 (1), 4 (1)	85°51'23.069"W
		Elkhart	6/1-6/11	Spring		1.2 (0.06)	100% (7)	1 (2), 2 (1), 3	85°59'41.235"W
			7/16-7/20	Summer		1.1 (0.05)	100% (2)	2 (1), 3 (1)	
		Upstream South Bend Dam	6/4-6/13	Spring		1.2 (0.03)	100% (5)	2 (1), 4 (4)	86°14'14.841"W 41°40'10.744"N
			7/2-7/13	Summer		1.2 (0.02)	40% (5)	0 (3), 4 (2)	
2013	Christiana Creek	Elkhart	6/4	Spring		1.1 (0.05)	100% (2)	2 (2)	85°58'8.191"W
			7/10-7/26	Summer		1.1 (0.06)	0% (7)	0 (7)	
	Elkhart River	Elkhart	5/30	Spring		1.2 (0.03)	100% (3)	2 (1), 3 (1), 4 (1)	85°57'25.518"W 41°40'37.516"N
			7/3	Summer		1.4	0% (1)	0 (1)	
	St. Joseph River	Bristol	6/10	Spring		1.2	100% (1)	4(1)	85°48'47"W 41°43'43"N
			7/8-8/5	Summer	0.3	1.3 (0.05)	41.7%	0 (7), 1 (1), 2	
		Elkhart	6/7-6/13	Spring		1.1 (0.02)	80% (5)	0 (1), 1 (1), 2 (1), 4 (2)	86°0'16.796"W 41°40'43.506"N
			7/19-7/30	Summer		1.1 (0.06)	0% (4)	0 (4)	
		Upstream South Bend Dam	6/11	Spring		1.2 (0.01)	67% (3)	0 (1), 1 (1), 2 (1)	86°13'48.041"W
			7/8-7/16	Summer	0.3 (0.03,	1.2 (0.05)	60% (15)	0 (6), 1 (2), 2 (1), 3 (5), 4 (1)	

	Stream	Sampling Site	Sampling Date(s)	Season	GSI (%)	Fulton's Condition Factor	Intersex (n) Prevalence	Severity*	Coordinates
		Downstream South Bend Dam	6/12-6/17	Spring		1.2 (0.08)	80% (5)	0 (1), 2 (2), 3 (1), 4 (1)	86°14'53"W 41°40'40"N
			7/8-7/31	Summer	0.3 (0.03, 9)	1.3 (0.03)	67% (12)	0 (4), 1 (2), 2 (1), 3 (3), 4 (2)	
2014	St. Joseph River	Bristol	5/9	Spring	0.8	1.2 (0.02)	100%	1 (8), 2 (3), 4 (1)	85°48'47"W
		Elkhart	5/9	Spring	0.8 (0.07,	1.2 (0.01)	83.3% (24)	0 (4), 1 (11), 2 (5), 3 (4)	85°59'52"W 41°40'41"N
2015	Elkhart River	Elkhart Above	8/11-8/12	Summer		1.3 (0.04)	83.3% (6)	0 (1), 3 (1), 4 (4)	85°49'58.745"W 41°32'23.975"N
	St. Joseph River	Bristol	8/20	Summer	(0.04, 9)	1.3 (0.06)	88.9% (9)	0 (1), 2 (1), 3 (2), 4 (5)	85°48'52.527"W 41°43'33.894"N
		Elkhart	8/20	Summer	(0.01, 12)	1.2 (0.03)	75% (12)	0 (3), 1 (1), 2 (1), 4 (7)	85°58'49.822"W 41°41'25.084"N
		Upstream South Bend Dam	8/20	Summer	0.2 (0.02, 12)	1.2 (0.02)	83.3% (12)	0 (2), 1 (4), 2 (1), 3 (1), 4 (4)	86°14'37"W 41°40'23"N
		Downstream South Bend Dam	6/12-6/17	Spring		1.2 (0.08)	80% (5)	0 (1), 2 (2), 3 (1), 4 (1)	86°14'53"W 41°40'40"N

* Severity index (0–4) (Blazer *et al.* 2007).

Table 2.2: Upstream land use characteristics for the POCIS deployment sites in 2014 and 2015, and the chemical masses captured and extracted from the POCIS deployed in these sites.

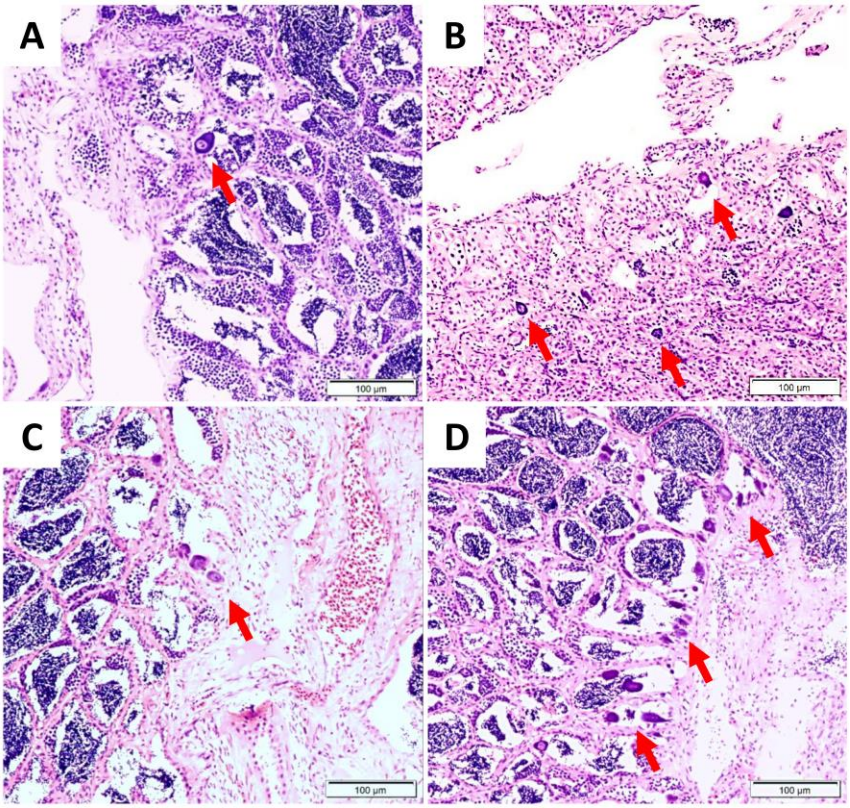
	2014					2015				
Land use	**RURAL 1**	**RURAL 2**	**URBAN 1**	**URBAN 2**	**URBAN 3**	**RURAL 1**	**RURAL 2**	**URBAN 2**	**URBAN 3**	**URBAN 4**
Urban (%)	9.8	8.6	11	14.4	13.4	9.8	8.6	14.4	13.4	9
Forest (%)	7.1	10.5	10	7.8	6.9	7.1	10.5	7.8	6.9	10.9
Agriculture (%)	75	65.5	62.2	66.2	60.8	75	65.5	66.2	60.8	61.1
Other (%)	8.1	15.4	16.8	11.6	18.9	8.1	15.4	11.6	18.9	19
Area (SqKm)	593.3	799.4	8792.7	1863.1	9463.7	593.3	799.4	1863.1	9463.7	5967.9
Compounds (ng/POCIS)	**RURAL 1**	**RURAL 2**	**URBAN 1**	**URBAN 2**	**URBAN 3**	**RURAL 1**	**RURAL 2**	**URBAN 2**	**URBAN 3**	**URBAN 4**
17B-estradiol	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
17A-estradiol	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
estrone	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Ethinyl estradiol	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Ibuprofen	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Trenbolone	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFOS	3.5	1.9	7.6	3.5	8.7	12.6	10.4	48.7	35.5	55.3
Triclosan	14.6	<LOQ	7.2	43.7	15.4	11.9	12.3	18.2	21.4	20.7
Triclocarban	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
PFOA	4.6	2.1	7.8	2.1	4.7	9.7	5.4	20.7	10.2	14.7
BPA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOQ	<LOD
BPS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
BPAF	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOD	<LOD
Progesterone	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Androstenedione	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

Compounds (ng/POCIS)	RURAL 1	RURAL 2	URBAN 1	URBAN 2	URBAN 3	RURAL 1	RURAL 2	URBAN 2	URBAN 3	URBAN 4
2,2'-dihydroxy-4-methoxy-benzophenone	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
2,4-dihydroxy-benzophenone	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOD	<LOD
methyl paraben	3.5	<LOQ	1.2	<LOQ	1.3	29.9	21.6	25.7	20.1	11.5
ethyl paraben	<LOD	<LOD	<LOD	<LOD	<LOD	1.0	3.8	<LOQ	<LOQ	1.0
propyl paraben	<LOQ	0.4	<LOQ	0.5	1.1	1.8	12.1	8.0	7.9	6.6
benzyl paraben	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	29.1	58.8	9.4	20.4
TBBPA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
4-N-nonylphenol	<LOD	<LOD	<LOQ	<LOD	<LOD	0.5	0.6	<LOD	<LOD	<LOD
Pesticides										
alachlor	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD
ametryn	1.8	4.2	2.4	1.5	2.3	6.3	8.6	12.9	6.2	7.0
atrazine	69.2	111.6	44.1	28.7	38.8	1283.5	1518.1	1684.3	1101.6	1226.3
atrazine-2-hydroxy	<LOD	<LOD	<LOD	<LOD	<LOD	231.2	219.4	287.6	180.5	189.6
atrazine-desethyl	7.6	17.3	14.6	5.5	6.7	379.1	383.9	509.4	286.3	279.8
azoxystrobin	9.5	2.8	2.1	2.1	2.6	69.3	7.1	35.2	8.2	3.8
bromacil	5.8	0.0	1.3	2.0	2.7	6.5	1.2	4.7	2.8	0.3
butachlor	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
DEET	26.0	24.2	29.5	10.0	24.0	599.6	76.6	241.7	109.5	237.1
diuron	10.1	16.6	3.0	6.2	3.2	36.1	68.7	74.6	17.5	6.0
hexazinone	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
imazapyr	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

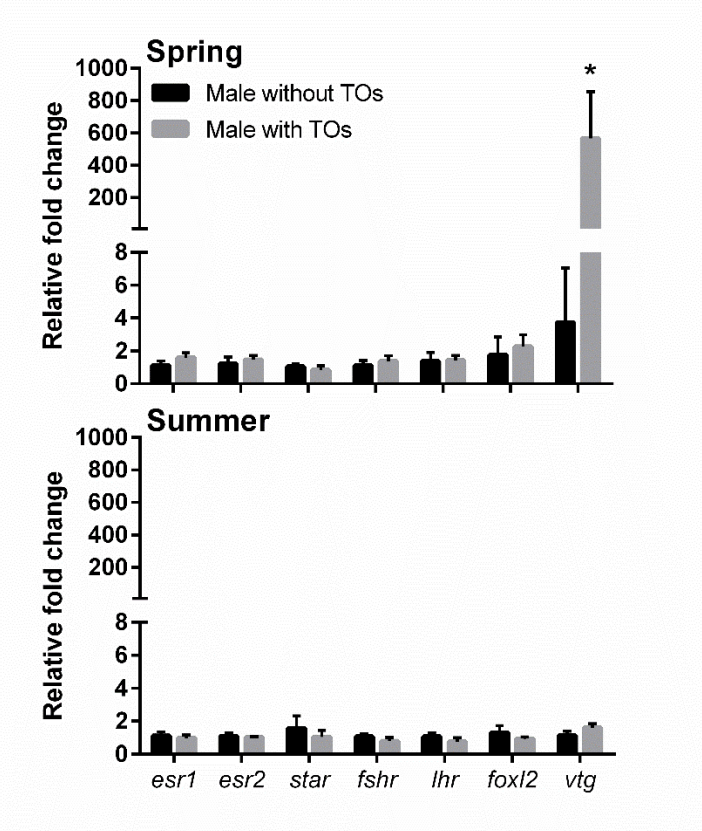
Compounds (ng/POCIS)	RURAL 1	RURAL 2	URBAN 1	URBAN 2	URBAN 3	RURAL 1	RURAL 2	URBAN 2	URBAN 3	URBAN 4
metolachlor	8.9	43.9	18.1	13.2	13.9	857.4	685.1	936.2	460.0	385.3
Monuron	4.8	15.3	1.8	3.1	2.0	29.5	53.3	54.5	15.4	3.9
Prometon	41.3	10.1	12.8	4.9	12.2	644.9	48.0	373.2	64.0	24.0
Prometryn	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Terbutryn	<LOQ	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD
Propazine	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	21.5	25.6	33.7	20.6	18.8
propiconazole	4.6	1.1	0.4	0.3	0.8	71.6	9.1	32.6	8.4	3.2
Simazine	3.9	9.4	18.9	7.6	13.0	74.6	107.2	438.7	153.8	152.6

Limits of detection (LOD) and quantification (LOQ) for the different analytes measured are identified in Supplementary Table 2.5.

2.7 Supplementary information



Supplementary Figure 2.1: Severity ranking system for evaluation of testicular oocytes (TOs) in testes of smallmouth bass sampled from the St. Joseph River and its tributaries. Five tissue sections were examined per sample and at least 4 fish were examined per condition. A) One oocyte per view (score 1). B) Less than 5 oocytes per view (score 2). C) Less than 5 oocytes per view with associations in between (score 3). D) Five or more oocytes per view with associations in between (score 4). Arrows point to oocytes.



Supplementary Figure 2.2: Relative mean ± standard error messenger RNA expression of testicular *esr1*, *esr2*, *star*, *fshr*, *lhr*, *foxl2* and hepatic *vtg* in smallmouth bass. The fold-change in transcript expression is relative to the expression level of the housekeeping gene (*β-actin*). Significant differences in messenger RNA expression between smallmouth bass testes with or without TOs is denoted by asterisks (*).

Supplementary Table 2.1: Parameters used for the LC-MS/MS analyses.

	Method 1		**Method 2**		**Method 3**	
Column	100 × 2.1 mm, 5 µm EVO		101 × 2.1 mm, 5 µm EVO		102 × 2.1 mm, 5 µm EVO	
Mobile phase A	acetic acid 0.15% in water		ethanolamine 2 mM in water		acetic acid 0.15% in water	
Mobile phase B	Methanol		methanol		methanol	
Flow rate (mL/min)	1		0.3		0.7	
Gradient	0 min	10%	0 min	5%	0 min	20%
	0.5 min	10%	0.5 min	5%	0.2 min	20%
	1.0 min	40%	1.0 min	40%	4.0 min	100%
	8.0 min	100%	8.0 min	100%	8.5 min	100%
	14.0 min	100%	10.0 min	100%	9.0 min	20%
	14.5 min	10%	10.5 min	10%	14.0 min	20%
	17.0 min	10%	13.0 min	5%		
Column oven Temperature (°C)	40		40		40	
Injection volume (µL)	20		20		20	
Source/polarity	ESI negative		ESI negative		ESI positive	
Detection mode	MS exact mass IDA for confirmation		MS exact mass IDA for confirmation		MS-MS -	
MS scan range (Da)	30-800		30-800		-	
MS accumulation time (s)	0.2		0.2		-	
MS declustering potential	-100		-100		-	
MS collision energy	-10		-10		-	
MS-MS collision energy	rolling collision energy		15		specific to compounds	
MS ion source gas 1	30		30		30	
MS ion source gas 2	30		30		30	
MS curtain gas	25		25		25	
MS temperature (°C)	500		500		450	

Supplementary Table 2.1 (continued)

	Method 1	**Method 2**	**Method 3**
MS ionspray voltage floating (V)	-4500	-4500	5500
MS-MS accumulation time (s)	0.1	0.1	0.05
IDA parameters:			
Mass range (Da)	140-600	140-600	-
Mass tolerance (ppm)	10	10	-
Maximum number of candidates per cycle	8	8	-

Supplementary Table 2.2: Parameters of the MS and MS-MS analyses. The method number refers to Supplementary Table 2.3

Compound	Method	Retention Time (min)	MS Exact Mass (Da)	MS-MS Precursor	MS-MS Product Ions Quantification	MS-MS Product Ions Confirmation	MS-MS Collision Energies	Internal Standard
17B-estradiol	2	5.81	271.170 ± 0.030	-	-	-	-	17B-estradiol labeled
17A-estradiol	2	5.90	271.170 ± 0.030	-	-	-	-	17B-estradiol labeled
estrone	2	5.76	269.155 ± 0.025	-	-	-	-	17B-estradiol labeled
ethinyl estradiol	2	5.87	295.170 ± 0.030	-	-	-	-	17B-estradiol labeled
ibuprofen	1	4.42	205.125 ± 0.025	-	-	-	-	ibuprofen labeled
simvastatin	2	7.85	417.270 ± 0.030	-	-	-	-	triclosan labeled
trenbolone	2	5.46	269.160 ± 0.020	-	-	-	-	17B-estradiol labeled
PFOS	2	4.24	498.935 ± 0.025	-	-	-	-	butyl paraben labeled
triclosan	1	5.09	286.955 ± 0.025	-	-	-	-	triclosan labeled
triclocarban	1	5.15	312.970 ± 0.030	-	-	-	-	triclocarban labeled
PFOA	2	3.83	412.970 ± 0.030	-	-	-	-	butyl paraben labeled
BPA	1	2.94	227.115 ± 0.025	-	-	-	-	BPA labeled
BPS	1	1.77	249.030 ± 0.030	-	-	-	-	BPA labeled
BPAF	1	3.91	335.055 ± 0.025	-	-	-	-	BPA labeled
progesterone	2	6.80	313.225 ± 0.025	-	-	-	-	17B-estradiol labeled
androstenedione	2	5.72	285.190 ± 0.020	-	-	-	-	17B-estradiol labeled
2,2'-dihydroxy-4-methoxybenzophenone	2	5.09	243.065 ± 0.025	-	-	-	-	triclosan labeled
2,4-dihydroxy-benzophenone	1	3.08	213.065 ± 0.025	-	-	-	-	triclosan labeled
methyl paraben	1	1.83	151.045 ± 0.015	-	-	-	-	methyl paraben labeled
ethyl paraben	1	2.22	165.020 ± 0.020	-	-	-	-	ethyl paraben labeled
propyl paraben	1	2.76	179.080 ± 0.020	-	-	-	-	propyl paraben labeled
benzyl paraben	1	3.46	227.075 ± 0.025	-	-	-	-	butyl paraben labeled
TBBPA	1	5.29	540.755 ± 0.025	-	-	-	-	triclosan labeled
4-N-Nonylphenol	2	8.44	219.180 ± 0.020	-	-	-	-	butyl paraben labeled
Pesticides								
alachlor	3	3.44	-	270.13	162.125 ± 0.015	147.105 ± 0.015	35	-
ametryn	3	2.83	-	228.13	186.085 ± 0.025	96.055 ± 0.025	35	-
atrazine	3	2.79	-	216.10	174.050 ± 0.020	104.005 ± 0.025	35	-
atrazine-2-hydroxy	3	0.42	-	198.13	86.040 ± 0.010	69.010 ± 0.010	35	-

Supplementary Table 2.2 (continued)

Compound	Method	Retention Time (min)	MS Exact Mass (Da)	MS-MS Precursor	MS-MS Product Ions Quantification	MS-MS Product Ions Confirmation	MS-MS Collision Energies	Internal Standard
atrazine-desethyl	3	1.71	-	188.07	146.025 ± 0.025	104.005 ± 0.025	35	-
azoxystrobin	3	3.20	-	404.12	372.095 ± 0.025	344.100 ± 0.030	35	-
bromacil	3	2.38	-	261.02	204.955 ± 0.025	187.930 ± 0.030	35	-
butachlor	3	4.06	-	312.17	162.125 ± 0.025	238.095 ± 0.025	35	-
DEET	3	2.83	-	192.14	91.055 ± 0.025	119.055 ± 0.025	35	-
Diuron	3	2.88	-	233.02	72.055 ± 0.025	46.065 ± 0.025	35	-
hexazinone	3	2.38	-	253.17	171.080 ± 0.030	71.065 ± 0.025	35	-
imazapyr	3	1.48	-	262.12	149.030 ± 0.030	217.090 ± 0.030	35	-
metolachlor	3	3.48	-	284.14	176.145 ± 0.015	252.115 ± 0.015	35	-
monuron	3	2.27	-	199.06	72.045 ± 0.025	46.065 ± 0.025	35	-
prometon	3	2.50	-	226.17	142.075 ± 0.025	86.035 ± 0.025	35	-
prometryn	3	3.16	-	242.14	158.045 ± 0.025	200.095 ± 0.025	25	-
terbutryn	3	3.21	-	242.14	186.085 ± 0.025	138.075 ± 0.025	35	-
propazine	3	3.11	-	230.12	146.025 ± 0.025	188.075 ± 0.035	35	-
propiconazole	3	3.63	-	342.08	158.975 ± 0.025	69.075 ± 0.025	35	-
simazine	3	2.39	-	202.08	104.005 ± 0.025	68.075 ± 0.025	35	-

Supplementary Table 2.3: Limits of detection (LOD) and quantification (LOQ), recoveries and blank concentrations for the different analytes measured from POCIS. LODs were calculated from the lowest standard detected and were equal to the concentrations giving a signal to noise ratio of 3.

Compounds	LOD	LOQ	Lab Spike Recovery	Field Blank POCIS 2014	Field Blank POCIS 2015
	ng/mL Injecte	ng/mL Injected	%	ng/mL Injected	ng/mL Injected
17B-estradiol	0.38	7.32	75%	<LOD	<LOQ
17A-estradiol	0.48	6.57	96%	<LOD	<LOD
estrone	0.14	1.03	89%	<LOD	<LOQ
ethinyl estradiol	0.12	0.93	87%	<LOD	<LOD
ibuprofen	0.68	5.17	57%	<LOD	<LOQ
trenbolone	0.96	6.70	95%	<LOD	<LOD
PFOS	0.01	1.47	89%	2.39	2.71
triclosan	0.02	17.16	119%	97.74	<LOQ
triclocarban	0.05	1.08	87%	<LOD	<LOD
PFOA	0.01	7.00	83%	<LOQ	8.42
BPA	0.05	55.80	94%	<LOD	<LOD
BPS	0.06	5.97	74%	<LOQ	<LOQ
BPAF	0.06	12.28	83%	<LOD	<LOQ
progesterone	43.11	411.33	191%	<LOD	<LOD
androstenedione	26.48	55.59	173%	<LOD	<LOD
2,2’-dihydroxy-4-methoxybenzophenone	0.03	6.67	86%	<LOD	<LOQ
2,4-dihydroxy-benzophenone	0.06	6.95	78%	<LOD	<LOD
methyl paraben	0.73	5.57	90%	17.14	11.32
ethyl paraben	0.10	5.96	96%	<LOQ	<LOQ
propyl paraben	0.01	0.94	96%	4.83	1.41
benzyl paraben	0.04	1.36	82%	<LOD	<LOD

Supplementary Table 2.3 (continued)

Compounds	LOD	LOQ	Lab Spike Recovery	Field Blank POCIS 2014	Field Blank POCIS 2015
	ng/mL Injecte	ng/mL Injected	%	ng/mL Injected	ng/mL Injected
TBBPA	0.01	74.88	71%	<LOD	<LOD
4-N-Nonylphenol	0.08	2.38	85%	<LOQ	<LOQ
Pesticides					
alachlor	0.23	4.17	100%	<LOD	<LOD
ametryn	0.07	5.91	103%	<LOD	<LOD
atrazine	0.04	10.34	95%	<LOD	<LOD
atrazine-2-hydroxy	0.02	7.65	117%	<LOD	<LOQ
atrazine-desethyl	0.06	4.73	98%	<LOD	<LOQ
azoxystrobin	0.02	5.62	105%	<LOD	<LOD
bromacil	0.19	4.77	100%	<LOD	<LOD
butachlor	0.03	8.37	100%	<LOD	<LOD
DEET	0.12	5.05	103%	7.8	174.87
Diuron	0.02	4.08	95%	<LOD	<LOD
hexazinone	0.21	13.09	106%	<LOD	<LOD
imazapyr	0.18	10.90	117%	<LOD	<LOQ
metolachlor	0.02	4.86	114%	<LOQ	<LOQ
monuron	0.08	5.40	108%	<LOD	<LOQ
prometon	0.03	10.69	90%	<LOD	<LOD
prometryn	0.07	31.85	99%	<LOD	<LOD
terbutryn	0.04	12.43	95%	<LOQ	<LOQ
propazine	0.10	25.36	96%	<LOD	<LOD
propiconazole	0.03	5.20	93%	9.4	<LOQ
simazine	0.05	11.76	94%	<LOD	<LOD

Supplementary Table 2.4: Primers used in real-time quantitative polymerase chain (qPCR) reactions with their product size, melting temperature (Tm), primer efficiency, reference and Genbank accession numbers.

Target gene	Primer sequence	Tm (°C)	Product Size (bp)	Primer Efficiency (%)	Reference	Sequence Accession no.
B-actin	5'-ACATCAAGGAGAAGCTGTGC-3' 5'-AGGATTCCATACCGAGGAAG-3'	55.3 53.6	183	94	Bangs *et al.* (unpublished)	KJ669301.1
esr1	5'-AAAATCATCAGTGACCGGAA-3' 5'-CATTATGGTGACCTCGGTGT-3'	51.9 54.5	121	100	Iwanowicz *et al.* (unpublished)	GU966636.1
esr2	5'-CCGACACCGCCGTGGTGGACTC-3' 5'-AGCGGGGCAAGGGGAGCCTCAA-3'	66.6 68	117	105	Developed in this study	KX253968
star	5'-TGTTGTCAGAGCGGAGAATG-3' 5'-AAAGTCCACCTGCGTCTGAG-3'	54.9 57.2	160	101	Jeffrey *et al.* 2014	
lhr	5'-CCTTCGTAGTTGTGTGCGTT-3' 5'-GGGAACCTTAAAAGCAGCAG-3'	55.5 54.1	179	100	Developed in this study	KX253969
fshr	5'-CAGTCCTCATCTTCACCGACTT-3' 5'-AGGCGTACAGGAAGGGGTT-3'	58.6 56.4	159	103	Developed in this study	KX253971
foxl2	5'-CAGGATAAAGTCCCGGAGAA-3' 5'-ATACCGGACAGAGTGAGACG-3'	53.7 55.9	116	100	Developed in this study	KX253970
vtg	5'-CAGAGTGAGATGGGCGTTG-3' 5'-CAGGCGTTTGTTGGGTGT-3'	55.8 56.3	177	99	Biales *et al.* 2007	

CHAPTER 3. OVARIAN STRUCTURE PROTEIN 1: A SENSITIVE MOLECULAR BIOMARKER OF GONADAL INTERSEX IN FEMALE JAPANESE MEDAKA AFTER ANDROGEN EXPOSURE

Reproduced from:

Abdel-moneim, A.; Mahapatra, C. T.; Azadeh Hatef; Sepúlveda, M. S., Ovarian Structure Protein 1: A Sensitive Molecular Biomarker of Gonadal Intersex in Female Japanese Medaka after Androgen Exposure. J. Env. Toxicol. Chem. 2015, 34, (9), 2087-2094.

3.1 Abstract

Intersex in gonochoristic fish can be induced after exposure to androgens and estrogens. The main objective of this study was to identify biomarkers that would be predictive of intersex in Japanese medaka (*Oryzias latipes*) after exposure to synthetic hormones. First, a gene was identified, ovarian structure protein 1 (*osp1*), with strong female-specific expression during gonadal differentiation. The authors hypothesized that *osp1* expression would decrease to male levels in females after the exposure of larvae (15-25 d post-fertilization, dpf) to 17β-trenbolone (TRB, 5 ng/L) and increased to female levels in males exposed to 17α-ethinylestradiol (EE_2, 5 ng/L) and that gonadal intersex would be induced later in life (60 dpf). Tissue distribution and cellular localization of OSP1 was investigated using Western blot and immunohistochemistry. The results indicate that this exposure regime delays testicular maturation in males and development of ovarian intersex in females. Although, decreased *osp1* expression in females exposed to TRB correlated to changes in ovarian phenotype, up-regulation of *osp1* was not observed in males exposed to EE_2. In addition, OSP1 was only observed in ovaries and localized in the cytoplasm and follicular layer of immature and mature oocytes. The authors conclude that *osp1* is a promising biomarker of androgen exposure and gonadal intersex in female medaka.

3.2 Introduction

Over the past decade, numerous studies have reported intersex in wild fish populations across the globe (Bahamonde *et al.,* 2013). This simultaneous presence of testicular and ovarian cells is rarely encountered in adult gonochoristic (fixed sex) fish (Devlin and Nagahama, 2002). In the United States, smallmouth bass (*Micropterus dolomieu*) males appear particularly susceptible to developing this condition in the form of testicular oocytes, with prevalences of > 80% in some areas (Bahamonde *et al.,* 2013). In females, nests of spermatogenic cells interspersed within ovarian tissue (Hecker *et al.,* 2002) have been reported in brown trout (*Salmo trutta*) and common carp (*Cyprinus carpio*) (Hinck *et al.,* 2007; Korner *et al.,* 2007). Laboratory and field in situ studies have shown that exposure to endocrine-disrupting chemicals (EDCs) can cause intersex, particularly if exposure occurs during the period of sex differentiation (Koger *et al.,* 2000; Kidd *et al.,* 2007). The EDCs enter the aquatic environment through various sources, including agricultural runoff and industrial and urban effluents, and exert their action by mimicking, blocking, and/or interfering with the function of endogenous hormones (Jobling and Tyler, 2003; Leet *et al.,* 2011). Two examples of EDCs that are known to disrupt reproductive development and function in fish are the synthetic androgen 17β-trenbolone (TRB) and the synthetic estrogen 17α- ethinylestradiol (EE_2) (Leet *et al.,* 2011). A metabolite of trenbolone acetate, TRB is commonly used as a growth promoter in beef, and reaches streams after crops are fertilized with animal manure (Jensen *et al.,* 2006). As the active ingredient in contraceptives, EE_2 is released into water bodies through wastewater sewage effluents (Martinez *et al.,* 2012).

The existence of biomarkers for the identification of intersex in teleosts is still in its infancy. Although vitellogenin (VTG) has been recognized as the biomarker of choice for estrogenic/antiestrogenic EDCs (Matozzo *et al.,* 2008), concentrations of this protein and/or of its messenger RNA (mRNA) for the diagnosis of intersex have not been predictive of gonadal intersex (Jobling *et al.,* 2006; Amberg *et al.,* 2010). A similar response has been observed for other genes such as the estrogen receptor (*esr*) and cytochrome P450 19 aromatase (*cyp19a*) (Zhao and Hu, 2012; Depiereux *et al.,* 2014). A recent study with Japanese medaka (*Oryzias latipes*) identified a novel gene, ovarian structure protein 1 (*osp1*), as highly expressed in both females and males exposed to EE_2,

more importantly, its expression was strongly correlated to the severity of male intersex (Zhao and Hu, 2012; Zhao *et al.*, 2014). The effects observed, however, were induced after long-term EE_2 exposures (30 - 90 d) or short-term exposures to EE_2 concentrations that were not environmentally relevant (74.8 ng/L). Effects of synthetic androgens on the expression levels of *osp1* and how changes in its expression after androgenic exposures are predictive of phenotypic changes in female gonads have yet to be identified.

Several fish models have been used to study the effects of EDCs on sex differentiation. Of all the commonly used laboratory small fish models, Japanese medaka has the most well-understood sex determination mechanism driven by the sex-determining gene double sex- and mab-3-related transcription factor-1 (*Dmrt1b/dmy*) located on the Y chromosome (Matsuda *et al.,* 2002; Wittbrodt *et al.,* 2002). Moreover, the sex of the medaka line SK2MC can be determined non-destructively very early during embryo development (5 d post-fertilization, dpf) because of the presence of leucophores along the body axis of males. In medaka, gonadal differentiation begins just before hatching (~9 dpf) and extends up to 19 dpf (Matsuda *et al.,* 2002; Iwamatsu, 2004). Short-term exposure to EDCs during this period of gonadal differentiation may cause irreversible, long-term effects on both structure and function of reproductive organs (Devlin and Nagahama, 2002).

The objectives of the present study were two-fold. First, we found a strong female-specific expression of *osp1* during the first 30 dpf of development when medaka is undergoing gonadal differentiation. Based on this data, we hypothesized that *osp1* expression would be significantly decreased in females after short-term exposure to a potent synthetic androgen (TRB) and increased in males after exposure to a potent synthetic estrogen (EE_2). Tissue distribution and cellular localization of OSP1 protein was investigated using Western blot and immunohistochemistry of gonadal tissues. Second, we investigated whether short-term exposures to environmentally relevant concentrations (~5 ng/L) of EE_2 or TRB during the period of gonadal differentiation (15-25 dpf) resulted in gonadal intersex at 60 dpf. Our results indicate that this exposure regime leads to delay in testicular maturation and development of ovarian intersex in males and females, respectively. Although decreased *osp1* expression in females exposed to TRB was correlated with changes in ovarian phenotype, no changes in the

expression of this gene were observed in males exposed to EE_2. We conclude that *osp1* is a promising molecular biomarker of androgen exposure in female medaka. Its expression levels are highly sensitive to short-term TRB exposure in a manner predictive of gonadal intersex development occurring later in life.

3.3 Material and Methods

3.3.1 Experimental fish and embryo collection

The SK2MC strain of see-through medaka were cultured at the Aquatic Ecology Laboratory (Purdue University, West Lafayette, IN, USA) with a 14:10-h light:dark photoperiod and a temperature 25 ± 1 °C. Broodstock fish were held in 35-L aquaria with recirculating water filtered through activated carbon. Water quality consisted of a dissolved oxygen level above 5 mg/L, pH 7-8, and total ammonia below detectable levels. Adult fish were fed ad libitum twice daily with a combination of hatched *Artemia* nauplii and commercial food (Tetramin). Fertilized eggs (< 24 hours post fertilization [hpf]) were collected either by brushing them off from females or from the bottom of tanks. They were immediately disinfected in a 0.005% bleach solution followed by a rinse in 0.0002% methylene blue. Embryos were then transferred to 6-well plastic plates (Corning) containing 10 mL of embryo medium consisting of a 5 g/L sodium chloride solution. The Purdue Animal Care and User Committee approved all the experimental protocols.

3.3.2 Developmental gene expression

The developmental expression of four genes (o*sp1, vtg, cyp19a* and *esr2*) was quantified during the first 30 dpf. *Osp1* is a novel gene with female-like expression pattern in Japanese medaka (Zhao and Hu, 2012); *vtg* is the precursor protein for egg yolk; *esr2* is a steroid receptor gene; and *cyp19a* or aromatase, is responsible for aromatizing androgens to estrogens in gonads. After hatching, larvae were transferred to a 150-mL glass beaker containing reverse osmosis (R/O) water with 0.00025 % Replenish (Seachem), a balanced salt mixture containing both soft (0.6-0.7% sodium, 0.12% potassium) and hard (13-14% calcium, 1.2% magnesium) chloride salts. Beakers were placed in a temperature-controlled chamber (25 ± 1 °C) with a 4:10-h light:dark

photoperiod. Larvae were collected at 5 dpf, 8 dpf, 10 dpf, 12dpf, 15 dpf, 20 dpf, 25 dpf and 30 dpf, flash-frozen in liquid nitrogen and stored at -80 °C for gene expression analyses.

Gene expression during development was quantified using real-time quantitative PCR (qPCR). Total RNA was extracted from whole larvae/juveniles (n = 4) using QIAzol (Qiagen) and further cleaned up by RNeasy MinElute Cleanup Kit (Qiagen). RNA concentrations, 260/280 and 260/230 ratios were quantified using a Nanodrop 1000 spectrophotometer and ratios were between 1.9 and 2.1, indicating the absence of contamination with proteins or reagents (Eldh *et al.*, 2012). To prevent genomic DNA contamination, all samples were digested with DNase I (Fermentas) according to the manufacturer's instructions. Total RNA (1 µg) treated with DNase was reverse transcribed to complementary DNA (cDNA) using a high–capacity reverse transcription kit (Applied Biosystems) according to manufacturer's instructions. Primer sequences (Supplementary Table 3.1) were designed and purchased from Integrated DNA. Reactions were performed on a Bio-Rad CFX96 system in a 96-well plate with 20 µL of total reaction mixture per well comprised of 10 µL of Master mix (iQ™ SYBR Green Supermix, Bio-Rad), 10 µM of forward and reverse primers, 50 ng of cDNA template, and nuclease-free water to fill the remaining volume. All reactions were performed in duplicate using the following conditions: initial template denaturation at 95 °C for 3 min; 40 cycles of 95 °C for 10 s; primers annealing at 58 °C for 30 s; product extension at 72 °C for 30 s; and final extension at 65 °C to 95 °C in increments of 0.5 °C for 5 s. We generated a melting curve for each run to confirm the specificity of the assay and used Bio-Rad CFX 2.1 software for real-time qPCR data acquisition and analysis. Four biological replicates were analyzed per gender and time point. The relative expression of each target gene (ΔCt) was calculated by normalizing to the reference gene (*β-actin)*. Subsequently, the relative expression (ΔCt) of all biological replicates were averaged and the expression of the target genes was quantified as normalized individual data points by estimating 2^ΔCt (Schmittgen and Livak, 2008).

3.3.3 OSP1 polyclonal antibody and Western blotting

Polyclonal anti-OSP1 antibodies were developed in rabbit against the 14-mer peptide (SKSHQGKGSAKGSR) located at the C- terminus of the OSP1 protein (see Supplementary Figure 3.1A) using the Custom Antibody Development Service from Genscript. The antibody was purified by affinity chromatography. To study the expression of OSP1 in gonadal tissues, Western blots were performed using ovarian and testicular tissue of adult Japanese medaka as described by Mahapatra and Rand (2012). Briefly, tissue lysates were prepared in lysis buffer on ice, centrifuged at 12,000 g for 10 min at 4 °C, and the supernatant was removed and placed in a fresh ice-cold tube. Protein concentration was determined using the Thermo Scientific BCA Protein Assay Kit. Equal concentrations of proteins were mixed with sodium dodecyl sulfate (SDS) sample buffer and denatured at 95 °C for 5 min. Samples were resolved in a 14% SDS–polyacrylamide gel electrophoresis gel and transferred onto a PVDF membrane (Bio-Rad). Membranes were blocked with Aqua Block (EastCoast Bio) for 1 h. Blots were then incubated in primary antibody, anti OSP1 (1:1000) and anti-β-ACTIN (1:1000; GenScript), overnight at 4 °C. After washing with phosphate buffered saline 0.1% Tween 20 (PBST) buffer, membranes were incubated with IRDYE 800 and IRDYE 700 secondary antibodies (1:15,000; Li-Cor) for 1 h at room temperature. Finally, membranes were washed again with PBST, followed by a quick rinse in phosphate-buffered saline (PBS) and viewed using an Odyssey infrared imager (Li-Cor).

3.3.4 Immunohistochemistry of OSP1

To study the localization of OSP1 in ovaries of adult medaka, immunostaining was performed using polyclonal anti-OSP1 antibody and a rabbit specific horseradish peroxidase/diaminobenzidine (HRP/DAB) detection IHC Kit (Abcam). Tissue sections were deparaffinized, rehydrated, incubated for 10 min in hydrogen peroxide and then treated with antigen retrieval solution (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) at 100 °C for 20 min. Slides were cooled down to room temperature, washed twice in PBST, and blocked for 1 h in protein block. They were incubated in an anti-OSP1 antiserum diluted 1:2500 with PBS containing protein block overnight at 4 °C. After 3 PBST washes, the slides were reincubated with a biotinylated goat anti-polyvalent

secondary antibody, followed by streptavidin peroxidase and chromogen dye. Sections were counterstained with hematoxylin, dehydrated, mounted, viewed and imaged at 200X using an Olympus CX41 microscope, Olympus DP70 digital camera and cellSens software ver 1.8 (all from Olympus). Negative controls were carried out by incubation without the primary antibody and always yielded negative results.

3.3.5 Test chemicals and exposure design

The TRB and EE_2 were purchased from Sigma. Stock solutions (1 mg/L) were prepared by dissolving the powdered chemicals in 1 mL methanol and then mixing them into 1 L MilliQ water. Working solutions of 5 ng/L TRB and EE_2 were prepared using sterile reverse osmosis water with 0.00025% Replenish. Solutions were prepared under aseptic conditions, stored at 4 °C, and then transferred to a temperature-controlled chamber (25 ± 1 °C) 24 h before the start of exposure to minimize larval stress. Larvae (15 dpf) were transferred to 6-well plastic plates with mesh inserts (Corning) with 1 to 2 larvae of the same gender per well. The mesh inserts have opaque sides to minimize stress and all inserts per plate are connected to a holder for efficient solution changes. Larvae were exposed to control and test solutions for 10 d, from 15 to 25 dpf. Solutions were changed three times per day to maintain a stable exposure concentration. Larvae were fed artificial rotifer (AZOO Corp.) three times a day after each water change and survival and abnormalities were monitored during each feeding. At the end of the exposure, some larvae were flash frozen in liquid nitrogen and stored at -80 °C for gene expression analyses of *osp1* and *vtg* transcription levels using qPCR as already described. The remaining larvae were reared in normal culture water to 60 dpf for histological analyses of gonads.

3.3.6 Chemical analysis

To determine the exposure concentrations of EE_2 and TRB, water samples from each exposure group were collected over a period of 24 h (3 water changes, total of 500 mL) before and after the renewal of test solutions and stored in 1 L amber glass bottles at 4 °C for up to 24 h. Samples were pooled for hormonal extraction. Sample preparation was performed as described by Gall *et al.* (2011) and chemical analyses conducted at the

Metabolite Profiling Facility, Bindley Biosciences Center, Purdue University using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). In brief, after the sample extract was reconstituted in 0.5 mL methanol, a 10-µL sample was analyzed for TRB and EE_2 using an Agilent 1200 series HPLC coupled to an Agilent 6460 triple quadrupole MS. Chromatography was performed using a XBridge Phenyl column (2.1 x 100 mm x 3.5 µm; Waters). A solvent system composed of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) was used under the following elution gradient: 0 min to 1 min, 30% B; 1 min to 5 min 100% B; 5 min to 10 min 100% B; 10 min to 12 min 30% B; 12 min to 15 min 30% B and at a column flow of 0.3 mL/min. The column effluent was directed to a Jet Stream ESI source, operating in positive ionization mode, with the nozzle and capillary voltage set at 1000 V and 3500 V respectively. Nebulizer pressure was set at 35 psi, drying gas (nitrogen) was set at 325 °C with a flow rate of 10 L/min, and the sheath gas was set at 250 °C with a flow rate of 7 L/min. The fragmentor voltage was 80 V for TRB and 60 V for EE_2. Multiple reaction monitoring (MRM) was used for selective detection. The quantitative mass transitions were 271.2 to 253.3 and 297.2 to 107.1, and the collision energies were 15 eV and 25 eV for TRB and EE_2, respectively. The retention times were 3.5 min for TRB and 6.1 min for EE_2, whereas the limits of detection were 1.7 ng/L and 3.4 ng/L, respectively.

3.3.7 Histological analyses of gonads

Juveniles (60 dpf) from control and exposed groups (TRB and EE_2; n = 9-11/group) were collected in histology cassettes and fixed in 10% neutral buffered formalin. Microtomy and standard hematoxylin and eosin staining were carried out at the Purdue Histology & Phenotyping Laboratory. Five sections covering the whole gonadal tissues of each individual fish were examined and imaged at a magnification of either 400 X or 200 X for testis and ovary sections, respectively. For each histology section, gonadal cell types were identified and manually counted. In testis, the gonadal cells of interest were spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa, whereas in ovary the cells examined were chromatin nucleolar oocytes and perinucleolar oocytes. The percentage of each gonadal cell type per section was blindly calculated and

averaged with the other sections to estimate the percentage per gonad. Anomalies and abnormally occurring cell types were also recorded.

3.3.8 Statistical analyses

All statistical analyses were performed using SPSS 21.0. First, all data were examined for normality using Shapiro-Wilk test prior to statistical analyses. Gene expression (qPCR) and percentage of gonadal cell types (histological analysis) of all groups are presented as mean ± standard error (SE). Response variables with normal distributions were analyzed using independent sample t-tests to determine differences between genders or exposed and controls. Nonnormal distributed response variables and percentages of gonadal cell types were statistically analyzed using Mann-Whitney U tests. In all cases, $p \leq 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Developmental expression of *osp1*, *vtg*, *cyp19a*, and *esr2*

To assess the developmental expression of 4 candidate genes during the sexual differentiation period, gene expression analysis was performed on males and females at various ages during the first 30 dpf. The reference gene, *β-actin*, showed constant expression at all development stages studied with a relative standard deviation (SD) value of 10.89%. Transcripts of all 4 genes were detected as early as 5 dpf regardless of gender (Figure 3.1). Gender-specific gene expression began at 12 dpf when females showed a significant increase in expression of *osp1* (~14-fold), *vtg* (~8-fold) and *cyp19a* (~10-fold), compared with males of the same age. An up-regulation in the expression of *osp1* was observed in female larvae starting at 12 dpf, which continued to 30 dpf, showing approximately 14-fold to 44-fold increase in expression, respectively, compared with males of the same age. Expression levels of *osp1* in males were extremely low (with Ct values always > 30, Figure 3.1A). In contrast, *vtg* did not show significant differential expression between genders across the developmental period studied except in 12 dpf females (Figures 3.1B). Expression of c*yp19a* increased with age in both genders, with higher expression in females only at 12 dpf and 30 dpf (Figure 3.1C).

Expression of *esr2* was not gender-specific at the different age groups tested (Figure 3.1D).

Because of the consistently higher expression of *osp1* in females, we characterized the regulatory motifs in the promoter region of *osp1* by sequencing the 4 kb upstream region (GenBank Acc. No. KM655767; Primer sequences in Supplementary Table 3.1) and analyzed it using several online software packages. Two probable promoter regions were found at – 632 and –1833 upstream of *osp1*. An Estrogen Response Element (ERE) was found at -3982 and several transcription factor binding sites were also located (Supplementary Figure 3.1A). These sequences included binding sites for general transcription factors (TATA box, CAAT box and C/EBP), in addition to binding sites for transcription factors involved in sex differentiation and cellular development (globin transcription factor 1, GATA-1; globin transcription factor 2, GATA-2; sex determining region Y, SRY; SRY-related HMG box transcription factor 5, SOX-5; cAMP-responsive element binding protein, CRE-BP; and E2 factor, E2F) (Supplementary Figure 3.1B).

3.4.2 Expression of OSP1 in gonads

To determine the OSP1 protein expression in the gonads, Western blot was performed using the tissue lysates from the testis and ovary of adult medaka (Figure 3.2A). Protein levels from gonadal extracts corresponded with *osp1* transcript levels; that is, high levels of OSP1 were detected in ovary, whereas no OSP1 was detected in testis. Overall, this result supports the hypothesis that *osp1* is a sex-specific gene that is predominantly expressed in females.

To determine the cellular localization of OSP1, immunohistochemical analysis was carried out using ovarian tissue sections from adult medaka. The protein was abundant in the cytoplasm of oogonia and perifollicular oocytes (Figures 3.2B and C). In mature follicles, OSP1 was localized primarily in the granulosa and theca cells of the follicular layers.

3.4.3 Changes in *osp1* expression after EDC exposure

Measured concentrations of TRB and EE_2 in the pre-exposure medium were 4.4 and 5.8 ng/L respectively. At 8 h of exposure, TRB and EE_2 concentrations in test media had decreased by 37% and by more than 41% to 2.8 and < 3.4 ng/L, respectively. Both compounds were below the detection limit in all control samples.

To assess changes in the expression of *osp1* and *vtg* after exposure to TRB and EE_2 for 10 d, qPCR was performed on 25 dpf male and female whole larvae. A significant down-regulation (~73 fold) in the expression of *osp1* was recorded in females exposed to TRB (Figure 3.3). No significant changes in *osp1* transcripts was observed in EE_2 exposed males as compared to controls (Figure 3.3). Levels of *vtg* did not show any significant changes in TRB-exposed females and EE_2-exposed males as compared to controls (data not shown).

3.4.4 Gonad histology

Histological analysis of gonad sections from 60 dpf males and females was performed to investigate the cellular effects of exposure to synthetic hormones on gonadal development of juvenile fish. Testes of males exposed to EE_2 exhibited a delay in gametogenesis compared to controls. Specifically, a significant increase in the percentage of spermatogonia and a significant reduction in the percentage of both primary and secondary spermatocytes was observed, and spermatids and spermatozoa were completely absent in the testes of EE_2-exposed males (Figures 3.4, 3.5A and 3.5B). In TRB-exposed females, 7 out of 10 individuals developed spermatogenic activity in their ovaries manifested by the presence of spermatogonia and spermatocyte nests interspersed between ovarian follicles (Figures 3.5C and 3.5D), indicating the development of intersex as a result of this short-term exposure to TRB. Percentages of oocytes, chromatin nucleolar oocytes and perinucleolar oocytes did not differ between the control and TRB-exposed females.

3.5 Discussion

Our findings suggest that *osp1* is a sensitive biomarker for androgen exposure with changes in its expression levels that correlate with the development of female gonadal intersex. The timing and expression level of *osp1* suggests its involvement in the sex differentiation process.

3.5.1 Developmental expression of *osp1, vtg, cyp19a* and *esr2*

Sexual dimorphic expression of *osp1* began at 12 dpf and continued to increase up to 30 dpf. Expression levels in females were high when compared with the negligible levels observed in males. This finding is in agreement with a previous study that observed an increase in transcription levels of *osp1* in female medaka between 9 dpf and 99 dpf, with a 13-fold increase in expression at 24 dpf (Zhao *et al.*, 2014). In contrast, *vtg* transcription levels in the present study showed high variability among individuals with no significant differences between genders except at 12 dpf. Similar variability in *vtg* levels has been reported in earlier studies, suggesting that it is a natural phenomenon for this gene during this developmental period (Biales *et al.*, 2007). Sharp sexual dimorphism in *vtg* expression is only observed later in life during vitellogenesis (Hara *et al.*, 2004). Aromatase or *cyp19a* plays a critical role in the sex differentiation process, with an up-regulation being essential for ovarian development and a down-regulation for testicular differentiation (Guiguen *et al.*, 2010). Our results are in agreement with this finding as aromatase transcription levels showed higher expression in females, although differences between genders were not as large and consistent throughout development compared to *osp1*. Low levels of *esr2* expression were found throughout the developmental period investigated with no significant differences between genders, which is in agreement with previous studies (Chakraborty *et al.*, 2011).

3.5.2 Expression and role of OSP1 in gonads

Results from the present study clearly show that OSP1 is a female-specific protein only expressed in the follicular layer of oocytes. This finding is in agreement with the in situ hybridization data published by Zhao and Hu (2012). This 214 amino acid OSP1 protein (23.5 kDa) has a N-terminal signal peptide sequence (Zhao and Hu, 2012) and is

thus predicted to be a secreted protein. Our attempts to find an OSP1 homolog in other species did not yield any obvious candidate.

3.5.3 Changes in *osp1* gene expression after estrogen and androgen exposure

Exposure of female medaka larvae to 4.4 ng/L TRB significantly decreased the transcription levels of *osp1* suggesting the high sensitivity of this gene to androgen exposure. In contrast, *osp1* did not increase in males exposed to 5.8 ng/L EE_2. This is in contrast to results obtained by Zhao and Hu (2012), in which males had transcription levels of *osp1* close to that of females after exposure to 74.8±10.2 ng/L of EE_2 for up to 90 d. Some induction in *osp1* expression in that study was also observed in males exposed to 5.6 ng/L and 16.1 ng/L EE_2 for 90 d. Together, these results suggest that induction of *osp1* expression in males requires exposure to high concentrations of EE_2 and/or for longer periods. The well-established biomarker of EDC exposure, *vtg*, showed no significant changes in transcription levels upon exposure to EE_2 and TRB. In contrast to our findings in the present study, significant induction in *vtg* expression was observed in male medaka larvae after exposure for 23 d to 100 ng/L EE_2 (Scholz *et al.*, 2004). Similarly, a significant down and up regulation in the expression of *vtg* was observed after medaka juveniles were exposed for 60 d to 50 ng/L TRB and 100 ng/L EE_2, respectively (Yamani, 2004). Our findings in the present study suggest that at least in medaka, *osp1* shows much higher sensitivity to EDCs exposure than *vtg*. Also, *osp1* showed higher sensitivity to TRB than to EE_2, suggesting that *osp1* is a successful biomarker of androgen exposure in short-term (10-d) assays.

3.5.4 Gonad histology

Significant changes in gonadal phenotype were observed as a result of EDC exposures. The delay in spermatocyte development in EE_2-exposed males and the appearance of spermatogonia in the ovaries of TRB-exposed females validate our hypothesis that short-term exposures to environmentally relevant concentrations of these synthetic sex steroids during a critical developmental period can result in long-term gonadal effects. These results are consistent with previous studies that have reported similar gonadal alterations after EDC exposure (Weber *et al.*, 2004; Silva *et al.*, 2012;

Zhao and Hu 2012; Zhao *et al.*, 2014), however, to our knowledge, the present study is the first to report these effects upon exposure to environmentally relevant doses of EDCs for a period of only 10 d. Very importantly, our observation of gonadal intersex in TRB-exposed female medaka at 60 dpf was preceded by an approximate 73-fold decrease in *osp1* expression 35 d earlier (in 25 dpf females) which emphasizes the utility of *osp1* as a sensitive biomarker capable of predicting disruptions to the sex differentiation process. In contrast, in males, this exposure regime resulted in no changes in the expression of *osp1* and in the absence of intersex. As already discussed (see change is *osp1* gene expression after estrogen and androgen exposure), *osp1* is inducible in medaka males, but after higher and longer EE_2 exposures (Zhao and Hu, 2012; Zhao *et al.*, 2014). Down-regulation of *dmrt1* expression as a result of EE_2 exposure provide a possible explanation to the observed delay in testicular maturation in EE_2-exposed males (Marchand *et al.*, 2000; Nagahama *et al.*, 2004). The *dmrt1* gene plays a critical role in the male sex differentiation processes through controlling male germ cell proliferation and the testicular development primarily through down-regulating the expression of aromatase causing inhibition to the estrogenic biosynthesis (Kobayashi *et al.*, 2004, Wang *et al.*, 2010).

3.5.5 Conclusions

In conclusion, the present study demonstrates that *osp1* is a promising biomarker of androgen exposure in female medaka. It is highly sensitive to androgens, and changes in its expression are predictive of gonadal intersex occurring at later ages. We also demonstrate that short-term exposure to ECDs during critical periods of medaka reproductive development can have long-term effects on gonadal development. Thus, the use of *osp1* in short-term *in vivo* assays using medaka larvae can be very useful for screening chemicals for androgenic effects.

Acknowledgment: We wish to thank S. Guffey, J. Serafin, C. Mapes, Y. Gao, J. Gao, P. Moraga, J. Leet and J. Zenobio for their assistance in fulfilling this work. We also thank B. Cooper of the Purdue University Metabolite Profiling Facility for assistance with HPLC-mass spectrometric analysis, and A. Zayed at Assiut University for his assistance

with histopathological analyses. We thank the Department of Forestry and Natural Resources at Purdue University, the Cultural Affairs and Mission Sector at the Egyptian Ministry of Higher Education, and the Grant Agency of the Czech Republic (GACR) P503/13/34049P (to A. Hatef) for providing funding for this study.

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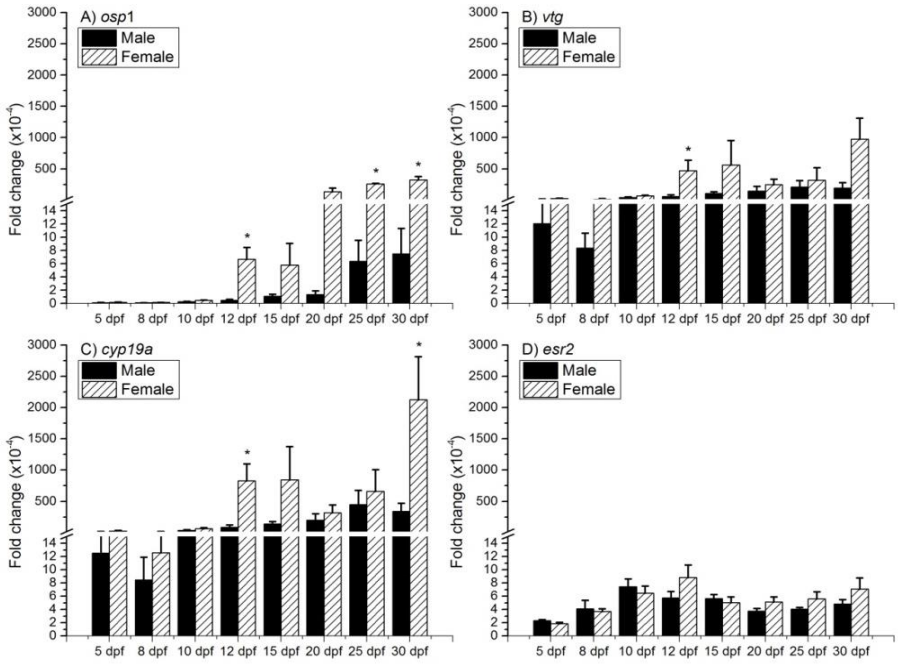


Figure 3.1: Relative mRNA expression of A) *osp1*, B) *vtg*, C) *cyp19a*, and D) *esr2* at 5, 8, 10, 12, 15, 20, 25 and 30 dpf in male and female Japanese medaka. Results are expressed as mean ± SE of four independent determinations (n = 4) quantified as normalized individual data points by estimating 2^ΔCt after normalizing gene expression to *β-actin*. Significant differences ($p < 0.05$) between genders of same age are denoted with asterisks.

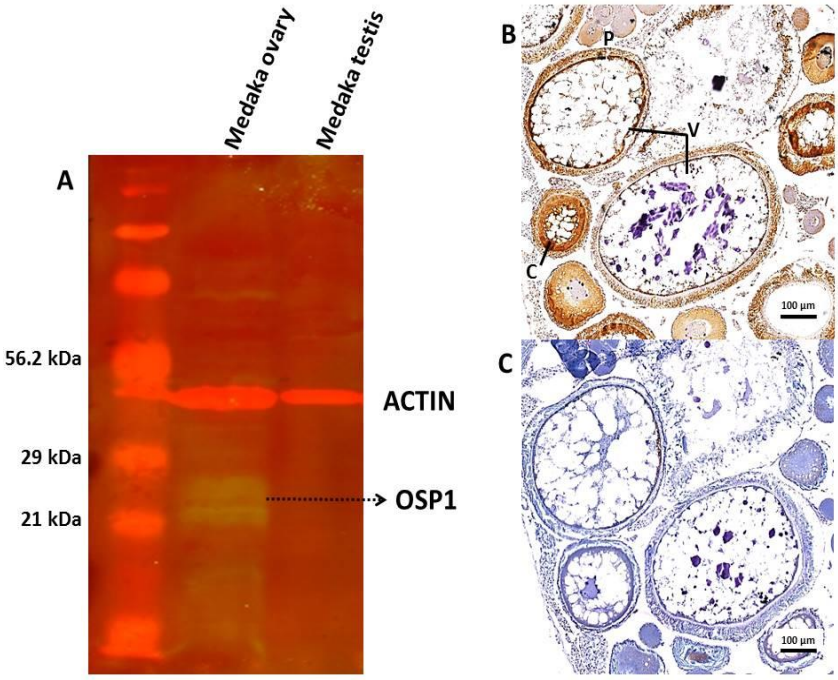


Figure 3.2: Expression and localization of OSP1 in adult Japanese medaka gonads. (A) OSP1 expression was determined by Western blotting of adult medaka ovary and testis. (B) Immunohistochemical localization of OSP1 in mature medaka ovary (50X), showing perinucleolar oocytes (P), cortical alveolar oocytes (C) and vitellogenic oocytes (V). (C) Negative control with no added anti-OSP1 antibodies (50X).

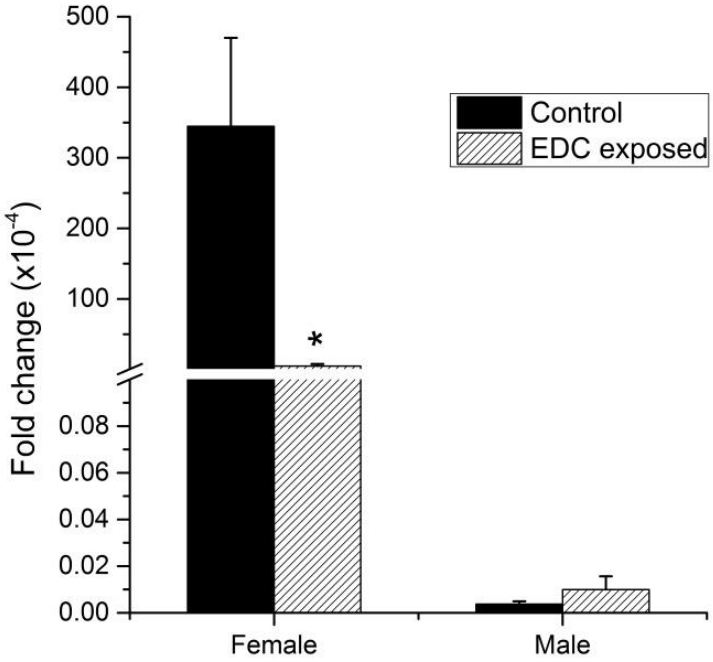


Figure 3.3: Relative *osp1* mRNA expression in control and EDC-exposed 25 dpf male (5 ng/L EE_2) and female (5 ng/L TRB) Japanese medaka. The fold-change (log scale) of *osp1* transcript expression in exposed group relative to the control within the same sex is shown. Results are expressed as mean ± SE of four independent determinations (n= 4) quantified as normalized individual data points by estimating 2^ΔCt after normalizing gene expression to *β-actin*. Asterisks indicate significant differences between control and exposed groups ($p < 0.05$).

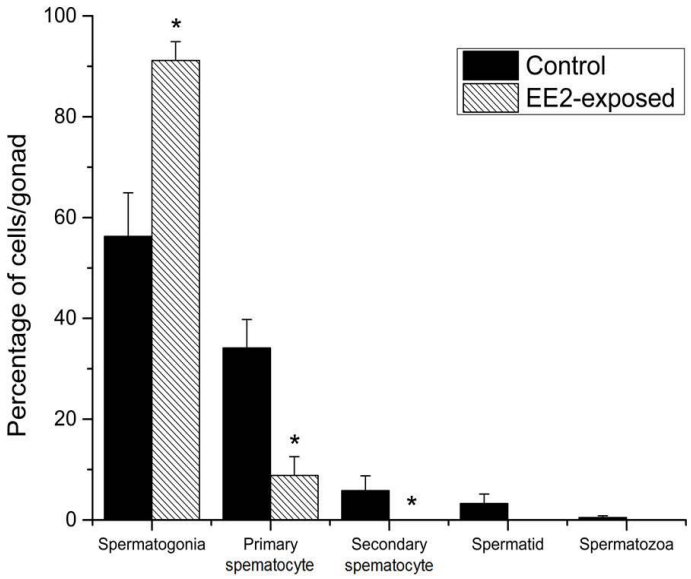


Figure 3.4: Average ± SE (n ≥ 9) percentage of gonadal cell types per gonad in control and EE_2-exposed 60 dpf male Japanese medaka. Significant differences ($p < 0.05$) between control and exposed groups are marked by asterisks.

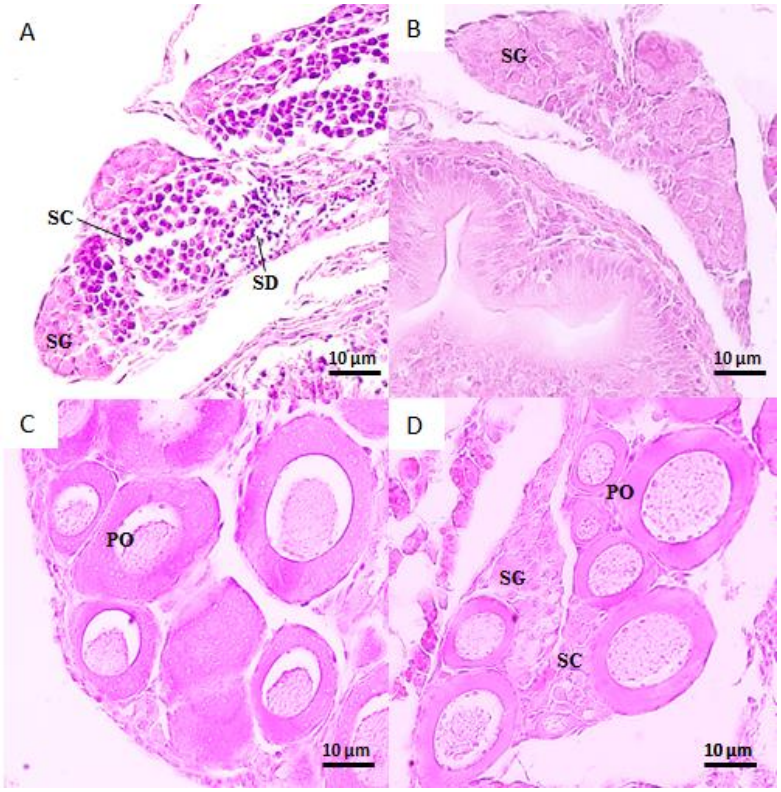
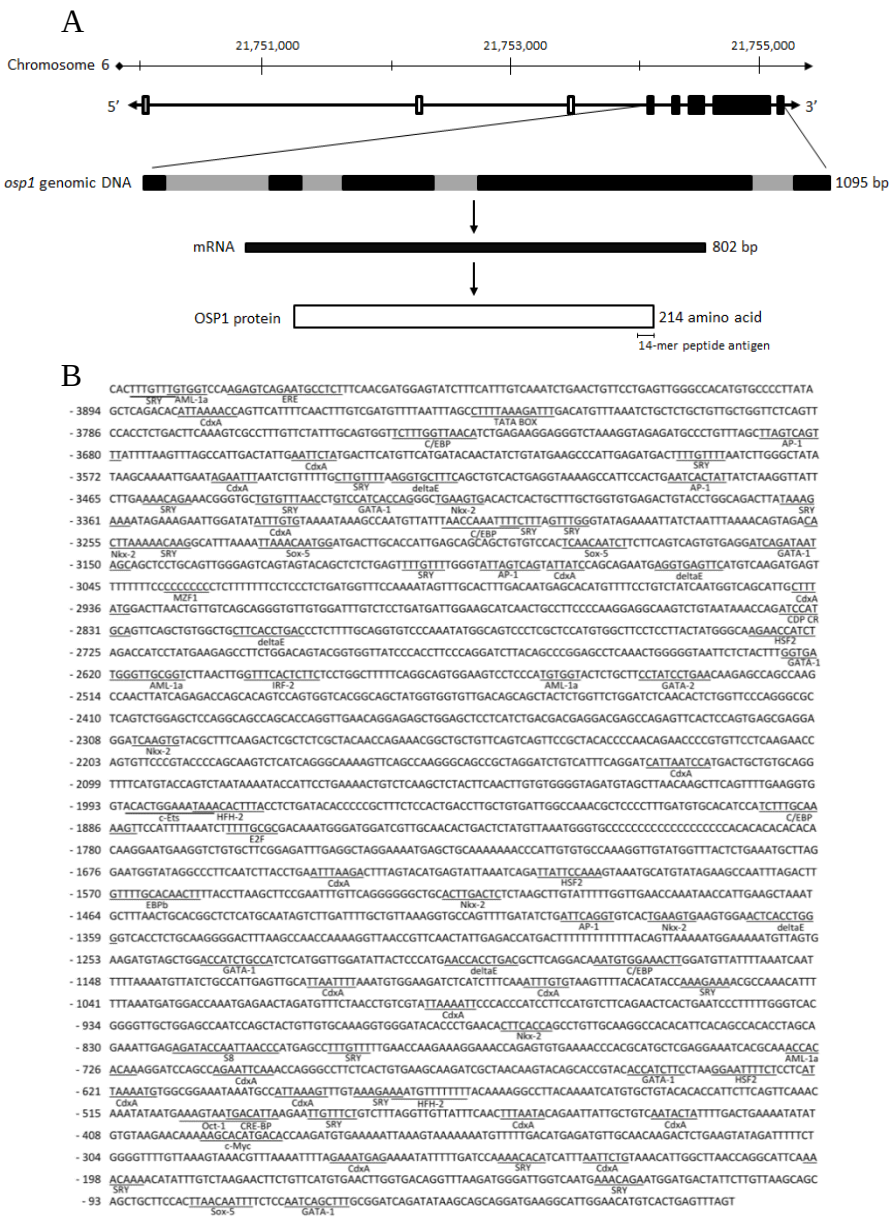


Figure 3.5: Hematoxylin and eosin staining of 60 dpf Japanese medaka gonads (400 X). (A) Immature testis of a 60 dpf control male containing spermatogonia (SG), spermatocytes (SC) and spermatids (SD). (B) Immature testis of a 60 dpf EE_2-exposed male filled with spermatogonia (SG). (C) Previtellogenic ovary of a 60 dpf control female containing perinuclear oocytes (PO). (D) Previtellogenic ovary of a 60 dpf TRB-exposed female containing perinuclear oocytes and spermatogenic nests harboring spermatogonia (SG) and spermatocytes (SC).

3.7 Supplementary information



Supplementary Figure 3.1: A) Graphical representation of *osp1* (▮ = exons, ▯ = introns), its promoter region, transcript and protein (illustration based on our findings and Zhao *et al.* [16]). ▯ are predicted promoter regions and ▮ is an estrogen response element (ERE) predicted using Neural Network Promoter Prediction program (NNPP, version 2.2) and Dragon Estrogen Response Element (ERE) Locator (version 6.0), respectively. Illustrated in OSP1 protein, is a 14-mer peptide used to develop polyclonal anti-OSP1. B) Shows predicted regulatory motifs in 4kb upstream *osp1* gene predicted using TFSEARCH program (version 1.3).

Supplementary Table 3.1: Primers used for PCR and qPCR amplifications with their product size and melting temperature (Tm).

Region amplified	Product size	Primer sequence	Tm (°C)
osp1 Promoter 1	717	5’-CAAGAACCAGTGTTCCCGTA-3’	57.46
		5’-TGGTCTCAATAGTTGAACGG-3’	55.11
osp1 Promoter 2	723	5’-CCGTTCAACTATTGAGACCA-3’	55.11
		5’-AATGGCATTTATTTCCGCCA-3’	56.34
osp1 Promoter 3	763	5’-TGTGCTGTACACACCATTCT-3’	57.07
		5’-AGACAAATCCACAACACCCT-3’	56.95
osp1 Promoter 4	800	5’-ACCAATTAACCCATGAGCCT-3’	56.77
		5’-GTTCCAATGCCTTCATCCTG-3’	56.76
osp1 Promoter 5	1100	5’-TGTATGGAAAGGTTGGGGTC-3’	54.60
		5’-TTGGAAACCATCAGAGGGAG-3’	54.30
osp1 Promoter 6	1098	5’-GCTGTGTCCACTCAACAATC-3’	53.90
		5’-TGCACAGCAGTCATGGATTA-3’	54.20
osp1 gene	714	5’-TGTGAATTGCCTCATGTCTG-3’	53.00
		5’-TTGTAGCGAGAGCGAGTCTT-3’	55.80
osp1	116	5’-CCCAAGGAAGTGTCCCAAAT-3’	55.1
		5’-ATAACCACCATACTGTCCAGAAGG-3’	56.2
β-actin	152	5’-ACATTGCCGCACTGGTTGTTGA-3’	60.1
		5’-TACGTAGCTGTCTTTCTGGCCCAT-3’	59.9
vtg	162	5’-TCCATGCTTGGCTTTGGCAGCA-3’	62.5
		5’-TCCGGCATTGCCCAGGACTTTA-3’	61.1
cyp19a	198	5’-ATCGGCATGAACGAGAAGGGA-3’	58.5
		5’-TGTGCAGCGCAAAAAGCCAA-3’	59.2
esr2	162	5’-TCCATTACTGTGCTGTGTGCCA-3’	59.1
		5’-TCTTGCGCCGGTTCTTGTCTAT-3’	58.8

CHAPTER 4. *IN VIVO* VISUAL REPORTER SYSTEM FOR DETECTION OF ESTROGENIC AND ANDROGENIC CONTAMINANT EXPOSURE USING TRANSGENIC SEE-THROUGH JAPANESE MEDAKA *ORYZIAS LATIPES*

4.1 Abstract

Estrogenic and androgenic contaminants interfere with the functioning of the endocrine system by mimicking or blocking the action of endogenous hormones, causing reproductive and developmental disturbances in many organisms. These contaminants are continuously released into the environment, creating an urgent need to develop time and cost effective tools for screening and testing chemicals for estrogenic and androgenic potential. Because sex differentiation and gonadal development are sex-hormone driven events, we hypothesized that a hormone responsive gene involved in these processes would be a good candidate for the development of a robust *in vivo* screening system for this group of contaminants. The main objective of this study was to develop a novel *in vivo* visual reporter system for rapid detection of contaminants with estrogenic/androgenic potential. First, we built a pOSP1-AcGFP (promoterOSP1-*Aequorea coerulescens* green florescence protein) Japanese medaka transgenic line with ovarian structural protein 1 (*osp1)* promoter region driving the expression of a reporter protein, AcGFP. *Osp1* is a gene with strong female-specific expression starting very early in development and its expression is highly responsive to estrogenic and androgenic hormonal exposures. Next, we tested the use of this transgenic line in an *in vivo* visual reporter system for identifying estrogenic contaminants. Overall, our results support the hypothesis that molecular biomarkers are sensitive tools that can be used for early detection of the effects of contaminants with estrogenic activity on fish and are ideal endpoints in wide-scale contaminant screening assays.

4.2 Introduction

Over the last few decades, studies have reported levels of estrogenic and androgenic contaminants in the environment. More importantly, these contaminants have

been associated with adverse reproductive outcomes in a variety of organisms, including humans (Falconer *et al.*, 2006; Frye *et al.*, 2012; Abdel-moneim *et al.*, 2015a; Bhandari *et al.*, 2015). These adverse outcomes have been primarily attributed to the ability of estrogenic and androgenic contaminants to mimic or block the action of endogenous hormones, particularly when exposures happen during the period of sex differentiation and gonadal development. In mice, exposures to xenoestrogens during embryonic/neonatal developmental stages had drastic effects on sperm quality and overall fertility of males when they reached adulthood (Delbes *et al.*, 2006). Similarly, numerous studies have reported the occurrence of gonadal intersex, a well-known adverse effect of estrogenic and androgenic exposures, among wild gonochoristic fish populations inhabiting water systems across the states (Hinck *et al.*, 2007; Abdel-moneim *et al.*, 2015a).

The US Environmental Protection Agency (EPA) identified the need for the development of a set of tools for testing endocrine disrupting chemicals (EDCs) over two decades ago, establishing the Endocrine Disruptors Screening Program (EDSP) (USEPA, 2016). Since then, the program has identified over 200 chemicals as needing immediate testing and it is likely that many more chemicals will require testing in the near future. Testing these chemicals using whole animals assays currently implemented in the EDSP program would require inordinate amounts of time and funds, particularly because the mechanism(s) of toxicity of most of these chemicals is unknown. *In vitro* assays provide a rapid and simple approach, but typically rely on specific modes of action, as in the estrogen receptor (ER) binding or the ER transcriptional activation cell assays, and are difficult to extrapolate to the whole animal level due to the complexity of endocrine systems (OECD, 2012). *In vivo* screening assays currently in use, as the fish short-term reproduction and the fish full life cycle assays, are based on endpoints such as vitellogenin (VTG) induction, morphological changes, gonadal histology, and reproductive success (e.g. fecundity). Although these endpoints can be applied in environment risk assessments, several limitations have been reported including the need for long exposure periods increasing costs; the need for large plasma volumes which might not be feasible in experiments using small fish models; and the limited sensitivity and specificity of endpoints involving changes in secondary sex characteristics (USEPA,

2007). In addition, histopathological changes, like gonadal intersex, can be easily overlooked resulting in false negative results (Zhao *et al.,* 2014). Therefore, there is a need for the development of novel screening assays, that are cost-effective, high-throughput and at the same time, measure endpoints that can be used to estimate both human and environmental risks.

Japanese medaka *Oryzias latipes* is a commonly used fish model for studying the effects of EDCs (Foran *et al.,* 2002; Balch *et al.,* 2004; Shima and Mitani, 2004) and it is included in the Organization for Economic Cooperation and Development (OECD) chronic test guidelines. It has several advantages over zebrafish (*Danio rerio*), including their well-understood sex determination mechanism (Postlethwait *et al.,* 2000; Wittbrodt *et al.,* 2002) and their early (3 d post fertilization, dpf) sexing methods. Genes involved in sexual differentiation and gonadal development in this species can provide excellent endpoints in human and environmental risk assessments, particularly if changes in their expression levels have direct effects on gonadal development and can be quantified non-invasively using a time/cost efficient approach.

Previous research has identified sensitive molecular biomarkers of estrogenic and androgenic contaminant exposures in medaka (Yamaguchi *et al.,* 2005; Kishi *et al.,* 2006; Chen *et al.,* 2008; Zhao and Hu, 2012; Abdel-moneim *et al.,* 2015b; Zhu *et al.,* 2015; Chen *et al.,* 2016). Further, transgenic lines targeting some of these genes have been developed and used as *in vivo* visual reporter systems (Kurauchi *et al.,* 2005; Zeng *et al.,* 2005; Zhao *et al.,* 2014). For instance, vitellogenin (*vtg*) was identified as good candidates for screening estrogenic chemicals after short exposure periods using adult fish (Yamaguchi *et al.,* 2005; Chen *et al.,* 2016); however, its use in early life stages has been less successful (Abdel-moneim *et al.,* 2015b). Choriogenin (*chg*) is more sensitive than *vtg* to estrogenic exposure in medaka and its expression is inducible at different developmental stages (Yu *et al.,* 2006; Chen *et al.,* 2008). A transgenic line with a reporter protein driven by the promoter of this gene was successfully implemented in an *in vivo* visual reporter system for the screening of estrogenic contaminants (Ueno *et al.,* 2004; Kurauchi *et al.,* 2005; Scholz *et al.,* 2005). Ovarian structure protein 1 (*osp1*) is a promising biomarker for both estrogenic and androgenic contaminant exposures (Zhao and Hu, 2012; Abdel-moneim *et al.,* 2015b). This protein has a sexually dimorphic

expression pattern, with an increase in expression in females as the ovary develops and negligible expression in males (Abdel-moneim *et al.*, 2015b). Further, exposure of male medaka to estrogenic contaminants causes induction in *osp1* expression to levels higher than *chg* and close to *osp1* expression levels in females (Zhao and Hu, 2012), whereas exposure of females to androgens elicits a significant a downregulation in *osp1* (Abdel-moneim *et al.*, 2015b). Importantly, changes in the expression levels of this gene were predictive of changes in gonadal development, including development of gonadal intersex (Zhao and Hu, 2012; Abdel-moneim *et al.*, 2015b). In 2014, Zhao *et al.* (2014) developed a medaka transgenic line harboring a gene coding for green fluorescence protein driven by a 2 kb promoter region of *osp1*. This *in vivo* visual reporter system provided higher sensitivity over histopathological approaches in evaluating the prevalence and severity of gonadal intersex in male medaka exposed to estrogenic compounds for 30 and 90 d. However, changes in GFP intensity during early developmental stages in response to very short exposures (< 48 h) to estrogenic or androgenic contaminants was not tested.

The long-term goal of our work is to develop a transgenic line of medaka for use as a screening tool for chemicals with the potential to act as estrogen and androgen agonists/antagonists. In this paper, we report the creation of a see-through transgenic Japanese medaka expressing *Aequorea coerulescens* green florescence protein (AcGFP) driven by the promoter region of *osp1*, a female-specific gene associated with gonadal intersex (pOSP1-AcGFP). The sensitivity and specificity of this transgenic line was evaluated after exposure of females to potent synthetic estrogens.

4.3 Materials and Methods

4.3.1 Test organism

One-cell stage embryos were collected from eight single parent crosses of see-through SK2MC medaka maintained at the Aquatic Ecology Laboratory (Purdue University, West Lafayette, IN, USA). Dividers separating genders were removed shortly after the morning feeding and eggs collected 1 h later to insure synchronized

fertilization. Healthy embryos were selected and disinfected with a rinse in 0.005% bleach solution, then transferred to 6-well plastic plates containing 10 mL embryo culture medium (Kinoshita *et al.*, 2009) supplemented with 0.0002% methylene blue. All developmental stages were kept in a 14:10-h light:dark photoperiod at a temperature of 25 ± 1 ºC. Water quality parameters, dissolved oxygen, total ammonia, and pH were maintained at > 5 mg/L, < 0.25 mg/L, and between 7 to 8, respectively. Adults and fingerlings were fed *ad libitum* twice daily with hatched *Artemia* nauplii in the mornings and dry food (Otohime, Nisshin Seifun, Japan) in the afternoon, whereas larvae were only fed dry food twice daily. All protocols involving fish care were approved by the Purdue Animal Care and Use Committee.

4.3.2 Generation of pOSP1-AcGFP1 construct

A 5′ flanking region (4128 bp) of the *osp1* proximal promoter region was amplified and inserted into the 5' Multiple Cloning Site of a pAcGFP1 vector (Clonetech, Mountain View, CA) using restriction digestion (Figure 4.1). In brief, Genomic DNA was extracted from mature ovaries using DNAzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Three overlapping fragments (0 to -1.63 kb, -1.63 to -2.83 kb and -2.83 to -4.13 kb) from the target 5′ flanking region, were amplified using a Taq DNA polymerase kit from 5PRIME (Hamburg, Germany). The PCR conditions used for all reactions were: initial template denaturation at 95 ºC for 2 min; 35 cycles at 94 ºC for 10 s; primer annealing at 55 ºC for 15 s; product extension at 68 ºC for 1 min; and final extension at 72 ºC for 5 min. The amplified fragments were then inserted into T-vectors, pMD-20 (Clonetech) and cloned into One Shot TOP10 Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA), according to the manufacturers' instructions. The plasmids were extracted from the cloned cells using PureLink Quick Plasmid Miniprep Kit (Invitrogen), gel purified and submitted to the Purdue genomic core facility for sequencing. Primer sets used for the amplification and sequencing analysis (Supplemental Table 4.1) were designed based on the sequence information of *osp1* proximal promoter region from our strain (Abdel-moneim *et al.*, 2015b) and medaka genome information (http://www.ensembl.org/Oryzias_latipes/Info/Index), and purchased from Integrated DNA Technologies. Fragments with verified sequences were

inserted into the pAcGFP1 vector according to their proximity to the AcGFP using appropriate restriction enzyme sites (see Figure 4.1).

4.3.3 Generation of pOSP1-AcGFP1 transgenic line

The pOSP1-AcGFP construct was linearized with the restriction enzyme *SalI* (New England Biolabs, Ipswich, MA), then microinjected into one-cell stage embryos (at a concentration of 12 ng/µl) before their first cleavage. The microinjection was performed using a microinjection apparatus (PV830 pneumatic picopump with vacuum, World precision instruments Inc., Sarasota, FL) and a micromanipulator (M3301R, World precision instruments Inc.). Needles with an outside diameter of 0.02 mm were pulled from alumsilicate glass capillary tubes (10 cm, outside/inside diameter of 1/0.64 mm; Sutter Inc., Novato, CA) using a P-1000 Micropipette Puller (Sutter Instrument, Novato, CA). Injections were performed while having eggs covered in saline medaka oocyte (SMO) medium (Iwamatsu 1983) to reduce the eggs' internal pressure. F_0 embryos were incubated in embryo-rearing medium (described in section 4.3.1) until hatching, then moved to a controlled recirculating system (Z-Hab System, Aquatic Habitats, Apopka, FL) for rearing. Once sexually mature, founder fish (F_0) were pair-mated with wild type SK2MC medaka and their offspring (F_1, $n > 12$) screened (~30 d post-hatch, dph) for germline transmission of pOSP1-AcGFP after isolating their genomic DNA from caudal fin clips using primers described in Supplementary Table 4.1. Positive juveniles were grown to adulthood and pair-mated with wild type SK2MC medaka to eliminate the possibility of having multiple insertions of the construct. The female offspring from each F_1 cross (F_2) were also screened by fin clipping and AcGFP fluorescence imaging. Positive candidates pair-mated wild type SK2MC medaka and their offspring (F_3) again screened for fluorescence. The line showing strongest stable fluorescence was chosen for the subsequent analyses.

AcGFP fluorescence imaging was performed using a Zeiss SteREO Discovery.V12 stereoscopic fluorescence microscope equipped with an AxioCam MRc camera and ZEN 2 lite (blue edition) software (all from Carl Zeiss, Oberkochen, Germany). Changes in the AcGFP fluorescence intensity were quantified using a TiS inverted microscope (Nikon, Melville, NY) equipped with Xenon Fluorescent Light

Source, a FITC filter cube (excitation: 460-500 nm; dichroic: 505 nm; emission: 510-560 nm), a QuantEM:512SC EMCCD camera (Photometrics, Tucson, AZ) and NIS-Elements AR v3.2 software.

4.3.4 Test chemicals and exposure design

Ethinyl estradiol (EE_2) was purchased from Sigma (St Louis, MO) and stored in powdered form at 4 ºC. A stock solution (10 mg/L) was prepared by dissolving the powdered chemical in 0.5 mL DMSO then mixed with 999.5 mL of autoclaved MilliQ water. A second stock solution (10 µg/L) was prepared by dissolving 1 mL of the first stock solution in 999 mL of autoclaved MilliQ water. A working solution of 500 ng/L was then prepared by mixing 50 mL of the second stock solution with 950 mL of Reconstituted Reverse osmosis (RO) water, respectively. RO water reconstitution was performed by adding 0.00025% Replenish. All solutions were stirred for a minimum of 2 h. Stock solutions were stored at 4 ºC for no longer than a month and working solutions were freshly prepared and kept in a temperature-controlled chamber at 25 ºC until use.

Embryos (7 dpf) were sexed by examining the presence of leucophores (only present in males) and females were reared under the conditions described in section 4.3.1. At 29 dph, juvenile females were taken out of the controlled recirculation system and kept in 250 mL beakers filled with RO water in an environmental chamber at the same photoperiod and temperature. Females were fasted for 24 h prior to imaging to minimize background fluorescence originating from the food in the gastrointestinal tract. After 24 h, females (30 dph) were anesthetized using MS-222 (Tricane, 226 mg/L) and imaged using a QuantEM:512SC EMCCD camera connected to a TiS inverted microscope and fluorescence intensity quantified as described in section 4.3.3. After imaging, single females were allowed to recover from anesthesia in 250 mL glass beakers filled with RO water, then beakers were randomly assigned to either the control or exposed (500 ng/L EE_2) groups and their holding medium was changed accordingly. All beakers were held in an environmental chamber at a 14:10-h light:dark photoperiod and 25 ± 1 ºC for 24 h without feeding. At the end of the exposure period, juveniles were taken out of the environmental chamber and again anesthetized and imaged to quantify their fluorescence intensity using the same method described in section 4.3.3.

4.3.5 Chemical analysis of EE_2

To prepare water samples for quantification, an internal standard (dansyl-d4-EE_2, 100 ng/mL, C/D/N Isotope, Pointe-Claire, Quebec, Canada) was added to the EE_2 stock solution and the mixture was derivatized using a dansyl chloride reaction protocol. In brief, 20 µL of sodium bicarbonate buffer (10 mM, pH adjusted to 10 with NaOH) was added to each sample, mixed for 1 min, followed by addition of 50 µL of dansyl chloride solution (3.0 mg/mL in acetone), then mixed for another minute. Prior to analysis, samples were placed in a 60 °C incubator for 15 min and cooled to room temperature. Analyses were performed on an Agilent 1200 high-performance liquid chromatography (HPLC) system using a Waters XBridge C8 (2.1x100 mm, 3.5 µm) column and the mobile phases A and B were H_20 with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. The following linear gradient elution was used: initial conditions 35% B, 0 - 8 min; 70% B, 8 - 8.5 min; and 90% B, 8.5 - 9.5 min. Column re-equilibration consisted of 35% B, 9.5 - 10.5 min and 35% B, 10.5 - 13.5 min. Flow rate was 0.3 mL/min and the retention time was 7.9 min. Samples were quantified by tandem mass spectrometry (MS/MS) utilizing an Agilent 6460 Triple Quadrupole MS (Agilent Technologies, Santa Clara, CA) with positive electrospray ionization (ESI), based on Multiple Reaction Monitoring (MRM). Transition was 530.6 to 171.0 for dansyl-EE_2 and 534.1 to 171.0 for dansyl-d4-EE_2, both with a collision energy of 18 V. Quantitation was based on a six-point standard EE_2 curve ranging from 0.1 to 1,000 ng/mL.

4.3.6 Statistical analysis

All statistical analyses were performed using SPSS 21.0 (IBM-SPSS Inc., Chicago, IL). Data were first examined for normality using the Shapiro–Wilks test. Fluorescence intensity levels showing normal distributions were compared using independent sample t tests to determine differences exposed and control groups. Groups showing non-normal distribution were statistically compared using Mann–Whitney U tests. P-values ≤ 0.05 were considered statistically significant. Fluorescence intensity parameters are presented as mean ± standard error (SE).

4.4 Results

4.4.1 Generation of pOSP1-AcGFP medaka transgenic line

The pOSP1-AcGFP microinjection process yielded 50 adult founder fish (F_0), with four males (8%) showing germ-line transmission. The rate of germ-line transmission to F_1 ranged between 9% and 25% between those four founders. Subsequently, separate transgenic lines were developed from each founder for over three generations, through which standard Mendelian ratios of transgene inheritance were observed. Imaging of F_3 fish showed AcGFP fluorescence only in the ovarian region (lower abdomen) of female fish (see Figures 4.2A, 4.2C, 4.2E, and 4.2G) with no fluorescence seen in males (not shown). AcGFP fluorescence in females was observed starting at 25 dph in the form of spheres (oocytes) appearing below the swim bladder and above the gastrointestinal tract (Figures 4.2E, 4.2G). The number and size of follicles increased as the fish developed occupying the majority of the lower abdominal area in adult females (Figures 4.2A-G).

4.4.2 AcGFP induction by EE_2 exposure (i*n vivo* visual reporter assay)

To identify the sensitivity of pOSP1-AcGFP transgenic line to estrogenic contaminants and its ability to be implemented in an *in vivo* visual reporter assay, we exposed 30 dph transgenic medaka females to 500 ng/L EE_2 for 24 h. The measured concentration of EE_2 in the first stock solution was 11.3 mg/L and hence EE_2 concentrations in the exposure medium was determined to be 565 ng/L. EE_2 concentration was below the detection limit in all control samples. A significant upregulation in fluorescence maximum intensity and intensity variance were observed in females exposed to EE_2 (Figure 4.3). No significant changes were in AcGFP mean and minimum intensities or in the measured fluorescing areas (Figure 4.3).

4.5 Discussion

In this study we developed a Japanese medaka see-through transgenic line with a AcGFP fluorescent reporter protein driven by a 4 kb promoter region of *osp1*, a promising molecular biomarker of estrogenic and androgenic contaminant exposure and

gonadal intersex development. We have also tested the sensitivity of our transgenic line to short-term (24 h) estrogenic exposure during early gonadal development and have recorded significant inductions in AcGFP fluorescence intensity.

4.5.1 pOSP1-AcGFP1 transgenic line

A sequence of 4128 bp upstream the *osp1* translation initiation site was used to drive the expression of AcGFP reporter protein. This proximal promoter region harbors predicted promoters for *osp1* expression, several transcription factor binding sites and an estrogen response element (ERE) located ~3.7 kb upstream of the *osp1* translation initiation site (Abdel-moneim *et al.*, 2015b). The latter likely impacts the responsiveness and specificity of our target gene or its reporter protein to estrogenic exposures. An earlier study developed a pOSP1-EGFP transgenic line using Japanese medaka, but only incorporated a ~2 kb promoter region to drive the expression of the reporter protein (Zhao *et al.*, 2014). In addition, the fluorescent reporter protein used in the development of the transgenic line herein, AcGFP, is a true monomeric protein well suited for fusion tag applications while harboring spectral properties similar to that of EGFP, the fluorescent reporter protein used in previous studies. Another advantage of the transgenic line developed herein is the ability to sex animals non-invasively very early in development, as males develop leucophores along the body axis starting at 3 dpf. This non-invasive sex marker allows us to incorporate females at early developmental stages in *in vivo* visual screening systems. The limitation of this sex marker is the high background fluorescence it produces in males; however, the screening system of interest in this study is based on changes in ovarian AcGFP florescence intensity in response to both estrogenic and androgenic contaminants.

4.5.2 AcGFP induction after a 24 h EE_2 exposure

Testing contaminants for their estrogenic potential in model fish species using long-term exposures extending from early development to maturity is a widely used approach (Balch *et al.*, 2004, Gray and Metcalfe, 1997). The endpoints adopted in these assays, such as morphological changes, gonadal histology, and reproductive success, are highly relevant as they can be used for ecological risk assessments, but involve several

limitations (USEPA, 2007; Zhao *et al.*, 2014). Utilizing these kind of assays to test large numbers of chemicals is cost and time prohibitive. For these reasons, scientists are striving to develop reliable *in vivo* assays that are responsive to short exposure times, while at the same time, use endpoints that are predictive of reproductive effects at higher biological levels (e.g., gonad abnormalities) (Zeng *et al.*, 2005; Kurauchi *et al.*, 2005; Pardo-Martin *et al.*, 2010; Zhao *et al.*, 2014). A transgenic Japanese medaka line under the control of *vtg1* gene promoter was able to induce GFP expression in the liver of adult males after 6 d of exposure to 500 ng/L 17-beta-estradiol (Zeng *et al.*, 2005). However, weak or no GFP induction was detected when adult males were exposed to weaker estrogenic compounds such as bisphenol A (up to 5 mg/L), nonylphenol (up to 1 mg/L) and methoxychlor (up to 20 µg/L). In this line, GFP expression under normal conditions was not observed in female livers until 70 dph, given the nature of *vtg1* promoter driving GFP expression. *Vtg* is an egg yolk precursor that is normally synthesized in the livers of adult oviparous females (Tyler *et al.*, 1996). Juveniles did not express GFP under normal conditions except some ectopic GFP expression recorded posterior to the gills. Kurauchi *et al.* (2005) reported GFP induction in the liver of Chgh-GFP transgenic medaka yolk sac larvae 24 h after onset of exposure to ~100 ng/L EE_2. The gender of the yolk sac larvae was determined by PCR analysis of the male-specific gene (*dmy*) using DNA extracted from whole larvae following the GFP expression quantification. Zhao *et al.* (2014) developed a transgenic line (pOSP1-EGFP) based on a biomarker showing higher sensitivity to estrogenic exposure over *chg* (Zhao and Hu, 2012). This transgenic line allowed monitoring intersex induction in male medaka at early developmental stages following 30 d exposure to 2.14 ng/L EE_2. The concentrations tested in this study were environmentally relevant, but the length of exposure is not suitable for wide scale screenings. Herein, we report significant AcGFP induction in the ovary of 30 dph pOSP1-AcGFP transgenic medaka 24 h after exposure to 500 ng/L EE_2.

In gonochoristic fish, early developmental stages are more sensitive to exogenous steroid exposure, as they undergo sex differentiation and gonadal development (Koger *et al.*, 2000; Liney *et al.*, 2005). Short-term exposures during this period of enhanced sensitivity can have drastic impacts on the individual's gonadal development, however, the majority of the well-established reproductive endpoints cannot be assessed at this

early stage of development. The use of *osp1* as a molecular biomarker for estrogenic and androgenic exposures can provide a solution to this issue. Significant changes in *osp1* expression in response to estrogenic and androgenic exposures have been recorded in early developing medaka (Zhao and Hu, 2012; Zhao *et al.*, 2014; Abdel-moneim *et al.*, 2015b). Importantly, the observed changes in gene expression were also predictive of gonadal intersex occurrence later-in-life. Implementing this sensitive molecular biomarker in transgenic Japanese medaka line provides an easy real-time tool to characterize changes in the expression of this molecular biomarker in females without the need to quantify changes in gene expression. The Japanese medaka transgenic line developed in this study shows AcGFP fluorescence in ovaries starting at 25 dph, this is more than two weeks after the start of *osp1* sexual dimorphic expression (Abdel-moneim *et al.*, 2015b). Appearance of AcGFP fluorescence at this age limits our ability to utilize this line in high throughput screening assays. In addition, the sensitivity of this transgenic line to estrogenic exposures has only been tested with a potent synthetic estrogen (EE_2) and at a concentration that lacks environmental relevance.

4.5.3 Conclusions

In this paper we describe the development of an *in vivo* visual reporter system for rapid detection of contaminants with estrogenic/androgenic potential. A see-through transgenic Japanese medaka expressing AcGFP driven by the promoter region of *osp1*, a sensitive molecular biomarker of estrogenic and androgenic contaminants, was used to test the sensitivity and specificity after exposure to potent synthetic estrogens. Further studies will be implemented to test the sensitivity and specificity of this transgenic line to putative androgenic contaminants. Once completed, this transgenic line and *in vivo* visual reporter system might provide regulatory agencies with a cost and time effective, predictive, wide-scale applicable tool for monitoring and testing EDCs. This tool would integrate advantages from both *in vitro* and *in vivo* testing methods by utilizing a simple and fast approach to quantify a sensitive endpoint at the whole animal level.

Acknowledgements: The authors thank M. Hensley, J. Zhang, G. Weisenbach, T. Vernon, A. Abdu for their assistance in fulfilling this work. We also thank B. Cooper of

the Purdue University Metabolite Profiling Facility for assistance with HPLC-mass spectrometric analysis. We thank the American College of Laboratory Medicine, the Department of Forestry and Natural Resources at Purdue University, and the Cultural Affairs and Mission Sector at the Egyptian Ministry of Higher Education for providing funding for this study.

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Zeng Z, Shan T, Tong Y, Lam SH, Gong Z. 2005. Development of estrogen-responsive transgenic medaka for environmental monitoring of endocrine disrupters. *Environ. Sci. Technol.* **39**:9001-9008.

Zhao Y, Hu J. 2012. Development of a molecular biomarker for detecting intersex after exposure of male medaka fish to synthetic estrogen. *Environ. Toxicol. Chem*. **31**:1765-1773.

Zhao Y, Wang C, Xia S, Jiang J, Hu R, Yuan G, Hu J. 2014. Biosensor medaka for monitoring intersex caused by estrogenic chemicals. *Environ. Sci. Technol*. **48**:2413-2420.

Zhu L, Wang H, Liu H, Li W. 2015. Effect of trifloxystrobin on hatching, survival, and gene expression of endocrine biomarkers in early life stages of medaka (*Oryzias latipes*). *Env. Toxicol.* **30**:648-655.

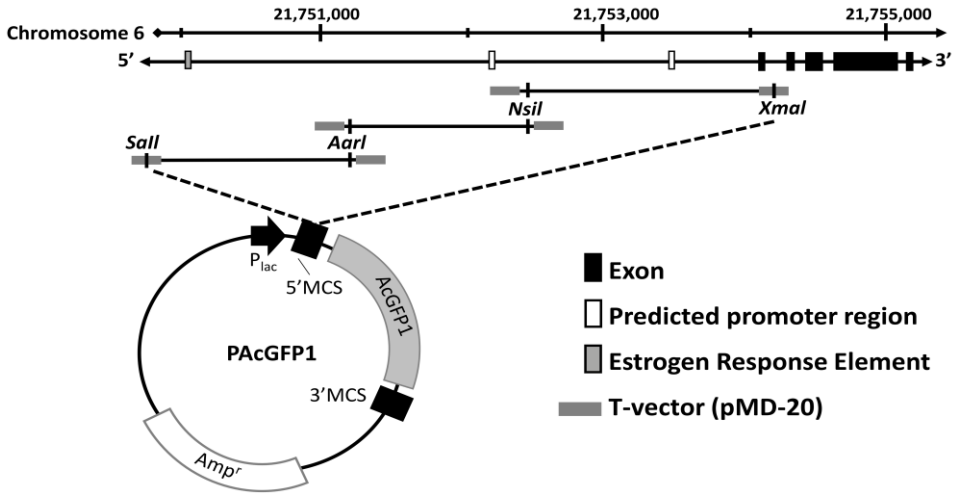

Figure 4.1: Graphical representation of *osp1* exons on chromosome 6, its targeted promoter region, the three amplified fragments, and their site of insertion in the PAcGFP1 vector.

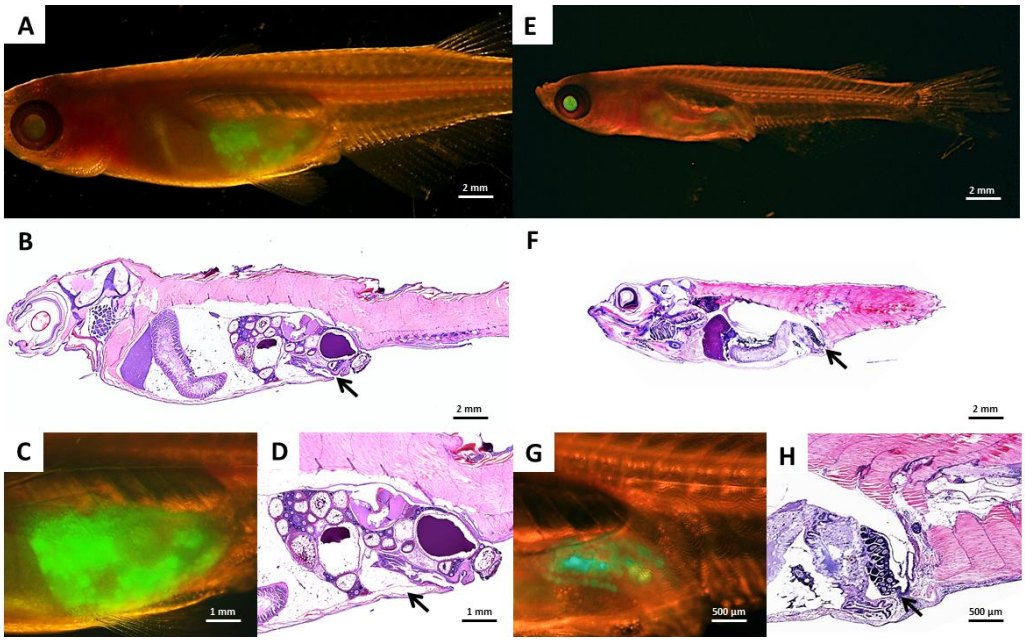

Figure 4.2: AcGFP expression in OSP1-AcGFP transgenic medaka at 70 dph (A and C) and 30 dph (E and G), and hematoxylin and eosin stained lateral sections in the same individuals showing the location of ovary (black arrow, B, D, F and H, respectively).

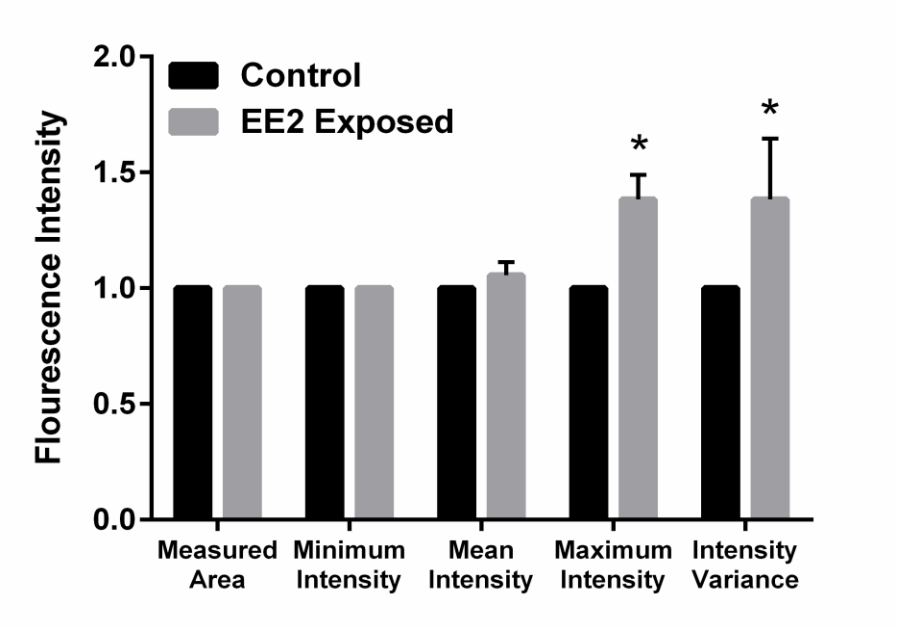

Figure 4.3: Relative changes in AcGFP fluorescence intensity in 30 dph transgenic female Japanese medaka controls in relation to EE_2 exposed (565 ng/L) for 24 h. Results are expressed as mean ± standard error of 3 independent determinations and asterisks indicate significant differences between control and EE_2-exposed groups ($p < 0.05$).

4.7 Supplementary information

Supplementary Table 4.1: Primers used in PCR reactions with their melting temperature (Tm) and product size.

Gene	Primer sequence	Tm (°C)	Product size (bp)
osp1-promoter1	5’-GCCCTTCAATCTTACCTGAA-3’	52.2	1695
	5’-TCTGCTGGTTGCTATTGAC-3’	52.5	
osp1-promoter2	5’-CCAAGGAGGCAAGTCTGTAA-3’	54.5	1339
	5’-CCCCTGAACAAATTCGGAAG-3’	53.8	
osp1-promoter3	5’-TGTATGGAAAGGTTGGGGTC-3’	54.6	1361
	5’-CCCATAGTAAGGAGGAAGCC-3’	54.4	
osp1-acgfp2	5’-ACCAATTAACCCATGAGCCT-3’	54.1	1476
	5’-ACAGGTAGTGGTTATCGGGC-3’	56.6	

CHAPTER 5. CONCLUSIONS

5.1 Chapter 1

Understanding the ecological consequences of gonadal intersex development in gonochoristic (fixed sex) fishes and amphibians has become increasingly important, given the fact that estrogenic and androgenic contaminants have developed into a persisting component of aquatic ecosystems. However, drawing conclusions from studies examining gonadal intersex in a single species from a single geographic region is challenging, thus we decided to synthesize the current knowledge of intersex in gonochoristic fishes and amphibians in order to find general patterns and help focus future research on the causes and consequences of estrogenic/androgenic contaminant exposure to aquatic organisms. Our findings from this literature review indicated that the majority of the manuscripts (84%) reporting gonadal intersex in fishes and amphibians were published after 1999, with the majority of cases coming from North America and Europe. Testicular oocytes (TOs) was the most commonly reported form of gonadal intersex. For fishes, smallmouth bass *Micropterus dolomieu* and largemouth bass *Micropterus salmoides* appear highly sensitive, whereas northern leopard frogs *Lithobates pipiens* and green frogs *Lithobates clamitans* have shown the highest reported cases of TOs among amphibians. Thus, their use has been recommended for monitoring and screening for contaminant exposure. We also concluded that establishing causal relationships linking ambient concentrations of contaminants with estrogenic/androgenic potential requires careful planning, including the use of suitable sample sizes, appropriate study site selection, and the use of long-term passive sampling approaches for the quantification of aquatic pollutants. Finally, our findings suggest that discovery of suitable biomarkers for the diagnosis of gonadal intersex will aid in the development of sensitive tools for identification of this condition.

5.2 Chapter 2

The development of TOs in gonochoristic fish species is a condition that has been widely reported across the US. No comprehensive analysis of TOs prevalence has been performed before in Indiana, particularly using a sentinel species such as smallmouth bass. Histological analysis has been the primary tool to identify this condition, but it bears several limitations including the fact that histopathological changes like TOs can be easily overlooked. Thus, our objectives in this study were to 1) quantify the prevalence and severity of TOs in smallmouth bass sampled from the St. Joseph River in northern Indiana, and 2) to develop molecular biomarkers for TOs. Our findings included high prevalence levels of TOs among smallmouth bass sampled from the St. Joseph River and tributaries in northern Indiana, a watershed previously identified as having medium to high potential to induce gonadal intersex. Prevalence and severity of TOs reached 100% in some sites, and significant decreases in prevalence and increases in severity of TOs occurrence were recorded after the spawning season. Vitellogenin (*vtg*) transcripts were up-regulated in livers of males with TOs sampled during the spawning season and positively correlated to plasma VTG levels, suggesting the suitability of using this gene and protein as a non-destructive molecular biomarker of gonadal intersex in this species. Finally, the use of passive sampling devices (POCIS) helped us quantify the levels of 43 contaminants in surface water at representative sites. Detectable levels of endocrine disruptors were recorded at multiple sites, but no correlations with the prevalence or severity of TOs was found.

5.3 Chapter 3

Gonadal intersex in gonochoristic fish species has been studied extensively in relation to exposure to estrogenic and androgenic contaminants, and as a result causal links have been established. However, identifying this reproductive endpoint at early stages of gonadal development in fish has been a challenge. This limitation hinders the applicability of this endpoint in wide-scale screening tools for monitoring and testing these types of contaminants. Our objective in this study was to identify a gene with a strong female-specific expression during early stages of gonadal development, and to

check its suitability as a biomarker predictive of gonadal intersex development as a result of exogenous hormone exposure. Using Japanese medaka (*Oryzias latipes*), we were successful in identifying a gene that can be utilized as a biomarker of exposure to estrogenic and androgenic contaminants and for development of gonadal intersex. Ovary structure protein 1 (*osp1)* is a gene with strong female-specific expression during gonadal differentiation with expression localized to the ovarian tissue. The expression levels of *osp1* in females decreased to male levels after exposing larvae (15-25 dpf, d post fertilization) to environmentally relevant levels of 17β-trenbolone. Importantly, declines in *osp1* expression in females correlated with the development of gonadal intersex at later life stages.

5.4 Chapter 4

Screening large numbers of contaminants for estrogenic/androgenic potential requires the development of novel screening assays that are cost-effective, high-throughput, and at the same time, quantify reproductive endpoints relevant for both human and environmental risk assessments. Thus, our main goal for this study was to develop an *in vivo* visual reporter system for rapid detection of contaminants with estrogenic/androgenic potential. *Osp1* was selected to drive the expression of a reporter protein (*Aequorea coerulescens* green florescence protein, AcGFP) in a Japanese medaka see- through transgenic line. We succeeded in using this transgenic line in an *in vivo* visual reporter system for identifying estrogenic contaminants, which supported our overall hypothesis that molecular biomarkers are sensitive tools that can be used as ideal endpoints in wide-scale estrogenic/androgenic contaminant screening assays.

5.5 Future Research

Our studies support the hypothesis that molecular biomarkers involved in sex differentiation and gonadal development are sensitive tools that can be used for early detection of exposure to estrogenic/androgenic contaminants in field and laboratory settings. More research is needed to assess the specificity and robustness of *vtg* for its

use as a non-invasive biomarker of gonadal intersex in smallmouth bass and in other sentinel freshwater fish species. Also, further investigations are needed to develop other molecular biomarkers that would show lower variability between seasons while maintaining high sensitivity and ecological relevance. More comprehensive studies investigating contaminant exposures and mixture effects in aquatic ecosystems are also required. The findings of these comprehensive studies, with the aid of sensitive biomarkers of gonadal intersex, will provide a clearer understanding of the etiology of gonadal intersex conditions.

Exposure of the pOSP1-AcGFP Japanese medaka transgenic line (developed in Chapter 4) to different concentrations of androgenic/estrogenic contaminants should be performed to further validate the sensitivity and suitability of this line as an *in vivo* visual reporter system for the rapid detection of contaminants with estrogenic/androgenic potential. If further tests prove successful, the transgenic line and *in vivo* visual reporter system developed in this study can provide regulatory agencies with a time and cost effective wide-scale screening assay for testing contaminants for estrogenic and androgenic potential.

We also discovered a gap in the knowledge of the molecular signaling pathways behind the development of gonadal intersex in gonochoristic fish species. Thus, we are currently trying to understand the pathways behind the development of this condition with the aid of transcriptomic approaches such as RNA-Seq. Exposure of Japanese medaka to a synthetic estrogen (ethinyl estradiol) at concentrations known to elicit gonadal intersex and complete sex reversal (male → female) are currently being conducted to increase our understanding of the molecular signaling mechanisms behind these gonadal changes. This study can also provide more candidates genes to be tested for their suitability as biomarkers for estrogenic contaminant exposure.

VITA

EDUCATION

Ph.D. Fisheries & Aquatic Sciences (Specialization: Aquatic ecotoxicology) 2016
Purdue University, College of Agriculture, West Lafayette, IN, USA

M.S. Applied Ecology (Specialization: Environmental Quality Evaluation and Ecotoxicology) 2011
University of Coimbra, Faculty of Science and Technology, Coimbra, Portugal.

M.S. Applied Ecology (Specialization: Ecology and Biology of Populations) 2011
University of Poitiers, Faculty of Fundamental and Applied Science, Poitiers, France.

D.V.M. (Specialization: Veterinary Medicine and Animal Surgery) 2007
Assiut University, Faculty of Veterinary Medicine, Assiut, Egypt.

RESEARCH EXPERIENCE

Graduate Research Assistant, Department of Forestry and Natural Resources, School of Agriculture, Purdue University. 2012−current
Research advisors: Sepúlveda, M. S.; Mahapatra, C. T.; Freeman, J.; Zhang, G.

Graduate Research Assistant, Department of Life Sciences, University of Coimbra, Portugal. 2010−2011
Research advisors: Ribeiro, R.; Moreira-Santos, M.

Research Assistant, Department of Veterinary Forensic Medicine and Toxicology, School of Veterinary Medicine, Assiut University. 2007−2009, 2011-2012

Research advisors: Abdel-nasser, M, Elzeky, M.

PUBLICATIONS

Peer-Reviewed Articles (published)

Abdel-moneim A, Coulter DP, Mahapatra CT, Sepúlveda MS. 2015. Intersex in fishes and amphibians: population implications, prevalence, mechanisms and molecular biomarkers. *Journal of Applied Toxicology*. 35(11):1228–1240.

Abdel-moneim A, Mahapatra CT, Hatef A, Sepúlveda MS. 2015. Ovarian structure protein 1: A sensitive molecular biomarker of gonadal intersex in female Japanese medaka after androgen exposure. *Journal of Environmental Toxicology and Chemistry*. 34(9):2087–2094.

Abdel-moneim A, Moreira-Santos M, Ribeiro R. 2014. A short-term sublethal toxicity assay with zebrafish based on preying rate and its integration with mortality. *Chemosphere* 120: 568–574.

CONFERENCE PRESENTATIONS

International/national meetings

Abdel-moneim A, Deegan D, Gao J, Sepúlveda MS. Sampling season, a factor influencing prevalence of gonadal intersex and molecular biomarkers in smallmouth bass. SETAC North America 37th Annual Meeting. 2016. Orlando, FL. Poster Presentation.

Abdel-moneim A, Sepúlveda MS. Molecular signaling pathways elicited by 17-α-ethinylestradiol in intersex and sex-reversed Japanese medaka. SETAC North America 37th Annual Meeting. 2016. Orlando, FL. Poster Presentation.

Godfrey A, **Abdel-moneim A**, Sepúlveda MS. Toxicity and thyroid disrupting effects of halogenated mixtures on zebrafish embryos. SETAC North America 37th Annual Meeting. 2016. Orlando, FL. Poster Presentation.

Abdel-moneim A, Mahapatra CT, Sepúlveda MS. *In vivo* visual reporter system for endocrine disrupting chemicals using transgenic see-through Japanese medaka *Oryzias latipes*. The 34th Annual Meeting of the Ohio Valley Chapter of the Society of Toxicology. 2016. Indianapolis, IN. Poster Presentation.

Abdel-moneim A. Hormones in the environment: Causes, consequences, and biomarkers of effects. SerPIE - One Health conference 2016. Huntsville, *al.* Platform presentation.

Abdel-moneim A, Mahapatra CT, Sepúlveda MS. Screening assay for non-invasive visualization of effects of endocrine disruptors using see- through Japanese medaka *Oryzias latipes*. Pharmaceutical and Personal Care Products in the Environment Conference. 2016. Champaign, IL. Poster Presentation.

Abdel-moneim A, Mahapatra CT, Sepúlveda MS. Toward an *in vivo* high- throughput screening assay for non-invasive visualization of effects of endocrine disruptors. 5th Young Environmental Scientist Meeting. 2016. Gainsville, FL. Platform Presentation.

Abdel-moneim A, Mahapatra CT, Sepúlveda MS. Molecular biomarkers for non-invasive visualization of effects of endocrine disruptors using see- through Japanese medaka *Oryzias latipes*. The 33rd Annual Meeting of the Ohio Valley Chapter of the Society of Toxicology. 2015. Highland Heights, KY. Poster Presentation.

Abdel-moneim A, Deegan D, Guffey SC, Mahapatra CT, De Perre C, Lee L, Sepúlveda MS. Gonadal intersex in smallmouth bass *Micropterus dolomieu* in northern Indiana: Prevalence, severity, molecular biomarkers and novel screening methods. SETAC North America 36th Annual Meeting. 2015. Salt Lake City, UT. Poster Presentation.

Abdel-moneim A, Mahapatra CT, Sepúlveda MS. A novel molecular biomarker for early prediction of the effects of exposure to endocrine disrupting chemicals in Japanese medaka *Oryzias latipes*. SETAC North America 35th Annual Meeting. 2014. Vancouver, BC, Canada. Poster Presentation. **3rd Place Phd Student Poster Award.**

Abdel-moneim A, Mahapatra CT, Sepúlveda MS. Development of molecular biomarkers for detection of gonadal sex reversal in Japanese medaka after exposure to synthetic estrogens and androgens. The 31th Annual Meeting of the Ohio Valley Chapter of the Society of Toxicology. 2013. Louisville, KY. Poster Presentation.

Abdel-moneim A, Mahapatra CT, Sepúlveda MS. Expression of genes involved in sex differentiation across several developmental stages in Japanese medaka (*Oryzias latipes*). International Association for Great Lakes Research 56th Annual Conference. 2013. West Lafayette, IN. Poster Presentation.

Local meetings

Abdel-moneim A, Mahapatra CT, Sepúlveda MS. Transgenic see- through Japanese medaka *Oryzias latipes* line for non-invasive visualization of effects of endocrine disruptors. ESE Symposium 2016. 2016. West Lafayette, IN. Poster Presentation. **1st place award - Graduate poster**

Abdel-moneim A, Degan D, Gao J, Sepúlveda MS. Vitellogenin – A biomarker for gonadal intersex showing seasonal variability in smallmouth bass. FNR Research Symposium. 2016. West Lafayette, IN. Poster Presentation.

Godfrey A, **Abdel-moneim A**, Sepúlveda MS. The effects of halogenated mixtures on zebrafish thyroid function. FNR Research Symposium. 2016. West Lafayette, IN. Poster Presentation.

Vernon T, **Abdel-moneim A**, Sepúlveda MS. Development of a fish transgenic line for use in environmental toxicology. Undergraduate Research & Poster Symposium. 2016. West Lafayette, IN. Poster Presentation.

Abdel-moneim A, Deegan D, Mahapatra CT, Sepúlveda MS. Prevalence of gonadal intersex in smallmouth bass *Micropterus dolomieu* in northern Indiana and differential expression of candidate biomarkers. ESE Symposium 2015. 2015. West Lafayette, IN. Poster Presentation.

Flores A, Smith C, Ready Z, **Abdel-moneim A**, Mashtare M, Sepúlveda MS. Tracking decomposition rates using a model mammal for wildlife forensic studies. ESE Symposium 2015. 2015. West Lafayette, IN. Poster Presentation**. Best Undergraduate Research Poster Award.**

Abdel-moneim A, Deegan D, Mahapatra CT, Sepúlveda MS. Gonadal intersex in smallmouth bass *Micropterus dolomieu* in northern Indiana: Prevalence and molecular biomarkers. FNR Research Symposium. 2015. West Lafayette, IN. Poster Presentation.

Abdel-moneim A, Coulter DP, Mahapatra CT, Sepúlveda MS. General patterns and research gaps in studies investigating intersex in wild populations of fish and amphibians. Health and Disease: Science, Culture and Policy Poster Session. 2015. West Lafayette, IN. Poster Presentation.

Abdel-moneim A, Deegan D, Mahapatra CT, Sepúlveda MS. Relationship between prevalence of gonadal intersex in smallmouth bass *Micropterus dolomieu* in northern Indiana and estrogen contaminant levels. Sigma XI Grad Student and Post-Doc Research Competition. 2015. West Lafayette, IN. Poster Presentation.

Abdel-moneim A, Mahapatra CT, Sepúlveda MS. Ovarian structure protein 1 (Osp1): A novel molecular biomarker for exposure to endocrine disrupting chemicals in Japanese medaka *Oryzias latipes*. FNR Research Symposium. 2014. West Lafayette, IN. Poster Presentation. **Best Phd Research Poster Award**.

HONORS AND AWARDS

The most recent and notable of these honors and awards are:

- Best graduate research poster award, ESE symposium. West Lafayette, IN (2016).
- Best research poster award, FNR symposium. West Lafayette, IN (2015).
- Best poster award (3rd place), SETAC North America 35th Annual Meeting. Vancouver, BC, Canada (2014).
- FNR graduate assistantship, Purdue University, West Lafayette, IN (2016).
- Graduate Tuition Scholarship (GTS), Purdue University, West Lafayette, IN (2012–2016).
- Governmental scholarship from the Egyptian government, administrated by the Egyptian Cultural and Educational Bureau (ECEB), Washington DC (2012–2016).
- Erasmus Mundus Scholarship Award, EMMC-EMAE European Consortium (2009-2011).
- University honors for B.V.Sc. students. Assiut University (2007).
- Student travel award, Young Environmental Scientist (YES) Meeting, Gainsville, FL (2016).

- Student travel award, offered by the Egyptian Cultural and Educational Bureau (ECEB) to attend 5th YES meeting. Gainesville, FL (2016).
- Student travel award, SETAC North America 36th Annual Meeting. Salt Lake City, UT (2015).

TEACHING EXPERIENCE

Graduate Teaching Assistant, Department of Forestry and Natural Resources, College of Agriculture, Purdue University. 2016

- Wildlife and Environmental Forensics class (FNR 59800-005_2016), 2 ETCS course.

Graduate Teaching Assistant, Department of Forestry and Natural Resources, College of Agriculture, Purdue University. 2016

- Aquatic Sampling Techniques class (FNR 35100_2016), 2 ETCS course with lecture and lab.

Teaching Assistant, Department of Veterinary Forensic Medicine and Toxicology, College of Veterinary Medicine, Assiut University. 2007−2009, 2011-2012

- Veterinary Toxicology class, 3 ETCS course with lecture and lab.
- Veterinary Forensic Medicine, 3 ETCS course with lecture and lab

Other teaching experiences:

- Graduate Instructional Development Certificate (GIDC), Center of Instructional Excellence (CIE), Purdue University (in process).
- Invited lecturer, presentation title “Fish health assessment”, Aquatic Sampling Techniques class (FNR 351), Purdue University. February 2nd, 2016.
- Invited lecturer, presentation title “Introduction to Fish Hematology and Blood Collection Techniques”, Aquatic Sampling Techniques class (FNR 351), Purdue University. February 4th, 2016.
- Invited lecturer, presentation title “Forensic serology and hematology”, Wildlife Forensic class (FNR 598), Purdue University. October 20th, 2015.
- Invited lecturer, presentation title “Ballistics and bullet trajectory”, Wildlife Forensic class (FNR 598), Purdue University. October 22th, 2015.

LEADERSHIP ROLES

- Student liaison at SETAC North America Science Committee (2014-present)
- Member at Large at SETAC North America Student Advisory Committee (NASAC) (2016-present)
- Associate member at SETAC NASAC (2015)
- Member of the Department of Forestry and Natural Resources (FNR) Graduate Student Council (2013-present)
- FNR Graduate Student Senator at Purdue's Graduate Student Government (PGSG) (2013-present)
- Member of the Graduate Students Affair Committee, Purdue University (2013-present)
- Treasurer of the Egyptian Student Association at Purdue (ESA-P) (2013-2014)

AFFILIATION WITH PROFESSIONAL ORGANIZATIONS

- Society of Environmental Toxicology and Chemistry (SETAC) (2014-present)
- Society of Toxicology (SOT) (2015-present)
- Ohio Valley Chapter of the Society of Toxicology (OV-SOT) (2013-present)
- Egyptian Society of Environmental Toxicology (ESET) (2010-present)
- Indiana Academy of Science (2015-present)