ABSTRACT

Title of Thesis: A NOVEL NON-LETHAL LAPAROSCOPIC APPROACH TO DETECT INTERSEX (TESTICULAR OOCYTES) IN LARGEMOUTH BASS (MICROPTERUS SALMOIDES) AND SMALLMOUTH BASS (MICROPTERUS DOLOMIEU)

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The appearance of testicular oocytes (TO) in wild fish populations has received considerable attention in the scientific literature and public media. Current methods to quantify TO are lethal; instead, a non-lethal alternative was examined. Laparoscopic insertion into the genital pore allowed internal visualization of the gonad and detection of TO by collecting five testis biopsies in smallmouth bass *Micropterus dolomieu* and largemouth bass *Micropterus salmoides*. Overall, biopsies quantified similar levels of TO detection and severity to conventional transverse sectioning with less than 10% mortality. Suitability of surgical anesthetics, tricaine methanesulfonate and electronarcosis were examined in laboratory and field applications. Electronarcosis had the added benefit of rapid sex identification and immediate release of female fish with minimal trauma, representing significant benefits when sampling small or compromised populations.

Laparoscopy may be useful for monitoring the prevalence and severity of TO in these fish species when lethal sampling is not a desired outcome.

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by

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DEDICATION

This thesis is dedicated to my sisters, Kailey and Lacy, and my brother, Clinton, and especially to my beloved father Brian “Highlander” MacLeod, whose bravery and intelligence continues to guide me through life. Thank you for everything.

To all MacLeods, “HOLD FAST AND ALWAYS SHINE BRIGHTLY”.

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EXECUTIVE SUMMARY

Over the past several decades, concern over numerous discoveries of gonochoristic (fixed-sex) fish species with gonadal abnormalities in the environment has spurred interest in the field of endocrine disruption. One of the most widely studied abnormalities is male fish with immature oocytes in their testes, commonly referred to as testicular oocytes (TO). The presence of TO is considered a pathological condition, believed to result from exposure during sexual differentiation or periods of gonadal recrudescence. Currently, the only available method for detecting TO is to sacrifice the fish, remove the testes, prepare tissue sections via routine histology and observe by light microscopy. This method is lethal, therefore often limits the frequency of sampling, the number of fish sampled, and the species permissible for sampling. When investigating threatened or endangered species, or in regions where fish are few in number, or where populations are already in decline, sacrificial methods are unfavorable and possibly prohibited.

To overcome these obstacles, a novel non-lethal, single-entry laparoscopic tissue sampling method was investigated as an alternative to the common lethal practices employed to detect TO. Laparoscopic insertion into the body cavity via the genital pore allows testis tissue collection via biopsy without penetration through the body wall. This single-entry method has been previously demonstrated to be effective for non-lethal sex identification of sexually immature male and female LMB. This was accomplished by internal visualization of the gonad with histologic confirmation using gonadal tissue collected by biopsy with high survival, rapid healing, and minimal infection. This study expands on that research by collecting multiple testis biopsies (n = 5) from each fish. This

minimally invasive method was employed to validate its use for sex determination and explore the possibility of detecting TO in largemouth bass *Micropterus salmoides* (LMB) and smallmouth bass *Micropterus dolomieu* (SMB). Both species are of commercial and cultural importance in the Mid-Atlantic region and along the East coast of the United States.

Single-entry laparoscopy and conventional transverse sectioning methods were compared for detection of TO using 79 SMB from 8 sites and 44 LMB from 3 sites. Overall, transverse sectioning resulted in higher detection of TO with the highest proportion of males detected with TO and the most severe sites occurring in SMB. In SMB, TO prevalence detected by transverse and biopsy sections was strongly correlated across sites ($r^2 = 0.81$) and severity was moderately correlated across sites ($r^2 = 0.59$). Transverse site severity determined by a common scoring method ranging from zero to four, ranked SMB as high as 2.2 ± 1.29 whereas, the most severe LMB site ranked as 0.6 ± 0.78. In SMB, multiple biopsy sampling detected TO in 66% of individuals, while transverse sectioning detected TO in 76% of individuals. The percent of individuals detected at each of the eight sites was generally comparable in biopsies (17% to 100%) to transverse sections (42% to 100%), these similar trends in TO detection across sites indicates the effectiveness of using biopsies. In SMB, the total proportion of individuals identified with TO using both sampling methods was 81% (64 of 79), the union being greater than the amount indicated by either method alone. In LMB, multiple biopsy sampling detected TO in 18% of individuals, while transverse sectioning detected TO in 45% of individuals. The percent of individuals detected at each of the three sites was often lower in biopsies (10% to 30%) to transverse sections (30% to 50%). In LMB, the

total proportion of individuals identified with TO using both sampling methods was 48% (21 of 44), the union minimally greater than the amount indicated by either method alone. Overall, transverse sectioning resulted in higher detection in both species; SMB had a higher proportion of males with TO and more severe cases than LMB. The positive correlation between the number of TO detected and the transverse severity rank index provides pathologists with a strong relationship for relating severity ranks to the number of TO in black bass species using non-lethal laparoscopic biopsy collection, and the method is potentially applicable in other fish species with similar urinary bladder elasticity.

This research indicates that single-entry laparoscopy may be useful for monitoring the prevalence and severity of TO in black bass species, especially when lethal sampling is not necessary. In SMB, fish at sites with low TO severity were more frequently detected by transverse sections, whereas sites with moderate severity were detected by both methods similarly. In LMB, severity was low at all sites sampled; consequently detection was low in biopsies. Male LMB (n = 20) post-operative recovery was high (90%) for survival and recovery of urinary bladder (≥ 28 days). Our results demonstrate that: (1) biopsies are capable of detecting TO and quantifying TO severity, (2) level of detection by both methods rises as severity increases, and (3) laparoscopic investigations for TO is applicable as a non-lethal alternative.

Hypotheses & Objectives

H_1: Non-lethal collection of testis by multiple biopsy using laparoscopic entry via the genital pore will effectively detect TO prevalence and severity with the same efficacy as conventional transverse sectioning.

H_2: Fish that experience laparoscopic entry and multiple biopsy will have full urinary bladder recovery and survive for 28 days with no signs of injury.

H_3: Fish that experience laparoscopic entry and multiple biopsy will release mature sperm when tested 28 days post-surgery.

These hypotheses were tested and evaluated based on the following objectives:

First Objective – The collection of representative tissue for the purpose of TO quantification requires that the technician is able to maneuver the biopsy forceps to retrieve tissue from the area where the nerves and blood vessels are concentrated, within the inner region of the testis where TO are concentrated. This means that the biopsy forceps must be properly navigated around the exterior of the testis to the area with the highest likelihood of TO, specifically located where the testis attaches via mesenteries to the swim bladder. Laparoscopic tissue collection of gonadal tissue has been used in one study, retrieving a single biopsy from each individual. Alternatively, the current study is based on the collection of five biopsies along the length of one lobe of the testis.

Second Objective – Satisfying the first objective is necessary for the application of this tool for detection of intersex, but if complications arise confounding healing or survival, then it has little added value compared to the speed and ease of sacrificing each organism. Thus, for this to be a viable application of laparoscopy it must be demonstrated that the fish quickly recovers. Nearly as dire as mortality is eliminating spawning capability of

males after laparoscopic intervention. It is important that collecting multiple biopsies of the testis will not reduce the male's ability to release mature sperm for fertilization of eggs.

CHAPTER 1: INTRODUCTION

ENDOCRINE DISRUPTION OVERVIEW

Over the past several decades, contaminants in the environment with adverse endocrine effects to aquatic organisms have become a global environmental concern (Ankley et al., 2007; Colborn et al., 1993; Hotchkiss et al., 2008). The ever-growing constellation of known or suspected endocrine disrupting chemicals (EDCs) runs the gamut from pesticides and herbicides to plasticizers, flame retardants, heavy metals and pharmaceuticals (Colborn et al., 1993; Diamanti-Kandarakis et al., 2009; Hotchkiss et al., 2008; Marty et al., 2011). These compounds in addition to natural and synthetic steroid hormones can have significant effects on fish and other aquatic organisms, through endocrine-mediated pathways (Davey et al., 2007; Eldridge et al., 2008; Klaper et al., 2006; Kugathas and Sumpter, 2011; Martinović et al., 2008; Martyniuk et al., 2011; Orton et al., 2011). Candidate EDCs reflect virtually all aspects of modern anthropogenic activity including agriculture, manufacturing, wastewater treatment, and solid waste disposal (Ciparis et al., 2012; Kusk et al., 2011; Vajda et al., 2008; Zhang et al., 2016). Generally, EDCs function by interfering with critical physiologic systems responsible for regulation of homeostatic and developmental processes; often this involves mimicking or hindering receptor binding of endogenous hormones causing physiological responses of incorrect magnitude and/or timing (review by Leet et al., 2011).

Exposure to EDCs, whether it is a naturally occurring hormone or synthetic chemical, will likely modulate the natural endocrine activity. The endocrine system is responsible for normal development, regulating nearly all aspects of growth, reproductive, metabolic and behavioral processes, therefore exposure may result in an

adverse physical, behavioral, and/or biochemical change (Kavlock et al., 1996). The focus of most studies on EDCs has been on reproductive steroids, thyroid hormones, and glucocorticoids (Scholz and Mayer, 2008). EDCs affect an extensive range of pathways and are not limited to agonism, by which a compound stimulates a receptor by binding to it, which activates a pathway or responsive genes; or antagonism, where the receptor is blocked or its responsiveness is reduced (Casanova-Nakayama et al., 2011). The endocrine system supports a vast array of regulatory systems and feedback mechanisms, including the hypothalamic-pituitary-gonadal axis as well as the hypothalamic-pituitary-thyroidal axis. In addition to those critical processes, the reproductive system communicates with the immune system and thus, may be modulated by EDCs (Ahmed, 2000; Casanova-Nakayama et al., 2011; Iwanowicz and Blazer, 2011; Shelley et al., 2013).

While the term EDC, encompasses all compounds that may interact with the endocrine system, compounds that stimulate the estrogen receptor (ER) or have analogous estrogenic effects (mimics), commonly referred to as “environmental estrogens”, have been the primary focus in the scientific literature as intersex causative agents (Harris et al., 1997; Jobling et al., 1998; Lange et al., 2009; Purdom et al., 1994; Schultz et al., 2003; Yanbin et al., 2014). Male fishes exposed to estrogenic endocrine disrupting chemicals (EEDCs) can lead to a multitude of developmental and reproductive effects. Non-reproductive effects such as modulating immune response have been associated with EEDCs (Ahmed, 2000; Casanova-Nakayama et al., 2011; Iwanowicz and Blazer, 2011; Iwanowicz and Ottinger, 2008; Robertson et al., 2009; Shelley et al., 2013; Wenger et al., 2014). EDCs enter waterways from point and non-point sources, including

various sources such as wastewater treatment plant (WWTP) effluent (Metcalfe et al., 2003; Kavanagh et al., 2004; Kolpin et al., 2002; Thorpe et al., 2009; Aerni et al., 2004) and agricultural runoff (Blazer et al., 2012; Kolodziej and Sedlak, 2007; Orlando et al., 2004; Sellin et al., 2010; Yonkos et al., 2014). Potential adverse effects to fish include, impairment and/or alteration of growth, maturation, sexual differentiation and expression of secondary sex characteristics, as well as temporal shifts in reproduction due to altered steroid regimes, retarded (or accelerated) gametogenesis, and altered spawning behavior (Harris et al., 2010; Jobling, 2002; Kidd et al., 2007; Nash et al., 2004).

INDICATORS OF ENDOCRINE DISRUPTION

Biomarkers

The effects of EEDCs in fishes have long been the focus as compared to other modes of endocrine disruption such as, androgen and thyroid hormones. The range of effects may vary between species and life history strategies and at many levels of biological significance based on several factors including the exposure chemical(s), exposure period and rate, developmental stage, season, and sex. The effects that have been associated with exposure to EEDCs have ecological significance and extend over several levels of biological organization (molecular, cellular, organ, individual and population; Ankley et al., 2009). The biomarkers commonly used are the measurable direct and indirect effects/responses spanning the several levels of biological organization up to the population level but are generally difficult to translate the likelihood of adverse effects beyond the individual level. The biomarkers often employed to detect feminization of male fish include, but are not limited to, gonadal abnormalities, gene upregulation potentially followed by production of female proteins, circulating sex

hormones in plasma (estrogen/testosterone ratio). Additionally, sexually dimorphic species may have altered secondary sex characteristics such as the male fathead minnow *Pimephales promelas* (FHM) with nuptial tubercles, black dot on dorsal fin (van Aerle et al., 2002; Johns et al., 2011; Länge et al., 2001; Leet et al., 2015).

Intersex (Testicular Oocytes)

Intersex is a condition defined by the presence of female germ cells, or oocytes, within the male gonad, referred to as testicular oocytes (TO; Nolan, 2001). In normally gonochoristic species (i.e. species that the phenotypic gender does not transform during the adult life of the animal, fixed-sex) the presence of TO is considered a pathological condition (Hecker et al., 2006) and has been used an indicator of exposure to exogenous estrogenic EDCs (Allen et al., 1999; Blazer et al., 2007, 2012; Jobling et al., 1998). Largemouth bass (LMB; *Micropterus salmoides*) are differentiated gonochoristic, meaning that primordial germ cells are programmed to differentiate into spermatogonia or oogonia (Johnston, 1951), therefore the occurrence of TO should not occur.

Reductions in TO prevalence (the proportion of individuals from a sampled population with at least one TO) and severity (the mean magnitude of TO observed in the sample population as determined by oocyte count, ranking/scoring and/or spatial distribution) in male smallmouth bass *Micropterus dolomieu* (SMB) between spring (pre-spawn) and summer (post-spawn) sampling suggest TO are shed during spawning and may not, by themselves, inhibit breeding (Blazer et al., 2007). Collectively, the literature suggests that individuals with low TO severity maintain normal reproductive function while those with higher severity are more likely to suffer adverse reproductive effects (Blazer et al., 2012; Harris et al., 2010; Jobling, 2002). Severity also tends to correlate

more strongly than does prevalence alone with anthropogenic land use characteristics known to introduce EDCs to surface waters (Blazer et al., 2007, 2012). Despite this knowledge, many studies report only TO prevalence without mention of severity, representing a significant limitation when comparing results between studies (Abdel-moneim et al., 2015; Bahamonde et al., 2013; Baldigo et al., 2006; Hecker et al., 2006; Hinck et al., 2009). Many definitions exist for the term, intersex. It may refer to masculinized females or as in this study, feminization of males exhibiting signs of feminine attributes such as TO and phenotypic sex changes. In European flounder *Platichthys flesus* Bateman et al., (2004) termed the condition "ovotestis", meaning that the tissue was predominately, testicular tissue with areas of oocytes concentrated. Intersex is a term most frequently used for male fish that display feminine attributes. Interestingly, female fish are rarely found with endocrine disruptive attributes as intense or frequent as males.

Vitellogenin (Vtg) Production

The phospholipoprotein vitellogenin (Vtg) is an egg yolk precursor protein necessary for normal oocyte maturation and development in most fish species with males expression generally at low to undetectable levels in the blood of normal males (Denslow et al., 1999; Devlin and Nagahama, 2002; Iwanowicz et al., 2009; Sumpter and Jobling, 1995). Generally, detection of TO in males is coupled with other ephemeral biomarkers of endocrine disruption, such as elevated plasma Vtg levels (Denslow et al., 1999; Devlin and Nagahama, 2002; Iwanowicz et al., 2009; Sumpter and Jobling, 1995). Vtg is produced in the liver and transported via blood and in the presence of exogenous EEDCs males will produce Vtg in a dose-dependent manner, this can cause damage to the liver

and kidneys during development (Hutchinson et al., 2006). For example, rainbow trout *Oncorhynchus mykiss* were caged for two weeks near a sewage treatment effluent outfall in UK rivers had upwards of 100,000-fold induction of Vtg (Purdom et al., 1994). The upregulation of hepatic Vtg gene and production of Vtg protein in males has been linked to exposure to EEDCs in several species, such as SMB, FHM, pearl dace *Margariscus margarita* (Blazer et al., 2014; Iwanowicz and Blazer, 2011; Kidd et al., 2007; Palace et al., 2006, 2009). The biological significance and consequences of long term Vtg induction in male fish are only partially characterized, it is a relatively energy intensive molecule that males do not produce under normal conditions, hence plasma Vtg has become a widely accepted biomarker of exposure to EEDCs (Blazer et al., 2012, 2014; Denslow et al., 1999; Sumpter and Jobling, 1995). Measurement of Vtg in fish can be an indicator of transient or continuous exposures to EEDCs or other types of EDCs that alter the protein synthesis pathways, and is relied on as a universal measure of endocrine disruption in many fish species.

Other Biomarkers & Indicators

Possibly the most compelling evidence for major adverse outcomes are the incidences where EEDCs reduced fertility, leading to collapse of a population (Jobling, 2002; Kidd et al., 2007; Nash et al., 2004; Palace et al., 2006, 2009). Nash et al., (2004) found that zebrafish *Danio rerio* exposed to 5 ng/L of 17α-ethynylestradiol (EE2) throughout an entire generation (egg to mature adult) had a 56% reduction in fecundity and complete population failure due to no fertilization. Induced infertility in male zebrafish was attributed to either nonfunctional testes due to undifferentiated or intersex gonads (Nash et al., 2004). Alternatively, the same concentration of EE2 had no effect on

the parent population (mature adults) with regard to reproductive success. The gonads in females and intersex males were fully-developed although significant malformations of the ovarian and sperm ducts preventing the release of sperm or eggs (Nash et al., 2004). Related effects have occurred in other fish species such as the common carp exposed to an environmentally relevant concentration of the industrial chemical intermediate alkylphenol, 4-tert-pentylphenol (TPP) of 0.14 mg/L, over several ages (-3, 0, or 3 dph, embryos, yolk sac larvae, or feeding larvae) prior to sexual differentiation to determine the presence of a labile period (Gimeno et al., 1997). Exposure to TPP at that concentration starting prior to the period of sexual differentiation and after, resulted in the formation of an oviduct that was considered irreversible, since it remained after transitioning the fish to clean water for 59 days, and no vas deferens were present in these fish (Gimeno et al., 1997). Furthermore, exposures for different durations before or during sexual differentiation significantly reduced the number of primordial germ cells in a dose-related response (Gimeno et al., 1997). Similar effects of TPP were observed in exposures of FHM (van Aerle et al., 2002) with no fertilization of eggs in both studies (van Aerle et al., 2002; Gimeno et al., 1997). Likewise, EE2 exposure for full life-cycle reduced fecundity, produced smaller males, and skewed sex ratios in zebrafish (Nash et al., 2004) and FHM (Länge et al., 2001).

A major aspect of endocrine disruption other than measurable physiological or morphological changes is the effect that it may cause on behavior. Zebrafish exposed to 1.1 ng/L EE2 delayed spawning by up to 15 days, and exposure at 10 ng/L resulted in no spawning during the entire exposure period of 177 days (Wenzel et al., 2001). Similarly, Balch et al. (2004) exposed Japanese medaka *Oryzias latipes* to 10 ng/L EE2 and placed

them with unexposed females and 84% of males did not copulate with low reproductive success. Interestingly, females exposed to the same concentration did not participate in reproductive behavior with unexposed males (Balch et al., 2004). In FHM, estradiol (E2) exposure has been shown to cause necrosis of germ cells and spermatozoa (Miles-Richardson et al., 1999a), in a subsequent study using nonylphenol, exposure of 1.1 or 3.4 µg/L changed the number and size of Sertoli cells and germ cell syncytia (Miles-Richardson et al., 1999b).

An emphasis on EEDCs is evident in the literature but many other non-estrogenic types of EDCs can cause complications for fish species. Jensen et al. (2004) found that FHM exposure to flutamide (anti-androgen) caused spermatocyte degeneration and necrosis as well as increased VTG in males. Anti-androgens can result in feminizing effects by preventing normal androgen action (Filby et al., 2007). Ankley et al. (2003) found that FHM exposed to the androgenic growth promoter, 17-trenbolone, expressed thinned germinal epithelia and heterogeneous spermatogenic activity. More recently, a diabetic medication, Metformin was demonstrated to cause the formation of TO in FHM (Niemuth and Klaper, 2015). This broad range of effects from several types of EDCs outlines the complexity of determining cause and effect in environmental monitoring.

INTERSEX OCCURRENCE IN THE ENVIRONMENT

The presence of intersex in wild fish populations has become of concern as evident by the considerable attention in the scientific literature and public media. The level of concern warranted towards continuing intersex investigations is a contentious topic among scientists, due to high uncertainty of the causative agents, the level of impact at the population level are unknown and/or complicated between species with different

life history strategies; suggesting that laboratory exposures with model species may not translate to wild species. A recent review of studies investigating intersex in gonochoristic fish populations indicates a primary focus (84% of published studies) in freshwater lotic systems (Abdel-moneim et al., 2015). Of 54 intersex species evaluated, those most commonly reported with TO were SMB (10% of reported cases), roach *Rutilus rutilus* (8%) and LMB (7%). The high frequencies of intersex in these particular species may reflect intensity of investigation effort, consider that roach were possibly most studied since they were the first species reported with high intersex incidence and severity (Jobling, 2002; Jobling et al., 1998; Rodgers-Gray et al., 2001). Jobling et al. (1998) found a higher prevalence of intersex in roach downstream of a major WWTP when compared to upstream populations. In severe cases, male wild roach had an ovarian cavity instead of a sperm duct, preventing them from reproducing (Jobling et al., 1998). Years subsequent to the previous observations, field collected male roach were found with severe cases of intersex and reduced milt (semen) quality (i.e., sperm motility, sperm density, and fertilization success), as compared with fish from less contaminated sites (Jobling, 2002).

Shortly after, several reports of intersex SMB and LMB throughout much of the United States were published (Anderson et al., 2003; Baldigo et al., 2006; Blazer et al., 2007, 2012; Hinck et al., 2009; Iwanowicz et al., 2009, 2016; Yonkos et al., 2014). Several large fish health surveys conducted in the United States frequently detected TO in SMB and LMB, while few other species were detected with TO and in few instances (van Aerle et al., 2002; Blazer et al., 2014; Hinck et al., 2009; Iwanowicz et al., 2016). The frequency of detecting TO in SMB has generally been higher than in LMB (Hinck et

al., 2009; Iwanowicz et al., 2016), in addition, SMB often express higher severity than LMB (Blazer et al., 2007, 2012, 2014; Iwanowicz et al., 2016; Yonkos et al., 2014). In the Mid-Atlantic region, some sites with SMB frequently detected with TO also had lower sperm count and less motility than a site with low prevalence (Blazer et al., 2012). Interestingly, TO severity and sperm motility were inversely correlated in this field survey (Blazer et al., 2012). Similar investigations have increased the variety of species with detectable TO such as, white perch *Morone americana* (Kavanagh et al., 2004), gudgeon *Gobio gobio* (van Aerle et al., 2001), greenside darters *Etheostoma blennioides* (Tetreault et al., 2011) and rainbow darters *E. caeruleum* (Fuzzen et al., 2015; Tanna et al., 2013).

Seasonality may be an important predictor of detecting the highest abundance of TO in several species, stressing the importance of consistent sampling for site comparisons. Prevalence and severity of intersex in seasonally sampled SMB from the summer of 2003 to fall 2004 were generally higher in spring (pre-spawn) as compared to summer and fall (post-spawn). The authors suggest the lower detection post-spawn may be due to sloughing from the epithelium since TO were occasionally detected in the lumen of the ducts (Blazer et al., 2007). In 2007 and 2009, darters collected seasonally downstream from WWTPs in Ontario, Canada were also found to have the highest prevalence of intersex pre-spawn in the spring (Tetreault et al., 2011). Along with the importance of temporal sampling for TO at the certain times of the year, is to consider the effects to other biomarkers such as gonadal steroidogenic activity, which is directly influenced by water temperature and photoperiod (Folmar et al., 2001).

Basal Rate of Intersex/Background Level

The complexity surrounding the mechanisms responsible for intersex in fish are not yet known, although several aspects that influence sex differentiation in fish range from anthropogenic releases of EDCs to endogenous steroid hormones to fluctuations in environmental conditions (temperature, pH, and photoperiod). Generally, studies will focus on the measurement of chemical constituents in surface water and rarely, in the sediments; yet a major contributor may be water temperature and has been suggested as a reason for perceived basal rates of intersex (Abdel-moneim et al., 2015). Temperature changes can have long-lasting impacts on gonad development in aquatic organisms with cases of complete sex reversal (Coulter et al., 2015; Goto-Kazeto et al., 2006; Navarro-Martín et al., 2011; Selim et al., 2009). Fish may have a proclivity for a specific sex but they all go through a sex differentiation where a bipotential gonad develops towards either testis or ovary (Devlin and Nagahama, 2002). Furthermore, high EDC concentrations and elevated water temperature have been shown to skew sex ratios in zebrafish, in an additive manner (Brown et al., 2015). Furthermore, the presence of TO in low numbers appears to be a normal phenomenon in numerous fish species (Bahamonde et al., 2013; Hecker et al., 2006).

TECHNIQUES TO DETECT TESTICULAR OOCYTES

As aspects of tissue collection (location on testes) and examination (orientation and amount of tissue observed) have significant implications on the likelihood of TO detection (i.e., prevalence) and on consequent expression of severity, some reconciliation of methods is necessary for meaningful interpretation of results. For example, many studies use multiple transverse sections taken through one or both testis lobes with

number of sections often varying substantially between and within studies (Anderson et al., 2003; Blazer et al., 2007; Fuzzen et al., 2015; Tanna et al., 2013; Vajda et al., 2008). Others use longitudinal sections and again vary in number of sections analyzed (Bateman et al., 2004; Hinck et al., 2009; Ingram et al., 2011; Kellock et al., 2014). With such a broad range in amount of tissue examined between methods, there is a consequent range in sensitivity to detect TO – analogous to improving analytical detection limits by increasing sample size and may have profound implications when severity is low. Method of reporting TO severity can introduce additional confusion (Bahamonde et al. 2013; Abdel-moniem et al. 2015). The first indexed system for scoring TO severity, developed for wild roach, employs values from 0 to 7 to indicate severity gradation based on number, spatial distribution, and developmental stage of observed TO (Jobling et al., 1998) and has been followed by many other scoring methods that are species-specific and have been previously covered in reviews (Abdel-moneim et al., 2015; Bahamonde et al., 2013; Hecker et al., 2006). The first ranking/scoring system for use in SMB (Blazer et al., 2007) was modified from that of Bateman et al. (2004) for European flounder and employs values of 0 to 4 for distribution score while the oocyte developmental stage score was excluded (since TO in SMB are not known to progress beyond the chromatin nucleolus stage) and relies only on spatial distribution and number of oocytes (Blazer et al., 2007). Each histological section receives a rank with the mean ranks per individual reported.

In the Mid-Atlantic region and along the East coast, recreational fresh-water sport fishing for SMB and LMB, also known as black bass, represents a significant revenue stream with numerous stakeholder interests (such as river guides, fishing suppliers,

lodging, restaurants, boat sales and service, etc). Within Atlantic Coastal Plain portions of Maryland (i.e., below the fall line of Western Shore rivers and the entirety of the Eastern Shore) LMB are the only black bass species and the primary target of freshwater recreational sport fishing. In the Piedmont region above the Fall Line of LMB and SMB can overlap. Generally LMB dominate in larger slower water bodies while SMB prevail in faster smaller systems (Love, 2011; Nack et al., 1993; Probst et al., 1984). However, SMB fish kills in the Potomac and Shenandoah Rivers over the last decade appear to have allowed an expansion of LMB beyond their historic range. This would seem to suggest that LMB are comparatively more tolerant to the constellation of stressors causing SMB fish kills, yet LMB are also known to demonstrate significant TO prevalence (Baldigo et al., 2006; Blazer et al., 2007, 2012, 2014; Hinck et al., 2009; Iwanowicz et al., 2009, 2016; Yonkos et al., 2014), and a simplified laparoscopic method for testis tissue collection in LMB has already been developed (Matsche, 2013). In addition to the aforementioned reasons, LMB were chosen rather than SMB for the survival study, as they are robust for use in a controlled lab setting, are more resilient to changes in water quality, and are more readily available from hatcheries. Although LMB are not generally a threatened or endangered species, sampling pressures in small bodies of water with small populations of mature fish can cause impacts to regional fish populations and trophic structures (Beamesderfer and North, 1995). To alleviate this additional sampling pressure on fish populations, especially from degraded/impacted regions, non-lethal techniques for monitoring of endocrine disruption should be employed where possible.

Intersex Detection via Non-lethal Approach

Currently, the preferred method for detecting the presence of TO is to sacrifice the fish, remove the testes, prepare tissue sections via routine histology and observe by light microscopy. This method is necessarily lethal and is therefore disadvantageous (and possibly prohibited) when used on threatened or endangered species or in regions where fish are few in number or where populations are already in decline. Traditional surgical and laparoscopic methods of tissue collection employ one or more incisions through the body wall into the abdomen, require sutures and post-operative care, and often result in high infection rates and subsequent mortality (Divers et al., 2009; Harms, 2005; Matsche, 2011). In contrast, Matsche (2013) pioneered a simplified laparoscopic technique using a single-entry via the urogenital pore as a non-lethal method of *in situ* gonad visualization, sex determination and biopsy tissue collection in LMB. This method is amenable to small fish (≥ 190mm total length), requires no sutures, and results in high (> 90%) survival (Matsche, 2013). Previously, the urogenital pore has been used in shovelnose sturgeon *Scaphirhynchus platorynchus* to view the gonad but without the ability to collect gonadal tissue (Wildhaber et al., 2005).

The scientific literature recognizes a need for methods to monitor populations using non-lethal techniques, to better characterize and track the state of fish populations, to effectively manage and rapidly assess impacts from natural and anthropogenic pressures as conditions fluctuate (Baker et al., 2013; Mills and Chichester, 2005). Investigations into TO onset or seasonal impacts in individual fish by repeated sampling may elucidate temporal trends. With many fish populations threatened by over-exploitation, impacted waters, and competition from invasive species, the development of

non-lethal effective monitoring techniques for abnormalities at the cellular level is paramount. To address this concern, we examined the effectiveness of laparoscopy as a non-lethal, minimally invasive, targeted sampling tool to validate sex determination and to explore TO detection in LMB and SMB. These species had the highest prevalence of TO in a survey throughout the U.S. (Hinck et al., 2009) and were the focus of a recent US wide reconnaissance study (Iwanowicz et al., 2016).

The purpose of the current study was to investigate whether non-lethal laparoscopic tissue collection of multiple biopsies from black bass could generate quantitatively similar levels of TO detection and severity to those generated by the more traditional examination of multiple transverse testis sections. Excising the testis before sectioning affords convenient access to the entire organ and opportunity for ample tissue collection. In contrast, laparoscopic tissue collection via the urogenital pore restricts both convenient access to some regions of the testis as well as the amount of tissue that can actually be collected. To be non-lethal the method must cause sufficiently minor damage to the fish to ensure rapid healing and recovery. And minimizing trauma to fish requires use of a very slender laparoscopic set-up, which can only accommodate small biopsy forceps. Therefore, the amount of tissue that can be collected within each biopsy is comparatively small. This constraint can be overcome by step-sectioning individual biopsies to produce sufficient tissue area for meaningful examination. Given these limitations, the objectives of this study were: (1) to create a non-lethal testis collection method that allows examination of quantitatively and qualitatively similar amounts of tissue to traditional transverse-sectioning; (2) to evaluate the effectiveness of the method at detecting TO in black bass; and (3) to determine the rate of survival of black bass

following application of laparoscopic tissue collection. The first two objectives were addressed by collecting testis tissue from individual male SMB and LMB both by multiple-biopsy and multiple-transverse section collection methods, and comparing TO detection results within individual fish and within sample populations indicated by site. Collection of SMB tissue served for biopsy model development (i.e., to determine the necessary number of biopsies/specimen and step-sections/biopsy to acquire sufficient tissue). This species was selected for model development because it is reported to have the highest TO prevalence and severity in many US freshwater systems and has well-established methods of TO detection and enumeration (Blazer et al., 2007, 2012, 2014, Iwanowicz et al., 2009, 2016). LMB collections served to challenge the resulting biopsy model. The third objective was addressed by maintaining several batches of LMB for up to one month after performing laparoscopic tissue collection to assess healing and survival.

Hypotheses & Objectives

H_1: Non-lethal collection of testis by multiple biopsy using laparoscopic entry via the genital duct will affectively detect TO prevalence and severity with the same efficacy as conventional transverse sectioning.

H_2: Fish that experience laparoscopic entry and multiple biopsy will have full urinary bladder recovery and survive for 28 days with no signs of injury.

H_3: Fish that experience laparoscopic entry and multiple biopsy will release mature sperm when tested 28 days post-surgery.

These hypotheses were tested and evaluated based on the following objectives:

First Objective – The collection of representative tissue for the purpose of TO quantification requires that the technician is able to maneuver the biopsy forceps to retrieve tissue from the area where the nerves and blood vessels are concentrated, within the inner region of the testis where TO are concentrated. This means that the biopsy forceps must be properly navigated around the exterior of the testis to the area with the highest likelihood of TO, located where the testis attaches via mesenteries to the swim bladder. Laparoscopic tissue collection of gonadal tissue has been used in one study, retrieving a single biopsy from each individual. Alternatively, the current study is based on the collection of five biopsies along the length of one lobe of the testis.

Second Objective – Satisfying the first objective is necessary for the application of this tool for detection of intersex, but if complications arise confounding healing or survival, then it has little added value compared to the speed and ease of sacrificing each organism. Thus, for this to be a viable application of laparoscopy it must be demonstrated that the fish quickly recovers. Nearly as dire as mortality, is eliminating spawning capability of males after laparoscopic intervention. It is important that collecting multiple biopsies of the testes will not reduce the male's ability to release mature sperm for fertilization of eggs.

CHAPTER 2: MANUSCRIPT

A NOVEL NON-LETHAL LAPAROSCOPIC APPROACH TO DETECT INTERSEX (TESTICULAR OOCYTES) IN LARGEMOUTH BASS (MICROPTERUS SALMOIDES) AND SMALLMOUTH BASS (MICROPTERUS DOLOMIEU)

ABSTRACT

The appearance of intersex in wild fish populations has received considerable attention in the scientific literature and public media. This study examined a non-lethal and less common method of single-entry laparoscopy by entering the body cavity via the genital pore without incising the body wall, as a minimally invasive sampling technique to collect gonadal tissue for detection of testicular oocytes (TO) in black bass *Micropterus* species. Single-entry laparoscopy and conventional transverse sectioning methods were compared for detection of TO using 79 smallmouth bass (SMB) *M. dolomieu* from 8 sites and 44 largemouth bass (LMB) *M. salmoides* from 3 sites. Overall, transverse sectioning resulted in a higher prevalence; SMB had the highest proportion of males detected with TO and the most severe cases compared to LMB. In SMB, TO prevalence detected by transverse and biopsy sections was strongly correlated across sites ($r^2 = 0.81$) and severity was moderately correlated across sites ($r^2 = 0.59$). This research indicates that single-entry laparoscopy may be useful for monitoring the prevalence and severity of TO in black bass species, especially when lethal sampling is not necessary. In SMB, fish at sites with low TO severity were more frequently detected by transverse sections, whereas sites with moderate severity were detected by both methods similarly. In LMB, severity was low at all sites sampled; consequently detection was low in biopsies. Our results demonstrate that: (1) biopsies are capable of detecting TO and quantifying TO severity,

(2) level of detection by both methods rises as severity increases, and (3) laparoscopic investigations for TO is applicable as a non-lethal alternative.

INTRODUCTION

Over the past several decades, concern over numerous discoveries of gonochoristic (fixed-sex) fish species with gonadal abnormalities in the environment has spurred interest in the field of endocrine disruption (Colborn et al., 1993; Ankley et al., 2007). One of the most widely studied abnormalities is male fish with immature oocytes in their testes, commonly referred to as testicular oocytes (TO) (Kavlock et al., 1996; Mills and Chichester 2005). The presence of TO is considered a pathological condition, believed to result from exposure during sexual differentiation or periods of gonadal recrudescence (Hecker et al., 2006; Mills and Chichester, 2005). The cause(s) of TO are still under investigation and the implications as a predictor of adverse outcomes in natural populations remains uncertain and has been previously reviewed elsewhere (Strüssmann and Nakamura 2002; Bahamonde et al., 2013; Abdel-moneim et al., 2015). Instead, the focus of this study is to compare and discuss commonly used methods to collect, examine (histological sectioning), and report TO in testis tissue; and investigate a new non-lethal method to do the same.

No matter what tissue collection or TO scoring method is employed, only a small fraction of the testis is actually observed. For example, five 6-μm transverse sections taken from a 6 cm long testis (average for a mature male LMB) allows observation of only 1/2,000 of the total tissue. Encountering a single TO suggests that there are likely many more. However, the converse is not supported, encountering no TO in five sections provides little confidence that none exist. Because the entire tissue cannot be observed

(for reasons of practicality), it is impossible to prove the absence of TO. With this in mind, it is important to understand that the two common metrics for TO reporting in the scientific literature (prevalence and severity) respond in different ways to inconsistent sampling efforts. Specifically, prevalence can only increase as additional sections are observed; more sections lead to an increased likelihood of encountering rare TO. In contrast, severity can be averaged across all observed sections resulting in an increasingly accurate reflection of TO abundance with increased sampling effort. For this reason, severity is a far preferable tool when comparing results both within and across studies. Severity has also been found to be more informative when investigating correlations with watershed land use characteristics (Blazer et al., 2012) and contaminant exposures (Alvarez et al., 2009).

Collectively, the literature suggests that fish with low TO severity maintain normal reproductive function while those with higher severity are more likely to suffer adverse reproductive effects (Blazer et al., 2012; Harris et al., 2010; Jobling, 2002). Despite the apparent benefit of reporting severity, many studies have only reported TO prevalence. This disparity represents a significant limitation when comparing and interpreting the literature (Hecker et al., 2006; Bahamonde et al., 2013; Abdel-moneim et al., 2015). To overcome this problem, the researcher must section tissues consistently and clearly report the number of sections within the study, as previously recommended by (Hecker et al., 2006). Since the majority of the tissue collected is never fully examined, a strategy that collects less tissue may provide the same information. If this could be accomplished without killing the fish, researchers could continue their investigations into the significance of TO without affecting the population adversely. Even after decades of

research on TO in wild fish populations, there continues to be high uncertainty as to cause(s) and the biological relevance of TO. Until we know what TO actually means, researchers should incorporate minimally invasive and non-destructive sampling strategies in future studies to reduce impact on sensitive wild populations.

Currently, the only available method for detecting TO is to, sacrifice the fish, remove the testes, prepare tissue sections via routine histology and observe by light microscopy. This method is lethal, therefore often limits the frequency of sampling and the number of fish sampled (generally 10 fish of each sex per site is considered permissible). When investigating threatened or endangered species, or in regions where fish are few in number, or where populations are already in decline, sacrificial methods are unfavorable and possibly prohibited. With many fish populations threatened by over-exploitation, degraded habitats, and competition from invasive species, the scientific and regulatory community recognizes an urgent need for non-lethal techniques to reduce pressure on fish populations; to better characterize and track the state of fish populations (National Research Council, 2007). This monitoring information will allow for rapid assessment and effective management of impacts to aquatic systems from natural and anthropogenic impacts (National Research Council, 2007).

Current non-lethal procedures consist of traditional surgery and less invasive laparoscopy. These sampling techniques have not been applied to tissue collection for the purpose of TO detection, possibly because they require skilled surgeons to make one or more incisions through the body wall and suture the incision(s), followed by post-operative care and subsequent higher likelihood of infection and mortality (Harms, 2005). These aspects deter common practice as they are time-consuming, expensive, and

require extensive training to perform. In contrast, Matsche (2013) pioneered a simplified single-entry laparoscopic technique by inserting the operating sheath through the urogenital pore and urinary bladder into the body cavity, as a non-lethal method of *in situ* gonad visualization, sex determination and biopsy tissue collection using largemouth bass *Micropterus salmoides* (LMB). This method is amenable to moderately small fish (≥ 190mm total length), requires no sutures, and resulted in high survival (> 90%) (Matsche, 2013).

To address the gap between easy-to-perform lethal collection strategies and difficult-to-perform non-lethal surgical methods, we examined the effectiveness of single-entry laparoscopy as a minimally invasive, moderate-to-perform, targeted sampling tool to validate sex determination and to explore TO detection in LMB and SMB, both species of commercial and cultural importance in the Mid-Atlantic region and along the East coast. The objectives of this study were to determine the extent that single-entry laparoscopy could: (1) detect TO in black bass; (2) provide quantitatively and qualitatively similar amounts and quality of tissue for observation, as transverse-sectioning; and (3) be deemed non-lethal as determined by the rate of survival. The first two objectives were addressed by using both tissue collection methods side-by-side from the same individual fish and comparing TO detection results. The third objective was addressed by maintaining several batches of LMB for up to one month after performing laparoscopic tissue collection.

METHODS

Specimen Collection

Male SMB (n = 79) were collected during 2014 and 2015 by boat electrofishing from several rivers in the Mid-Atlantic region (Sites B through H) and by electrofishing and hook-&-line in a single river in Vermont (Site A). Resulting SMB sample numbers per site ranged from seven to 14 fish. Male LMB (n = 44) were collected by boat electrofishing from two sites within the Potomac River system (2014) and from a lake in Georgia (2015) (Table 1). Resulting LMB samples per site ranged from ten to 24 fish. SMB was selected for model development because it is reported to have the highest TO prevalence and severity in many US freshwater systems and has well-established methods of TO detection and enumeration (Blazer et al., 2007, 2012, 2014, Iwanowicz et al., 2009, 2016). LMB collections served to test the resulting biopsy model. Black bass species have been recorded to have the highest prevalence of TO in a survey throughout the US (Hinck et al., 2009) and were the focus of a recent reconnaissance study in the Northeast US (Iwanowicz et al., 2016).

Collection of fish occurred in the early spring prior to spawning season and only fish of sufficient size to be reproductively mature (total length ≥ 250 mm) were included. Specific collection locations of wild-caught fish are not reported because it was not the intention of the study to compare TO results of field-collected fish populations with those of reference populations. Rather, the design was meant to compare effectiveness of tissue collection and examination method (biopsy vs. transverse) at TO quantification. Additional male LMB were sourced from a hatchery in Kentucky (n = 24; Smartfish Farms, Auburn KY) exclusively for use in the first recovery and survival study. The

second survival study used LMB from the Potomac River system that were also included for assessment of TO.

Tissue Collection

In accordance with University of Maryland Institutional Animal Care and Use Committee (IACUC; Project Reference No: R-13-16) requirements all fish were anesthetized prior to tissue collection. Fish were anesthetized either in a water bath with buffered tricaine methanesulfonate (100 mg/L Finquel®, Argent Laboratories, Redmond, WA) or with a self-constructed portable electro-immobilization system (Hudson et al., 2011; Matsche, 2013). Once immobilized, fish were measured for total length (mm) and weight (g) (Table 1) before testis tissue was collected. In all instances, fish were anaesthetized and then sacrificed by decapitation prior to testis excision. On sacrifice, fish were dissected, sex was confirmed, and testes were weighed and placed in fixative. Fish condition ($k = [\text{body weight}/\text{length}^3] \times 100$) and gonadosomatic index (GSI = [gonad weight/body weight] x 100; LMB only) were calculated to compare fish responses across sites.

Biopsy and Transverse Tissue Collection

Only LMB testes were biopsied by single-entry laparoscopic surgery on live specimens. Tissue collection by laparoscopy followed the methods of Matsche (2013) with several modifications (see *Supporting Material* for detailed laparoscopic methods). The laparoscopic instrument set (www.meditinc.com) consisted of a 2.7 mm 30° rigid endoscope housed within a 4.8 mm x 190 mm operating sheath, portable LED light source, blunt obturator, and 1.7 mm flexible oval biopsy forceps (Figure 1A). The imaging system was comprised of a 35 mm lens attached to an ImagePro USB endoscope

camera (Medit, Inc.) and viewed from a laptop computer. The operating sheath was inserted into the urogenital opening (Figure 1A), the urinary bladder was perforated using the biopsy forceps and the instrument was advanced into the body cavity. Once inside the body cavity, five biopsies were collected approximately equidistant along the length of the left testis lobe (Figure 1B) guided by video imagery (Figure 1C). The LMB that were intended for post-biopsy survival studies (hatchery and a subset of eight wild-collected fish from the Potomac River system) were allowed to recover (~1 week) in water free of anesthetic immediately after completion of the laparoscopic procedure before being returned to holding tanks or raceways for approximately one month. Therefore, for these fish, fixation (10% neutral buffered formalin) and transverse sectioning of testis occurred approximately one month after biopsy.

In some LMB, biopsies were collected during necropsy by removal of the ventral body wall and using the biopsy forceps independent of the operating sheath and endoscope, due to time constraints. For SMB, biopsies (5/specimen) were collected from excised testes either immediately after sacrifice (Site A) or from archived tissue (Sites B - H) several months after fixation in 10% neutral buffered formalin and transverse sectioned (details in Table 1). Care was taken to ensure biopsies were collected approximately equidistant along the length of the left testis lobe when possible (Site A). However, relative location of biopsies from archived tissues was indeterminable. To be clear, laparoscopy was not used to collect biopsies from any SMB in this study because these fish were part of a larger study requiring rapid sacrifice and preservation of tissues.

Generally, transverse testis sections were generated by cutting segments of 3 mm to 5 mm thickness from five equidistant regions along the length of the left testis. For all

LMB and field-processed SMB (Site A), transverse segments were cut adjacent to sites of biopsy collection. The number of transverse sections from SMB was generally five but occasionally varied to as few as three and as many as seven sections. After adequate fixation, tissues were processed for routine histological evaluation (Figure 2). Briefly, preserved tissue was dehydrated in alcohol, embedded in paraffin wax, sectioned at 6-μm, glass-mounted and stained with hematoxylin and eosin (Presnell et al., 1997). A single set of transverse sections were cut from each fish and examined by light microscopy (Olympus BX51, Tokyo, Japan). Because biopsies were significantly smaller than transverse sections, for the comparison all five biopsies from each fish were embedded together and six step sections, spaced at 30 μm to 120 μm (to represent the entire tissue thickness), were cut, mounted on two glass slides (3 sections/slide) and stained. Histological tissues from 15 LMB (total of 343 biopsies and 75 transverse sections) were digitally photographed, tissue outlines traced, and areas calculated using ImageJ (US National Institutes of Health, Bethesda, Maryland, USA; http://imagej.nih.gov/ij/) to compare the relative amount of observable area from each collection method.

Testicular Oocyte Quantification and Comparison

Histological sections of multiple transverse segments and of multiple biopsies were examined for TO. Briefly, tissue sections of adequate quality from each fish were scanned for the presence of TO under low and moderate magnification (4× and 10× objectives, respectively) with confirmation of presumptive oocytes made under high magnification (40× objective). Results were used to estimate site TO prevalence (calculated as the proportion of individuals from each site with at least one TO) and severity (discussed below) in transverse sections and biopsies.

Transverse severity was reported by TO enumeration and by ranking. For TO enumeration, all observable TO were counted in each transverse section and the mean number of TO/transverse section for each fish was calculated. The same transverse sections were then scored by ranking as described by Blazer et al. (2007) designed for use with SMB. Briefly, the hilus region (indentation where vasculature, nerves and ducts enter the organ; Figure 2B) was examined under moderate magnification (10× objective) and TO observed within a single field of view were ranked as follows: focal distribution (score 1), occurrence of a single oocyte; diffuse distribution (score 2), more than one spatially distinct oocytes; cluster distribution (score 3), more than one but fewer than five closely associated oocytes; and zonal distribution (score 4), multiple oocyte clusters (see Blazer et al. 2007 for detailed description). Both of these methods resulted in five severity scores (TO enumeration and ranking) per fish that were averaged to produce a mean severity score for each individual.

Biopsy severity was calculated by TO enumeration only and each unit scored was mildly different from transverse severity. Each biopsy section composed of 5 biopsies (opposed to a single transverse section) was enumerated for TO resulting in five severity scores per fish that were averaged resulting in a mean severity score for each individual. It should be recognized that each of these sections included all five spatially distinct biopsies so the unit of observation (five biopsies) is qualitatively and quantitatively different than a single transverse section (Figure 2). Due to these and other differences associated with tissue collection, blocking and sectioning methods, no severity ranking system was applied to biopsied tissues.

Survival and Healing

In the first survival study, the viability of laparoscopy was assessed on live hatchery-reared LMB by: assessing healing following perforation of the urinary bladder and biopsy collection, and monitoring of survival over a 28-day period. Fish were anesthetized using tricaine methanesulfonate, placed on a table between two angled blocks to facilitate insertion and gills were kept wet using wetted paper towels. The males were identified via endoscopy and five biopsies were obtained from each fish (n = 24) equidistantly along the length of the left lobe of the testis. A subset (n = 7) of fish were sacrificed immediately as controls and a second subset (n = 5) were sacrificed after one week to investigate testis condition and reparation of the urinary bladder integrity. The remainder (n = 12) were held for sacrifice at 28 days to further investigate testis condition, repair of the perforated urinary bladder and establish likelihood of long-term survival. Fish used in this study (285 mm to 360 mm total length; 350 g to 811 g total wet weight) were obtained in early March 2014, tagged with a passive integrated transponder, and maintained in temperature controlled 2000 L circular tanks receiving aerated de-chlorinated city water in a flow-through system with a biofilter.

In the second survival study, laparoscopy was performed on wild-caught LMB from the Potomac River system (Potomac and Anacostia rivers) with the goal of assessing potential effects to the spawning capability of the male fish. Field-collected LMB were anesthetized using electronarcosis and laparoscopically viewed to definitively establish sex. Females (360 mm to 480 mm total length; 397 g to 1531 g total wet weight) were identified by laparoscopic viewing and received no additional treatment, thereby leaving the urinary bladder intact. In males (280 mm to 400 mm total length; 284 g to

1021 g total wet weight) five biopsies were collected from the left testis lobe. Fish were segregated by sex and held for a short recovery period (~1 week) before pairing in flow-through spawning raceways to monitor for survival.

STATISTICAL ANALYSIS

Quantitative data (e.g., morphometric characteristics; TO counts), if normally distributed, were analyzed by one-way analysis of variance (ANOVA) followed by Holm-Šídák pairwise multiple-comparison test for significance. Where data failed assumptions of normality or homogeneity of variance, estimations used REML (restricted maximum likelihood), Kruskal-Wallis one-way ANOVA on ranks was performed, and significant differences were discerned by Dunn's pairwise multiple-comparison test for site differences. A linear regression (r^2) was used to test for the relation of TO metrics between transverse and biopsy methods at $p = 0.05$. All analyses were performed using SigmaStat version 12.0 (Systat Software, Inc., San Jose, CA, USA) with statistical significance reported.

Figure 1. Laparoscopic testis tissue collection from largemouth bass *Micropterus salmoides*: **A**) external view of examination sheath inserted into the urogenital pore; **B**) testis sampling via biopsy forceps (body-wall removed and surgical clamp employed for demonstration only); **C**) image capture of video from laparoscope showing protruding biopsy forceps approaching testis.

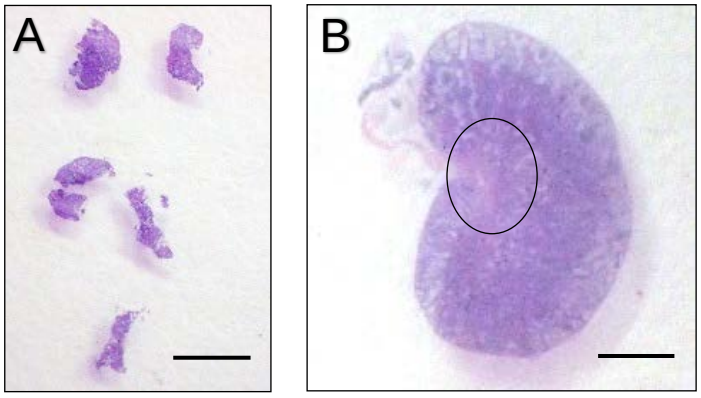

Figure 2. A cluster of five biopsies (**A**) collected from distinct regions along the length of a largemouth bass *(Micropterus salmoides)* testis was selected as an equivalent unit of observation to a single transverse testis section (**B**) for investigation of testicular oocytes (TO) presence and abundance (6-µm sections glass-mounted and stained with hematoxylin and eosin; bar = 2 mm).

RESULTS

Morphometric Parameters

Morphometric measures of SMB varied significantly between many of the sites for weight, total length, and condition factor (Table 1). In general, the population from Vermont (Site A) was significantly shorter and in several instances lighter than the Mid-Atlantic populations (Table 1). Morphometric characteristics of LMB varied significantly between all sites (Table 2). The LMB from the Potomac and Anacostia River sites were generally larger than the Georgia site (Table 2). Fish collected from the Anacostia River had a higher condition factor than Georgia fish but were not significantly different from Potomac River fish (Table 2).

A sub-set of samples analyzed for observable area showed that biopsy-collected tissues (n = 343) were reasonably consistent with a mean area of 2.3 mm^2 and standard deviation of 0.86 mm^2. The resulting average unit of observation for biopsies was 11.5 mm^2; the sum of five biopsies being equivalent to the cross-sectional area of a testis with a diameter of 3.7 mm. By comparison, measured transverse sections (n = 75) had a mean area of 19.0 mm^2 with standard deviation of 9.42 mm^2; equivalent to the cross-sectional area of a testis with a diameter of 6.0 mm. On average, biopsies yielded 61% as much observable area as the transverse sections (recall that the biopsy forceps are a fixed dimension while transverse sections reflect the actual cross-sectional area of the testis).

Smallmouth Bass – Biopsy Method Validation

At all eight SMB sample sites, TO were detected using both tissue collection procedures. Overall, multiple biopsy sampling detected TO in 52 of 79 individuals (66%) while transverse sectioning detected TO in 60 of 79 individuals (76%). Percent TO

prevalence at the eight sites was generally comparable in biopsies (17% to 100%) to transverse sections (42% to 100%) demonstrating similar trends in detection of TO occurrence (Table 1; Figure 3A; sites ordered from low to high TO prevalence). The biopsy method tended to yield lower results than did the transverse method at those sites where TO prevalence was generally low (e.g., Sites A, B, and C), but mostly matched and on one occasion exceeded transverse TO detection where severity was high. Despite these differences, a strong positive correlation was indicated in TO prevalence between the two methods ($r^2 = 0.81$; Figure 4A). It should be noted that TO presence in individual SMB was frequently detected by only one tissue collection method. Of the 60 individuals identified with TO in transverse sections, 12 were not observed to possess TO in corresponding biopsies (possibly the result of missing the hilus region). Likewise, of the 52 individuals identified with TO in biopsies, four were not observed to possess TO in corresponding transverse sections. Therefore, the total proportion of individuals identified with TO using both methods was actually 81% (64 of 79), the union being greater than the amount indicated by either method alone.

Site TO severity tended to agree between biopsy and transverse sectioning methods. Biopsy site severities ranged from an average of 0.03 to 14.9 based on TO enumeration only, while transverse site severities ranged from an average of 0.1 to 9.6 and 0.1 to 2.2, based on TO enumeration and ranks, respectively (Table 1; Figure 3B). TO enumeration averaged across all sites were nearly identical between biopsy and transverse severities (3.5 and 3.4, respectively; Table 1). Notable exceptions include Site G where transverse sections indicated substantially greater severity than did biopsies, and Site H where the reverse was true. Both instances were the result of one or two

individuals from the site yielding disproportionately high numbers of TO in one method versus the other. This demonstrates the influence of individual fish on results when sample size is small. Despite the small number of sample sites (n = 8), there was a reasonably strong positive correlation between site severities by TO enumeration (r^2 = 0.59; Figure 4C). Agreement was weaker when comparing biopsy and transverse severity results of individual SMB by TO enumeration (r^2 = 0.44; data only included for fish if TO detected by at least one method; Figure 4B). Comparing the two methods used to score severity for transverse sections (i.e., TO enumeration vs. ranking) across individuals and site means, produced strong positive correlations (r^2 = 0.79 and r^2 = 0.92, respectively; see *Supporting Material*).

Overall, the suite of methods (tissue collection procedures, histological sectioning methods, and severity metrics) used to estimate TO severity ranged broadly across sample sites, with Sites A and B standing out as minimally affected and Sites E through H markedly more severe (Table 1). While the purpose of the study was not to identify differences between sample sites, one way ANOVA and pairwise testing of severity data is a reasonable tool to assess the relative capabilities of the suite of methods employed to detect statistically significant differences, especially given the high variability between individuals and differences in tissue collection. Multiple pairwise comparisons of TO severity across the eight sites found numerous statistically significant differences (Table 1). Severity results from biopsy-collected tissues indicate five instances where sites were significantly different from one-another (Table 1; see *Supporting Material* for detailed statistical results). Severity results from transverse-sectioned tissues detected four out of five of these site differences and missed one, but indicated an additional two as differing

significantly (Table 1). Therefore, measures of site severity between methods varied in detection of statistical significance in three instances, two favoring transverse sectioning, one favoring biopsy.

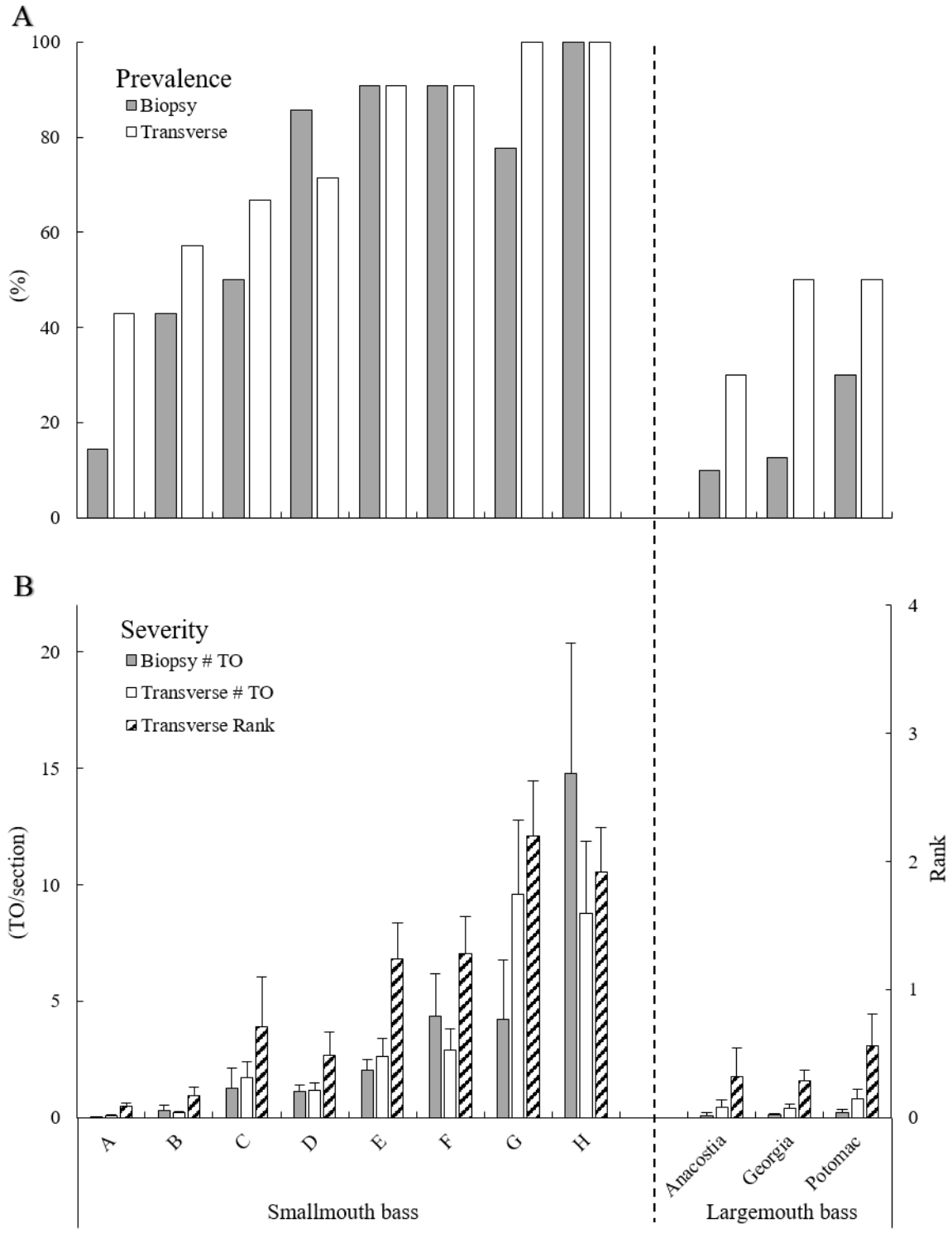

Figure 3. Comparison of testicular oocyte (TO) prevalence (**A**) and severity (**B**) following biopsy and transverse tissue collection methods applied to individual field collected smallmouth bass *Micropterus dolomieu* and largemouth bass *Micropterus salmoides*; prevalence (% with detected TO) within sample populations sorted from low to high for each species; severity by site reported as mean number of TO per section within individual fish (biopsy and transverse sections; left y-axis) and by severity ranking following methods of Blazer et al. (2007)(transverse sections only, right y-axis); Error bars = Standard Error.

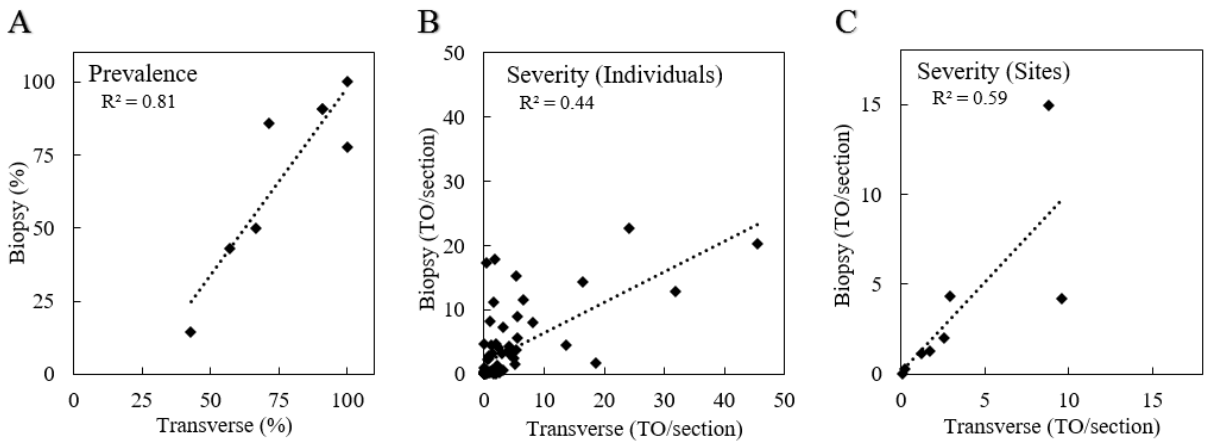

Figure 4. Relationships of testicular oocyte (TO) prevalence (**A**), individual severity (**B**), and site severity (**C**) between biopsy and transverse testis tissue collection methods applied to individual field collected smallmouth bass *Micropterus dolomieu* (n=79) from eight sample populations; prevalence: % of individuals within each sample population with detected TO; individual severity: mean number of TO/section (data only included in analysis if TO were detected by at least one method); site severity: mean of severity ranks from all individuals within a sample population; linear regression.

Largemouth Bass – Laparoscopic Surgery

At all three LMB sample sites, TO were detected using both tissue collection procedures (Table 2; Figure 3A). Overall, multiple biopsy sampling detected TO in 8 of 44 individuals (18%) while transverse sectioning detected TO in 20 of 44 individuals (45%). Percent TO prevalence at the three sites was consistently lower in biopsies (10% to 30%) than transverse sections (30% to 50%). In only one specimen were TO found by biopsy collection but not by transverse sectioning. In contrast, 13 individuals identified with TO were only detected via transverse sectioning. Therefore, the total proportion of individuals identified with TO using both methods was actually 48% (21 of 44), the union being greater than the amount indicated by either method alone. Prevalence at all LMB sites were similar to those of the least impacted SMB sites (i.e., Sites A and B).

Site TO severity for all three LMB sites were low between biopsy and transverse sectioning methods. Biopsy site severities ranged from an average of 0.1 to 0.2 based on TO enumeration only, while transverse site severities ranged from an average of 0.4 to

0.8 and 0.3 to 0.6, based on TO enumeration and ranks, respectively (Table 2; Figure 3B). Although biopsy site severity estimates were consistently lower than transverse estimates, the differences were not found to be statistically significant. Comparison of LMB biopsy and transverse severity results from individual fish (data only included for fish if TO detected by at least one method) indicated a strong positive correlation between methods ($r^2 = 0.76$; Figure 5). This correlation is somewhat surprising given the generally low severity values and limited frequency of simultaneous TO detection by both methods. Biopsy-generated severity estimates for the three LMB populations resemble those of the least impacted SMB populations studied (i.e., Sites A and B). Transverse section-generated severity estimates were modestly higher and approached levels similar to the moderately severe SMB populations (i.e., Site C).

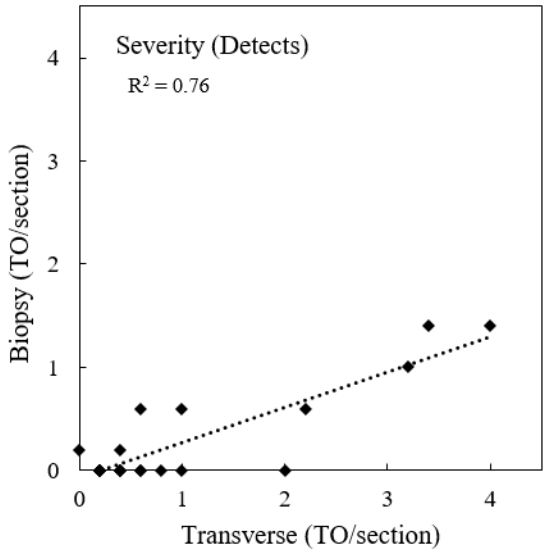

Figure 5. Relationship between estimates of testicular oocyte (TO) severity following application of biopsy and transverse tissue collection methods to individual field collected largemouth bass *Micropterus salmoides* (data only included in analysis if TO were detected by at least one method); linear regression.

Survival and Recovery

Duration of initial efforts lasted up to 23 min/specimen to collect 5 biopsies but with experience were reduced to as few as 4 min (generally 5 to 8 min). Endoscopic identification of females was often immediately obvious (presence of oocytes) and in the rare circumstance required perforation of the urinary bladder to observe the entire gonad. The collection of biopsies (i.e. laparoscopy) was never necessary to confirm sex since all females were close to spawning. After appropriate training and with practice, the time necessary to perform the laparoscopic procedure improved dramatically.

Survival of male LMB following laparoscopic biopsy collection was 90% (18 of 20) after 28 d. One fish was found dead at 6 d and another was sacrificed at 14 d after losing equilibrium, possibly from a punctured swim bladder. Urinary bladders were intact (determined by gentle application of axial pressure) at 28 d in all but one of the surviving fish. No biopsy-associated lesions were apparent on testes under gross examination but hemorrhage was evident within body cavities in 30% of surviving fish (see *Supporting Material*). Addressing spawning capability was not achieved because the females did not lay eggs during the allotted 28-day period.

Table 1. Morphometric characteristics and testicular oocyte prevalence and severity measures in male smallmouth bass *Micropterus dolomieu* collected throughout the Mid-Atlantic region and one site in Vermont (Site A) using transverse sectioning and biopsy collection methods. Values followed by the same letters are not significantly different. All metrics were analyzed for significance by Dunn's test, except for condition factor using the Holm-Šídák method.

					Testicular Oocyte				
					Prevalence		Severity		
Site	*n*	Length[a] (cm)	Weight[a] (g)	Condition Factor[a,b]	Biopsy	Transverse	Biopsy (#TO/section)	Transverse (#TO/section)	Transverse Rank
A	14	32.7 ± 3.32 X	495 ± 158.8 X	1.4 ± 0.13 WXY	14%	43%	0.03 ± 0.07 X	0.1 ± 0.11 W	0.1 ± 0.10 W
B	7	39.2 ± 6.08 XYZ	810 ± 334.7 XY	1.3 ± 0.11 XY	43%	57%	0.3 ± 0.59 XY	0.2 ± 0.23 WX	0.2 ± 0.18 WX
C	12	39.3 ± 3.92 YZ	862 ± 270.5 XY	1.3 ± 0.15 WXY	50%	67%	1.3 ± 2.19 XY	1.7 ± 3.30 WXYZ	0.7 ± 1.04 WXYZ
D	7	37.0 ± 3.01[c] XY	773 ± 219.1[c] XY	1.5 ± 0.11[c] YZ	86%	71%	1.1 ± 1.06 XYZ	1.2 ± 1.87 WXYZ	0.5 ± 0.63 WXYZ
E	11	40.1 ± 6.64 YZ	897 ± 389.1 XY	1.3 ± 0.07 W	91%	91%	2.0 ± 1.51 YZ	2.6 ± 2.91 XYZ	1.2 ± 0.94 XYZ
F	11	43.1 ± 6.12 YZ	1214 ± 469.7 YZ	1.4 ± 0.09 XYZ	91%	91%	4.3 ± 6.13 YZ	2.9 ± 3.33 XYZ	1.3 ± 0.96 XYZ
G	9	40.5 ± 4.44 YZ	1079 ± 369.2 YZ	1.6 ± 0.06 Z	78%	100%	4.2 ± 7.73 XYZ	9.6 ± 8.59 Z	2.2 ± 1.29 Z
H	8	45.4 ± 3.73 Z	1458 ± 310.1 Z	1.5 ± 0.08 Z	100%	100%	14.9 ± 15.74 Z	8.7 ± 6.84 Z	2.1 ± 0.92 Z
Mean		39.7 ± 3.83	949 ± 295.9	1.4 ± 0.10	69%	78%	3.5 ± 4.87	3.4 ± 3.73	1.0 ± 0.81

[a]Length, weight, condition factor, and severity indices presented as mean ± standard deviation.
[b]Condition factor calculated by the formula (body weight/total length^3) x 100.
[c]Metric data absent for 1/7 fish.

Table 2. Morphometric characteristics and testicular oocyte prevalence and severity measures in male largemouth bass *Micropterus salmoides* collected in the Potomac River system and a lake in Georgia. Values followed by the same letters are not significantly different. All metrics were analyzed for significance by Dunn's test, except GSI by Holm-Šídák method.

						Testicular Oocyte				
						Prevalence		Severity		
Site	*n*	Length[a] (cm)	Weight[a] (g)	Condition Factor[a,b]	GSI[a]	Biopsy	Transverse	Biopsy (#TO/section)	Transverse (#TO/section)	Transverse Rank
Anacostia	10	34.4 ± 8.10 YZ	488 ±227.5 Z	1.40 ± 0.21 Z	0.3 ± 0.16 Z	10%	30%	0.1 ± 0.32 Z	0.4 ± 1.02 Z	0.3 ± 0.71 Z
Potomac	10	35.1 ± 4.28[c] Z	571 ± 238.3[c] Z	1.31 ± 0.08[c] YZ	0.3 ± 0.15 Z	30%	50%	0.2 ± 0.46 Z	0.8 ± 1.24 Z	0.6 ± 0.78 Z
Georgia	24	29.8 ± 1.95 Y	325 ± 67.7 Y	1.21 ± 0.08 Y	0.4 ± 0.15 Z	17%	50%	0.1 ± 0.32 Z	0.4 ± 0.82 Z	0.3 ± 0.42 Z
Mean		32.4 ± 2.36	470 ± 127.9	1.3 ± 0.10	0.3 ± 0.06	19%	43%	0.1 ± 0.06	0.5 ± 0.23	0.4 ± 0.17

[a]Length, weight, condition factor, GSI, and severity indices presented as mean ± standard deviation.
[b]Condition factor calculated by the formula (body weight/total length^3) x 100.
[c]Metric data absent for 3/10 fish.

DISCUSSION

Method Development – Biopsies versus Transverse Sections

In the present study, we investigated the applicability of single-entry laparoscopy in black bass to quantify levels of TO detection and severity (non-lethally) to those generated by examination of multiple transverse testis sections (lethally). Our results show that in black bass single-entry laparoscopy can quantify similar levels of TO detection and severity to those generated by examination of multiple transverse testis sections with less than 10% mortality. These results validate the potential for single-entry laparoscopy to detect TO, non-lethally. Furthermore, electronarcosis had the added benefit of rapid sex identification allowing for immediate release of female fish with minimal trauma, representing significant benefits when sampling small or compromised populations. The technique also falls in line with the sentiments of the 3 R's paradigm of "reducing, refining and/or replacing" lethal whole-animal toxicological testing and especially resonates when applied to ecologically and economically important native species (National Research Council, 2007).

Benefits of non-lethal laparoscopic tissue collection should be weighed against disadvantages. Application of the method requires prescriptive training of technical personnel and purchase of equipment not typically used in field settings. The biopsy tool collects tissues of consistent shape and dimension, but, even when employed correctly, may not consistently encounter the hilus region where TO are generally most abundant (Figure 2B), a limitation that does not occur in transverse sectioning (Blazer et al., 2007; Ingram et al., 2011). Moreover, orientation is lost when biopsies are collected, which necessitates step-sectioning through the entire biopsy to increase the likelihood of

observing the hilus region if the biopsy captured from it. These challenges of biopsy-based TO detection illustrate the importance of technical training for effective laparoscopic tissue collection and proper histological sectioning methods to detect TO. Furthermore, practice is essential to minimize fish handling time to ensure high rates of survival.

In addition to necessary training and equipment purchases, the wide reaching applicability of laparoscopy hinges on demonstrating its results correspond to existing methods. It is important to understand that laparoscopy was only performed on LMB, thus references to "the biopsy method" means that the biopsy forceps used for laparoscopic procedures were used separately, directly taking biopsies from the testes during necropsy or after fixation. Although, the collection of fixed biopsies reflect a best-case scenario, they also illustrate that small biopsies of the testis can detect TO and the results may be comparable to existing data sets.

Results of the present study indicate that the biopsy method reliably estimated TO prevalence at the majority of SMB sites, yielding similar results as transverse findings. The few instances where transverse detection modestly exceeded biopsy findings, were at sites with low severity. Site severities were low for all three LMB sites, this likely contributed to the consistent underestimation of TO prevalence using biopsies for this species. The low severity in LMB may translate to minimal concern for adverse impacts to the populations. Collectively, TO occurred in > 80% of SMB, with five of eight sites having severity ranks ≥ 0.6. In contrast, TO prevalence was only 43% in LMB with all site severity ranks ≤ 0.6. Hinck et al., (2009), in a retrospective survey of eight US river basins (1995 - 2004), report TO prevalence of 33% in SMB (n = 70) compared to 18% in

LMB (n = 390) (severity not reported). The relatively lower TO prevalence and severity observed in LMB compared to SMB mirrors similar findings within the scientific literature (Blazer et al., 2007, 2012, 2014, Iwanowicz et al., 2009, 2016; Yonkos et al., 2014). More recently, in a comprehensive reconnaissance of TO in black bass on national wildlife refuges in the US Northeast, prevalence of TO averaged 85% in SMB (n = 118) compared to 27% in LMB (n = 173) (Iwanowicz et al., 2016). Site severity in SMB was generally high, with ranks exceeding 0.6 at 11 of 12 sites (range 0.3 to 2.2), but was generally low for LMB with ranks exceeding 0.6 at only one of 27 sample sites (range 0 to 1.0). Currently there is not a consensus on the biological relevance but the consistent disparity between the abundance of TO found in SMB compared to LMB in this study and others referenced, is clear. This observation may mean that LMB deserve a more sensitive scale, such as a ratio of TO enumeration per observable area. It is apparent that each species may respond differently and a lower magnitude of response may have the same implications as a more severe occurrence in a different species or it may not.

Diversity in current and previous practices employed to characterize TO prevalence and severity cause considerable ambiguity when comparing results from different studies. Approaches for fish include sub-sampling transversely from anterior, middle, and posterior regions of both testis lobes (Jobling et al., 1998; Vajda et al., 2008; Yonkos et al., 2014); sub-sampling transversely at five equidistant locations along one lobe (Blazer et al., 2007, 2012); or sampling longitudinally from one or both lobes (Ingram et al., 2011; Kellock et al., 2014). Tissues collected by these various methods can then be processed and observed individually or step-sectioned multiple times. Doing so produces multiple observable sections from proximate regions. This results in

substantial inconsistencies in area of tissue observed. The primary method of SMB TO quantification was formulated by Blazer et al. (2007) using five transverse sections and ranking severity on a scale from zero to four based on TO number and spatial distribution. An important component of this method is the ability to produce severity results for individual fish by averaging severity ranks of all sections observed. This serves to "normalize" severity results despite variability in tissue collection method and analysis (e.g., number of sections observed). Previously, Anderson et al. (2003) reported TO prevalence in SMB but did not indicate number of transverse sections observed and then used a scale based on oocyte enumeration (ranked from zero to three) to report severity. Surprisingly, severity was only reported as the frequency of each score instead of averaging site severity (Anderson et al., 2003). Since then, Baldigo et al., (2006) reported TO prevalence (but not severity) in SMB and LMB without indicating orientation of tissue collection or number of sections observed. More recently, Yonkos et al., (2014) reported TO prevalence and severity in LMB by observing two to 14 sections per specimen (generally six) and applied the distribution-based ranking method to score severity. These and other black bass studies based results on transverse sections, the most common method used. In contrast, Kellock et al., (2014) applied the distribution-based ranking method to longitudinal sections of both testis lobes from LMB. After examining entire longitudinal sections (estimated as equivalent in observable area to approximately 10 transverse sections) a single rank was assigned indicating the most severe region observed. Since only one distinct histological section was ranked, no average of multiple ranks could be calculated to adjust for the amount of area observed. Therefore, results tended to be higher than reported elsewhere for LMB. Prevalence and severity values

derived in this manner are appropriate for intra-experimental comparison, but cannot be compared explicitly to other studies that employ the distribution-based ranking scale to multiple transverse sections.

The biopsy method employed for the current study collected consistent sizes of tissue. On average, enumeration of TO from five biopsies sectioned five times yielded quantitatively similar values to five equidistant transverse sections across sites (3.4 TO per site vs. 3.5 TO per site, respectively; Table 1). This was somewhat unexpected considering biopsy-generated sections yielded only 61% of the amount of observable area on average to those generated by transverse sections. Biopsies were also presumed to less frequently obtain tissue from the hilus region where TO are most abundant and may account for the difference in detection. Despite these handicaps, results across sites were sufficiently similar to produce a strong positive correlation in TO prevalence between the two tissue collection methods ($r^2 = 0.81$; Figure 4A). Severities across sites measured by TO enumeration were reasonably well correlated between biopsy and transverse sections ($r^2 = 0.59$; Figure 4C). Together, these findings suggest that biopsy-generated results from this and future studies may be comparable to transverse-generated results in the literature with an understanding of the uncertainty. It should also be noted that in transverse sections, determination of severity by TO enumeration was essentially interchangeable with ranking (i.e., $r^2 = 0.92$), suggesting that TO enumeration may be preferable to ranking since it can be compared to other studies including biopsy-generated results. Indices provide a simplified understanding of continuous data and could be used in concert with reporting TO enumeration by ranking a range of TO.

Reporting severity using TO enumeration and a ranking index could increase transparency while providing the desired, easy to understand results (index values).

When TO severity was low (i.e., where TO were rare and dispersed), the lessor amount of observable area from biopsy-generated samples may be a disadvantage. Biopsies detected fewer individuals than transverse sections when the site severity rank was (≤ 0.5). Therefore, modification of the laparoscopic method may be necessary to improve detection at less severe sites. Specifically, individual biopsies could be serial sectioned to produce more observable area possibly increasing the likelihood of encountering rare TO. This limitation may be irrelevant in SMB where the level of TO severity that is presumed to be indicative of adverse population-level effect is well above a site severity rank of 0.5. Instances of a site with a rank severity of > 0.5 may warrant management action and/or additional investigation.

The direction of environmental monitoring is moving toward reducing lethal whole-organism sampling and focusing on molecular and cellular biomarkers. For example, application of *-omics* in aquatic ecotoxicology is growing exponentially, providing mechanistic insights into biological responses to contaminants and other environmental stressors (Simmons et al., 2015). Initially developed for laboratory screening assays, methods ranging from simple analysis of single compounds (e.g., aromatase, endogenous hormones) to complex bioinformatic approaches (e.g., gene arrays, proteomics, metabolomics) are increasingly being employed in field investigations of endocrine disruption and other toxicological effects in fish (Blum et al., 2008; Larkin et al., 2003; Martyniuk et al., 2009, 2011; Sabo-Attwood et al., 2007; Sanchez et al., 2009). Although, biomarkers that require organ tissue can be assessed

over time if non-lethal methods are employed and at this time, it can only be accomplished by complex surgical methods. Single-entry laparoscopy may ameliorate these issues, but is predicated on demonstrating that it remains non-lethal after collection of other tissues (not only testis, but also liver, kidney, head kidney, etc.). Additionally, it may provide the opportunity to repetitively sample the same individual fish to determine trends in fluctuating biomarkers, either through controlled laboratory exposures or field deployments (Denslow et al., 2012). A common limitation and cause of uncertainty in animal research is sample size. Single-entry laparoscopy could be used to sample more individuals without concern of detrimental effects to the population from over-sampling.

Ultimately, the benefit of laparoscopic testis tissue collection requires demonstration that it does not alter reproductive capability or cause sterility through fibrosis and scarring. This could be investigated by performing spawning studies at various intervals after laparoscopic tissue collection. In addition, laparoscopic collection of other tissues and organs must be investigated to determine the potential of adverse effects. A monitoring scheme employing laparoscopy could elucidate mechanisms of exposure or other aspects of interest by repeated, frequent sampling on large numbers of fish. Single-entry laparoscopy may be applicable to other species; it has yet to be tested. The authors hope that incorporation of this technique will shift the traditional paradigm of lethal sampling in field and laboratory studies, toward non-lethal monitoring strategies.

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CHAPTER 3: DISCUSSION

Repeatedly, studies have attempted to determine cause and effect using a biophysical change in an organism and tying that response to a chemical concentration in the water, sediment or food. A clear example in the realm of EDCs, is birth control, comparing a water grab sample to the occurrence of TO in wild fish populations, without knowledge of life history or when the TO may have formed. These investigations have some credibility if properly designed but often studies are too short and the scale too small. Commonly, the water chemistry is correlated to the induction of Vitellogenin and with TO, these designs fail to acknowledge the uncertainty of mechanisms that control TO formation. The chemistry portion requires extensive resources and effort especially if the study is comprehensive and attempts to characterize the full extent and suite of possible compounds and even with this exhaustive approach an 'unknown' causative agent cannot be measured. Whereas, monitoring responses in resident organisms (generally fish) will identify the occurrence of the 'unknown' endocrine disruptor if exposure elicits a response. Biomarkers that require organ tissues are generally collected by whole organism sacrifice. Lethal collection methods provide a snap-shot into the status of the organism but removes the possibility of gaining insight into changes over time, eliminating the possibility of tracking individuals in a longitudinal study. Some of the effects measured may be caused in during a crucial period that would otherwise be missed. The current paradigm of relating environmental chemistry with organism responses in the field and exposures in a controlled laboratory may greatly benefit from the inclusion of longitudinal studies on individuals. Recognizing this gap of information acquisition, our first step was to develop a non-lethal approach to collect testis tissue for

histological evaluation of intersex, and then we compared the results to lethal approaches. This study represents the first non-lethal quantification of both the presence and degree of intersex (TO) in individual fish (specifically black bass) by small biopsies.

Method Development

SMB site prevalence and severity offer insight into the competence of both methods to detect TO across a wide-range of impacted sites. Prevalence between methods at each site were largely in concordance, identifying the approximately the same number of individuals with TO, albeit, occasionally different individuals. Generally, prevalence was similar by both methods, differing by 10% across all SMB individuals and a strong correlation by sites ($r^2 = 0.81$; Figure 4A). The tendency of lower detection by biopsy compared to transverse sections occurred primarily at sites with generally low prevalence (e.g. Sites A, B, C). This was expected considering the nuances of using biopsy forceps to collect tissue from the central region of the testis in addition to the uncertainty of detection at sites where occurrence of TO was low, which is demonstrated by two individuals detected by biopsy (Site A and B) that transverse did not detect. Understandably, at sites with low severity, detection will also be low considering that a small fraction of the testis observed yet it is used to determine whether a fish is intersex or not. Along these lines, we observed tissue from the same testis in two ways, effectively doubling the amount viewed thus increasing detection yielding a higher proportion with intersex, totaling to 81% instead of 76% by transverse or 66% by biopsy alone. The nature of the tissue collected by each method is clearly diverse but fortunately it appears that five sections of five biopsies provide a similar amount of tissue for observation and yields quantitatively similar numbers of TO/section compared to five transverse sections

for SMB (mean of 3.5 and 3.4, respectively; Table 1). Site transverse rank severity in SMB ranged from 0.1 to 2.2 and is similar to findings in the literature (Blazer et al., 2007; Iwanowicz et al., 2016).

Overall, in SMB using mean TO count for comparison to the degree of severity in biopsied tissues matched well with the conventional system for ranking transverse sections, producing reasonably strong correlations when individual differences are not pronounced by using a ranking method ($r^2 = 0.59$; Figure 4B). While individual fish often received highly divergent ranks, mean site severity generally agreed when site severity rank was ≥0.5 and samples sizes were ≥7 fish. A notable exception was site A with 14 fish that produced a mean transverse severity rank and number of TO (0.1 for both) that was nearly three times higher than the biopsy mean number of TO (0.03). Because both rankings were very low, this still reflected less than a 0.1 unit difference between the methods. Similarly, where severity was high, individuals varied with a couple highly divergent by biopsy yet mean site severity generally agreed with large standard deviations, which complicates statistical analysis.

In a recent survey study of 28 water bodies for SMB and LMB, only 20 sites were detected with TO; and only one site had 100% prevalence which corresponded to the highest severity rank (Iwanowicz et al. 2016), neither of which were found of that magnitude at LMB sites sampled in this study, highest occurrence was 50%. This severity is comparatively low to findings in SMB. Biopsies in LMB produced a less consistent result, which may be attributed to previously mentioned caveats of the difficulties involved in collecting tissue from the correct area and sectioning the tissue through that area. Since all of the LMB populations sampled had low mean severity, the variability

was high and detection with biopsies was low (Figure 3; Table 2). Additional research on LMB may better characterize the range of severity, and if high severity (similar to SMB) is not found it may be necessary to develop an alternative ranking system in addition to reporting mean TO per individual that would be more applicable, if these populations exist.

Species Comparison

Overall, results appear to indicate that the non-lethal sampling method can more reliably estimate TO prevalence and severity in SMB than in LMB. In SMB biopsy detection was generally comparable to transverse sections with increasing severity, whereas LMB severity was low across all sites complicating interpretation and species translation (Figure 3A & B). LMB prevalence and severity by both methods were in the low range of the SMB sites (Table 1 & 2). LMB severity (number TO/section) fell below the top 6 SMB sites and was where prevalence was most variable between methods, suggesting that effectiveness of TO detection in LMB by biopsy may be enhanced in severe populations (Table 1 & 2). Thus, laparoscopy may lend itself to a LMB severe scenario, where monitoring temporal trends and minimizing population effects of lethal sampling would be greatly beneficial. Although this comparison may not come to fruition, considering the observations of higher incidence and severity in SMB versus LMB in this study reiterates findings previously documented (Blazer et al., 2007; Iwanowicz et al., 2016). In a recent U.S. wide reconnaissance of SMB and LMB, all LMB site severity ranks (number TO not reported) ranged between zero (no detects) to about one, whereas SMB ranged from about 0.3 to 2.5 (Iwanowicz et al. 2016). Those findings for both TO measures largely confirm the species difference observed in this

study. These species generally occupy different habitats, suggesting that TO development may be influenced by the different environmental conditions and chemical exposures confounding this, at the one site with co-occurrence of each species both prevalence and severity were statistically higher in SMB (Iwanowicz et al. 2016).

Considerations and Complications

Recall that the conventional technique for intersex detection in black bass species requires sacrificing the fish, excising the testes and cutting at least five transverse sections for histopathology (Blazer et al., 2007). In the current study, we proposed a non-lethal method of collecting multiple biopsies along the length of the testes, paraffin embedding the 5 biopsies together, and making up to 6 step-sections of the collected tissue. Each step-section of the 5 biopsies functioned as our unit (comparable to a single transverse section) for estimating severity, based exclusively on the number of oocytes found, not the spatial distribution to reduce confounding factors.

Intersex has been detected and characterized in several teleost fish species using a variety of techniques and methodologies; ranging from altering orientation of histologic sections, number and spatial distribution of sections observed for occurrence and, where appropriate, a ranking scheme to describe the nature and/or severity of the condition. Consider that by only observing 5 histologic transverse sections of 6 μm thickness, the proportion of tissue observed in a 5.0 cm long testis is ≤ 0.06%. Viewed in this light, the likelihood of encountering oocytes in only 5 transverse sections is probabilistic in nature. In specimens where TO are abundant, detection may be common. However, where TO are rare, encounters are unlikely. Looked at another way, when a single or relatively few TO are detected, it is probable that there are dozens, possibly hundreds more.

Blazer et al. (2007) describes a severity rank exceeding 0.5 is considered sufficient to provide at least 90% probability of detection in five sections and a severity rank of 0.2 sufficient to provide 70% probability of detection in five sections based on 150 individuals with TO. Applying this logic more broadly to site mean severity (including non-detects) reveals a similar trend across sites. In SMB, two sites with severity ranks below 0.5 (sites A & B) detected fewer individuals with TO by biopsy than all sites above 0.5 (Table 1, Figure 3). The site with the lowest severity across all three measures and the lowest prevalence for both methods (site A) contained individuals with severity ranks ranging from zero to 0.2, indicating a low likelihood of detection with 5 transverse sections and obviously lower for the 5 biopsy units (Table 1). The difficulty of detecting TO below severity rank of 0.5 may indicate minimal impacts to the individuals/population and may not be of concern considering it may be related to background levels. Following this logic, biopsy detection was low when site mean severity rank was below 0.5 (sites A & B) but the concern may also be low, whereas, above 0.5 detection is improved and the concern becomes higher. If a severity rank of 0.5 is appropriate for this interpretation then it may be considered the demarcation between regions of concern. Sites with impacted populations above this threshold may be threatened by other stressors, in turn the addition of sampling pressures by researchers may exasperate the situation, in this case a non-lethal tool, such as laparoscopy provides an alternative.

Since the biopsy tool collects a consistent tissue size, it does not collect tissue in proportion to the size of the organ. This is in contrast to transverse sections that, for larger testes, necessarily present more area for examination. There may also be a

meaningful difference in the distribution of oocytes within the testes of the two species that makes detection of TO in biopsied tissue less likely in LMB compared to SMB. For example, the distribution of oocytes were observed to be concentrated primarily in the center of transverse sections near the confluence of nerves and blood vessels, where the gonad attaches via mesenteries to the swim bladder in LMB and SMB as previously reported in the literature (Hinck et al., 2009). Similar observations of TO predominantly centrally located were noted in the present study as well. Because the biopsy tool can only collect a specific size (and shape) of tissue, it is imperative that the region collected actually contain the central testis region where TO prevalence is highest. Obviously in fish with testes of greater diameter this is more difficult. This serves to highlight the challenges of using biopsies for TO detection and emphasize the importance of a trained technician (easily achieved with minimal guidance) targeting the biopsy collection. Another potentially confounding factor of using laparoscopy is the loss of orientation of the biopsied tissue once removed for fixation. Interior and exterior surfaces of transverse sections are readily identifiable under gross observation. Therefore correct orientation during blocking is virtually guaranteed. In the case of biopsied tissues, this orientation is entirely lost. As a consequence, even for correctly targeted biopsies that contain tissue for the central region, multiple step-sections must be taken to increase the probability of actually encountering this region of concern.

Ideally, researchers adapt comprehensive stereological methods or volumetric estimates (e.g. number of TO per unit volume of testes), as these would address the variability of testes size. It is clearly an unrealistic goal considering the increased cost and labor required but it emphasizes the importance of experimental design to reduce

uncertainty where possible. The paradox between prevalence and severity is that increased sectioning may serve to increase prevalence whereas severity incorporates this increased sampling effort, this inherent bias instills the importance of consistent sectioning for relatable prevalence results. Severity measures appear to be the most informative tool to gauge the level of impact except in cases where site mean severity excludes individuals not detected which may artificially inflate the perceived population severity. The discrepancy between the two methods employed for reporting prevalence of intersex within a population clearly indicates that not detecting TO in a proportion of a population does not preclude the possibility of encountering some if the entire testes was examined, which is not economically feasible and not necessarily warranted. This concept of likely underestimating prevalence and possibly severity, has been discussed previously in the literature (Hinck et al., 2009; Blazer et al., 2012) because sample sizes are often small with respect to the population and possibly more importantly, the percent of gonad tissue examined is very low even though the entire testes is collected. The importance of refining the reporting of results by including other measures than prevalence and severity is evident, considering the difficulties of reconciling transverse sections, and now, biopsies of testes. A semi-recent method to observe TO in fresh or fixed tissue states high fidelity with traditional histology and is faster, cheaper, and potentially higher detection (Tanna et al., 2013). This method is most applicable to small amounts of tissue presenting an opportunity to combine with laparoscopy as a nearly real-time screening tool in the field and as the authors suggest, relating the results to other molecular and/or organismal level (Tanna et al., 2013).

The literature is laden with reports of intersex prevalence yet exclude evaluation of the severity, for this purpose and to reduce impact on the compromised population, laparoscopy offers an alternative to traditional lethal approaches. Considering the perceived importance of prevalence and its inherent bias on observational effort (as a binary measure that can only increase with sampling effort), excluding severity diminishes the value and relatability of those results, when possible, section all individuals equally and clearly state the number and proportion of the testes sectioned, and has been iterated previously (Hecker et al. 2006). These strategies will enhance interpretation and validity of statistical results. Standardization and transparency enhances the confidence in the development and interpretation of severity measures, since level of effort is incorporated in mean severity observational biases associated with the number of sections viewed is avoided. In this regard, it may be useful for future intersex studies to report both severity measures (number of TO/section and ranks). The literature suggests that the direction of the field is in reducing the use/sacrifice of organisms and focusing on molecular and cellular biomarkers, while this endeavor may provide much needed indicators and increase the weight of evidence, accepted histological approaches using standardized number of sections across all individuals will enhance our understanding.

It is important to note that the SMB data for each site may not be indicative of the status of each population because the tissue was sampled post-fixation and transverse processing, thereby some of the individuals may have had no tissue leftover or very little that could have an impact on the overall site prevalence and severity, thus site locations were not stated. Although the biopsied tissue may not reflect results from live tissue

collections, it was a necessary step to understand the potential of detection. Whereas the majority of LMB were biopsied during live surgery, which includes most of the individuals within the Potomac regions (Potomac, Anacostia rivers). Another aspect of consideration for LMB is many of the other fish were biopsied post-mortem (Georgia) with a subset collected post-fixation. These disparities contributes to the uncertainty yet laparoscopy appears to be applicable for intersex detection and severity in SMB, less so in LMB due to low severities encountered in the field.

Concern of laparoscopic intervention affecting reproduction is warranted and an attempt was made as part of the second survival study with wild LMB, although due to unforeseen complications the females never released eggs thwarting any attempts of fertilization post-biopsy collection. The facility housing the fish sourced water from an underground storage tank that was a couple degrees colder than the surface waters during collection. The most probable explanation is that the reduction in water temperature was a cue to suppress spawning.

Comparison of Three Methods – Case Study

It is obvious that many factors must be considered and the uncertainty characterized to adequately compare all three sampling methods employed in this study. Considering that the Blazer et al., (2007) semi-quantitative severity index for SMB has been the most reported method for intersex ranking for black bass, this study attempted to reconcile the other methods to it. In order to do this a few assumptions had to be made and they vary between longitudinal and biopsy step-sections. Since the specimen is in a different orientation and the sections includes the entire length of the tissue, this potentially increases detection if sectioned in the correct area. This resonates with the site

prevalence of 75%, 50%, and 17% for longitudinal, transverse, and biopsy sections, respectively. Longitudinal sections have been reported in the literature, generally with high severity comparatively for LMB (Kellock et al., 2014), which are generally low using the Blazer et al., (2007) severity ranking system. When all three methods were employed on the same individuals at one site, severity by TO enumeration were 7, 0.4, and 0.1 for longitudinal, transverse, and biopsy sections, respectively. Longitudinal sections have been reported for detection of intersex in cases where the testes was very small but severity was not included in the assessment (Hinck et al., 2009). Kellock et al. (2014), reports severity based on Blazer's ranking method but does not calculate the mean of each rank, thus the observed area is not factored into the calculation and median site severities ranged from 2 to 3.5, ranks which are rarely encountered. For instance if a testis is long and the longitudinal section is used then the area viewed, will be several times higher as compared to transverse sections, this is problematic when interpreting severity ranks between both published methods. The longitudinal mean severity is derived from each individual severity; not each individual mean severity like the other methods. Since longitudinal severity reporting does not differentiate the entire area observed, the severity value derived cannot properly be compared to the other methods and therefore not shown. The number of TO along with basic statistics for the number encountered is often reported alongside the rank for the studies that use longitudinal sections, this may be useful for reconciliation between methods, although when transverse section ranking is used, those studies rarely publish the number of TO instead focusing on prevalence of TO and in some cases, a severity ranking system (Blazer et al. 2007; Bateman et al. 2004; Kellock et al. 2014; Nolan et al. 2001).

The discrepancy between the three methods employed for reporting prevalence of intersex within a population clearly indicates that not detecting TO in a proportion of a population does not preclude the possibility of encountering some if the entire testis was examined, which is not economically feasible and not necessarily warranted. This concept of likely underestimating prevalence and possibly severity has been discussed previously in the literature (Hinck et al., 2009; Blazer et al., 2012) because sample sizes are often small with respect to the population and possibly more importantly, the percent of gonad tissue examined is very low even though the entire testis is collected but not processed. The importance of refining the reporting of results by including other measures than prevalence and severity is evident, considering the difficulties of reconciling longitudinal, transverse sections, and now, biopsies of testis.

Non-Lethal Techniques For Monitoring Populations – Other Applications

Since survival was high and healing was rapid during the 28-day studies, it appears that laparoscopy may lend itself to a method of monitoring several physiological attributes of fish that the public as well as scientists want to protect instead of relying on other model species with different life-history strategies. Information could be collected for observational studies as well as for experimental studies such as during an exposure in experimental pond or controlled system. The use of laparoscopy via incision into the body wall has been used to monitor endangered or imperiled fish populations such as shortnose sturgeon (Matsche et al., 2013) and shovelnose sturgeon (Divers et al., 2009) but these instances required multiple incisions to collect biopsies. Compared to conventional surgical procedures, the use of laparoscopy in this fashion reduces complications with the caveat of the dangers associated with creating an incision and

subsequent suture. Examination within the body cavity via the genital duct have been limited to one instrument, enabling viewing but not the ability to collect tissue (Kynard and Kieffer, 2002; Ortenburger et al., 1996; Wildhaber et al., 2005). Recently, Matsche, (2013) pioneered this method of laparoscopic entry via the genital pore utilizing an examination sheath containing a telescope and biopsy forceps on immature LMB for gender identification via microscopic viewing of a single gonad biopsy.

Impact to Wild Fish Populations

Some species have individuals within somewhat steady populations that have been highly impacted by EDCs such as wild roach in the UK. These populations seem to sustain their population dynamic even though a relatively high proportion of males have been found with major reproductive abnormalities (delayed spermatogenesis, reduced sperm viability, etc. Jobling, 2002). In contrast, wild carp have been found with intersex males as defined by presence of TO were compared to males with normal testis within the same population and found that despite having TO, comparatively higher levels of plasma VTG, and reduced testosterone levels, spermatogenesis was not impacted (Soto et al., 2003). These seemingly confounding results are frequently reported and may be a result of low detection from sampling a small portion of the testes.

In contrast, other species have been found to have signs of EDC exposure that were related to major incidents such as fish kills. At times, some of these have been attributed to a compromised immune system due to EDC exposure causing a cascade of negative effects. While many of the physiological processes affected by EEDCs discussed pertain to reproduction and development, additional mechanisms have been elucidated associated with the ER that may mediate immune responses and/or modulate

immunotoxicity (Ahmed, 2000; Inadera, 2006; Ndebele et al., 2004b; Salo et al., 2007). The arylhydrocarbon receptor (AhR) is widely recognized as a means of contaminant-induced immunotoxicity and the ER has been implicated as a signaling pathway for some AhR agonists (Matthews, 2006; Abdelrahim, 2006; Kerkvliet, 2002, 1995). Casanova-Nakayama et al., (2011) found evidence suggesting that estrogenic compounds may have a role in the immunomodulatory role in fish; based on several reasons, piscine immune organs have ERs, estrogen exposure can modulate immune gene expression, and during exposure the susceptibility to pathogens increases (Casanova-Nakayama et al., 2011).

When bearing in mind the impact EEDCs may have on an organism, population, and community, it is integral to consider the other influences that may exacerbate the effects of EEDCs singularly, especially during critical and sensitive developmental periods. Salmonids exposed to the EDCs o,p-DDE and Aroclor 1254 during early-life stages are known to result in long-term immunomodulation (Milston et al., 2003; Iwanowicz et al., 2005). Recently, a widely prescribed anti-diabetic medicine, metformin, was demonstrated to act as an endocrine disrupter at environmentally relevant concentrations (Niemuth and Klaper, 2015). FHM exposed to concentrations of metformin measured in wastewater treatment plant (WWTP) effluent for full life-cycle beginning at early life stage were highly intersex gonads in males, smaller size of treated males, and a reduction in fecundity for treated pairs (Niemuth and Klaper, 2015).The complex dynamic of interactions between the endocrine system and all other systems that maintains an organisms functionality is becoming clearer, even still within vertebrates much of the interplay is largely unknown from a holistic view. The overall evolutionary conservation of estrogens and associated receptors and pathways within vertebrates

exemplifies the importance of understanding the complexities from molecular interactions at the cellular level to the actions of the organism. Elucidating the pathways and receptors that are essential to life may provide the connection to predicting the outcome of exposure to the complex mixtures of compounds in the environment.

Future Considerations

It is interesting to note that only the first five good quality biopsy units were used for prevalence and severity, the implication is that on some specimens but not most, extra step-sections were observed and had oocytes outside the number of steps in consideration for the purpose of being consistent. This also goes for some of the transverse and longitudinal sections where up to three step-sections were observed, depending on the quality of the section; yet only the first good quality section was included in the data presented because it is often not considered cost-effective, it iss easier, and quicker. The addition of that data may slightly increase the precision of detection but until several testes from intersex males of both species are spatially mapped for TO, the uncertainty associated with the data should be characterized. To map the testis would require, step-sectioning the entire testis and stitching that data back together for a visual representation of the distribution of oocytes from posterior to anterior entirely. In addition, to validate the use of laparoscopy for detection of intersex, the relationship between methods developed for SMB sample set should be validated in LMB at sites with known high intersex severity.

REFERENCES

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

Abdelrahim, M. (2006). 3-Methylcholanthrene and Other Aryl Hydrocarbon Receptor Agonists Directly Activate Estrogen Receptor. Cancer Research *66*, 2459–2467.

van Aerle, R., Nolanusan, M., Jobling, S., Christiansen, L.B., Sumpter, J.P., and Tyler, C.R. (2001). Sexual disruption in a second species of wild cyprinid fish (the gudgeon, Gobio gobio) in United Kingdom freshwaters. Environmental Toxicology and Chemistry *20*, 2841–2847.

van Aerle, R., Pounds, N., Hutchinson, T.H., Maddix, S., and Tyler, C.R. (2002). Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow, Pimephales promelas. Ecotoxicology *11*, 423–434.

Aerni, H.-R., Kobler, B., Rutishauser, B.V., Wettstein, F.E., Fischer, R., Giger, W., Hungerbühler, A., Marazuela, M.D., Peter, A., Schönenberger, R., et al. (2004). Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents. Analytical & Bioanalytical Chemistry *378*, 688–696.

Ahmed, S.A. (2000). The immune system as a potential target for environmental estrogens (endocrine disrupters): a new emerging field. Toxicology *150*, 191–206.

Allen, Y., Matthiessen, P., Scott, A.P., Haworth, S., Feist, S., and Thain, J.E. (1999). The extent of oestrogenic contamination in the UK estuarine and marine environments — further surveys of flounder. Science of The Total Environment *233*, 5–20.

Alvarez, D.A., Cranor, W.L., Perkins, S.D., Schroeder, V.L., Iwanowicz, L.R., Clark, R.C., Guy, C.P., Pinkney, A.E., Blazer, V.S., and Mullican, J.E. (2009). Reproductive Health of Bass in the Potomac, Usa, Drainage: Part 2. Seasonal Occurrence of Persistent and Emerging Organic Contaminants. Environmental Toxicology and Chemistry *28*, 1084–1095.

Anderson, M.J., Cacela, D., Beltman, D., Teh, S.J., Okihiro, M.S., Hinton, D.E., Denslow, N., and Zelikoff, J.T. (2003). Biochemical and toxicopathic biomarkers assessed in smallmouth bass recovered from a polychlorinated biphenyl-contaminated river. Biomarkers *8*, 371–393.

Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., Henry, T.R., Denny, J.S., Leino, R.L., Wilson, V.S., et al. (2003). Effects of the androgenic growth promoter 17-β-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environmental Toxicology and Chemistry *22*, 1350–1360.

Ankley, G.T., Brooks, B.W., Huggett, D.B., and Sumpter, and J.P. (2007). Repeating History: Pharmaceuticals in the Environment. Environ. Sci. Technol. *41*, 8211–8217.

Ankley, G.T., Bencic, D.C., Breen, M.S., Collette, T.W., Conolly, R.B., Denslow, N.D., Edwards, S.W., Ekman, D.R., Garcia-Reyero, N., and Jensen, K.M. (2009). Endocrine disrupting chemicals in fish: Developing exposure indicators and predictive models of effects based on mechanism of action. Aquatic Toxicology *92*, 168–178.

Bahamonde, P.A., Munkittrick, K.R., and Martyniuk, C.J. (2013). Intersex in teleost fish: Are we distinguishing endocrine disruption from natural phenomena? General and Comparative Endocrinology *192*, 25–35.

Baker, M.R., Gobush, K.S., and Vynne, C.H. (2013). Review of factors influencing stress hormones in fish and wildlife. Journal for Nature Conservation *21*, 309–318.

Balch, G.C., Mackenzie, C.A., and Metcalfe, C.D. (2004). Alterations to gonadal development and reproductive success in japanese medaka (Oryzias latipes) exposed to 17α-ethinylestradiol. Environmental Toxicology and Chemistry *23*, 782–791.

Baldigo, B.P., Sloan, R.J., Smith, S.B., Denslow, N.D., Blazer, V.S., and Gross, T.S. (2006). Polychlorinated biphenyls, mercury, and potential endocrine disruption in fish from the Hudson River, New York, USA. Aquat. Sci. *68*, 206–228.

Bateman, K.S., Stentiford, G.D., and Feist, S.W. (2004). A RANKING SYSTEM FOR THE EVALUATION OF INTERSEX CONDITION IN EUROPEAN FLOUNDER (PLATICHTHYS FLESUS). Environmental Toxicology and Chemistry *23*, 2831.

Beamesderfer, R.C.P., and North, J.A. (1995). Growth, Natural Mortality, and Predicted Response to Fishing for Largemouth Bass and Smallmouth Bass Populations in North America. North American Journal of Fisheries Management *15*, 688–704.

Blazer, V.S., Iwanowicz, L.R., Iwanowicz, D.D., Smith, D.R., Young, J.A., Hedrick, J.D., Foster, S.W., and Reeser, S.J. (2007). Intersex (testicular oocytes) in smallmouth bass from the Potomac River and selected nearby drainages. Journal of Aquatic Animal Health *19*, 242–253.

Blazer, V.S., Iwanowicz, L.R., Henderson, H., Mazik, P.M., Jenkins, J.A., Alvarez, D.A., and Young, J.A. (2012). Reproductive endocrine disruption in smallmouth bass (Micropterus dolomieu) in the Potomac River basin: spatial and temporal comparisons of biological effects. Environmental Monitoring and Assessment *184*, 4309–4334.

Blazer, V.S., Iwanowicz, D.D., Walsh, H.L., Sperry, A.J., Iwanowicz, L.R., Alvarez, D.A., Brightbill, R.A., Smith, G., Foreman, W.T., and Manning, R. (2014). Reproductive health indicators of fishes from Pennsylvania watersheds: association with chemicals of emerging concern. Environ Monit Assess *186*, 6471–6491.

Blum, J.L., James, M.O., Stuchal, L.D., and Denslow, N.D. (2008). Stimulation of transactivation of the largemouth bass estrogen receptors alpha, beta-a, and beta-b by

methoxychlor and its mono- and bis-demethylated metabolites in HepG2 cells. The Journal of Steroid Biochemistry and Molecular Biology *108*, 55–63.

Brown, A.R., Owen, S.F., Peters, J., Zhang, Y., Soffker, M., Paull, G.C., Hosken, D.J., Wahab, M.A., and Tyler, C.R. (2015). Climate change and pollution speed declines in zebrafish populations. PNAS *112*, E1237–E1246.

Casanova-Nakayama, A., Wenger, M., Burki, R., Eppler, E., Krasnov, A., and Segner, H. (2011). Endocrine disrupting compounds: Can they target the immune system of fish? Marine Pollution Bulletin *63*, 412–416.

Ciparis, S., Iwanowicz, L.R., and Voshell, J.R. (2012). Effects of watershed densities of animal feeding operations on nutrient concentrations and estrogenic activity in agricultural streams. Science of The Total Environment *414*, 268–276.

Colborn, T., Saal, F.S. vom, and Soto, A.M. (1993). Developmental Effects of Endocrine-Disrupting Chemicals in Wildlife and Humans. Environmental Health Perspectives *101*, 378–384.

Coulter, D.P., Höök, T.O., Mahapatra, C.T., Guffey, S.C., and Sepúlveda, M.S. (2015). Fluctuating water temperatures affect development, physiological responses and cause sex reversal in fathead minnows. Environ. Sci. Technol. *49*, 1921–1928.

Davey, J.C., Bodwell, J.E., Gosse, J.A., and Hamilton, J.W. (2007). Arsenic as an Endocrine Disruptor: Effects of Arsenic on Estrogen Receptor–Mediated Gene Expression In Vivo and in Cell Culture. Toxicol. Sci. *98*, 75–86.

Denslow, N.D., Chow, M.C., Kroll, K.J., and Green, L. (1999). Vitellogenin as a Biomarker of Exposure for Estrogen or Estrogen Mimics. Ecotoxicology *8*, 385.

Denslow, N.D., Griffitt, R.J., and Martyniuk, C.J. (2012). Advancing the Omics in aquatic toxicology: SETAC North America 31st Annual Meeting. Ecotoxicology and Environmental Safety *76*, 1–2.

Devlin, R.H., and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture *208*, 191–364.

Diamanti-Kandarakis, E., Bourguignon, J.-P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., and Gore, A.C. (2009). Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. Endocrine Reviews *30*, 293–342.

Divers, S.J., Boone, S.S., Hoover, J.J., Boysen, K.A., Killgore, K.J., Murphy, C.E., George, S.G., and Camus, A.C. (2009). Field endoscopy for identifying gender, reproductive stage and gonadal anomalies in free-ranging sturgeon (Scaphirhynchus) from the lower Mississippi River. Journal of Applied Ichthyology *25*, 68–74.

Eldridge, J.C., Stevens, J.T., and Breckenridge, C.B. (2008). Atrazine Interaction with Estrogen Expression Systems. In Reviews of Environmental Contamination and Toxicology Vol 196, D.M. Whitacre, ed. (Springer US), pp. 147–160.

Filby, A., Thorpe, K., Maack, G., and Tyler, C. (2007). Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. Aquatic Toxicology *81*, 219–231.

Folmar, L.C., Denslow, N.D., Kroll, K., Orlando, E.F., Enblom, J., Marcino, J., Metcalfe, C., and Guillette Jr, L.J. (2001). Altered Serum Sex Steroids and Vitellogenin Induction in Walleye (Stizostedion vitreum) Collected Near a Metropolitan Sewage Treatment Plant. Archives of Environmental Contamination and Toxicology *40*, 392–398.

Fuzzen, M.M.L., Bennett, C.J., Tetreault, G.R., McMaster, M.E., and Servos, M.R. (2015). Severe intersex is predictive of poor fertilization success in populations of rainbow darter (Etheostoma caeruleum). Aquatic Toxicology *160*, 106–116.

Gimeno, S., Komen, H., Venderbosch, P.W., and Bowmer, T. (1997). Disruption of sexual differentiation in genetic male common carp (Cyprinus carpio) exposed to an alkylphenol during different life stages. Environmental Science & Technology *31*, 2884–2890.

Goto-Kazeto, R., Abe, Y., Masai, K., Yamaha, E., Adachi, S., and Yamauchi, K. (2006). Temperature-dependent sex differentiation in goldfish: Establishing the temperature-sensitive period and effect of constant and fluctuating water temperatures. Aquaculture *254*, 617–624.

Harms, C.A. (2005). Surgery in Fish Research: Common Procedures and Postoperative Care. Lab Animal *34*, 28–34.

Harris, C.A., Henttu, P., Parker, M.G., and Sumpter, J.P. (1997). The Estrogenic Activity of Phthalate Esters in Vitro. Environmental Health Perspectives *105*, 802–811.

Harris, C.A., Hamilton, P.B., Runnalls, T.J., Vinciotti, V., Henshaw, A., Hodgson, D., Coe, T.S., Jobling, S., Tyler, C.R., and Sumpter, J.P. (2010). The Consequences of Feminization in Breeding Groups of Wild Fish. Environmental Health Perspectives *119*, 306–311.

Hecker, M., Murphy, M.B., Coady, K.K., Villeneuve, D.L., Jones, P.D., Carr, J.A., Solomon, K.R., Smith, E.E., Kraak, G.V.D., Gross, T., et al. (2006). Terminology of Gonadal Anomalies in Fish and Amphibians Resulting from Chemical Exposures. In Reviews of Environmental Contamination and Toxicology, D.G.W. Ware, ed. (Springer New York), pp. 103–131.

Hinck, J.E., Blazer, V.S., Schmitt, C.J., Papoulias, D.M., and Tillitt, D.E. (2009). Widespread occurrence of intersex in black basses (Micropterus spp.) from US rivers, 1995–2004. Aquatic Toxicology *95*, 60–70.

Hotchkiss, A.K., Rider, C.V., Blystone, C.R., Wilson, V.S., Hartig, P.C., Ankley, G.T., Foster, P.M., Gray, C.L., and Gray, L.E. (2008). Fifteen Years after "Wingspread"—Environmental Endocrine Disrupters and Human and Wildlife Health: Where We are Today and Where We Need to Go. Toxicol. Sci. *105*, 235–259.

Hudson, J.M., Johnson, J.R., and Kynard, B. (2011). A Portable Electronarcosis System for Anesthetizing Salmonids and Other Fish. North American Journal of Fisheries Management *31*, 335–339.

Hutchinson, T.H., Ankley, G.T., Segner, H., and Tyler, C.R. (2006). Screening and Testing for Endocrine Disruption in Fish—Biomarkers As "Signposts," Not "Traffic Lights," in Risk Assessment. Environ Health Perspect *114*, 106–114.

Inadera, H. (2006). The immune system as a target for environmental chemicals: Xenoestrogens and other compounds. Toxicology Letters *164*, 191–206.

Ingram, D.R., Miller, D.L., Ingram, T.R., and Tannehill, J.E. (2011). Intersex Condition of Shoal Bass in the Flint River, Georgia. Journal of Aquatic Animal Health *23*, 189–194.

Iwanowicz, L.R., and Blazer, V.S. (2011). An overview of estrogen-associated endocrine disruption in fishes: evidence of effects on reproductive and immune physiology. 266–275.

Iwanowicz, L.R., and Ottinger, C.A. (2008). Estrogens, estrogen receptors and their role as immunoregulators in fish. 277–322.

Iwanowicz, L.R., Lerner, D.T., Blazer, V.S., and McCormick, S.D. (2005). Aqueous exposure to Aroclor 1254 modulates the mitogenic response of Atlantic salmon anterior kidney T-cells: Indications of short- and long-term immunomodulation. Aquatic Toxicology *72*, 305–314.

Iwanowicz, L.R., Blazer, V.S., Guy, C.P., Pinkney, A.E., Mullican, J.E., and Alvarez, D.A. (2009). Reproductive health of bass in the Potomac, USA, drainage: part 1. Exploring the effects of proximity to wastewater treatment plant discharge. Environmental Toxicology and Chemistry *28*, 1072–1083.

Iwanowicz, L.R., Blazer, V.S., Pinkney, A.E., Guy, C.P., Major, A.M., Munney, K., Mierzykowski, S., Lingenfelser, S., Secord, A., Patnode, K., et al. (2016). Evidence of estrogenic endocrine disruption in smallmouth and largemouth bass inhabiting Northeast U.S. national wildlife refuge waters: A reconnaissance study. Ecotoxicology and Environmental Safety *124*, 50–59.

Jensen, K.M., Kahl, M.D., Makynen, E.A., Korte, J.J., Leino, R.L., Butterworth, B.C., and Ankley, G.T. (2004). Characterization of responses to the antiandrogen flutamide in a short-term reproduction assay with the fathead minnow. Aquatic Toxicology *70*, 99–110.

Jobling, S. (2002). Wild Intersex Roach (Rutilus rutilus) Have Reduced Fertility. Biology of Reproduction *67*, 515–524.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., and Sumpter, J.P. (1998). Widespread Sexual Disruption in Wild Fish. Environmental Science & Technology *32*, 2498–2506.

Johns, S.M., Denslow, N.D., Kane, M.D., Watanabe, K.H., Orlando, E.F., and Sepúlveda, M.S. (2011). Effects of estrogens and antiestrogens on gene expression of fathead minnow (Pimephales promelas) early life stages. Environ. Toxicol. *26*, 195–206.

Johnston, P.M. (1951). The embryonic history of the germ cells of the largemouth black bass, Micropterus salmoides salmoides (Lacépède). J. Morphol. *88*, 471–542.

Kavanagh, R.J., Balch, G.C., Kiparissis, Y., Niimi, A.J., Sherry, J., Tinson, C., and Metcalfe, C.D. (2004). Endocrine Disruption and Altered Gonadal Development in White Perch (Morone americana) from the Lower Great Lakes Region. Environmental Health Perspectives *112*, 898–902.

Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac, M.J., Maczka, C., et al. (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ Health Perspect *104*, 715–740.

Kellock, K.A., Trushel, B.E., Ely, P.C., Jennings, C.A., and Bringolf, R.B. (2014). Survey of Intersex Largemouth Bass from Impoundments in Georgia USA. Transactions of the American Fisheries Society *143*, 565–572.

Kerkvliet, N. (2002). T Lymphocytes Are Direct, Aryl Hydrocarbon Receptor (AhR)-Dependent Targets of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD): AhR Expression in Both CD4+ and CD8+ T Cells Is Necessary for Full Suppression of a Cytotoxic T Lymphocyte Response by TCDD. Toxicology and Applied Pharmacology *185*, 146–152.

Kerkvliet, N.I. (1995). Immunological effects of chlorinated dibenzo-p-dioxins. Environmental Health Perspectives *103*, 47.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., and Flick, R.W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences *104*, 8897–8901.

Klaper, R., Rees, C.B., Drevnick, P., Weber, D., Sandheinrich, M., and Carvan, M.J. (2006). Gene Expression Changes Related to Endocrine Function and Decline in Reproduction in Fathead Minnow (Pimephales promelas) after Dietary Methylmercury Exposure. Environmental Health Perspectives *114*, 1337–1343.

Kolodziej, E.P., and Sedlak, D.L. (2007). Rangeland Grazing as a Source of Steroid Hormones to Surface Waters. Environ. Sci. Technol. *41*, 3514–3520.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., and Buxton, H.T. (2002). Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999−2000: A National Reconnaissance. Environ. Sci. Technol. *36*, 1202–1211.

Kugathas, S., and Sumpter, J.P. (2011). Synthetic Glucocorticoids in the Environment: First Results on Their Potential Impacts on Fish. Environmental Science & Technology *45*, 2377–2383.

Kusk, K.O., Krüger, T., Long, M., Taxvig, C., Lykkesfeldt, A.E., Frederiksen, H., Andersson, A.-M., Andersen, H.R., Hansen, K.M.S., Nellemann, C., et al. (2011). Endocrine potency of wastewater: Contents of endocrine disrupting chemicals and effects measured by in vivo and in vitro assays. Environmental Toxicology and Chemistry *30*, 413–426.

Kynard, B., and Kieffer, M. (2002). Use of aiborescope to determine the sex and egg maturity stage of sturgeons and the effect of borescope use on reproductive structures. J. Appl. Ichthyol *18*, 505–508.

Lange, A., Paull, G.C., Coe, T.S., Katsu, Y., Urushitani, H., Iguchi, T., and Tyler, C.R. (2009). Sexual reprogramming and estrogenic sensitization in wild fish exposed to ethinylestradiol. Environmental Science & Technology *43*, 1219–1225.

Länge, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., and Sumpter, J.P. (2001). Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (Pimephales promelas). Environmental Toxicology and Chemistry *20*, 1216–1227.

Larkin, P., Sabo-Attwood, T., Kelso, J., and Denslow, N.D. (2003). Analysis of Gene Expression Profiles in Largemouth Bass Exposed to 17-β-Estradiol and to Anthropogenic Contaminants that Behave as Estrogens. Ecotoxicology *12*, 463–468.

Leet, J.K., Gall, H.E., and Sepúlveda, M.S. (2011). A review of studies on androgen and estrogen exposure in fish early life stages: effects on gene and hormonal control of sexual differentiation. J. Appl. Toxicol. *31*, 379–398.

Leet, J.K., Sassman, S., Amberg, J.J., Olmstead, A.W., Lee, L.S., Ankley, G.T., and Sepúlveda, M.S. (2015). Environmental hormones and their impacts on sex differentiation in fathead minnows. Aquatic Toxicology *158*, 98–107.

Love, J.W. (2011). Habitat Suitability Index for Largemouth Bass in Tidal Rivers of the Chesapeake Bay Watershed. Transactions of the American Fisheries Society *140*, 1049–1059.

Martinović, D., Blake, L.S., Durhan, E.J., Greene, K.J., Kahl, M.D., Jensen, K.M., Makynen, E.A., Villeneuve, D.L., and Ankley, G.T. (2008). Reproductive toxicity of vinclozolin in the fathead minnow: Confirming an anti-androgenic mode of action. Environmental Toxicology and Chemistry *27*, 478–488.

Marty, M.S., Carney, E.W., and Rowlands, J.C. (2011). Endocrine Disruption: Historical Perspectives and Its Impact on the Future of Toxicology Testing. Toxicol. Sci. *120*, S93–S108.

Martyniuk, C.J., Kroll, K.J., Porak, W.F., Steward, C., Grier, H.J., and Denslow, N.D. (2009). Seasonal relationship between gonadotropin, growth hormone, and estrogen receptor mRNA expression in the pituitary gland of largemouth bass. General and Comparative Endocrinology *163,* 306–317.

Martyniuk, C.J., Spade, D.J., Blum, J.L., Kroll, K.J., and Denslow, N.D. (2011). Methoxychlor affects multiple hormone signaling pathways in the largemouth bass (Micropterus salmoides) liver. Aquatic Toxicology *101,* 483–492.

Matsche, M.A. (2011). Evaluation of tricaine methanesulfonate (MS-222) as a surgical anesthetic for Atlantic Sturgeon Acipenser oxyrinchus oxyrinchus: MS-222 anesthesia in Atlantic sturgeon. Journal of Applied Ichthyology *27,* 600–610.

Matsche, M.A. (2013). A portable electro-immobilization and laparoscopy system for sex determination and gonadal biopsy in largemouth bass *Micropterus salmoides*: Endoscopic sex determination in *Micropterus salmoides*. Journal of Fish Biology *83,* 1391–1400.

Matsche, M.A., Rosemary, K.M., Brundage, H.M., and O'Herron, J.C. (2013). Reproductive demographics, intersex, and altered hormone levels in shortnose sturgeon, Acipenser brevirostrum, from Delaware River, USA. J. Appl. Ichthyol. *29,* 299–309.

Matthews (2006). Estrogen receptor and aryl hydrocarbon receptor signaling pathways. Nuclear Receptor Signaling.

Metcalfe, C.D., Miao, X.-S., Koenig, B.G., and Struger, J. (2003). Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environmental Toxicology and Chemistry *22,* 2881–2889.

Miles-Richardson, S.R., Kramer, V.J., Fitzgerald, S.D., Render, J.A., Yamini, B., Barbee, S.J., and Giesy, J.P. (1999a). Effects of waterborne exposure of 17 β-estradiol on secondary sex characteristics and gonads of fathead minnows (Pimephales promelas). Aquatic Toxicology *47,* 129–145.

Miles-Richardson, S.R., Pierens, S.L., Nichols, K.M., Kramer, V.J., Snyder, E.M., Snyder, S.A., Render, J.A., Fitzgerald, S.D., and Giesy, J.P. (1999b). Effects of Waterborne Exposure to 4-Nonylphenol and Nonylphenol Ethoxylate on Secondary Sex Characteristics and Gonads of Fathead Minnows (Pimephales promelas). Environmental Research *80,* S122–S137.

Mills, L.J., and Chichester, C. (2005). Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? Science of The Total Environment *343,* 1–34.

Milston, R.H., Fitzpatrick, M.S., Vella, A.T., Clements, S., Gundersen, D., Feist, G., Crippen, T.L., Leong, J., and Schreck, C.B. (2003). Short-Term Exposure of Chinook Salmon (Oncoryhnchus tshawytscha) to o,p´-DDE or DMSO during Early Life-History

Stages Causes Long-Term Humoral Immunosuppression. Environmental Health Perspectives *111*, 1601–1607.

Nack, S.B., Bunnell, D., Green, D.M., and Forney, J.L. (1993). Spawning and Nursery Habitats of Largemouth Bass in the Tidal Hudson River. Transactions of the American Fisheries Society *122*, 208–216.

Nash, J.P., Kime, D.E., Van der Ven, L.T.M., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., and Tyler, C.R. (2004). Long-Term Exposure to Environmental Concentrations of the Pharmaceutical Ethynylestradiol Causes Reproductive Failure in Fish. Environmental Health Perspectives *112*, 1725–1733.

National Research Council (2007). Toxicity testing in the 21st century: A vision and a strategy.

Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., and Piferrer, F. (2011). DNA Methylation of the Gonadal Aromatase (cyp19a) Promoter Is Involved in Temperature-Dependent Sex Ratio Shifts in the European Sea Bass. PLoS Genet *7*.

Ndebele, K., Tchounwou, P.B., and McMurray, R.W. (2004a). Coumestrol, bisphenol-A, DDT, and TCDD modulation of interleukin-2 expression in activated CD+ 4 Jurkat T cells. International Journal of Environmental Research and Public Health *1*, 3–11.

Ndebele, K., Tchounwou, P.B., and McMurray, R.W. (2004b). Coumestrol, Bisphenol-A, DDT, and TCDD Modulation of Interleukin-2 Expression in Activated CD+4 Jurkat T Cells. International Journal of Environmental Research and Public Health *1*, 3–11.

Niemuth, N.J., and Klaper, R.D. (2015). Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. Chemosphere *135*, 38–45.

Nolan, M. (2001). A histological description of intersexuality in the roach. Journal of Fish Biology *58*, 160–176.

Orlando, E.F., Kolok, A.S., Binzcik, G.A., Gates, J.L., Horton, M.K., Lambright, C.S., Gray, L.E., Soto, A.M., and Guillette, L.J. (2004). Endocrine-Disrupting Effects of Cattle Feedlot Effluent on an Aquatic Sentinel Species, the Fathead Minnow. Environmental Health Perspectives *112*, 353–358.

Ortenburger, A.I., Jansen, M.E., and Whyte, S.K. (1996). Nonsurgical videolaparoscopy for determination of reproductive status of the Arctic charr. Can Vet J *37*, 96–100.

Orton, F., Rosivatz, E., Scholze, M., and Kortenkamp, A. (2011). Widely Used Pesticides with Previously Unknown Endocrine Activity Revealed as in Vitro Antiandrogens. Environmental Health Perspectives *119*, 794–800.

Palace, V.P., Wautier, K.G., Evans, R.E., Blanchfield, P.J., Mills, K.H., Chalanchuk, S.M., Godard, D., McMaster, M.E., Tetreault, G.R., Peters, L.E., et al. (2006).

Biochemical and histopathological effects in pearl dace (Margariscus margarita) chronically exposed to a synthetic estrogen in a whole lake experiment. Environmental Toxicology and Chemistry *25*, 1114–1125.

Palace, V.P., Evans, R.E., Wautier, K.G., Mills, K.H., Blanchfield, P.J., Park, B.J., Baron, C.L., and Kidd, K.A. (2009). Interspecies differences in biochemical, histopathological, and population responses in four wild fish species exposed to ethynylestradiol added to a whole lake. Canadian Journal of Fisheries & Aquatic Sciences *66*, 1920–1935.

Presnell, J.K., Schreibman, M.P., and Humason, G.L. (1997). Humason's Animal tissue techniques (Johns Hopkins University Press).

Probst, W.E., Rabeni, C.F., Covington, W.G., and Marteney, R.E. (1984). Resource Use by Stream-Dwelling Rock Bass and Smallmouth Bass. Transactions of the American Fisheries Society *113*, 283–294.

Purdom, C.E., Hardiman, P.A., Bye, V.V.J., Eno, N.C., Tyler, C.R., and Sumpter, J.P. (1994). Estrogenic Effects of Effluents from Sewage Treatment Works. Chemistry and Ecology *8*, 275–285.

Robertson, L.S., Iwanowicz, L.R., and Marranca, J.M. (2009). Identification of centrarchid hepcidins and evidence that 17β-estradiol disrupts constitutive expression of hepcidin-1 and inducible expression of hepcidin-2 in largemouth bass (Micropterus salmoides). Fish & Shellfish Immunology *26*, 898–907.

Rodgers-Gray, T.P., Jobling, S., Kelly, C., Morris, S., Brighty, G., Waldock, M.J., Sumpter, J.P., and Tyler, C.R. (2001). Exposure of Juvenile Roach (*Rutilus rutilus*) to Treated Sewage Effluent Induces Dose-Dependent and Persistent Disruption in Gonadal Duct Development. Environmental Science & Technology *35*, 462–470.

Sabo-Attwood, T., Blum, J.L., Kroll, K.J., Patel, V., Birkholz, D., Szabo, N.J., Fisher, S.Z., McKenna, R., Campbell-Thompson, M., and Denslow, N.D. (2007). Distinct expression and activity profiles of largemouth bass (Micropterus salmoides) estrogen receptors in response to estradiol and nonylphenol. J Mol Endocrinol *39*, 223–237.

Salo, H.M., Hébert, N., Dautremepuits, C., Cejka, P., Cyr, D.G., and Fournier, M. (2007). Effects of Montreal municipal sewage effluents on immune responses of juvenile female rainbow trout (Oncorhynchus mykiss). Aquatic Toxicology *84*, 406–414.

Sanchez, B.C., Ralston-Hooper, K.J., Kowalski, K.A., Dorota Inerowicz, H., Adamec, J., and Sepúlveda, M.S. (2009). Liver proteome response of largemouth bass (Micropterus salmoides) exposed to several environmental contaminants: Potential insights into biomarker development. Aquatic Toxicology *95*, 52–59.

Scholz, S., and Mayer, I. (2008). Molecular biomarkers of endocrine disruption in small model fish. Molecular and Cellular Endocrinology *293*, 57–70.

Schultz, I.R., Skillman, A., Nicolas, J.-M., Cyr, D.G., and Nagler, J.J. (2003). Short-term exposure to 17 alpha-ethynylestradiol decreases the fertility of sexually maturing male rainbow trout (Oncorhynchus mykiss). Environ. Toxicol. Chem. *22*, 1272–1280.

Selim, K.M., Shinomiya, A., Otake, H., Hamaguchi, S., and Sakaizumi, M. (2009). Effects of high temperature on sex differentiation and germ cell population in medaka, Oryzias latipes. Aquaculture *289*, 340–349.

Sellin, M.K., Snow, D.D., and Kolok, A.S. (2010). Reductions in hepatic vitellogenin and estrogen receptor alpha expression by sediments from an agriculturally impacted waterway. Aquatic Toxicology *96*, 103–108.

Shelley, L.K., Osachoff, H.L., van Aggelen, G.C., Ross, P.S., and Kennedy, C.J. (2013). Alteration of immune function endpoints and differential expression of estrogen receptor isoforms in leukocytes from 17β-estradiol exposed rainbow trout (Oncorhynchus mykiss). General and Comparative Endocrinology *180*, 24–32.

Simmons, D.B.D., Benskin, J.P., Cosgrove, J.R., Duncker, B.P., Ekman, D.R., Martyniuk, C.J., and Sherry, J.P. (2015). Omics for aquatic ecotoxicology: Control of extraneous variability to enhance the analysis of environmental effects. Environ Toxicol Chem *34*, 1693–1704.

Soto, A.M., Calabro, J.M., Prechtl, N.V., Yau, A.Y., Orlando, E.F., Daxenberger, A., Kolok, A.S., Guillette, L.J., le Bizec, B., Lange, I.G., et al. (2003). Androgenic and Estrogenic Activity in Water Bodies Receiving Cattle Feedlot Effluent in Eastern Nebraska, USA. Environmental Health Perspectives *112*, 346–352.

Strüssmann, C.A., and Nakamura, M. (2002). Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. Fish Physiology and Biochemistry *26*, 13–29.

Sumpter, J.P., and Jobling, S. (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ Health Perspect *103*, 173–178.

Tanna, R.N., Tetreault, G.R., Bennett, C.J., Smith, B.M., Bragg, L.M., Oakes, K.D., McMaster, M.E., and Servos, M.R. (2013). Occurrence and degree of intersex (testis-ova) in darters (Etheostoma SPP.) across an urban gradient in the Grand River, Ontario, Canada. Environ. Toxicol. Chem. *32*, 1981–1991.

Tetreault, G.R., Bennett, C.J., Shires, K., Knight, B., Servos, M.R., and McMaster, M.E. (2011). Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges. Aquatic Toxicology *104*, 278–290.

Thorpe, K.L., Maack, G., Benstead, R., and Tyler, C.R. (2009). Estrogenic Wastewater Treatment Works Effluents Reduce Egg Production in Fish. Environ. Sci. Technol. *43*, 2976–2982.

Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Woodling, J.D., and Norris, D.O. (2008). Reproductive Disruption in Fish Downstream from an Estrogenic Wastewater Effluent. Environ. Sci. Technol. *42*, 3407–3414.

Wenger, M., Shved, N., Akgül, G., Caelers, A., Casanova, A., Segner, H., and Eppler, E. (2014). Developmental oestrogen exposure differentially modulates IGF-I and TNF-α expression levels in immune organs of Yersinia ruckeri-challenged young adult rainbow trout (Oncorhynchus mykiss). General and Comparative Endocrinology *205*, 168–175.

Wenzel, S.D.A., Schäfers, C., Vollmer, G., Michna, H., and Diel, P. (2001). Research efforts towards the development and validation of a test method for the identification of endocrine disrupting chemicals. Contract *6*, 15.

Wildhaber, M.L., Papoulias, D.M., DeLonay, A.J., Tillitt, D.E., Bryan, J.L., Annis, M.L., and Allert, J.A. (2005). Gender identification of shovelnose sturgeon using ultrasonic and endoscopic imagery and the application of the method to the pallid sturgeon. Journal of Fish Biology *67*, 114–132.

Yanbin, Z., Chen, W., Shuang, X., Jieqiong, J., Rui, H., Guanxiang, Y., and Jianying, H. (2014). Biosensor Medaka for Monitoring Intersex Caused by Estrogenic Chemicals. Environmental Science & Technology 140203084006000.

Yonkos, L.T., Friedel, E.A., and Fisher, D.J. (2014). Intersex (testicular oocytes) in largemouth bass (Micropterus salmoides) on the Delmarva Peninsula, USA. Environ Toxicol Chem *33*, 1163–1169.

Zhang, C., Li, Y., Wang, C., Niu, L., and Cai, W. (2016). Occurrence of endocrine disrupting compounds in aqueous environment and their bacterial degradation: A review. Critical Reviews in Environmental Science and Technology *46*, 1–59.