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The Effects of a Model Androgen 5α-dihydrotestosterone on Mummichog (Fundulus heteroclitus) Reproduction Under Different Salinities

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The Effects of a Model Androgen 5α-dihydrotestosterone on Mummichog (*Fundulus heteroclitus*) Reproduction Under Different Salinities

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General Abstract

Endocrine disrupting substances (EDSs) have the potential to disrupt sensitive hormone pathways, including those involved in development and reproduction. Both fresh and estuarine water bodies receive inputs of EDSs from a variety of sources, including sewage effluent, industrial effluent, and agricultural runoff. Based on limited available literature, freshwater species appear to respond to lower levels of EDSs than estuarine or marine species. To address this potential difference, a short-term reproductive bioassay was carried out under low and high salinity conditions using mummichog (*Fundulus heteroclitus*), a euryhaline species that is native to the east coast of North America. The goal of the study was to determine the response of mummichog at multiple biological levels when exposed to an androgenic EDS and whether salinity affected the response. A second goal was to adapt a population model for mummichog to predict impacts from anthropogenic or environmental stressors on population status. A model androgen, 5-alpha dihydrotestosterone (DHT), was used because androgenic EDSs are not as well-studied as estrogenic EDSs. In the high salinity group egg production was significantly reduced (39-49%) in all exposure concentrations, while in low salinity conditions there were no significant differences in egg production based on DHT treatment. However, egg production in the low salinity control group was reduced by 55% relative to the high salinity control group. Additionally, DHT reduced sex steroid production in males and females at both salinities. This indicates that androgenic EDSs such as DHT may reduce the reproductive capacity of mummichog, and that osmotic stress from low salinity conditions likely diverted energy from reproductive output in the low salinity group. These results were applied in a mummichog population model which was adapted from a fathead minnow (*Pimephales promelas*) model. Observed changes in fecundity were employed in this predictive model to estimate the long term
effects of exposure to anthropogenic (DHT) or environmental (low salinity) stressors on population status. Different model projections were developed based on the unique spawning patterns of the mummichog subspecies, the southern species *Fundulus heteroclitus heteroclitus*, and the northern subspecies *Fundulus heteroclitus macrolepidotus*. Results indicated that the reduction in fecundity over a 20 year period due to DHT exposure can lead to a reduction of 50% of carrying capacity in the southern subspecies and a near population collapse in the northern subspecies due to their shorter spawning season. In its current form, the model demonstrates that exposure to anthropogenic or environmental stressors (in this case DHT and low salinity) can have equally negative impacts on the population viability of mummichog. Future studies will focus on refinement of the model to account for temporal sensitivity over multiple generations as well as the effects of migration.
Chapter 1  General Introduction
1.1 Endocrine Disrupting Substances (EDSs)

Endocrine disrupting substances (EDSs) have received a significant amount of attention both from the general media and the scientific community as research has indicated that exposure to EDSs can negatively impact the health and performance of organisms, including development and reproduction (Martin and Voulvoulis 2009). EDSs include a wide range of anthropogenic and naturally occurring substances that have the potential to disrupt sensitive hormone pathways of the hypothalamic-pituitary-gonadal (HPG) axis (Martin and Voulvoulis 2009). The hypothalamic-pituitary gonadal axis is a complex system regulated by a series of positive and negative feedback loops that control development and reproductive processes (Norris 2007).

Neural inputs, which can include temperature and photoperiod, will trigger the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, which will travel to the anterior lobe of the pituitary gland where it will induce the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the blood stream (Norris 2007). LH and FSH act cooperatively; LH is responsible for initiating ovulation in females and stimulates the process of steroidogenesis, i.e. the production of androgens such as testosterone (T) in Leydig cells in males (Norris 2007). FSH will work together with T in males to initiate spermatogenesis (i.e. the process of sperm production), and in females FSH stimulates the growth and differentiation of primordial follicles into primary follicles and oocytes (Norris 2007). LH and FSH also work cooperatively in females in the steroidogenesis of androgens, most of which are then converted to estrogens (Norris 2007). Androgens and estrogens act as feedback inhibitors of GnRH, which will in turn stop the secretion of FSH and LH (Fig. 1.1; Norris 2007). Androgens and estrogens are lipophilic steroids that exert their actions by diffusing through cell membranes and binding to either androgen or estrogen receptors or by interacting through the plasma membrane via
Fig. 1.1 A diagram depicting the reproductive system of fish, including the hypothalamic-pituitary gonadal axis. GnRH = gonadotropin releasing hormone; LH = luteinizing hormone; FSH = follicle-stimulating hormone; T = testosterone; E2 = 17β-estradiol; 11-KT = 11-ketotestosterone; + and – represent positive and negative feedback loops (Kime 1998; Peters 2005).
receptors or membrane-bound kinase messaging systems (Parker 1993; Mangelsdorf et al. 1995). The HPG axis is very sensitive to perturbation in its complex signaling system; therefore, small amounts of hormone mimics or agonists (EDSs) can have significant and far reaching impacts on reproductive and developmental systems (Norris 2007).

EDSs that specifically act as estrogen and androgen (ant)agonists can elicit powerful effects on reproductive function at low concentrations (Kelce et al. 1994; Laws et al. 1995; Sharpe et al. 2004). For example, in a whole-lake study that exposed fathead minnow (*Pimephales promelas*) to a chronic, environmentally relevant dose (5-6 ng/L) of 17α-ethinylestradiol (EE2: a potent estrogen used in oral contraceptives), male fish exhibited feminization and intersex conditions, females displayed altered oogenesis, and the entire population suffered a near collapse (Kidd et al. 2007). Additionally, EDSs can disrupt hormone synthesis, transport, metabolism and clearance, which can cause organizational and functional changes that may be permanent or transient (Lister and Van Der Kraak 2001; Sharpe et al. 2004).

### 1.2 Sources of EDSs

EDSs enter both fresh and estuarine water bodies through a variety of sources (Martin and Voulvoulis 2009). One major source is treated sewage effluent, which contains pharmaceuticals that can act as estrogen- and androgen- mimicking compounds (Milla et al. 2011; Yang et al. 2012). Other examples of EDS sources include pulp mill effluent (Parks et al. 2001), livestock runoff (Gall et al. 2011; Lister et al. 2011), and agricultural runoff (which contains fungicides such as vinclozolin that have anti-androgenic properties) (Kiparissis et al. 2003; Lister et al. 2011). For example, a study that evaluated the effects sewage-treatment works (STW) effluent
output in an estuary containing flounder (*Platichthys flesus*) found evidence of estrogenic compounds in the effluent based on the unexpectedly high plasma concentrations of vitellogenin, an egg-yolk precursor protein (Lye et al. 1997). Bleached kraft mill effluents have been shown to contain androgenic compounds via studies that have shown masculinizing effects on female mosquitofish (*Gambusia affinis*; Deaton and Cureton II 2011). With a myriad of sources that enter the environment and contain EDSs, it is important to further the understanding of the effects of EDSs in order to accurately predict their potential ecological impacts.

### 1.3 Androgenic EDSs

To date, the majority of EDS research has focused on estrogens (Milla et al. 2011). Estrogenic receptor response studies, often using the potent and well-known estrogen EE2, have been conducted on many fish species in both field and lab experiments (e.g. Lin and Janz 2006; Kidd et al. 2007; Zha et al. 2008). This estrogen has been shown to reduce fecundity and impair other important reproductive endpoints such as sex steroid levels and mating behavior across a range of teleost species (Lin and Janz 2006; Kidd et al. 2007; Milla et al. 2011). For example exposure to EE2 has resulted in reduced egg production in freshwater Chinese rare minnow (*Gobiocypris rarus*) and zebrafish (*Danio rerio*) at concentrations as low as 0.2 ng EE2/L (Zha et al. 2008) and 1 ng EE2/L, respectively (Lin and Janz 2006). Studies have also shown that male Japanaese medaka (*Oryzias latipes*) exposed to 10 ng EE2/L failed to copulate with females (Balch et al. 2004), while a similar study with male fathead minnows demonstrated that EE2 exposure reduced male-male competition and their ability to maintain spawning substrates (Salierno and Kane 2009).
In contrast, there have been much fewer studies that have evaluated the impacts of androgenic substances. Androgens regulate a wide range of physiological processes in male vertebrates, including fish gonad differentiation, development of secondary sex characteristics, and modulation of behavior (Borg et al. 1993; Brantley et al. 1993a, b; Borg 1994; Kobayashi and Nakanishi 1999; (Margiotta-Casaluci and Sumpter 2011). Androgens that are commonly used as model compounds include 17β-trenbolone (TB; Cripe et al. 2010), which is a metabolite of trenbolone acetate used in the livestock industry to promote growth, and methyl testosterone (MT), a model androgen that is sometimes used to treat testosterone deficiency in men (Sharpe et al. 2004). A study that exposed sheepshead minnow (Cyprinodon variegates) to TB showed that reproductive capacity of the fish was reduced, while a study exposing mummichog (Fundulus heteroclitus) to MT showed reduced plasma sex steroid concentrations in both sexes (Sharpe et al. 2004). Another common androgen used in studies is the androgen 5α-dihydrotestosterone (DHT). DHT is a naturally occurring sex steroid and is one of the most important androgens in male vertebrates, with the exception of teleosts in which 11-ketotestosterone (11-KT) and testosterone (T) are the major circulating male androgens (Margiotta-Casaluci and Sumpter 2011). Although DHT is not found in high concentrations in the environment, it is non-aromatizable (i.e. not transformed into an estrogenic compound once taken up into the body) and therefore a pure androgen (Shilling and Williams 2000). Studies employing DHT in fish exposure studies have shown measurable effects from DHT on reproductive endpoints. For example, a study that exposed female fathead minnow to DHT resulted in intersex conditions of ovaries as well as exhibition of male secondary sex characteristics (Margiotta-Casaluci and Sumpter 2011). Another study demonstrated that mummichog exposed to DHT resulted in reduced plasma estradiol levels (Rutherford 2011). Based on these findings, we selected DHT
as the model androgenic compound in this study to evaluate its potential impacts on fish reproductive capability.

1.4 The influence of salinity on fish response to EDSs

In addition to the focus on estrogenic compounds, most research has also focused on the impacts of EDSs in freshwater systems. However, it is also important to understand the impacts of EDSs in estuarine habitats, as they are of vital economic and ecological importance. They are breeding and rearing grounds for many fish species and are both a year-round and transitional habitat for important commercial species, such as salmon, sturgeon and eels (Franco et al. 2006; Sarria et al. 2011). Moreover, most estuaries are highly urbanized and important to economies because of ship ports and other commercial endeavors, which have resulted in high level inputs of municipal and industrial effluents (Sarria et al. 2011). A local example is New Bedford Harbor (Massachusetts, USA) which was heavily contaminated by industrial effluents between the 1940s and 1970s with heavy metals, organic contaminants, and polychlorinated biphenyls (PCBs), many of which have the potential to act as EDSs (Nacci et al. 1999; Voyer et al. 2000). To optimally protect these environments, it is important to understand the impacts of EDSs under estuarine conditions.

The salinity of the water is directly related to how fish osmoregulate and may also be linked to how EDSs are taken up into the body. Under freshwater conditions, osmoregulation occurs through the gill and oral tissues with little water ingested, while marine and estuarine fish drink water to compensate for the dehydrating effects of saline water and diffuse ions out of the gills (Black 1957). Salinity has been implicated as an environmental condition that can influence the
response of fish to contaminants. For example, in a study where mummichog (*Fundulus heteroclitus*; an estuarine species) embryos were exposed to a petrogenic contaminant (water soluble fraction of No. 2 fuel oil) under optimal conditions of salinity and temperature (20 ppt salinity, 20°C) the effects were only mildly toxic (Linden et al. 1979). However, under suboptimal conditions (10 ppt salinity and 20°C, 30 ppt salinity and 30°C) embryos exposed to the same concentration of contaminant experienced 85-87% mortality (Linden et al. 1979). In another study in which different salinity levels were used to assess the acute toxicity of zinc (Zn) in mummichog and the mangrove killifish (*Kryptolebias marmoratus*; an estuarine species), it was found that the LC50 values for Zn generally increased (and thus toxicity decreased) at higher salinity levels for both species (Bielmyer et al. 2012). This indicated that both species had a higher tolerance to Zn when exposed above their isotonic points, i.e. the point at which equal solute concentrations occur within the fish and its outside environment (9 ppt for mummichog 15 ppt for mangrove killifish) (Bielmyer et al. 2012).

Based on limited available research, there also seems to be a difference in the concentration of EDSs required to elicit effects between freshwater and estuarine teleosts. For example, exposure to EE2 has caused reduced egg production in freshwater Chinese rare minnow (*Gobiocypris rarus*) and zebrafish (*Danio rerio*) at concentrations as low as 0.2 ng EE2/L (Zha et al. 2008) and 1 ng EE2/L, respectively (Lin and Janz 2006). However, a study on mummichog reported a reduction in egg production at exposure concentration of 100 ng EE2/L (Peters et al. 2007), while follow-up studies found no impact, even when fish were exposed to concentrations up to 3,000 ng EE2/L (Bosker et al. unpublished). In two other studies using estuarine model species, male mummichog (Peters et al. 2007) and sheepshead minnow (*Cyprinodon variegates*; Folmar
et al. 2002) exposed to EE2 showed inductions of vitellogenin (an egg yolk precursor protein) at 100 ng EE2/L, while induction occurred at significantly lower levels in freshwater species (<6.5 ng EE2/L) (Hutchinson et al. 2006). A similar pattern was also observed when fish were exposed to 17β-trenbolone; sheepshead minnow showed reduction in egg production at 5 μg/L (Hemmer et al. 2008), while fathead minnow responded to the same compound at concentrations 100-fold lower (Ankley et al. 2003). Based on the potential influence of salinity on fish response to EDSs, a component of this study will examine the effect of DHT on fish reproductive endpoints under high and low salinity conditions to determine if there is a difference in response.

1.5 Standard 21-day reproductive bioassay

The methodology used for this study is a short-term fish reproductive bioassay. Fish laboratory bioassays are an important method to determine the impacts of EDSs on the aquatic environment because fish are considered sensitive bioindicators for pollution in aquatic environments (Lee et al. 2004; Milla et al. 2011). Fish have been selected for this purpose not only because of the environmental relevance of aquatic exposures, but also because there is a high degree of evolutionary conservation in the basic aspects of the HPG axis (Ankley and Johnson 2005). This similarity in the HPG axis among vertebrates allows for the possibility of extrapolating EDS effects to other species (Ankley and Johnson 2005). Many national and international organizations, such as the Environmental Protection Agency (EPA) and the Organization for Economic Cooperation and Development (OECD), use short-term reproduction bioassays to evaluate the potential of chemicals to act as EDSs in fish and to develop criteria and guidance documents for the identification of EDSs (Dang et al. 2011).
The in vivo 21-day fish reproductive bioassay is a Tier 1 standard approach to EDS screening in terms of environmental exposure and tests have been developed for a variety of fish species (Milla et al. 2011). In these tests a reproductively mature teleost species (typically of a small-bodied variety) is exposed to a range of concentrations of a particular compound for 21 days. Following the exposure, the fish are screened for disruption within the HPG axis by evaluating endpoints at different levels of biological organization. Some of the most common indicators include vitellogenin induction, gonadal histology, and fecundity (Dang et al. 2011). It should be noted that the Tier 1 21-day reproductive bioassay is not intended to quantify or confirm endocrine disruption or to provide quantitative assessment of risk. The purpose of this Tier 1 test is to determine if the measured endpoints reflect disturbances in the HPG axis and, based on a weight-of-evidence approach, whether such disturbances warrant further testing to determine the endocrine-related effects in Tier 2 testing (EPA 2007). The ultimate goal of this two-tiered approach is to assess the risk of the contaminant to humans and wildlife and involves more elaborate testing that encompasses critical life stages as well as a broader range of dosing (EPA 2007). However, the EPA has also advised that researchers should proceed from first-order models that can be developed from Tier 1 screening to more specific models derived from Tier 2 testing (Ankely et al. 1997; Miller and Ankley 2004). This approach has been used to estimate potential population levels effects based on short-term reproductive bioassays (Miller and Ankley 2004; Bosker et al. 2010).

### 1.6 Modeling population level effects from EDS exposure

It is important to understand the effects of EDSs on fish reproductive capability, and ultimately on their population viability over time. It has been shown that low-level chronic exposures to
EDSs can cause population-level collapses. For example, after a 3 year whole-lake study in which in fathead minnow were exposed to an environmentally relevant dose of EE2 (5-6 ng/L) the entire population suffered a near collapse (Kidd et al. 2007). Although this study has provided a first confirmation that EDSs can cause population level collapses, such population-level exposure studies are relatively rare while laboratory and field studies that examine the effects of EDSs at the individual level are much more common (Miller and Ankley 2004). In response to this, a predictive and density-dependent population model has been developed for fathead minnow from short-term reproductive bioassays (Miller and Ankley 2004). This model combines elements of a Leslie matrix and a logistic equation to predict changes in a closed population at carrying capacity (Miller and Ankley 2004). The modeling approach is relatively simple, requiring only a species-specific life history table, an estimate of carrying capacity, and the effects of how a particular stressor affects the vital rates at the individual level (Miller and Ankley 2004). The application of this simple model allows for an assessment of a particular stressor. For the current study, this modeling approach was adapted for the use of mummichog and used to evaluate the effects of long-term exposure to an anthropogenic (DHT) or environmental (low salinity) stressor at the population level.

1.7 Mummichog as a toxicological model

In order to increase our understanding of how fish reproductive endpoints will respond to an androgenic endocrine disruptor under different salinities, mummichog were chosen as the toxicological model species for this study. There are several reasons why mummichog were selected for this study. Firstly, mummichog are an abundant small-bodied fish indigenous to estuaries along the eastern North American coastline from Newfoundland to northern Florida,
making them an environmentally relevant species to study the impacts of contaminants under estuarine conditions (Kneib 1986). Secondly, they are an established model species for environmental toxicology (Burnett et al. 2007) with short-term reproductive bioassays that have been developed and refined for this species (Peters et al. 2007; Bosker et al. 2007). Using these bioassays, mummichog have been employed extensively in toxicology studies to examine the effects of environmental contaminants and model compounds on aspects of its development and reproduction (e.g. Peters et al. 2007; Bosker et al. 2010; Lister et al. 2011). Thirdly, their hardy nature and sexual dimorphism (Fig. 1.2) make them excellent specimens to work with in field and laboratory experiments (Kneib 1986). Finally, mummichog can tolerate a wide range of salinities, from freshwater to full strength seawater (Griffith 1974). The preferred range of salinities reported for mummichog ranges from 22-57 ppt saltwater (Fritz and Garside 1975); however, estuarine mummichog inhabit low salinity conditions on a daily tidal basis and have evolved mechanisms to adjust osmoregulation in such environments (Wood and Grosell 2009). These characteristics, in particular their ability to tolerate a wide range of salinities, make them an ideal species to examine the impacts of an EDS under different salinities.

1.8 Objectives and Hypotheses

The main objectives of this study are to determine whether the response of mummichog reproductive endpoints to an androgenic EDS, in this case DHT, is affected by the salinity of the holding medium (i.e. exposure under low and high salinities) and to determine how such a response may translate to the population level. These objectives were carried out in two phases. First, we used a 21-day reproductive bioassay to determine the effects of DHT on mummichog reproduction under different salinities (Chapter 2).
Fig. 1.2 Sexual dimorphism of mummichog (*Fundulus heteroclitus*): note the yellow belly and dark dorsal spot of the male (left) and the uniform silvery-gray color of the female (right; Smith 1985).
This study tested the following null hypotheses:

(1) there will be no difference between fish exposed to DHT under low salinity and estuarine conditions,

(2) there will be no difference in the effects of DHT on reproductive endpoints in exposed and unexposed fish, and;

(3) there will be no difference in the effect of salinity on reproductive endpoints in fish.

The second phase of this study refined a population projection model originally developed for fathead minnow (Miller and Ankley 2004) to predict population-level effects of mummichog exposed to anthropogenic and environmental stressors (Chapter 3). The data collected in Chapter 2 are applied in this model as a case study to predict the effects of chronic DHT and low salinity exposures on mummichog population viability.
1.9 References


Rutherford RJG. 2011. Improving understanding of endocrine-active compounds in pulp and paper mill condensates using a mummichog (*Fundulus heteroclitus*) bioassay. *MSc Thesis Wilfrir Laurier University, Waterloo, ON.*


Chapter 2  The impact of model androgen 5α-dihyrdotestosterone on mummichog (Fundulus heteroclitus) under different salinities
2.1 Abstract

Endocrine disrupting substances (EDSs) have the potential to perturb sensitive hormone pathways, particularly those involved in development and reproduction. Both fresh and estuarine water bodies receive inputs of EDSs from a variety of sources, including sewage effluent, industrial effluent and agricultural runoff. Based on limited literature, freshwater species appear to respond to lower levels of EDSs than estuarine or marine species. Therefore, effects elicited by EDSs in freshwater teleosts may not be an accurate representation of how EDSs affect teleosts in estuarine and marine environments. To address this potential difference, a short-term reproductive bioassay was carried out under low and high salinity conditions using mummichog (*Fundulus heteroclitus*), a euryhaline species that is native to the east coast of North America. The goal of the study was to determine the response of mummichog when exposed to an androgenic EDS and whether salinity affected the response. A model androgen, 5-alpha dihydrotestosterone (DHT), was selected for this experiment because androgenic EDSs are not as well-studied as estrogenic EDSs, although both pose risks to the environment. Impacts on reproduction were evaluated at multiple biological levels, including physiological (sex steroid levels), organismal (gonad size and gonad morphology), and functional (egg production) endpoints. In the high salinity group, egg production was significantly reduced in all exposure concentrations. In the low salinity group, there were no significant differences based on DHT treatment; however, all treatments including the control were significantly reduced relative to the high salinity control group. Other reproductive endpoints, such as reductions in sex steroid production, showed similar responses in both salinities. This study indicates that DHT may reduce the fecundity of mummichog, which is in stark contrast to studies that have found that exposure of mummichog to estrogenic 17α-ethinylestradiol (EE2) show no impact on fecundity.
The study also indicates that osmotic stress from low salinity conditions likely diverted energy from reproductive output in the low salinity group. This study demonstrates that mummichog fecundity is sensitive to androgenic endocrine disruption while also underscoring the importance of how changes in environmental variables can impact reproduction.
2.2 Introduction

Endocrine disrupting substances (EDSs) have received a significant amount of attention both from the general media and the scientific community as research has indicated that exposure to EDSs can negatively impact the health and performance of organisms, including development and reproduction (Martin and Voulvoulis 2009). EDSs include a wide range of anthropogenic and naturally occurring substances that have the potential to disrupt sensitive hormone pathways which regulate developmental and reproductive functions (Martin and Voulvoulis 2009). EDSs that specifically target estrogen or androgen receptors may act as agonists or antagonists and can elicit powerful effects at low concentrations (Kelce et al. 1994; Laws et al. 1995; Sharpe et al. 2004). For example, in a whole-lake study that exposed fathead minnow (Pimephales promelas) to a chronic, environmentally relevant dose (5-6 ng/L) of 17α-ethinylestradiol (EE2: a potent estrogen used in oral contraceptives), male fish exhibited feminization and intersex conditions, females displayed altered oogenesis, and the entire population suffered a near collapse (Kidd et al. 2007). Additionally, EDSs can disrupt hormone synthesis, transport, metabolism and clearance, which can cause organizational and functional changes that may be permanent or transient (Lister and Van Der Kraak 2001; Sharpe et al. 2004).

Estrogenic and androgenic (ant)agonists are a group of commonly occurring EDSs and enter both fresh and estuarine water bodies through a variety of sources (Martin and Voulvoulis 2009). One major source is treated sewage effluent, which contains pharmaceuticals that can act as estrogen and androgen mimicking compounds (Milla et al. 2011). Other examples of sources of EDSs include pulp mill effluent (Parks et al. 2001) livestock runoff (Gall et al. 2011; Lister et al. 2011), and agricultural runoff (which contains certain fungicides with have anti-androgenic
properties) (Kiparissis et al. 2003; Lister et al. 2011). With a myriad of sources that enter the
environment and contain EDSs, it is important to further the understanding of the effects of
EDSs in order to accurately predict their potential ecological impacts.

To date, most research has focused on the impacts of EDSs in freshwater systems. However it is
also important to understand the impacts of EDSs in estuarine habitats since both environments
receive inputs of these substances. Estuarine environments are of vital economic and ecological
importance; they are breeding and rearing grounds for many fish species and are both a year-
round and transitional habitat for important commercial species, such as salmon, sturgeon and
eels (Franco et al. 2006; Sarria et al. 2011). Most estuaries are highly urbanized and important to
economies because of ship ports and other commercial endeavors, resulting in high level inputs
of municipal and industrial effluents (Sarria et al. 2011). To optimally protect these
environments, it is important to understand the impacts of EDSs under estuarine conditions.

Under freshwater conditions, osmoregulation occurs through the gill and oral tissues with little
water ingested, while marine and estuarine fishes drink water to compensate for the dehydrating
effects of saline water (Black 1957). This difference in osmoregulation and uptake of
contaminants may impact the effect of EDSs on fish exposed under fresh or marine and estuarine
conditions. Based on limited data in the current literature, the salinity of the water under which
fish are exposed appears to affect the concentration level of EDS that elicits a reproductive
response from fish. For example, exposure to EE2 has resulted in reduced egg production in
freshwater Chinese rare minnow (*Gobiocypris rarus*) and zebrafish (*Danio rerio*) at
concentrations as low as 0.2 ng EE2/L (Zha et al. 2008) and 1 ng EE2/L (Lin and Janz 2006),
respectively. However, a study on mummichog (Fundulus heteroclitus; an estuarine species) reported a reduction in egg production at an exposure concentration of 100 ng EE2/L (Peters et al. 2007), while follow-up studies found no impact, even when fish were exposed to concentrations up to 3,000 ng EE2/L (Bosker, unpublished data.). In two other studies using estuarine model species, male mummichog (Peters et al. 2007), and sheepshead minnow (Cyprinodon variegatus) (Folmar et al. 2002) exposed to EE2 showed inductions of vitellogenin (an egg yolk precursor protein) at concentrations ≥100 ng EE2/L, while induction occurred at significantly lower levels in freshwater species (≤6.5 ng EE2/L; Hutchinson et al. 2006). A similar pattern was also observed when fish were exposed to 17β-trenbolone (a synthetic androgen used as a growth promoter in cattle); sheepshead minnow exposed under estuarine conditions to 17β-trenbolone showed reduction in egg production at 5 μg/L (Hemmer et al. 2008), while fathead minnow exposed under freshwater conditions responded to the same compound at concentrations 100-fold lower (Ankley et al. 2003).

There has only been one side-by-side comparison of freshwater and estuarine species using a short-term laboratory test. The study compared the effects of pulp mill effluent (PME; which can cause endocrine disruption) on fathead minnow and mummichog reproduction. However the effluent had little influence on the reproductive endpoints of either fish, with the only notable finding showing that egg production was increased in mummichog at the lowest PME concentration (Melvin et al. 2009).

This current study will be the first to directly compare the reproductive effects of an EDS under low (2 ppt) and high (16 ppt) salinities on mummichog, a euryhaline teleost, using an in vivo 21-
day fish reproductive bioassay. The 21-day fish reproductive bioassay is a standardized approach to screening chemicals for evidence of endocrine disruption and tests have been developed for a variety of fish species (Milla et al. 2011). Mummichog were chosen as the toxicological model species for this study as they are an established model species for environmental toxicology (Burnett et al. 2007). The mummichog is an abundant small-bodied fish indigenous to estuaries along the eastern North American coastline from Newfoundland to northern Florida, making them an environmentally relevant species to study the impacts of contaminants under estuarine conditions (Kneib 1986). Mummichog have been used extensively in environmental toxicology studies to examine the effects of environmental contaminants and model compounds on aspects of its development and reproduction (Peters et al. 2007; Bosker et al. 2010; Lister et al. 2011). Additionally, mummichog can tolerate a wide range of salinities, from freshwater to full strength seawater (Griffith 1974). The preferred range of salinities reported for mummichog ranges from 22-57 ppt saltwater (Fritz and Garside 1974), however, estuarine mummichog are exposed to low salinity conditions on a tidal basis and have evolved mechanisms to adjust osmoregulation in such environments (Wood and Grosell 2009). These characteristics make them an ideal species to examine the impacts of an EDS under different salinities.

To date, the majority of EDS research has focused on estrogens (Milla et al. 2011), while there have been much fewer studies that have evaluated the impacts of androgenic substances. In order to help to fill the gap in knowledge of androgenic EDSs, the androgen 5α-dihydrotestosterone (DHT) was selected for the EDS in this study. DHT is a naturally occurring sex steroid and is one of the most important androgens in male vertebrates, with the exception of teleosts in which 11-ketotestosterone (11-KT) and testosterone (T) are the major circulating male
androgens (Margiotta-Casaluci and Sumpter 2011). Androgens regulate a wide range of physiological processes in male vertebrates, including fish gonad differentiation, development of secondary sex characteristics, and modulation of behavior (Borg et al. 1993; Brantley et al. 1993a, b; Borg 1994; Kobayashi and Nakanishi 1999; Margiotta-Casaluci and Sumpter 2011). Although DHT is not found in high concentrations in the environment, it is non-aromatizable (i.e. not transformed into an estrogenic compound) and therefore can be used as a model androgen (Shilling and Williams 2000).

The primary objective of this study was to better understand whether salinity impacts the effects of DHT on mummichog reproduction. In addition, this study will also further our understanding about the impacts of DHT (regardless of salinity) and salinity (regardless of DHT) on the reproductive capability of mummichog. The following null-hypotheses will be evaluated: (1) there will be no difference between fish exposed to DHT under freshwater and estuarine water conditions, (2) DHT will have no effect on reproductive endpoints and (3) exposure under low and high salinity will not result in differences in mummichog reproductive endpoints.
2.3 Methods

2.3.1 Chemical

5-α-Dihydrotestosterone (DHT) was purchased from Steraloids Inc. (Newport, RI, USA). DHT was stored at 4°C in 100% ethanol (Decon, King of Prussia, PA, USA) at stock concentrations of 1, 10, and 100 μg DHT/mL ethanol, which translates to exposure levels of 0.05, 0.5, and 5 μg DHT/L in 20 L aquaria.

2.3.2 Fish

The study was conducted within the natural spawning season of the mummichog, which falls between April and September (Kneib 1986). All fish were handled according to protocol approved by University of Connecticut Institutional Animal Care and Use Committee (protocol number A12-019). Adult mummichog were collected using seine nets and minnow traps from Barn Island Wildlife Management Area in Stonington, Connecticut, USA (71°52' W, 41°20' N) in April of 2012. Barn Island, which is bounded to the south by Little Narragansett Bay, was selected for the specimen collection site because it is Connecticut’s largest coastal property managed for wildlife conservation and has limited human disturbance. Fish were transported to the Atwater Aquatic Facilities laboratory at the University of Connecticut Storrs campus and acclimated to filtered 16 parts per thousand (ppt) saline water. Deionized (DI) water was mixed with Instant Ocean Sea Salt (Blacksburg, VA, USA) to reach desired salinity levels. Fish were kept at ambient temperature (18-21°C), a summer photoperiod of 16 h light: 8 h dark, and dissolved oxygen (DO) at >80%. Fish were kept in four 246-L holding tanks and fed dense culture pellets (2/16-3/16”) and tropical fish flake food twice daily (Aquatic Eco-systems, Apopka, FL, USA). Basic water quality parameters such as dissolved oxygen (DO; >80%), pH
(6.9-7.7), temperature (18-21°C), salinity (1-2.5 ppt for low group; 14.4-16.5 for high group), hardness (425 ppm), nitrate (0.06-1.6 mg/L), nitrite (0-1.96 mg/L) and ammonia (1.1-8.5 mg/L) were tested daily. Salinity and temperature were monitored using a YSI 30 salinity, conductivity, and temperature meter (YSI, Yellow Springs, OH, USA). DO was monitored using a DO 200 Ecosense meter (YSI, Yellow Springs, OH, USA) and pH was monitored with a pH pen (Fisher, Scottsdale, AZ, USA). Other water quality parameters were measured using basic test strips designed for monitoring aquaria (Mardel, Glendale Heights, IL, USA) or analyzed by the University of Connecticut Center for Environmental Science and Engineering nutrients lab. In order to ensure proper water quality levels during the holding period, one-third of the water was changed out of each tank on either a daily or every-other-day basis.

After two weeks under constant brackish water conditions, fish were divided into two groups for a two week period of salinity acclimation. Half of the fish were acclimated to low salinity conditions (2 ppt) while the other half remained at brackish (16 ppt) water conditions. The salinity levels were incrementally adjusted, with a maximum salinity decrease of 3 ppt per day until the desired level was reached.

### 2.3.3 Short-term reproductive bioassay

After the five week acclimation period in the holding tanks, the 14-day pre-exposure phase of the experiment began. This phase mimicked all aspects of the actual experiment with the exception of the exposure to DHT. Fish (3 fish/sex/aquaria) were randomly allocated among the 64 20-L aquaria, with 32 low salinity and 32 brackish aquaria. Each aquarium was fitted with a removable plastic 3.175 mm mesh bottom (Aquatic Eco-systems, Apopka, FL, USA) for egg
collection and aerated to maintain adequate DO levels (>80%). Fish were fed the same diet of commercial crushed trout pellets and tropical fish flakes, but feeding frequency was reduced to once per day as the tanks were unfiltered.

During the pre-exposure phase water was changed daily beginning at approximately 9 am; fish were moved to a temporary aquarium with a small dip net. The mesh bottom was then removed from the original aquarium and the bottom of the aquarium was gently dredged with a small dip net to collect eggs. Water from the aquarium was replaced with clean water and the fish were placed back in their original aquarium. Eggs were counted and weighed daily during this phase to assess average egg production per day.

To optimize statistical power, tanks were selected based on standardized selection criteria (Bosker et al 2009 and 2010). Power was calculated based on a critical effect size of 40% reduction in egg production, which has been modeled to potentially reduce the population of fathead minnow, a small-bodied fish (Miller and Ankley 2004). Based on the standardized criteria, tanks with limited to no egg production were removed, and tanks that experienced mortalities during the pre-exposure phase were excluded from the study, except for two tanks that had five fish (3 F and 2 M in both cases). Based on these criteria, the number of 20 L aquaria in the experiment was reduced from 32 to 24 per salinity, for a total of 48 aquaria. Aquaria were randomly assigned to the different treatments (n=4 chemical treatments, with n=6 aquaria per treatment and 3 fish/sex/aquarium).
Eggs were collected, counted and weighed daily during the exposure phase using the same methods as previously described during the pre-exposure phase. Following egg collection each day, aquaria were dosed with 1 mL of stock solution. Prior to dosing, the stock solutions were stirred for approximately 5 min. Fish were exposed for 21 days to 0 (solvent control), 0.05, 0.5 and 5 μg DHT/L, a range of concentrations which had shown effect in previous studies conducted with DHT (Panter et al. 2004; Margiotta-Casaluci and Sumpter 2011; Rutherford 2011; Bosker et al., unpublished data). In order to facilitate sampling at the end of the experiment, the start of the exposure period was staggered by two days. Over the course of the pre-exposure and exposure phase there were 4 deaths in the experimental tanks: 3 at the 0.5 μg/L concentration (1 male, 1 female under low salinity; 1 male under high salinity) and one control (male, low salinity). Mortalities during the exposure were low and accounted for <2%.

### 2.3.4 Fish sampling and reproductive endpoints

After 21 days of exposure, the experiment was terminated and fish were anesthetized by placing them in 0.1g/L methanesulfonate (TMS; Aquatic Ecosystems, Apopka, FL, USA). Fish were weighed (±0.01g) and length was measured (±1 mm), to determine condition factor (CF; body wt./body length X 100). Next, fish were euthanized by spinal severance, and gonad and liver tissue were removed and weighed (±0.001g) to determine gonadal and liver somatic indices (GSI and LSI; tissue wt./body wt. X 100). The interior gonad section was placed in chilled Medium 199 (M199) prior to preparation for in vitro incubation, and the posterior section was placed in a histology cassette and immersed in Davidson’s fixative (Fournie et al. 2000) for 24-48 h prior to routine histologic processing for paraffin embedment and histologic sectioning.
2.3.5 ELISA for *in vitro* sex steroid production

Gonadal tissue was prepared for *in vitro* steroid level analysis following a protocol established by MacLatchy et al. (2003). Male and female gonad sections were divided into pieces weighing 19-25 mg and placed in 1 mL of M199 in *in vitro* well plates in duplicate. Gonadal tissue was incubated for 18 h at 18°C in an incubator (RevSci, Schafer, MN, USA), after which the incubation medium was collected with a pipette with minimal disturbance to the gonadal tissue and stored in microcentrifuge tubes at -20°C until steroid levels were measured using ELISA.

Commercial ELISA kits (Cayman Chemical, Ann Arbor, MI, USA) were used to measure *in vitro* production of T in males and females, 11-KT in males, 17β-estradiol (E2) in females (n=14-19 fish per treatment). Samples were run in duplicate and re-assayed when duplicate samples had a coefficient of variation (CV) ≥20% or concentrations exceeded standard curve values. If the same issue occurred after re-assaying then samples were disregarded from the analysis. Intra- and inter-assay variability was assessed by creating a mixture of incubation medium from control fish for males and females and adding 3 sets of duplicates to each 96-well ELISA plate (Table 2.1). Minimum detectable steroid concentrations were 54 pg/mL for T, 25 pg/mL for 11-KT, and 41pg/mL for E2.

2.3.6 Histological analysis

Following the immersion fixation of gonadal tissue in Davidson’s fixative for 24-48 h, the gonadal samples were transferred to 10% neutral buffered formalin and stored on an orbital shaker until they were processed. Histologic sections were prepared by the Histology Laboratory of the Connecticut Veterinary Medical Diagnostic Laboratory at the University of Connecticut.
Table 2.1
Mean values of intra-and inter-assay variability for *in vitro* steroid production in mummichog (*Fundulus heteroclitus*) using commercial ELISA kits.

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Male Intra-assay</th>
<th>Male Inter-assay</th>
<th>Female Intra-assay</th>
<th>Female Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>29.3%</td>
<td>35.8%</td>
<td>27.05%</td>
<td>12.7%</td>
</tr>
<tr>
<td>11-ketotestosterone</td>
<td>39.1%</td>
<td>20.0%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Estradiol</td>
<td>-</td>
<td>-</td>
<td>18.80%</td>
<td>25.0%</td>
</tr>
</tbody>
</table>
Tissues were processed routinely for paraffin embedment, sectioned it at 3-4 µm, mounted on glass slides and stained it with hematoxylin and eosin, then covered with glass cover slips (Sheehan and Hrapchak 1980). Gonads were sectioned sagittally with 2-5 sections per specimen.

Testicular maturity was assessed using a modification of the Environmental Protection Agency’s Histopathology Guidelines for the Fathead Minnow (Pimephales promelas) 21-day reproduction assay (USEPA 2006). Because all of the males were found to be reproductively viable and at approximately the same testicular maturity level (stage 2-3: mid to late spermatogenic), a morphometric approach was taken based on the proportion of gonadal tissue occupied by mature gametes (spermatids and spermatozoa) relative to total germinal epithelium (Yonkos et al. 2010). The less mature gametes (spermatagonia and spermatocytes) have a high proportion of cytoplasm and a low proportion of chromatin, and therefore stain predominantly eosinophilic (purple or pink) (Yonkos et al. 2010). The more developed gametes (spermatids and spermatozoa) are characterized by dense chromatin and scant cytoplasm and thus are strongly basophilic (blue) (Fig. A1 in Appendix A). Seminiferous chambers within the testes encompass predominantly one gamete type and so those that stain basophilic indicate that they contain the more mature gametes and those that stain eosinophilic contain the less mature gametes. This difference in staining properties was used to compare the proportion of mature gametes to the total germinal epithelium.

Histologic sections were examined by light microscopy using an Olympus BX60 microscope at 4X magnification (Tokyo, Japan). Digital images were captured with a Diagnostic Instruments Insight camera (4 MP) and SPOT version 5.0 imaging software (Sterling Heights, Michigan,
USA). Images were assessed using Adobe Photoshop Creative Suite 4 (San Jose, CA, USA) to determine the percentage of basophilic area relative to the entire germinal epithelium. This was carried out by separating the basophilic (blue) portion of the image from the entire histological section and using the threshold tool to convert the blue portion to black in order to read the pixel density on a set area. This pixel density was divided by the pixel density of the entire gonadal section in order to calculate the given area of the mature gametes (blue portion).

Female gonadal staging followed a modified approach to the EPA’s Histopathology Guidelines by classifying and counting the follicles as either exhibiting a vitellogenic yolk stage (includes early and late vitellogenic stages as well as mature stages) or a pre-vitellogenic stage (includes oogonia, nuclolar, perinucleolar and cortical alveolar oocytes; Fig. A2 in Appendix A) using an Olympus CX21 microscope at 4X magnification (Tokyo, Japan). The percent of vitellogenic follicles relative to the entire collection of oogenic cells per ovarian section was recorded. Follicles that were not sectioned properly (e.g. consisted primarily of chorion, otherwise known as zona radiata) were disregarded from assessment.

2.3.7 Solid phase extraction water chemistry analysis

Water samples (0.5L) were collected from aquaria (3 replicates per concentration per salinity) 1 hr post-dosing. Subsequent samples were collected over a 24-hr time period from control and high-dose aquaria per salinity 2, 4, 8, and 24 hr post dosing. Water samples were frozen at -20°C until the time of analysis. Prior to analysis, method detection limit (MDL) and precision/accuracy studies were performed to determine the MDL (0.02 ng/mL) with a precision/accuracy percent recovery (135% with 4.9 uncertainty) of DHT. Due to increased
particulates in the water, samples collected at 4, 8, and 24 h post-tank dose were filtered under vacuum pressure using fine porosity filter paper (Fisher, Scottsdale, AZ, USA) prior to sample preparation.

To prepare for analysis, 200 mL of the samples were spiked with surrogate standard, 5α-dihydrotestosterone-D3 (Cerilliant, Round Rock, TX, USA). Other laboratory controls included duplicate, method blank, laboratory control sample (LCS), matrix spike (MS) (LCS and MS were each spiked with 0.5 ng/ml DHT), as well as initial (original source: Steraloids Inc., Newport, RI, USA) and continuing calibration verification of DHT (CCV; alternate source: Cerilliant, Round Rock, TX, USA); these QA/QC procedures were performed every 16 samples. Oasis HLB extraction cartridges (60 mg, Waters, Milford, MA, USA) were washed 5 times with 4 mL of HPLC grade methanol and 1.5 times with 4 mL DI water by means of vacuum suction to remove excess particulate matter. Samples (200 mL) were extracted at a flow rate of 5 mL/min under vacuum pressure (10-12 psi). After the samples were processed through the cartridges, the cartridges were rinsed 3 times with 4 mL of DI water to remove excess salt from the cartridges. The DHT was then eluted from the cartridges with 10 mL of HPLC grade methanol. Eluates were filtered with PTFE 0.22 μm syringe filters (13 mm diameter, MicroSolv, Eatontown, NJ, USA) to remove excess particulates. Following this, eluates were placed in a 40°C water bath and reduced in volume from 10 mL to 0.2 mL under high purity nitrogen flow (thereby concentrating the original 200 mL sample 1000-fold). The internal standard, testosterone-d3 (Cerilliant, Round Rock, TX, USA), was added to the elutates prior to the sample analysis for a total sample volume of 220 μL.
Final DHT samples were analyzed by a Waters Acquity UPLC-MS-MS system (Milford, MA, USA) equipped with a triple quadropole mass spectrometer. Sample extracts (5μL) were injected into a reverse phase column (Waters Acquity UPLC BEH C 18, 1.7 μm, 2.1x50 mm column, Wexford, Ireland) at a flow rate of 0.65 mL/min. Separations of DHT from the internal standard (T-d3) and surrogate (DHT-d3) were achieved using gradient conditions (Table 2.2)

Detection and quantification of analytes of interest (DHT, T-d3, DHT-d3) were achieved using the following mass spectrometry parameters in Multi-Reaction Monitoring mode (MRM): ionization mode electrospray positive (ES+); DHT m/z 291 → m/z 255 (cone voltage 50 V, collision voltage 24V); T-d3 m/z 292 → m/z 96.8 (cone voltage 40 V, collision voltage 26); DHT-d3 m/z 295 → m/z 259 (cone voltage 50 V, collision voltage 24 V). Final injection volume was 5 μL and total run-time was 5 min. Quantification was carried out by internal calibration using a set of 7 calibration standards of known concentrations ranging from 0.005 ng/ml to 10.0 ng/ml. Non-detects were below 0.02 ng/ml (MDL).

2.3.8 Statistics

Statistical analyses were performed with the software Minitab version16 (State College, PA, USA). All data were tested for normality using standardized residuals with the Anderson-Darling test and tested for homogeneity of variance with Bartlett’s test and Levene’s test. Those that did not pass these tests were log transformed to meet the assumptions. Standardized residuals ≥3 or ≤-3 were deemed outliers and removed from analysis (Simon 2004).
Table 2.2
Gradient LC conditions used to separate the analytes of interest: DHT, the internal standard (T-d3) and the surrogate (DHT-d3) using a UPLC-MS-MS system, where A is 0.1% formic acid in water and B is 0.1% formic acid in acetonitrile.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate</th>
<th>%A</th>
<th>%B</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0.65</td>
<td>65</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.65</td>
<td>65</td>
<td>35</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>0.65</td>
<td>2</td>
<td>98</td>
<td>6</td>
</tr>
<tr>
<td>4.5</td>
<td>0.65</td>
<td>2</td>
<td>98</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>0.65</td>
<td>65</td>
<td>35</td>
<td>6</td>
</tr>
</tbody>
</table>
To assess endpoints that used individual fish as the unit of replication (length, weight, LSI, CF, GSI, steroid levels, histology) a two-way nested analysis of variance (ANOVA) was used with “DHT exposure level” and “salinity” as factors and the aquaria within each treatment group as the nested term. To calculate the F-statistics for each combination of factors, the adjusted mean squares (MS) of each factor was divided by the MS of the nested term. Egg production was assessed using a two-way ANOVA with “DHT exposure level” and “salinity’ as factors. It was first determined whether there was a significant interaction of DHT and salinity. If no significant interaction was found, it was determined whether there were significant effects of DHT or salinity. In the case of a significant difference, a Tukey’s post-hoc multiple comparison test was used to determine difference among treatments. All results are reported as means ± standard error of the mean (SEM).
2.4 Results

2.4.1 Somatic endpoints

**Males**

No significant interaction between salinity and DHT treatment was found in length, weight, LSI, or CF of males (Table 2.3 and 2.4). However, the DHT treatment, regardless of salinity, significantly affected the CF of males (Table 2.4). CF in the 0.05 µg DHT/L (1.22) was found to be significantly higher than control (1.15), 0.5 µg DHT/L (1.17), and 5 µg DHT/L (1.13) (Tukey’s post-hoc test, p<0.001). DHT treatment alone did not significantly affect length, weight, or LSI (Table 2.4). Salinity alone did not significantly affect any of the endpoints (Table 2.4).

**Females**

No significant interaction between salinity and DHT treatment was found in length, weight, LSI, or CF of females (Table 2.3 and 2.5). However, the DHT treatment, regardless of salinity, significantly affected the LSI of females (Table 2.5). LSI in the DHT 0.05 µg DHT/L (5.36%) was found to be significantly higher than 5.00 µg DHT/L (4.42%) (Tukey’s post-hoc test, p<0.001). The LSI of either treatment group was not significantly different from control (4.97%) or 0.50 µg DHT/L (5.08%) (Tukey’s post-hoc test, p>0.05). Females in the high salinity group were found to have a significantly higher CF (1.27) than those in the low salinity group (1.20), regardless of DHT treatment (Tukey’s post-hoc test, p=0.002). DHT treatment alone did not significantly affect length, weight, or CF and salinity alone did not significantly impact length, weight, or LSI (Table 2.5).
Table 2.3
Summary statistics of mummichog (Fundulus heteroclitus) exposed to 5α-dihydrotestosterone (DHT) (0, 0.05, 0.5, 5.0 for 21 days at high (16 ppt) and low (2 ppt) salinities.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Salinity</th>
<th>Treatment</th>
<th>(n_{(fish)})</th>
<th>Length (mm)</th>
<th>Weight (g)</th>
<th>LSI (%)</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>16 ppt</td>
<td>0.00</td>
<td>16</td>
<td>78.0 ± 1.58</td>
<td>5.58 ± 0.39</td>
<td>3.03 ± 0.76</td>
<td>1.15 ± 0.02</td>
</tr>
<tr>
<td>M</td>
<td>16 ppt</td>
<td>0.05</td>
<td>18</td>
<td>79.6 ± 1.71</td>
<td>6.24 ± 0.47</td>
<td>3.43 ± 0.81</td>
<td>1.22 ± 0.02</td>
</tr>
<tr>
<td>M</td>
<td>16 ppt</td>
<td>0.50</td>
<td>18</td>
<td>80.9 ± 1.01</td>
<td>6.33 ± 0.24</td>
<td>3.31 ± 0.16(^b)</td>
<td>1.19 ± 0.02</td>
</tr>
<tr>
<td>M</td>
<td>16 ppt</td>
<td>5.00</td>
<td>17</td>
<td>81.1 ± 1.72</td>
<td>6.04 ± 0.37</td>
<td>2.91 ± 0.71</td>
<td>1.13 ± 0.02</td>
</tr>
<tr>
<td>M</td>
<td>2 ppt</td>
<td>0.00</td>
<td>17</td>
<td>79.9 ± 1.54</td>
<td>5.95 ± 0.35</td>
<td>3.16 ± 0.20</td>
<td>1.15 ± 0.02</td>
</tr>
<tr>
<td>M</td>
<td>2 ppt</td>
<td>0.05</td>
<td>18</td>
<td>82.5 ± 1.71</td>
<td>7.04 ± 0.36</td>
<td>3.78 ± 0.22</td>
<td>1.24 ± 0.02</td>
</tr>
<tr>
<td>M</td>
<td>2 ppt</td>
<td>0.50</td>
<td>18</td>
<td>81.6 ± 1.42</td>
<td>6.31 ± 0.35</td>
<td>3.42 ± 0.17</td>
<td>1.15 ± 0.02</td>
</tr>
<tr>
<td>M</td>
<td>2 ppt</td>
<td>5.00</td>
<td>18</td>
<td>79.9 ± 1.05</td>
<td>5.94 ± 0.33</td>
<td>3.34 ± 0.19</td>
<td>1.14 ± 0.03</td>
</tr>
<tr>
<td>F</td>
<td>16 ppt</td>
<td>0.00</td>
<td>19</td>
<td>88.8 ± 1.51</td>
<td>9.08 ± 0.45</td>
<td>5.07 ± 0.21</td>
<td>1.27 ± 0.02</td>
</tr>
<tr>
<td>F</td>
<td>16 ppt</td>
<td>0.05</td>
<td>18</td>
<td>87.5 ± 1.26</td>
<td>8.57 ± 0.39</td>
<td>5.57 ± 0.29</td>
<td>1.27 ± 0.03</td>
</tr>
<tr>
<td>F</td>
<td>16 ppt</td>
<td>0.50</td>
<td>17</td>
<td>87.2 ± 1.61</td>
<td>8.42 ± 0.55</td>
<td>4.66 ± 0.25</td>
<td>1.25 ± 0.03</td>
</tr>
<tr>
<td>F</td>
<td>16 ppt</td>
<td>5.00</td>
<td>19</td>
<td>84.4 ± 1.45</td>
<td>7.72 ± 0.39</td>
<td>4.23 ± 0.22</td>
<td>1.26 ± 0.03</td>
</tr>
<tr>
<td>F</td>
<td>2 ppt</td>
<td>0.00</td>
<td>18</td>
<td>86.9 ± 0.99</td>
<td>7.87 ± 0.31</td>
<td>4.85 ± 0.19</td>
<td>1.17 ± 0.03(^c)</td>
</tr>
<tr>
<td>F</td>
<td>2 ppt</td>
<td>0.05</td>
<td>18</td>
<td>85.7 ± 1.33</td>
<td>7.62 ± 0.40</td>
<td>5.19 ± 0.20</td>
<td>1.19 ± 0.03</td>
</tr>
<tr>
<td>F</td>
<td>2 ppt</td>
<td>0.50</td>
<td>16</td>
<td>89.0 ± 1.71</td>
<td>8.57 ± 0.53</td>
<td>5.47 ± 0.36</td>
<td>1.19 ± 0.03</td>
</tr>
<tr>
<td>F</td>
<td>2 ppt</td>
<td>5.00</td>
<td>18</td>
<td>88.0 ± 1.88</td>
<td>8.26 ± 0.55(^a)</td>
<td>4.63 ± 0.30</td>
<td>1.24 ± 0.03</td>
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Values are means ±SEM
Letters reflect the values based on the removal of outliers: \(^a\)n=17, \(^b\)n=16, \(^c\)n=17
Table 2.4
Nested two-way ANOVA of somatic endpoints of male mummichog (*Fundulus heteroclitus*) exposed to 5α-dihydrotestosterone (DHT) (0, 0.05, 0.5, 5 µg/L) for 21 days at high (16 ppt) and low (2 ppt) salinities. The nested aquaria factor was used as the error term to assess the significance of the other fixed-effects factors. Weight and LSI data has been Log$_{10}$ transformed to meet assumptions of ANOVA.

<table>
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<tr>
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<th>p-value</th>
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<td>0.49</td>
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<tr>
<td>Error</td>
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<td>3430</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.) Weight of males (g)</td>
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<td></td>
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<td>C.) LSI of males(%)</td>
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<tr>
<td>Error</td>
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<td>0.96</td>
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<td></td>
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<tr>
<td>D.) CF of males</td>
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<td></td>
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<td>p&lt;0.001</td>
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<td>0.53</td>
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<tr>
<td>Error</td>
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<td>0.71</td>
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Table 2.5
Nested two-way ANOVA of somatic endpoints of female mummichog (*Fundulus heteroclitus*) exposed to 5α-dihydrotestosterone (DHT) (0, 0.05, 0.5, 5 µg/L) for 21 days at high (16 ppt) and low (2 ppt) salinities. The nested aquaria factor was used as the error term to assess the significance of the other fixed-effects factors. Weight and LSI data has been Log$_{10}$ transformed to meet assumptions of ANOVA.

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<td>0.02</td>
<td>0.39</td>
<td>0.76</td>
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<td>0.02</td>
<td>1.18</td>
<td>0.28</td>
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<td>1.00</td>
<td>0.40</td>
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<td>p&lt;0.001</td>
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<td>Error</td>
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<td>0.69</td>
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<td>C.) LSI of females(%)</td>
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<td>1.16</td>
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2.4.2 In vitro hormone levels

Males

Testosterone

In vitro T production in males was not significantly affected by the interaction of salinity and DHT treatment (Fig. 2.1, Table 2.6), nor was there an effect of DHT treatment or salinity alone (Table 2.6).

11-ketotestosterone

No significant interaction between salinity and DHT treatment was found in in vitro 11-KT production in male fish (Fig. 2.2, Table 2.6). However, a significant effect from treatment (regardless of salinity) was found on in vitro 11-KT production (Table 2.6). 11-KT production in the 5 µg DHT/L (112 pg/mg tissue) were significantly lower compared to control (239 pg/mg tissue), 0.05 µg DHT/L (277 pg/mg tissue) and 0.5 µg DHT/L (213 pg/mg tissue) (Tukey’s post-hoc test, p<0.001 for all comparisons). Salinity did not significantly affect the in vitro 11-KT production (Table 2.6).

Females

Testosterone

In vitro T production in females was not significantly affected by the interaction of salinity and DHT treatment, but there was a significant effect found from treatment regardless of salinity (Fig. 2.3, Table 2.6). T production in the 0.5 µg DHT/L (200 pg/mg tissue) decreased significantly relative to the control (339 pg/mg tissue) and 0.05 µg DHT/L (275 pg/mg tissue) (Tukey’s post-hoc test, p<0.001 for both comparisons), while T production in 5 µg DHT/L (223
Fig. 2.1 Mean (±SE) *in vitro* testosterone (T) production in male mummichog (*Fundulus heteroclitus*) exposed to 0, 0.05, 0.5, 5 µg/L 5α-dihydrotestosterone (DHT) at high (16 ppt; light gray) and low (2 ppt; dark gray) salinity for 21 days, \( n = 16-18 \). No significant differences based on the combined treatment of salinity and DHT treatment, salinity (independent of DHT treatment), DHT treatment (independent of salinity) were found (Tukey’s post-hoc test, \( p>0.05 \)).
**Fig. 2.2** Mean (±SE) *in vitro* 11-ketostestone (11-KT) production in male mummichog (*Fundulus heteroclitus*) exposed to 0, 0.05, 0.5, 5 µg/L 5α-dihydrotestosterone (DHT) at high (16 ppt; light gray) and low (2ppt; dark gray) salinity for 21 days, *n* = 14-18. Paired bars that do not share the same letter are significantly different based on DHT treatment alone (Tukey’s post-hoc test, *p*<0.001).
pg/mg tissue) decreased only relative to control (Tukey’s post-hoc test, \(p<0.001\)). Salinity did not significantly affect the in vitro T production in females (Table 2.6).

Estradiol

No significant interaction between salinity and DHT treatment was found in in vitro E2 production in female fish, but there was a significant effect found from treatment regardless of salinity (Fig. 2.4, Table 2.6). E2 production in 0.5 \(\mu\)g DHT/L (2450 pg/mg tissue) and 5 \(\mu\)g DHT/L (2372 pg/mg tissue) declined significantly relative to the control (3964 pg/mg tissue) and 0.05 \(\mu\)g DHT/L (3747 pg/mg tissue) (Tukey’s post-hoc test, \(p<0.001\) for both comparisons). Salinity did not significantly affect the in vitro E2 production (Table 2.6).

2.4.3 Histology

Males

The interaction of DHT treatment and salinity had a nearly significant effect on the percent of mature gametes (spermatids and spermatozoa) present within male mummichog with \(p=0.049\) (Fig. 2.5, 2.6, Table 2.7), however, the Tukey’s post-hoc test failed to detect any significant differences among the groups (all \(p>0.05\)). Neither salinity nor DHT treatment alone had a significant effect on the percent of mature spermatozoa (Table 2.7).

Females

The percent of vitellogenic follicles relative to the entire collection of oogenic cells per ovarian section was not significantly affected by the interaction of salinity and DHT treatment (Fig. 2.7, 2.8, Table 2.8), nor was there an effect from DHT treatment or salinity alone (Table 2.8).
Fig. 2.3 Mean (±SE) *in vitro* testosterone (T) production in female mummichog (*Fundulus heteroclitus*) exposed to 0, 0.05, 0.5, 5 µg/L 5α-dihydrotestosterone (DHT) at high (16 ppt; light gray) and low (2 ppt; dark gray) salinity for 21 days, \( n = 16-19 \). Paired bars that do not share the same letter are significantly different based on DHT treatment alone (Tukey’s post-hoc test, \( p<0.001 \)).
Fig. 2.4 Mean (±SE) in vitro estradiol (E$_2$) production in female mummichog (*Fundulus heteroclitus*) exposed to 0, 0.05, 0.5, 5 µg/L 5α-dihydrotestosterone (DHT) at high (16 ppt; light gray) and low (2ppt; dark gray) salinity for 21 days, $n = 16$-19. Paired bars that do not share the same letter are significantly different based on DHT treatment alone (Tukey’s post-hoc test, p<0.001).
Table 2.6
Nested two-way ANOVA of in vitro hormone production in mummichog (*Fundulus heteroclitus*) exposed to 5α-dihydrotestosterone (DHT) (0, 0.05, 0.5, 5 µg/L) for 21 days at high (16 ppt) and low (2 ppt) salinities. The nested aquaria factor was used as the error term to assess the significance of the other fixed-effects factors. Values reflect that the data has been \( \log_{10} \) transformed.

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<th>P-value</th>
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<td></td>
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<td><strong>B. 11-ketotestosterone production in males (pg/mg gonad tissue)</strong></td>
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<td>0.96</td>
</tr>
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<td>Error</td>
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Fig. 2.5 Mean (±SE) percentage of spermatids and spermatozoa relative to the entire germinal epithelium in male mummichog (*Fundulus heteroclitus*) exposed to 0, 0.05, 0.5, 5 µg/L 5α-dihydrotestosterone (DHT) at high (16 ppt; light gray) and low (2ppt; dark gray) salinity for 21 days, *n* = 16-18.
Fig. 2.6 Histology of mummichog (*Fundulus heteroclitus*) testes (4X) exposed to 5α-dihydrotestosterone (DHT) for 21 days at high (16 ppt) and low (2 ppt) salinities. A (0 µg/L), B (0.05 µg/L), C (0.5 µg/L) and D (5 µg/L) are representative testes of males held under high salinity. E (0 µg/L), F (0.05 µg/L), G (0.5 µg/L) and H (5 µg/L) are representative testes of males held under low salinity. All males were found to be reproductively viable and at approximately the same testicular maturity level (stage 2-3: mid to late spermatogenic). Seminiferous chambers within the testes encompass predominantly one gamete type and so those that stain basophilic indicate that they contain the mature gametes (spermatids and spermatozoa) and those that stain eosinophilic contain the less mature gametes (spermatagonia and spermatocytes).
Table 2.7
Nested two-way ANOVA of the percent of mature gametes (spermatids and spermatozoa) relative to the germinal epithelium of male mummichog (*Fundulus heteroclitus*) exposed to 5α-dihydrotestosterone (DHT) (0, 0.05, 0.5, 5 µg/L) for 21 days at high (16 ppt) and low (2 ppt) salinities. The nested aquaria factor was used as the error term to assess the significance of the other fixed-effects factors.

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<td>1.09</td>
<td>0.35</td>
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<tr>
<td>Error</td>
<td>92</td>
<td>0.85</td>
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</table>
Fig. 2.7 Mean (±SE) percentage of percent of vitellogenic follicles (includes early vitellogenic, late vitellogenic, and mature oocytes) relative to non-vitellogenic follicles of female mummichog (*Fundulus heteroclitus*) exposed to 0, 0.05, 0.5, 5 µg/L 5α-dihydrotestosterone (DHT) at high (16 ppt; light gray) and low (2 ppt; dark gray) salinity for 21 days, $n = 12$. 
Fig. 2.8 Histology of mummichog (*Fundulus heteroclitus*) ovaries (4X) exposed to 5α-dihydrotestosterone (DHT) for 21 days at high (16 ppt) and low (2 ppt) salinities. A (0 µg/L), B (0.05 µg/L), C (0.5 µg/L) and D (5 µg/L) are representative ovaries of females held under high salinity. E (0 µg/L), F (0.05 µg/L), G (0.5 µg/L) and H (5 µg/L) are representative ovaries of females held under low salinity. Assessment indicates that all females were reproductively active based on the presence of vitellogenic follicles. Vitellogenic yolk is indicated by eosinophilic (dark purple) staining, less mature follicles have a lower affinity for the eosinophilic stain and therefore are a lighter purple.
Table 2.8
Nested two-way ANOVA of the percent of vitellogenic follicles (includes early vitellogenic, late vitellogenic, and mature oocytes) relative to non-vitellogenic follicles of female mummichog (*Fundulus heteroclitus*) exposed to 5α-dihydrotestosterone (DHT) (0, 0.05, 0.5, 5 µg/L) for 21 days at high (16 ppt) and low (2 ppt) salinities. The nested aquaria factor was used as the error term to assess the significance of the other fixed-effects factors. Values reflect that the data has been $\log_{10}$ transformed.

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</tr>
<tr>
<td>Treatment</td>
<td>3</td>
<td>0.10</td>
<td>0.97</td>
<td>0.41</td>
</tr>
<tr>
<td>Salinity</td>
<td>1</td>
<td>0.06</td>
<td>1.96</td>
<td>0.17</td>
</tr>
<tr>
<td>Treatment*Salinity</td>
<td>3</td>
<td>0.01</td>
<td>0.20</td>
<td>0.90</td>
</tr>
<tr>
<td>Tanks (Treatment, Salinity)</td>
<td>39</td>
<td>1.73</td>
<td>1.21</td>
<td>0.27</td>
</tr>
<tr>
<td>Error</td>
<td>46</td>
<td>1.69</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4.4 Gonadal somatic index

*Males*

GSI in males was not significantly affected by the interaction of salinity and DHT treatment (Fig. 2.9, Table 2.9), nor was there an effect of DHT treatment or salinity alone (Table 2.9).

*Females*

No significant interaction between salinity and DHT treatment was found in female GSI; however, there was a significant difference based on treatment alone (Fig. 2.10, Table 2.9). The GSI of the 5 µg DHT/L treatment (10.3%) increased significantly relative to the 0.05 µg DHT/L treatment (7.77%; Tukey’s post-hoc test, p=0.004). Neither of these treatment groups were significantly different from control (8.89%) or 0.5 µg DHT/L (9.20%) treatments (Tukey’s post-hoc test, p >0.05). Salinity alone did not significantly affect GSI (Table 2.9).

2.4.5 Egg production

There was a significant interaction of DHT treatment and salinity found in egg production (Fig. 2.11, Table 2.10). Egg production experienced a significant decline in all treatment groups relative to the control treatment of the high salinity group (81.9 eggs/aquaria/day). Although egg production was significantly reduced in the low salinity group relative to the control of the high salinity group, there were no significant differences among the low salinity treatments (Fig. 2.9, Table 2.10, Tukey’s post-hoc test, p>0.05). Egg production in the low salinity control group (36.6 eggs/aquaria/day) was reduced by 55% relative to the high salinity control group.
Fig. 2.9 Mean (±SE) GSI male mummichog (*Fundulus heteroclitus*) exposed to 0, 0.05, 0.5, 5 µg/L 5α-dihydrotestosterone (DHT) at high (16 ppt; light gray) and low (2ppt; dark gray) salinity for 21 days, \( n = 16-18 \).
Fig. 2.10 Mean (±SE) GSI female mummichog (*Fundulus heteroclitus*) exposed to 0, 0.05, 0.5, 5 µg/L 5α-dihydrotestosterone (DHT) at high (16 ppt; light gray) and low (2ppt; dark gray) salinity for 21 days, *n* = 16-19. Paired bars that do not share the same letter are significantly different based on DHT treatment alone (Tukey’s post-hoc test, *p*=0.004).
Table 2.9
Nested two-way ANOVA of GSI of mummichog (Fundulus heteroclitus) exposed to 5α-dihydrotestosterone (DHT) (0, 0.05, 0.5, 5 µg/L) for 21 days at high (16 ppt) and low (2 ppt) salinities. The nested aquaria factor was used as the error term to assess the significance of the other fixed-effects factors.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of squares</th>
<th>F ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A.) GSI of males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>3</td>
<td>0.93</td>
<td>0.22</td>
<td>0.88</td>
</tr>
<tr>
<td>Salinity</td>
<td>1</td>
<td>0.01</td>
<td>0.01</td>
<td>0.94</td>
</tr>
<tr>
<td>Treatment*Salinity</td>
<td>3</td>
<td>3.66</td>
<td>0.88</td>
<td>0.46</td>
</tr>
<tr>
<td>Tanks (Treatment, Salinity)</td>
<td>40</td>
<td>55.4</td>
<td>0.85</td>
<td>0.71</td>
</tr>
<tr>
<td>Error</td>
<td>91</td>
<td>147</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B.) GSI of females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>3</td>
<td>119</td>
<td>4.50</td>
<td>0.009</td>
</tr>
<tr>
<td>Salinity</td>
<td>1</td>
<td>1.24</td>
<td>0.14</td>
<td>0.71</td>
</tr>
<tr>
<td>Treatment*Salinity</td>
<td>3</td>
<td>62.1</td>
<td>2.35</td>
<td>0.087</td>
</tr>
<tr>
<td>Tanks (Treatment, Salinity)</td>
<td>40</td>
<td>353</td>
<td>0.92</td>
<td>0.61</td>
</tr>
<tr>
<td>Error</td>
<td>93</td>
<td>895</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2.11 Mean (±SE) daily egg production/aquaria of mummichog (*Fundulus heteroclitus*) exposed to 0, 0.05, 0.5, 5 µg/L 5α-dihydrotestosterone (DHT) at high (16 ppt; light gray) and low (2 ppt; dark gray) salinity for 21 days, \( n = 16-18 \). Means that do not share the same letter are significantly different (p<0.05).
Table 2.10
Two-way ANOVA of average daily egg production/aquaria of mummichog (*Fundulus heteroclitus*) exposed to 5α-dihydrotestosterone (DHT) (0, 0.05, 0.5, 5 µg/L) for 21 days at high (16 ppt) and low (2 ppt) salinities.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of squares</th>
<th>F ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average daily egg production/aquaria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>3</td>
<td>4610</td>
<td>6.30</td>
<td>0.003</td>
</tr>
<tr>
<td>Salinity</td>
<td>1</td>
<td>4404</td>
<td>18.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment*Salinity</td>
<td>3</td>
<td>2943</td>
<td>4.02</td>
<td>0.020</td>
</tr>
<tr>
<td>Error</td>
<td>40</td>
<td>9759</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4.6 Water chemistry

Water analysis showed that fish were exposed to DHT in the expected range of concentrations and that there was no contamination of control aquaria with DHT. Recovery after the first hour of the 5 µg DHT/L treatments was 98.7% and 93.3% in the high and low salinity groups, respectively, thereafter declining steadily over the 24-h period (Table 2.11). DHT recovery concentrations after the first hour at the 0.5 µg DHT/L treatment at high and low salinities was 54.0% and 40.7%, respectively, and 0.05 µg DHT/L treatments were 80.0% and 73.3%, respectively (Table 2.11). Surrogate recovery of DHT-D3 fell within the expected range of 50-150% and criteria for all other QA/QCs were met.
Table 2.11
Mean (±SE) measured water concentrations (µg/L) of DHT during the exposure period. DHT concentrations were measured using solid phase extraction with UPLC-MS-MS. n=3.

<table>
<thead>
<tr>
<th>Salinity</th>
<th>Nominal treatment concentration (µg/L)</th>
<th>Exposure time post-treatment (h)</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>16 ppt</td>
<td>0.00</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td>0.03 ± 0.01</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.50</td>
<td>0.27 ± 0.01</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.00</td>
<td>4.93 ± 0.23</td>
<td>4.38 ± 0.31</td>
<td>3.77 ± 0.20</td>
<td>1.54 ± 0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 ppt</td>
<td>0.00</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td>0.04 ± 0.00</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.50</td>
<td>0.20 ± 0.01</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.00</td>
<td>4.67 ± 0.72</td>
<td>4.03 ± 0.96</td>
<td>2.00 ± 0.41</td>
<td>0.95 ± 0.67</td>
</tr>
</tbody>
</table>

ND, not detected; *n=2; x = samples not taken
2.5 Discussion

2.5.1 The influence of salinity on DHT treatment

This is the first study to directly compare the impacts of an androgenic EDS on the reproductive endpoints of a single species at different salinities. Egg production, an endpoint which can be directly related to the population viability of a species (Miller and Ankley 2004) was significantly impacted by the interactive effects of DHT and salinity. In the high salinity group, egg production declined significantly in all treatment levels relative to the control group by 39-49%. This equals or exceeds our pre-determined critical effect size of 40% (Miller and Ankley 2004). However, in the low salinity treatment, there was no significant effect from the DHT treatment, though baseline egg production, as determined within the low salinity control group, was significantly reduced by 55% compared to the control of the high salinity group. A previous study conducted with mummichog in which they were exposed to various concentrations of EE2 for 21 days under high (16ppt) and low (0 ppt) salinity conditions showed no effects of EE2 treatment on egg production, but did see a similar reduction of ~60% in egg production in fish exposed in low salinity compared to high salinity (Bosker et al, unpublished data). These findings suggest that stress related to salinity may have similar impacts on egg production as a potent EDS.

Unlike egg production, the responses of other reproductive endpoints to DHT were not significantly affected by salinity treatment as was hypothesized. This may be due to the sensitivity of fecundity, which has been shown to be one of the most sensitive endpoints when fish are exposed to EDSs. In a review study of EDSs used in fish 21-day reproductive bioassays, it was found that fecundity was the most sensitive endpoint in tests using fathead minnow (30
tests evaluated) and the second most sensitive endpoint in tests using Japanese medaka (*Oryzias latipes*; 22 tests evaluated) (Dang et al. 2011). Egg production is therefore a highly sensitive endpoint which helps to explain why it was the only endpoint affected by salinity.

The impact that low salinity had on egg production is important to note because exposures of mummichog to low salinity environments are scenarios which can be encountered in their natural environments. Estuaries are highly dynamic environments and wildlife that inhabit them are subject to the effects of storms, waves, run-off, fluctuation of water temperatures, currents, winds and solar radiance (Bindoff et al. 2007; Nicholls et al. 2007). For example, mummichog begin to spawn in the late spring to early summer and this time period coincides with periods of the highest annual rainfall in the northeast (Able and Palmer 1988). Furthermore, the occurrence of spring freshets (high volume outputs of freshwater into estuaries due to spring thaw) can also have a drastic effect on the salinity of estuaries on a diurnal as well as monthly basis (Hoff and Ibra 1977). Fluctuations in salinity by as much as 20 ppt over a month have been reported in a Massachusetts estuary (Hoff and Ibra 1977). In addition to natural fluctuations in salinity, the addition of large effluent volumes from municipal and industrial waste can have the dual effect of reducing salinity as well as inputting anthropogenic contaminants such as EDSs into an estuarine system. For example, in a study that examined the salinity fluctuations as a result of discharge effluent from household and industrial effluent Mangueria Bay, Brazil, it was found that the salinity surrounding the area of a main discharge location was consistently lower than reference sites (Niencheski and Zepka Baumgarten 2007). A study in Boston Harbor (MA, USA) showed that the input of treated sewage effluent also resulted in a decreased salinity (Singell et al. 2000).
Salinity is one of several environmental cues that play a role in the success of the fish reproductive cycle (Nguyen et al. 2003; Weltzein et al. 2004; Pham et al. 2010). Changes in salinity can cause stress due to the demands of maintaining osmotic balance (Nordlie 2006; Martin et al. 2009). Thus, mummichog in the low salinity group may have experienced difficulty in striking a balance between osmoregulation and reproduction. Landlocked freshwater mummichog have been reported (Klawe 1957; Denoncourt et al. 1978; Samaritan and Schmidt 1982) to have evolved a fundamentally different cell type in the gill from their estuarine counterparts in order to achieve active \( \text{Na}^+ \) uptake (Laurent et al. 2006). Although mummichog are capable of adapting to low salinities and changing their gill physiology, it is an energetically costly conversion (Laurent et al. 2006). A study that examined the respiratory rates of the euryhaline western mosquitofish (\textit{Gambusia affinis}) found a significant increase in respiration rates when fish were exposed to 0 ppt salinity as compared with 10 ppt, indicating that these low salinity conditions require a higher osmotic investment (Akin and Neill 2004). To avoid the conversion to freshwater-gill physiology, mummichog will maintain the saltwater-gill epithelial morphology in salinities as low as 1 ppt (Laurent et al. 2006; Wood and Grosell 2009). This means that in the current experiment the mummichog in the low salinity group (2ppt) likely shared the same gill physiology as their high-salinity counterparts but invested more energy into osmoregulation (Blewett et al. 2013; Wood and Grosell 2009), which resulted in less energy resources being available for reproduction.

Maintaining homeostasis under stressful conditions can divert a substantial portion of the organism’s energy budget and leave less energy available for other functions (e.g. locomotion, growth and reproduction; Sibly and Calow 1998; Martin et al. 2009). As an example, the
osmotic stress caused by low salinity conditions in some marine finfish species may inhibit ovulation and reduce egg quality during spawning season (Kjørsvik et al. 1990; Pham et al. 2010) due to the sensitivity of these processes to environmental factors (Wen and Lin 2001). Therefore, tradeoffs between responses to salinity stress and investments such as reproduction can influence the occurrence, community structure, and reproductive effort of species (Dunson and Travis et al. 1991).

The depressed reproductive output of the low salinity mummichog in this study relative to the high salinity group may be a result of energy tradeoffs between osmotic regulation under stress-inducing low salinity conditions and reproductive output. This finding is corroborated by a field study that examined the impacts of a salinity gradient on fecundity of three euryhaline small-bodied fish inhabiting fresh and brackish marshes in Louisiana, USA. Reproductive output of sailfin molly (Poecilia latipinna), western mosquitofish and least killifish (Heterandria formosa), as judged by the developmental stage of embryos removed from each gravid female, was assessed in fresh, intermediate, and brackish water (Martin et al. 2009). Reproductive output of all three species increased significantly with increasing salinity (Martin et al. 2009). Similar studies have also demonstrated that western mosquitofish (Stearns and Sage 1980; Brown-Peterson and Peterson 1990) and sailfin mollies (Trexler and Travis 1990) have a higher reproductive output in high salinity habitats than those from lower-salinity habitats. These studies provide evidence that the balance between reproductive output and osmotic regulation are more balanced at higher salinities in the three species. Conversely, lower level endpoints likely do not require as much energy to maintain and therefore may not respond as easily to a stressor. For example, maturing sockeye salmon (Ochorhynchus nerka) exposed to freshwater (0 ppt), iso-
osmotic water (13 ppt) and saltwater (28 ppt) for 5 days showed that there were no significant differences in the plasma T and E2 levels following the experiment (Cooperman et al. 2010). Moreover, the same review study that demonstrated the sensitivity of fecundity to EDS exposure also showed that changes in endpoints such as GSI and sex steroids are generally induced at exposure levels higher than those affecting fecundity in fathead minnow (Dang et al. 2011).

Although information regarding the exact energy expenditure of teleost sex steroid production relative to egg production is not available, it is well established that in most vertebrates female egg production is a more energetically costly endeavor than male sperm production (Randall et al. 2002). This information may allow for the inference that sex steroid production is also less costly than egg production.

Environmental factors, such as salinity can influence a fish’s response to contaminants (Abraham 1985). For example, in a study where mummichog embryos were exposed to a 15% water soluble fraction of No. 2 fuel oil under optimal conditions of salinity and temperature (20 ppt salinity, 20°C) the effects were only mildly toxic. However, under suboptimal conditions (10 ppt salinity and 20°C, 30 ppt salinity and 30°C ) embryos exposed to the same 15% WSF solution experienced 85-87% mortality (Linden et al. 1979). In another study in which different salinity levels were used to assess the acute toxicity of zinc (Zn) in mummichog and the mangrove killifish (*Kryptolebias marmoratus*), it was found that the LC50 values for Zn generally increased (and thus toxicity decreased) with increasing salinities for both species (Bielmyer et al. 2012). This study indicated that each species has a higher tolerance to Zn when exposed above their isotonic points, i.e. the point at which equal solute concentrations occur within the fish and its outside environment (9 ppt for mummichog, 15 ppt for mangrove killifish) (Bielmyer et al.
2012). These studies indicate that environmental variables play an important role in mediating the effects of some contaminants.

The salinity of water in which fish are exposed to an EDS may also be linked to how it is taken up into a fish’s body. In a study that examined the uptake of radiolabeled EE2 in mummichog it was determined that diffusion through the lipid-rich gills is the likely entry point for EE2 under high (16ppt) and freshwater (0ppt) salinities (Blewett et al. 2013). The highly lipophilic properties of EE2, with a large octanol-water partition coefficient (logK\text{ow} 4.12), make it probable that the gills are the rate-limiting step of EE2 uptake and from there the EE2 likely travels to different organs via the circulatory system (Blewett et al. 2013). Although the logK\text{ow} for DHT is not available, it shares lipophilic properties with EE2 and therefore its route of uptake in mummichog is likely similar to the route used by EE2. Since mummichog in the high and low salinity groups most likely shared the same saltwater-gill physiology, it would be expected they had nearly equal amounts of DHT uptake through the gills. Despite the shared route of uptake through the gills, it was found that EE2 levels of mummichog in the 16 ppt salinity group were 3-fold higher than those of the freshwater group (Blewett et al. 2013). This difference in EE2 accumulation between the two salinities is likely due to the differences in osmoregulatory demands between the two salinity groups; whereas mummichog in the freshwater group primarily osmoregulate via the active uptake of ions through the gills, the mummichog in the 16 ppt salinity will uptake water through mouth as well as passing it through the gill. Additional EE2 uptake via direct drinking of water is supported by the finding that the gut accounted for 10-20% of EE2 uptake in 16 ppt group (Blewett et al. 2013). Based on these findings, it is likely that the mummichog in the high salinity group of this study experienced an increased uptake of
DHT in contrast to the low salinity group which had lower drinking water demands for osmoregulation. This potential difference in DHT uptake may help explain the significant decline in egg production in all exposure groups in the high salinity group whereas egg production was uniformly depressed in the low salinity group across all treatment levels including the control, presumably due to stress of osmoregulation under low salinity conditions. However, further studies using radiolabeled DHT would be required to verify any potential difference in DHT uptake in mummichog.

Based on the results from the current study, it seems that in term of fecundity the exposure to low salinity conditions has a similar impact as exposure to the potent androgen DHT. These findings suggest that environmental conditions can strongly influence the reproductive capability of mummichog. Salinity, among other environmental conditions such as temperature and dissolved oxygen, are important because they act as environmental cues that help to regulate internal signals that control growth and reproduction (Pham et al. 2010). Due to the dynamic nature of estuaries, other environmental conditions can also cause stress, thereby impacting reproductive capability. For example, in a laboratory study that evaluated the effects of hypoxia on reproductive capability of gulf killish (*Fundulus grandis*), a close relative of the mummichog, it was found that after they had been exposed to hypoxic conditions for one month the females initiated spawning later than control fish and also produced a significantly lower number of eggs (Landry et al. 2007). A follow-up field study found significantly reduced gonad size in gulf killifish exposed to moderate to severe diel hypoxia as well as reduced 11-KT and E2 plasma levels (Cheek et al. 2009). The impact on lower-level endpoints indicates that hypoxia presents a high stress to the fish. The process of steroidogenesis necessitates oxygen-requiring enzymes,
thus hypoxic conditions likely suppress the activity of steroidogenic enzymes in the gonads, potentially explaining the lower sex steroid levels (Cheek et al. 2009). Temperature can also affect reproductive capability. Raised temperature from nuclear power plant effluent caused early maturity and faster rates of gameto- and gonadogenesis and oocyte degeneration in common roach (*Rutilus rutilus*) that occurred in near the effluent outflow compared with another subset of roach living in cooler waters of the same embayment (Luksiene and Sandström 1994). In a laboratory study that examined the effects of temperature on age and size of sexual maturity (defined as first egg production) it was found that fathead minnow exposed to warmer temperatures (30°C) with *ad libitum* feeding treatments resulted in decreased length and age at maturity (Dhillon and Fox 2004). While the warm temperatures may allow the fish to direct more energy to reproductive output at a younger age, this benefit may be outweighed by the costs to growth and survival (Dhillon and Fox 2004). These studies thus provide evidence of the powerful effect that environmental variables can have on reproductive capability.

It will be vital to understand the combined impacts of environmental stressors, such as hypoxia, and fluctuations in salinity and temperature on fish reproduction. Wild fish populations exposed to anthropogenic stressors such as EDSs do not experience them singularly, rather, such stressors are often accompanied by changes in environmental variables. It is therefore important for future work in the EDS field to examine how fish endocrine-mediated endpoints respond to the combined effects of EDSs and environmental variables.
2.5.2 Effects of DHT on mummichog reproduction

We assessed the impacts of a model androgen as a potential EDS in this study because there have been much fewer studies that have evaluated the impacts of androgenic substances relative to the extensive collection of studies examining estrogenic substances as endocrine disruptors (Milla et al. 2011). Although salinity either by itself or in conjunction with DHT largely did not have a significant effect of the reproductive endpoints of mummichog in this study (aside from egg production), sex steroid production (11-KT in males, and T and E2 in females) was impacted by DHT exposure independent of the effects of salinity. Changes in steroid production are considered reliable biomarkers because they can reflect direct (inhibition of enzymes involved in biosynthesis or degradation) and indirect (feedback inhibition or stimulation) on the HPG axis (Ankley et al. 2008).

The assessment of in vitro production of steroid levels in the mummichog of this study is comparable to other studies that have evaluated the effects of androgenic EDSs with other fish species. In males, there was a significant decline in 11-KT levels in the highest exposure group relative to all other treatment groups, while testosterone levels were not affected by DHT exposure. A review paper on 13 short-term reproductive tests using fathead minnow exposed to different EDSs showed that T and 11-KT levels or production are variable in relation to declines in fecundity (Dang et al. 2011). This lack of connection has been supported in another study that found that the quantitative relationship between male biomarkers in small-bodied fish and fecundity is weak (Bosker et al. 2010). 11-KT production in this study was only impacted at the highest level of DHT exposure while egg production was significantly reduced at all levels in the
high salinity group, thus reinforcing the conclusions of other studies that male biomarkers, including sex steroid levels and production, do not necessarily correlate with fecundity.

Female mummichog exposed to DHT in this study showed a significant decline in the in vitro production of both T and E2 in the medium and high exposure (0.50 and 5 μg DHT/L) groups. This response pattern in in vitro steroid production in females was comparable to other studies and had a more definitive response pattern than males. In a study in which mummichog were exposed to 17α-methyltestosterone (MT), females experienced a significant decline in T and E2 after 7 and 14 days of exposures (Sharpe et al. 2004). Similar results were also seen in juvenile fathead minnows, with females showing a significant decrease in T when exposed to DHT (2 and 200 ng DHT/L) (Margiatto-Casaluci and Sumpter 2011). In female gulf killifish T levels were unchanged while the E2 levels showed a significant decline under severe hypoxic conditions (Cheek et al. 2009). The relationship between fecundity and both T and E2 production in female fish has been shown to be relatively strong (Ankley et al 2008; Bosker et al 2010). In a review study, it was determined that female biomarkers have the highest potential to work as signposts for reproductive effects via endocrine disruption when screening for potential EDSs (Bosker et al. 2010). By comparison, male biomarkers have a higher occurrence of false negatives and therefore are not particularly reliable as indicators of reproductive effects at the functional level (Bosker et al. 2010). The results of this experiment help to support the conclusion that focusing on female reproductive endpoints provides a better means to predict the impacts of EDSs on reproduction.
There were no clear patterns concerning changes in the somatic indices, GSI or gonad histology in this study. This finding is typical of other studies that have evaluated the impacts of androgenic EDSs at these levels. For example, in a study that exposed juvenile fathead minnow to DHT (20 and 200 ng DHT/L), there were no observed effects on condition factor in either sex (Margiotta-Casaluci and Sumpter 2011). Similarly, in a study that exposed mummichog to treated effluent from pulp and paper mills, which are known to contain androgenic substances, there was no effect on GSI for males or females (Bosker et al. 2009). Based on these similar studies these endpoints do not appear to be sensitive endpoints for endocrine disruption.

The lack of response in gonad histology is somewhat surprising, as it has been shown to be a very sensitive endpoint for freshwater teleost species (Dang et al. 2011). In the current study, no significant changes were observed, although the proportion of vitellogenic follicles in the females of the low salinity control group was less than that of the high salinity control group, a trend which is similar to egg production. The lack of response in males is similar to a study that evaluated the GSI of F0 and F1 generations of male sheephead minnow to 17β-trendbolone and found that testes were normal at all exposure concentrations (Cripe et al. 2010). Therefore, while gonad histology is considered a sensitive endpoint, its response to endocrine disruption may vary by species or contaminant.

Biomarkers such as somatic indices, GSI, sex steroid hormones and histology are used in short-term reproductive bioassays to assess the potential of contaminants to negatively impact fish reproductive systems (Bosker et al. 2010). However, changes in these biomarkers are not always a clear indication of the effects at higher level endpoints such as survival and fecundity. For
example, a study where largemouth bass (*Micropterus salmoides*) exposed to pulp mill effluent resulted in reduced gonad size, but experienced no changes in fecundity (Sepúlveda et al. 2003). Findings like these show evidence of false positives, i.e. cases in which biomarker responses are not accompanied by functional responses (Hartung 2009; Bosker et al. 2010). While some biomarkers showed a response to DHT in this study, several biomarkers provided false negatives, meaning that they did not change to reflect the significant reduction in egg production experienced by all treatment levels in the high salinity group.

In the current study mummichog exposed under 16ppt salinity to concentrations as low as 0.05 µg DHT/L experienced a significant reduction in egg production. This response is an interesting contrast to some studies that evaluated the impact of estrogenic EDSs on mummichog. A study that examined the effects of EE2 on mummichog reproduction found a significant decline in fecundity (100 ng EE2/L) (Peters et al. 2007). However, four follow-up studies that re-evaluated the effects of EE2 using exposures ranging from 30-3,000 ng EE2/L on mummichog reproduction found no significant effects on fecundity (Bosker et al. unpublished). A lack of response was also found at a lower-level when gene expression of estrogen receptor-related receptors (ERRs), a group of nuclear receptors similar to those of estrogen receptors, was measured in adult male mummichog injected with EE2 (Tarrant et al. 2006). Alternatively, other studies have identified responses of lower levels biomarkers in mummichog exposed to estrogens. For example, reproductively inactive male mummichog injected with EE2 (5mg/Kg) experienced a significant increase in vtg expression (Greytak et al. 2010). Male mummichog exposed to 500 ng EE2/L showed a significant increase in vtg expression while females experienced a significant decline in plasma E2 levels at the same exposure level (MacLatchy et
Finally, E2 exposure resulted in a 20% reduction in male mummichog reproductive competence, measured by the presence and abundance of spermatozoa within collecting tubules and ductus deferens (Yonkos et al. 2010). Therefore, it is clear that estrogens can affect lower level endpoints in mummichog, yet such effects do not appear to translate to higher-level responses. Based on this marked difference in mummichog fecundity during exposure to estrogens (no response) and androgens (significant decline), it is important to understand why mummichog are capable of normal reproductive output when exposed to estrogens, while their egg production is severely impaired when exposed to an androgens. To date, androgen receptor(s) in mummichog have not been characterized or sequenced; therefore, the mechanisms of action (MOA) by which androgens function in mummichog are not well understood (Lister et al. 2011).

There are several MOAs by which DHT can affect the levels endogenous steroids. Although the goal of this study was not to determine the MOAs of DHT, potential MOAs can be inferred based on the changes in sex steroid biosynthesis. Effects could be mediated through classical binding to the androgen receptor and subsequent signaling pathways within the HPG axis. Other potential MOA explanations include changes to steroidogenesis, alterations to steroid transport, or changes in metabolism (Sharpe et al. 2004). The androgen receptor is a nuclear receptor to which androgenic steroids bind in order to transcribe mRNA that will be used to produce proteins that will be used in male-specific behaviors, sexual development and maturation (Lindzey et al. 1994; Zhou et al. 1994). DHT has been shown to have a higher AR binding affinity than 11-KT in several teleost species, including fathead minnow, zebrafish, rainbow trout, and stickleback (Margiatto-Casaluci and Sumpter 2011; Wilson et al. 2007; Jorgenson et
al. 2007; Takeo and Yamashita 2000; Olsson et al. 2005). Once DHT is bound to the AR the response will vary by species based on AR isoforms that have different characteristics (Wells and Van Der Kraak 2000). For example, in some species, such as Japanese eel (Anguilla japonica) and rainbow trout, DHT was a stronger inducer of AR-mediated transcription than 11-KT, which is the main circulating androgen in teleost species normally responsible for this function (Ikeuchi et al. 1999; Takeo and Yamashita 2000). However, in other species, such as stickleback and mosquitofish, 11-KT was the most potent inducer of AR-mediated transcription (Olsson et al. 2005; Katsu et al. 2007). These findings suggest that different androgens mediate the androgenic response differently by species, potentially based on the specificity of AR isoforms (Margiatto-Casaluci and Sumpter 2011; Sperry and Thomas 2000). The specificity of AR isoforms therefore makes it difficult to determine the effects of androgenic EDSs on different teleost species and the mechanisms by which they act.

In the cases where an androgenic EDS targets the AR, it is expected to act as either an agonist and mimic the action of an endogenous androgen or act as an antagonist and block the intended action of the endogenous androgen (Luccio-Camelo and Prins 2011). In this study, exposure to DHT caused a significant decline of T and E2 in females, the latter of which is synthesized from T by the enzyme aromatase. Since both endogenous steroids were reduced by DHT, it may be that DHT had an effect at the level of steroidogenesis, which is the process responsible for generating steroids from cholesterol. In other words, DHT may have produced an effect before reacting with either the AR. A similar barrier may be occurring in the steroidogenesis process in males, yet further into the process as T is unaffected but 11-KT is reduced at the highest exposure group. Based on these suppressed endogenous sex steroid levels, DHT may act an
antagonist in mummichog. However, this theory would require further testing identifying the mechanism of action before such a conclusion is drawn.

As aforementioned, biomarkers are intended to provide evidence of effects at the functional level (such as survival and fecundity), but as evidenced from the above examples, they are not necessarily indicative of such changes. In order to discern the potential differences in mummichog response between androgens and estrogens it is important to conduct studies that measure a range of biological endpoints, especially high-level endpoints such as fecundity and fertilization success.

Based on current literature, estuarine and freshwater teleost species respond to (anti-) estrogenic and androgenic EDSs (Peters et al. 2007; Yonkos et al. 2010; Margiatto-Casaluci and Sumpter 2011). However, a distinct difference between estuarine and freshwater teleost models is that typically estuarine model species require higher levels of EDS exposure to elicit effects than their freshwater counterparts. For example, ovarian histological analysis of sheepshead minnow exposed to TB revealed a substantial increase in atretic oocytes, which coincided with significant reductions in egg production at concentrations ≥ 870 ng TB/L (Cripe et al. 2010). In contrast, histological analysis of female fathead minnow exposed to ≥ 20 ng DHT/L experienced significant occurrences of testicular tissue in the ovaries (Margiotta-Casaluci and Sumpter 2011). Regarding estrogens, mummichog will continue to spawn at concentrations of 3,000 ng EE2/L while freshwater species such as fathead minnow, Japanese medaka, and zebrafish respond with a decrease in egg production at much lower concentrations of EE2 (0.1-10 ng EE2/L; Balch et al. 2004; Nash et al. 2004; Parrot and Blunt 2005; Lister et al. 2011). However, an interesting
aspect of the current study is that egg production was significantly reduced at 0.05 µg DHT/L (or 50 ng DHT/L), a low concentration more typically seen to elicit effects in freshwater species, rather than estuarine species as is the case here. This further warrants an investigation into the mechanisms of mummichog response to both androgens and estrogens.

To conclude, mummichog at 16 ppt salinity are highly sensitive to a model androgenic EDS in terms of fecundity, a response that is very dissimilar to studies that have evaluated the effects of estrogenic EDSs on this species. This portion of the study leaves us with two interesting questions that deserve follow-up investigations. The first asks why mummichog egg production varies so drastically between androgenic and estrogenic EDS exposures. To understand this potential difference in sensitivity, follow up studies should seek to discern the physiological modes of action of estrogenic and androgenic EDSs within mummichog. The second asks if mummichog sensitivity to androgenic EDSs is similar to that of freshwater species, which typically respond at much lower concentrations than their estuarine counterparts. Follow-up analysis to this question should use lower concentrations of DHT (<0.05 µg DHT/L) to determine a lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) in terms of fecundity with a side-by-side comparison of mummichog and a model freshwater species such as fathead minnow. Continued research into these areas will help to elucidate how endocrine disruption occurs and help to distinguish differences in the response between freshwater and estuarine species.
Appendix A
**Fig. A1** Histology of mummichog (*Fundulus heteroclitus*) testis representative of the high salinity control group, enlarged to show detail (40X). SG = spermatagonia, primary SC = primary spermatocyte, secondary SC = secondary spermatocyte, ST = spermatids, SZ = spermatozoa.
Fig. A2 Histology of mummichog (*Fundulus heteroclitus*) ovary representative of the high salinity control group, enlarged to show detail (10X). CN = chromatin nucleolar follicle, PN = perinucleolar oocyte, CA = cortical alveolar oocyte, EV = early vitellogenic oocyte, LV = late vitellogenic oocyte, M = mature/spawning oocyte.
2.6 References


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Rutherford RJG. 2011. Improving understanding of endocrine-active compounds in pulp and paper mill condensates using a mummichog (Fundulus heteroclitus) bioassay. MSc Thesis Wilfrir Laurier University, Waterloo, ON.


Chapter 3    Modeling population level effects of mummichog (*Fundulus heteroclitus*) exposed to 5α-dihydrotestosterone
3.1 Abstract

In this study a mummichog (*Fundulus heteroclitus*) population model was adapted from a similar fathead minnow (*Pimephales promelas*) model to predict population-level changes based on exposure to an anthropogenic or environmental stressor from a short-term toxicity test. This model combines elements of a Leslie matrix and a logistic equation to predict density-dependent changes in a closed population at carrying capacity. The model employed changes in mummichog fecundity from a short-term reproductive bioassay in which the fish were exposed to varying concentrations (0.05, 0.5, and 5 μg /L) of a model androgenic endocrine disruptor, 5α-dihydrotestosterone (DHT), as well as changes in fecundity from low salinity conditions (2 ppt) with no DHT exposure. Different model projections were developed based on the unique spawning patterns of mummichog subspecies, the southern species *Fundulus heteroclitus heteroclitus*, and the northern subspecies *Fundulus heteroclitus macrolepidotus*. After 20 years of reduced fecundity due to DHT exposure, the model predicted a 50% reduction in carrying capacity of the southern subspecies while the northern subspecies was expected to have a near population collapse due to their shorter spawning season. The long-term effects of low salinity conditions on mummichog showed a similar population projection to that assessing the effects of long-term androgen exposure. The model can be refined to account for variations in changes in sensitivity to the stressor (chemical or environmental), migration, and fecundity, but in its current form it shows that exposure to an anthropogenic or environmental stressor (in this case DHT and low salinity) can negatively impact the population viability of mummichog.

Employing data from short-term toxicity tests to population-level models is a credible and cost-effective means of developing ecological risk assessments.
3.2 Introduction

Anthropogenic endocrine disrupting substances (EDSs) enter the environment through a number of pathways and have been linked to reproductive and developmental impacts in exposed wildlife (Martin and Voulvoulis 2009). Effluent from wastewater treatment plants is one of the key routes of wildlife exposure because pharmaceuticals, such as 17α-ethynylestradiol (EE2), a potent estrogen used in oral contraceptives, are not sufficiently broken down in the treatment process (Ternes et al. 1999). As a result, 17β-estradiol (E2) equivalents have been measured in quantities as high as 147 ng/L in final treated effluent and 17 ng/L in receiving surface waters (Kirk et al. 2002; Tilton et al. 2002; Furuichi et al. 2004). Wastewater treatment effluent is not necessarily limited to the (anti-)estrogenic variety, it can also include (anti-)androgenic EDSs (Söffker and Tyler 2012). Other sources of androgenic EDSs include agricultural runoff and pulp and paper mill effluent (Yokos et al. 2010; Lister et al. 2011). Furthermore, effluent and run-off output are not consistent mixtures and its contents can vary overtime (Altenburger et al. 2004; Cleuvers 2004).

Field assessments as well as in vivo laboratory exposures of various fish species to EDSs have shown effects from the expression of vitellogenin (VTG) mRNA and protein in male fish (a biological marker associated with oocyte maturation in females) to reduced gonad size and fecundity and skewed sex ratios (Colborn et al. 1993; Munkittrik and Van Der Kraak 1994 Jobling et al. 1998; Yonkos et al. 2010). Importantly, a study has demonstrated that exposure to chronic, low concentrations of EE2 can lead to a population collapse in small-bodied fish (Kidd et al. 2007). Understanding the effects of EDSs on individuals as well as population status is essential to accurately
predict ecological effects because such information is applied to ecological risk assessments (Arcand-Hoy and Benson 1998).

Although many laboratory and field studies have examined the effects of EDSs at the individual level, there has been relatively little research done on how such exposures might affect an entire population (Miller and Ankley 2004). The prohibitive expense and complexity of chronic exposure tests that would better describe population-level effects are generally a limiting factor (Miller and Ankley 2004). An alternative means to deriving potential population-level effects from exposure to an EDS is through a short-term reproductive bioassay. The in vivo 21-day fish bioassay is a standard approach to screening for EDSs and tests have been developed for a variety of fish species (Milla et al. 2011). In these tests a reproductively mature teleost species (typically of a small-bodied variety) is exposed to a range of concentrations of a particular compound for 21-28 days. The test derives a great deal of information about the potential of a contaminant to act as an EDS by determining the impacts of the contaminant on reproductive endpoints, including fecundity and fertility.

Data from these short-term reproductive tests have been used to predict population-level responses by using models (Miller and Ankley 2004; Bosker et al 2010a). These population models need to use species-specific life history data in order to make useful assumptions of population-level effects (Miller and Ankley 2004). In the current study, we used a population risk assessment model developed by Miller and Ankley (2004) that is based on a short-term fathead minnow (Pimephales promelas) reproductive bioassay in which the fish were exposed to 17β-trenbolone, an androgen receptor agonist used to stimulate cattle growth. This model
combines elements of a Leslie matrix and a logistic equation to predict changes in a closed population at carrying capacity (Miller and Ankley 2004). The modeling approach is relatively simple, requiring only a life history table, an estimate of carrying capacity, and the effects of how a particular stressor affects the vital rates at the individual level (Miller and Ankley 2004). The application of this simple model allows for an assessment of a particular stressor, such as an EDS, at the population level.

The model developed by Miller and Ankley (2004) is focused on freshwater environments. However, the sensitivity of fish to EDSs can differ based on environmental conditions (Jin et al. 2011), and responses are species-specific (Lister et al. 2011). It is important to monitor the effects on estuarine systems because they are of vital economic and ecological importance; they are breeding and rearing grounds for many fish species and are both a year-round and transitional habitat for important commercial species (e.g. salmon, sturgeon and eels) (Franco et al. 2006; Sarria et al. 2011). Estuaries are also important to economies because of ship ports and other commercial and recreational endeavors. As a result, many estuaries are heavily impacted by major population centers and industries which input high levels of municipal and industrial effluents (Sarria et al. 2011).

The goal in the current study is to adapt the aforementioned population model for mummichog (Fundulus heteroclitus) to predict population-level effects from chronic exposure to anthropogenic and environmental stressors. Mummichog are an abundant small-bodied fish indigenous to estuaries along the eastern North American coastline from Newfoundland to northern Florida, which makes them an environmentally relevant species to study the impacts of
contaminants under estuarine conditions (Kneib 1986). In addition, mummichog can tolerate a wide range of salinities, from freshwater to full strength seawater which makes them a suitable laboratory organism for evaluating the effects of an EDS under different salinities (Griffith 1974). Their abundant numbers, non-migratory nature, general hardiness, and ease of maintenance in the laboratory make mummichog an agreeable toxicological model both in the lab and field (Burnett et al. 2007). For this reason, mummichog have been used extensively in environmental toxicology studies to examine the effects of environmental contaminants and model compounds on aspects of its development and reproduction (Peters et al. 2007; Bosker et al. 2010a,b; Lister et al. 2011). Additionally, a short-term reproductive bioassay has been developed, refined, and validated for mummichog (Peters et al 2007; Bosker et al. 2010a). These characteristics make mummichog an excellent candidate for which to develop a species specific population model.

An important component to the population model assessment is that it assumes a closed population with a pre-breeding census (Miller and Ankley 2004). This assumption is plausible for the fathead minnow, for which this population model was originally developed, as this species occurs in freshwater bodies that can be isolated from the effects of immigration and emigration (Kidd et al. 2007). Although mummichog typically occur in estuaries that are open systems rather than closed, the proposed population model remains a suitable fit for this species because of their high site fidelity. In a mark and recapture study, 96.6% of marked fish remained within 200 m of their original marking sites within a large riverine estuary system (Skinner et al. 2005). This finding supports other studies that demonstrated similar site fidelity of mummichog in different environments, including small tidal creeks and pannes (Murphy 1991; Sweeney et al.
Due to their high site fidelity, mummichog are assumed to accurately reflect local environmental conditions and have therefore been used in environmental monitoring programs, such as those used to assess the effects of pulp and paper mill discharge (Courtenay et al. 2002; Skinner et al. 2005).

In the present study we used concentration-response data from a short-term mummichog reproductive bioassay that exposed fish to a model androgenic EDS, 5α-dihydrotestosterone (DHT), under low (2 ppt) and brackish (16 ppt) salinities. The objectives of this study are to (1) modify a population projection model for mummichog, an important estuarine model species, (2) use the population model to assess the impacts of an anthropogenic stressor, in this case the potent androgen DHT, on a mummichog population under brackish conditions and (3) to determine the potential effects of a prolonged environmental stressor, in this case low salinity conditions, on population viability.
3.3 Methods

3.3.1 Development of a mummichog population model

The model used in the current study is based on the original model developed by Miller and Ankley (2004). Their methods for developing the model are outlined in Appendix A. To assess the impacts of anthropogenic and environmental stressors on population status, a density dependent population model was used (Eq. (1)).

\[ n(t+1) = \exp\left( -\frac{rP_t}{K} M^* n_t \right) \]  (Eq. 1).

In Eq. (1), \( n \) is the population age structure at time \( t \), \( P_t \) is the scalar population size at time \( t \), \( r \) is the intrinsic rate of increase, \( K \) is the carrying capacity and \( M^* \) is the Leslie matrix containing the vital rates of survivorship and fertility that have been adjusted to include age-specific changes in these parameters over time step \( t \) resulting from an exposure (Ankley and Miller 2004). Therefore, by using Eq. (1), the effect of a stressor on a given population can be assessed with respect to a population existing in a given geographic area with a given carrying capacity (Miller and Ankley 2004).

3.3.2 Model application

To refine the model for mummichog, life history characteristics (fertility and mortality rates, carrying capacity) were collected and analyzed from available literature. The length of annual reproductive period of mummichog varies with latitude, but will typically begin in spring (March-May) and end in the later summer or fall (July-September) (Hardy 1978, based on Kneib 1986). Most mummichog attain sexual maturity during their second year, although some may mature and spawn during their first year; egg production typically increases with age and size (Hardy 1978; Kneib and Stiven 1978). It should be noted that a density-dependent logistic matrix
model can be used with a Leslie matrix based on either a size-class or an age-class (Miller and Ankley 2004). The current model was based on size-class which is appropriate as fecundity in fish is typically related to size and various size classes do not contribute to population fecundity equally (Bagenal 1978; Kneib 1986; Nikolsky 1986).

There are two known subspecies of mummichog: the southern subspecies *Fundulus heteroclitus heteroclitus*, and the northern subspecies *Fundulus heteroclitus macrolepidotus*. The subspecies distinction is made based on differences in egg morphology, gene frequencies and spawning behaviors with populations divided along coastal northern New Jersey (Morin and Able 1983).

Age-specific vital rates of the southern subspecies *Fundulus heteroclitus heteroclitus* were used to construct a life table (Table 3.1) and a Leslie matrix model (Fig. 3.1), based on a pre-breeding census and survival and fecundity rates found in the literature (Meredith and Lotrich 1979; Kneib 1986; Abraham 1985; Skinner et al. 2005). In using birth pulse fertility values and a prebreeding census with an annual time step, the fertility values for each size-class are calculated as the product of survival from birth to age 1 year and reproductive output of an individual upon reaching its i\textsuperscript{th} birthday (Caswell 2001; Miller and Ankley 2004). Birth pulse fertility values were used because mummichog have an annual breeding season that lasts between 3-5 months, depending on location (Kneib 1986) and a prebreeding census was chosen so that the number of eggs within the water body would not need to be estimated when comparing the model to field samples (Miller and Ankley 2004).

Variation in mummichog egg production was not available for either subspecies, so to incorporate uncertainty of model projections to variations in fecundity rates, the method used by Miller and Ankley (2004) was employed. The egg production of fathead minnow, the small-
Table 3.1
Life table for the southern mummichog subspecies (*Fundulus heteroclitus heteroclitus*) vital rates derived from field populations (Kneib 1986; Abraham 1985; Meredith and Lotrich 1979; Skinner et al. 2005).

<table>
<thead>
<tr>
<th>Size class (SL mm)</th>
<th>Survival (per year)</th>
<th>Fecundity (eggs/breeding season)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;36</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>36-45</td>
<td>0.005</td>
<td>105</td>
</tr>
<tr>
<td>46-55</td>
<td>0.0023</td>
<td>372</td>
</tr>
<tr>
<td>&gt;56</td>
<td>0.001058</td>
<td>1284</td>
</tr>
</tbody>
</table>
Fig. 3.1 Leslie matrix for southern mummichog subspecies (*Fundulus heteroclitus heteroclitus*) derived from field studies (Kneib 1986; Abraham 1985; Meredith and Lotrich 1979; Skinner et al. 2005) and developed using birth pulse survival and fertility rates and a prebreeding census (Miller and Ankley 2004).
bodied fish used in the Miller and Ankley model, varies by less than 10% of the mean number of eggs produced per female (Duda 1989). Assuming a similar variation for female mummichog, egg production was varied by 10% of mean fertility rates at DHT exposure concentrations of 0.05 μg DHT/L, the lowest concentration at which an effect was seen, using fertility rates of both subspecies.

Mortality of young-of-year mummichog may be as high as 99.5% (Meredith and Lotrich 1979). Within the population model, the fecundity of breeding fish and the survival of age 0 to age class 1 is represented by the fertility parameters of the model as the Leslie matrix uses a prebreeding census (Caswell 2001; Miller and Ankley 2004). Once mummichog have reached a size of >36 mm standard length, mortality rates stay relatively stable among all size classes at approximately 54% (Table 3.1, Fig. 3.1; Meredith and Lotrich 1979; Skinner et al. 2005).

Mummichog are the most abundant member of their genus in some areas, but their schooling numbers can be highly variable (Hilebrand and Schroeder 1928; Huver 1973). In the case of this population model, carrying capacity was based on field data that reported mummichog populations ranging from 130,000-136,000 individuals along 3 km of a Delaware tidal creek (Meredith and Lotrich 1979). A mid-range of 133,000 was selected as the population carrying capacity for mummichog over a range of 3km of tidal creek for this population model. Assuming a population at carrying capacity is fitting because field experiments have suggested that mummichog populations are food limited (Weisberg and Lotrich 1982). Therefore, a population at carrying capacity assumes sufficient resources for the population to invest energy into normal reproductive output. Based on a population at carrying capacity with a presumed
logistic population growth, the population model simulations were initiated with the population at the stable size distribution as calculated by the Leslie matrix. The stable size distribution was determined by inputting the Leslie matrix into an online eigenvector and eigenvalue calculator (Brünner 2003). Using this approach, a population of 133,000 *F. h. heteroclitus* at stable size distribution consists of 96,973 in size class 36-45 mm, 27,963 in size class 46-55 mm and 8,064 in size class >56 mm at the beginning of each time step.

### 3.3.3 Effects of exposure to stressors on population status

This density-dependent logistic matrix model was applied to predict the population dynamics for mummichog exposed to the model androgen DHT. The concentration-response data at the individual level was based on the 21-day short-term reproductive bioassay that exposed reproductively-mature mummichog to DHT concentrations ranging from 0.05-5 μg DHT/L. Adult survival was unaffected in this study; however, fecundity of the fish at brackish salinity (16 ppt) was significantly reduced at test concentrations ≥0.05 μg DHT/L (Table 3.2). For each population simulation, the population of mummichog began at carrying capacity (133,000 individuals at stable size distribution) and was subsequently exposed to a given concentration of DHT. The effects of DHT exposure on population structure was projected over a 20 year period.

In addition to the effect of a potent EDS on fecundity, the effect of exposure to low salinity conditions (2ppt; no exposure to DHT) on fecundity was also compared to fecundity under brackish (16ppt; no exposure to DHT) conditions. On average, egg production in brackish conditions was 81.9 eggs/aquaria/day ±5.3 (3 females/aquaria). By comparison, egg production
Table 3.2
Effects on female mummichog fecundity of exposure to varying concentrations of 5α-dihydrotestosterone under 16 ppt salinity and low salinity (2 ppt) with no DHT exposure.

<table>
<thead>
<tr>
<th>Stressor</th>
<th>Decrease in fecundity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure to DHT (ug/L)</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>38.8</td>
</tr>
<tr>
<td>0.50</td>
<td>46.6</td>
</tr>
<tr>
<td>5.00</td>
<td>49.1</td>
</tr>
<tr>
<td>Holding at low salinity</td>
<td></td>
</tr>
<tr>
<td>2ppt</td>
<td>55.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values for decrease in fecundity are relative to the control group for 16 ppt
in low salinity conditions was significantly lower at 36.6 eggs/aquaria/day ±3.4 (Tukey’s post-hoc test, p = 0.0016). Assuming that the high salinity conditions support the carrying capacity of 133,000, this shows that low salinity conditions deplete egg production by 55.3% (Table 3.2). The population projection simulation of mummichog began with *F. h. heteroclitus* Leslie matrix at carrying capacity of 133,000 at stable size distribution and was subsequently exposed to low salinity conditions. The effect of low salinity exposure on population structure was projected over a 20 year period.

### 3.3.4 Subspecies differences

There are two subspecies of mummichog, a southern and northern variety, each with distinct spawning patterns. An additional Leslie matrix was developed to reflect the longitudinal variability in spawning of the two subspecies. The original matrix (Fig. 3.1) was based on a spawning period of the southern subspecies *Fundulus heteroclitus heteroclitus*, which tend to have longer spawning periods that are synchronous with spring tides at new moon and full moon phases (Taylor et al. 1979). That is, this subspecies may spawn for 4-8 times in the breeding season with each spawning session lasting up to 5 days (Taylor et al. 1979). In contrast to the well-studied spawning events of *F. h. heteroclitus*, there is less information available regarding the spawning habits of the northern subspecies, *Fundulus heteroclitus macrolepidotus*. The northern subspecies spawning habit is asynchronous with moon phases and appears to be more closely correlated with water temperature (McMullin et al. 2009). *F. h. macrolepidotus* tend to spawn daily for a shorter period of time (e.g. 5 June – 9 July for a population in St. John, New Brunswick, Canada) and produce less eggs per spawn (n= 12 eggs/female in St. John population) (McMullin et al. 2009). Although data concerning egg production for different size classes in the
northern species is not available, this information was estimated by calculating the percent of egg production in the lower sizes classes relative to the largest size class in the *F. h. heteroclitus* population and using those different percentages to calculate the number of eggs spawned in the size classes of the *F. h. macrolepidotus* population, assuming $n=12$ eggs/day/female for the largest size class (McMullin 2009). The spawning period used for the *F. h. macrolepidotus* was 35 consecutive days, which is based on the study of McMullin et al. (2009). The survivability parameters were not altered between the different populations. A new life table (Table 3.3) and Leslie matrix (Fig. 3.2) were developed for the population assessment of the northern subspecies.

While the same carrying capacity of 133,000 was used for this population projection, the stable age distribution was adjusted to reflect the new Leslie matrix. The stable size distribution at the beginning of each time step for the *F. h. macrolepidotus* consists of 79,604 in size class 36-45 mm, 36,583 in size class 46-55 mm and 16,812 in size class >56 mm at the beginning of each time step. This approach was applied to investigate the sensitivity of model projections to variability in fertility in different subspecies of mummichog.
Table 3.3
Life table for the northern mummichog subspecies (*Fundulus heteroclitus macrolepidotus*) vital rates derived from field populations (Kneib 1986; Abraham 1985; Meredith and Lotrich 1979; Skinner et al. 2005; McMullin et al. 2009)

<table>
<thead>
<tr>
<th>Size class (SL mm)</th>
<th>Survival (per year)</th>
<th>Fecundity (eggs/breeding season)</th>
</tr>
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<tbody>
<tr>
<td>&lt;36</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>36-45</td>
<td>0.005</td>
<td>42</td>
</tr>
<tr>
<td>46-55</td>
<td>0.0023</td>
<td>151</td>
</tr>
<tr>
<td>&gt;56</td>
<td>0.001058</td>
<td>420</td>
</tr>
</tbody>
</table>
Fig. 3.2 Leslie matrix for the northern mummichog subspecies (*Fundulus heteroclitus* *macrolepidotus*) derived from field studies (Kneib 1986; Abraham 1985; Meredith and Lotrich 1979; Skinner et al. 2005; McMullin et al. 2009) and developed using birth pulse survival and fertility rates and a prebreeding census (Miller and Ankley 2004).
3.4 Results

The Leslie matrix for the southern subspecies mummichog (*F. h. heteroclitus*) (Fig. 3.1) yielded an intrinsic rate of increase of 0.47. The projected population trend of a southern subspecies mummichog (*F. h. heteroclitus*) existing at a carrying capacity of 133,000 and exposed to varying levels of DHT (0.05, 0.5, 5 μg DHT/L) was calculated from Eq. (1) (Fig. 3.3). The reduction in fecundity due to exposure to all DHT concentrations resulted in marked population declines. After 10 years, the population exposed to the lowest concentration had been reduced to 51% of carrying capacity while populations exposed to 0.50 and 5.00 μg DHT/L were reduced to 39.4% and 35.7% of carrying capacity, respectively. After 20 years reduction in fecundity due to continuous exposure to DHT (with the assumption of no outside recruitment) the mummichog populations at all DHT exposure concentrations were reduced to 50% or less of carrying capacity (Fig. 3.3).

To assess the sensitivity of the model to changes in fertility, the fertility rate was changed with a 10% increase and decrease around the mean fertility values (Table 3.4). This resulted in minimal changes in the overall estimated population size, with a maximum deviation of 6% above projected mean population size and a minimum deviation of 7.8% below projected mean population size after 10 years (Table 3.4). After 20 years of exposure the maximum deviation was 7.3% above and the minimum 10.4% below the projected mean population size (Table 3.4).

By comparison, the Leslie matrix for the northern subspecies mummichog (*F. h. macrolepidotus*) (Fig. 3.2) derived from the northern population in McMullin et al. (2009) yielded an intrinsic rate of increase of 0.00095. This projection shows that a population exposed to any of the
Fig. 3.3 Population projection for a southern mummichog subspecies (*F. h. heteroclitus*) population existing at a carrying capacity of 133,000 and subsequently exposed to varying levels of $5\alpha$-dihydrotestosterone (DHT). One control and three exposure concentrations were evaluated: (A) control, (B) 0.05 μg DHT/L, (C) 0.5 μg DHT/L, (D) 5 μg DHT/L.
Table 3.4
Sensitivity of model projections to fertility rates within the Leslie matrix. Fertility rates of the northern and southern subspecies were varied from 90% to 110% of their values found within the Leslie matrix. Projections for a mummichog (*Fundulus heteroclitus*) population exciting at a carrying capacity of 133,000, exposed to 0.05 μg DHT/L, was calculated after 10 and 20 years of exposure.

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>Population size after years of exposure to DHT</th>
<th>Fertility rates set to 90% of matrix values</th>
<th>Fertility rates unchanged</th>
<th>Fertility rates set to 110% of matrix values</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. h.</em> heteroclitus</td>
<td>10 years 62,553</td>
<td>67,851</td>
<td>72,221</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 years 59,145</td>
<td>66,040</td>
<td>71,281</td>
<td></td>
</tr>
<tr>
<td><em>F. h.</em> macrolepidotus</td>
<td>10 years 12,016</td>
<td>15,251</td>
<td>18,730</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 years 933</td>
<td>1,816</td>
<td>3,246</td>
<td></td>
</tr>
</tbody>
</table>
concentrations of DHT used in this study suffered a near collapse after 10 years of continuous exposure with no outside recruitment, with populations ranging from 5.3-11.5% of carrying capacity (Fig. 3.4). After 20 years of exposure, the populations were reduced to 0.3-1.4% of carrying capacity. Similar to the southern subspecies, varying the fertility 10% around the mean fertility values did not have a great impact on projected population status (Table 3.4).

The impact of long-term exposure to low salinity conditions results in a similar population projection as the mummichog exposed to a potent androgen (Fig. 3.5). After 10 years of continuous exposure to low salinity conditions the population is reduced to 48% of carrying capacity; after 20 years it is further reduced to 40% (Fig 3.5).
Fig. 3.4 Population projection for a northern mummichog subspecies (*F. h. macrolepidotus*) population existing at a carrying capacity of 133,000 and subsequently exposed to varying levels of 5α-dihydrotestosterone (DHT). One control and three exposure concentrations were evaluated: (A) control, (B) 0.05 μg DHT/L, (C) 0.5 μg DHT/L, (D) 5 μg DHT/L.
**Fig. 3.5** Population projection for a southern mummichog subspecies (*F. h. heteroclitus*) population existing at a carrying capacity of 133,000 and subsequently exposed to low salinity conditions (2 ppt, solid line). Dashed lines represent the confidence intervals of low salinity egg production, based on SEM.
3.5 Discussion

The results from this model predict that a closed population of mummichog with a continuous reduction in fecundity due to DHT exposure (≥0.05 μg/L) will experience large population declines in both subspecies, *F. h. heteroclitus* and *F. h. macrolepidotus*, with the latter potentially suffering a complete population collapse due to its lower reproductive output.

Although DHT used in the current case study is not an environmentally-relevant androgenic contaminant (Runnals et al. 2010), other androgenic EDSs can be present in aquatic systems from a variety of sources. For example, complex effluents such as pulp and paper mill effluent and treated wastewater effluent can contain chemicals that can act as EDSs through the androgen receptor by measuring effects on androgenic-dependent traits or direct testing for androgens (Parks et al. 2001; Lister et al. 2011; Yang et al. 2012). Additionally, these complex effluents typically also contain other EDSs with different modes of action such as anti-androgenic and (anti-)estrogenic compounds (Yang et al. 2012). Studies examining the effects of complex effluent on fish reproductive endpoints in short-term reproductive bioassays and field studies have found declines in sex steroid production, alterations in gonad morphology, increased incidence of intersex conditions, and reduced fecundity (Bortone et al. 1989; Munkittrik and Van Der Kraak 1994; Yonkos et al. 2010; Tetreault et al. 2011). For example, a study assessing the effects of pulp mill effluent (PME) on mummichog reproduction found that a 30% concentration of PME caused a reduction of egg production while lower concentrations (3% PME) caused an increase in egg production (Bosker et al. 2010b). Mosquitofish (*Gambusia affinis*) in the Brazos River (TX USA) downstream from wastewater effluent outflows and agricultural runoff were found to have masculinized secondary sex characteristics and significantly lower total fecundity than fish at reference sites (Deaton and Cureton II 2011). Given the presence of these EDSs in
aquatic systems and their negative impacts to fish reproduction, it is important to understand how such contaminants can affect fish at the population level.

In addition to the effects from anthropogenic inputs, changes in environmental variables, such as salinity and temperature can create stressful environments for fish which may impair reproductive functions. For example, in a field study that evaluated reproductive output of small-bodied euryhaline fish, including sailfin molly (*Poecilia latipinna*), western mosquitofish and least killifish (*Heterandria formosa*), along a salinity gradient, it was found that fish in freshwater had significantly lower egg production than those at intermediate or brackish salinities (Martin et al. 2009). In addition to natural fluctuations in salinity, aquatic environments that receive anthropogenic inputs of large effluent volumes from municipal and industrial waste can also experience the added stressors of reduced salinity and increased temperature (Niencheski and Zepka Baumgarten 2007).

The simple model that was adapted for mummichog in this study is based on standard life characteristics of mummichog. Although mummichog are a well-researched model estuarine species, some of their life-history characteristics have either not been explored or are subject to high variation. Therefore, there are several factors to take into consideration when interpreting this model.

Firstly, this population model assumes a closed system and therefore does not account for any potential immigration or emigration of mummichog from adjacent populations. Although mummichog exhibit high site fidelity, it is likely that some individuals may leave or join a population (Skinner et al. 2005). If data is available for migration between mummichog...
populations, Eq. (1) can be amended to include a vector that accounts for migrants and immigrants among the different size-classes at each time step (Miller and Ankley 2004).

A second important consideration is a change in fecundity based on population density and growth rates. Mummichog density has been found to be negatively correlated to growth rates; when held at 1/8 of their natural density they experienced growth rates three times the rate of the natural population (i.e. a population at carrying capacity), conversely, when they were enclosed at four times their natural density, their growth rate was only half of their natural growth rate (Weisberg and Lotrich 1986). Importantly, fecundity was reduced when fish density exceeded carrying capacity, while it was unaffected when fish were held at densities below the carrying capacity (Weisberg and Lotrich 1986). The relationship between growth rates and density is important because fecundity rates have been based on size classes in this model, thus if density varies from year to year so too will the fertility rates. Based on Kneib and Stiven (1978), from which fecundity rates were derived for this model, larger fish have longer spawning periods with higher reproductive output. Therefore more detailed field studies are required to understand the dynamics of density, growth rate, and fecundity year to year in order to make better assumptions of how these variables may change in response to a stressor. More detailed information for the northern subspecies *F. h. macrolepidotus* is also required since fertility rates based on age or size class were not available and had to be inferred based upon the southern subspecies *F. h. heteroclitus*.

Finally, another major assumption of this model is that mummichog will react with the same sensitivity to anthropogenic and environmental stressors in each new generation. When
environmental conditions are constant, the exposed population may change its genetic structure overtime as an evolutionary mechanism to adapt to local conditions (Futuyama 1986; Nacci et al. 1999). For example, a study examining the sensitivity of mummichog to dioxin-like compounds (DLCs), contaminants that are extremely toxic to the early development of many fish species (Walker and Peterson 1991; Safe 1994; Grinwood and Dobbs 1995), found that mummichog from the extremely polluted New Bedford Harbor (Massachusetts, USA) were much less sensitive to DLCs than mummichog from a clean reference site (Nacci et al. 1999). This shows that reduced sensitivity to DLCs is an inherited trait that is correlated with increased survivability following exposures to DLCs and thus supports the idea that mummichog are capable of adapting to high concentrations of toxic and persistent contaminants (Nacci et al. 1999).

Conversely, a three generation laboratory exposure study of sheepshead minnow (*Cyprinodon variegatus*; also an estuarine species) to constant levels of 17β-trenbolone found that successive generations became more sensitive to the effects of 17β-trenbolone (Cripe et al. 2010). These studies show that fish adaptability to particular contaminants may be compound- or species-specific. In order to understand the effects of EDSs or environmental stressors on a mummichog population overtime, it may be necessary to complete a multi-generation laboratory assay and incorporate the change (if any) of sensitivity of successive generations to stressors in the projected population model effects. Such a study could also discern size/age-class specific responses to stressors.

Despite these important considerations, the model nonetheless provides a basic understanding of the potential effects of stressors on mummichog population viability. Understanding population level effects from anthropogenic or environmental stressors is important because this information is applied to ecological risk assessments (Arcand-hoy and Benson 1998). The most accurate way
to predict long-term population-level effects is by conducting an ecosystem-based study. For example, a whole lake study in an Experimental Lake (34 ha) in Canada showed that a 3-year chronic exposure of fathead minnow to low concentrations of EE2 (5-6 ng EE2/L) resulted in a near extirpation of the species (Kidd et al. 2007). This study was the first to confirm that EDSs can cause population-level collapses and thus provided valuable information regarding the potential far-reaching effects of EDSs. However, the main disadvantage of this type of study is the extensive time and monetary commitment (Miller and Ankley 2004). In fact, the Experimental Lake Area in Canada is one of the few long-term ecosystem-level aquatic systems aquatic research facilities in the world and its future funding has been a subject of contention (Babbage 2013). Thus, opportunities for controlled large-scale toxicity studies are relatively uncommon. Conversely, short-term reproductive bioassays provide a more cost-effective means of gathering information about the effects of stressors on reproductive endpoints. In these tests, information about changes in sex steroid levels, somatic indices, fecundity and survival rates are collected at the individual level (Miller and Ankley 2004). This data can be incorporated into the logistic Leslie matrix model employed in this study to derive population-level effects from any species for which there exists a short-term reproductive test that evaluates some type of stressor (anthropogenic or environmental) and the appropriate life-history information.

To conclude, we successfully modified a population model for mummichog and demonstrated that exposure to an anthropogenic or environmental stressor (in this case DHT and low salinity) has the potential to negatively impact the population viability of mummichog. While this method provides several advantages (e.g. affordable, short-term, available for a variety of species), there are options for refinement that would serve to make more reliable predictions
about the extent of population-level effects. More detailed field studies will provide site-specific data regarding changes in population density and fecundity rates while more comprehensive laboratory exposure studies will provide data concerning changes in sensitivity based on size/age classes and generational sensitivities. Nonetheless, in its current form this population model is a useful and economical means of deriving population-level effects from short-term reproductive bioassays that can be applied to risk assessment.
Model development

The Leslie matrix was developed to project population growth over time using fecundity and survival rates of individual age classes within the population (Miller and Ankley 2004). The equation

\[ n_{t+1} = M n_t \]  

(1)

denotes that the vector of population age structure at time \( t + 1 \) \( (n_{t+1}) \) is calculated from the product of the Leslie matrix and the vector of population age structure at time \( t(n_t) \). Using birth pulse survival probabilities, birth pulse fertilizes, and a prebreeding census, the Leslie matrix can be written as:

\[
\begin{bmatrix}
F_1 & F_2 & F_3 & \ldots & F_{i=n} \\
S_1 & 0 & 0 & \ldots & 0 \\
0 & S_2 & 0 & \ldots & 0 \\
\vdots & \vdots & \vdots & \ddots & \vdots \\
0 & \ldots & 0 & \ldots & S_{i=n-1} \\
\end{bmatrix}
\]

(2)

where \( S_i \) is the survival from age \( i \) at time \( t \) to age \( i+1 \) at tie \( t+1 \) and \( F_i \) is the fertility of age group \( i \). To identify the stable age distribution, the eigenvector was calculated using an online eigenvector calculator (Brünner 2003). The basic Leslie matrix projects exponential population growth when the population is at a stable age distribution (Miller and Ankley 2004). The dominant eigenvalue of the Leslie matrix represents the finite rate of increase. The natural log of the dominant eigenvalue of the Leslie matrix results in the intrinsic rate of increase, which is an estimate of the per capita rate of population increase associated with a population represented by the rates in the matrix (Miller and Ankley 2004). The application of the Leslie matrix model
alone is limited in utility because it projects only exponential growth and does not account for the density of the population (Miller and Ankley 2004; Burgman et al. 1993). Density-dependent competition dictates population size by way of regulating fecundity and survival rates. The logic of this phenomenon states that limited food and resources are available within a population and the amount of these resources can only support a certain population level which makes exponential growth unlikely (Miller and Ankley 2004).

To account for this limitation of the Leslie matrix, the logistic equation can be employed to describe logistic rather than exponential population growth. The logistic equation has been used for projecting population growth in many studies and is widely accepted as model for density-dependent population growth (Maynard-Smith 1968; Burgman et al. 1993; Gotelli, 1998). Combining the simple Leslie matrix with a discrete time form of the logistic equation creates a simple density-dependent matrix model that can be written as (May 1974; Miller and Ankley 2004):

\[ P_{t+1} = P_t \exp(r) P_t \quad (3) \]

And

\[ P_{t+1} = P_t \exp(r - rP_t/K) \quad (4) \]

Where \( P_t \) is the scalar population size at time \( t \), \( P_{t+1} \) is the scalar population size at time \( t+1 \), \( r \) is the intrinsic rate of increase, and \( K \) is the carrying capacity (Miller and Ankley 2004). To describe logistic growth the Leslie matrix of Eq. (2) can be multiplied by \( \exp(r - rP_t/K) \) resulting in the equation:

\[ n(t + 1) = \exp (- (rP_t)/K) Mn \quad (5) \]
The matrix model of Eq. (5) exhibits the stability characteristics of a stable age distribution that is identical to the discrete time form of the logistic Eq. (4) (May 1974; Miller and Ankley 2004). The parameters required for the logistic matrix model of Eq. (5) include a combined life and fecundity table and an estimate of carrying capacity based on field data (Miller and Ankley 2004). This model provides a link between the life table parameters, the Leslie matrix, and the logistic growth equation (Miller and Ankley 2004).
3.6 References


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Chapter 4  General discussion
The occurrence of endocrine disrupting substances (EDSs) in the aquatic environment is an important issue that has been examined extensively over the past decades (Martin and Voulvoulis 2009). However, while there is an abundance of information regarding the effects of estrogenic EDSs in freshwater teleost species, it is apparent that estuarine species and androgenic EDSs are not as well-studied (Milla et al. 2011). This research was undertaken with the purpose of helping to fill this gap in knowledge by examining the effects of an androgenic EDS on a euryhaline species under low and estuarine salinity conditions. The objectives of the study were (1) to determine the response of mummichog (Fundulus heteroclitus) reproductive endpoints to a model androgen, 5α-dihydrotestosterone (DHT) and to see if these responses varied under low or estuarine salinity and (2) to use this data to model population-level effects.

The main findings of my thesis work are that mummichog fecundity rates are sensitive to both DHT and low salinity conditions. In the high salinity group, egg production declined significantly in all treatment levels relative to the control group by 39-49%. In the low salinity treatment, there was no significant effect from the DHT treatment, but baseline egg production as determined within the low salinity control group was significantly reduced by 56 % compared to the control of the high salinity group. The depressed reproductive output of the low salinity mummichog in this study may be a result of energy tradeoffs between osmotic regulation under stress-inducing low salinity conditions and reproductive output. These findings suggest that stress related to salinity may have similar effects on egg production as a potent EDS. The significant effect of low salinity on mummichog reproduction underscores the importance of considering environmental variables as stressors to reproductive function. For example, in a study that examined the effects of raised temperature from nuclear power plant effluent, it was
found that common roach (*Rutilus rutilus*) occurring in the enclosed area near the effluent outflow experienced early maturity and faster rates of gameto- and gonadogenesis as well as oocyte degeneration than another subset of roach living in cooler waters of the same embayment (Luksiene and Sandström 1994). Another study that evaluated the effects of DO oscillations in estuarine populations of gulf killish (*Fundulus grandis*), a close relative of the mummichog, found that testes and ovaries of fish in sites that experienced moderate to severe diel hypoxia were significantly smaller than those of fish at a reference site that experienced little to no diel hypoxia (Cheek et al. 2009). This effect also translated to hormone levels, with significant reductions in 11-KT under moderate to severe diel hypoxia and significant reductions in E2 at severe hypoxia (Cheek et al. 2009). These studies demonstrate that environmental variables can have a strong influence on the reproductive potential of fish.

An interesting aspect of this study is the marked effect of DHT, at levels as low as 0.05 μg DHT/L, on fecundity. This presents an interesting contrast to the lack of effects estrogenic 17α-ethinylestradiol (EE2) has had on mummichog fecundity (Bosker et al. unpublished), suggesting that mummichog may be more sensitive to androgenic EDSs than their estrogenic counterparts. Future studies should examine the mechanism of actions behind the (lack of) response of mummichog to estrogenic and androgenic contaminants.

The predictive mummichog population model which was adapted from Ankley and Miller (2004) showed steady population declines in both the southern *F. h. heteroclitus* and northern *F. h. macrolepidotus* subspecies at all DHT exposures in the high salinity group. Although the model can be further refined to account for uncertainties in variation of generational and size/age-class
sensitivity to stressors, migration and fertility rates, it nonetheless shows a basic understanding of the potential effects of stressors on mummichog population viability.

Based on the information learned from this project, I have identified two important future areas of research. Firstly, a side-by-side study should be conducted in which mummichog are exposed to an estrogen or an androgen. This research should confirm whether mummichog are more sensitive to androgens than estrogens, and describe the different mechanisms of action of androgens and estrogens. Information derived from such a study may be useful in verifying whether mummichog are reliable indicators of detecting the effects of androgenic and/or estrogenic EDSs. A second area of future work should evaluate the effects of EDSs in conjunction with changes in environmental variables. Important environmental variables include the level of dissolved oxygen (normoxic versus hypoxic conditions), different temperature levels (e.g. to simulate global warming scenarios or the effects of high effluent input), as well as a combination of these environmental stressors. Employing data from these studies in predictive population models, as was done in this case, will help to create reliable ecological risk assessments. The proposed future work may eventually add to the collective effort of developing mitigation methods for endocrine disruption in both estuarine and freshwater systems.
4.1 References


