ENDOCRINE MODULATORS AND THE DEVELOPMENT OF SEXUALLY DIMORPHIC CHARACTERS IN WEAKLY ELECTRIC FISH, *MORMYRUS RUME PROBOSCIROSTRIS*

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Abstract

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The African weakly electric fish Mormyrus *rume proboscirostris* is an excellent preparation to study organization and activation of sexually dimorphic characters. These characters are readily observable and include the waveform of the fish's electric organ discharge (EOD) and the morphology of basal anal-fin rays. Fish used in this study were bred in our laboratory, and therefore allowed for accurate accounting of their developmental stage. This thesis investigated the organizational role of the non-aromatizable androgen, 17α-methyldihydrotestosterone (MDHT) in the development of sexually dimorphic characters in *M. r. proboscirostris*. The results served as a baseline for comparison with the effects of the organometal tributyltin (TBT), an aromatase inhibitor, on the expression of these characters. We tested the following predictions: (1) early-life exposure at 17-26 days post fertilization to MDHT (1.5-3 mg/L) will result in androgen-specific organization of sexually dimorphic characters at 4 and 24 months post exposure, (2) adult exposure to TBT (10 µg/L) will elicit androgen-specific activational transformations of these same characters, and (3) the aromatase inhibiting effects of TBT will adversely affect the fish's reproductive potential. The results

revealed: (1) the female gonadal phenotype is organized prior to that of the male suggesting that mormyrids are undifferentiated gonochorists. (2) Activation of secondary characters (EOD duration and basal fin-ray expansion) likely occurred immediately following MDHT treatment as evident in juveniles. (3) Exposure of adults to TBT mimicked MDHT affects as shown in the expression of male-typical EOD duration and anal-fin morphology in females. In males, the purportedly stable first EOD phase lengthened, suggesting that TBT induced reduction of ATP synthesis affected membrane permeability. (4) The reproductive potential was adversely affected by TBT exposure, as both oocytic development and spermatogenesis appeared to be suppressed. The results strongly support the notions that the endocrine system in *M. r. proboscirostris* retains a high degree of plasticity into adulthood, and that organization and activation are not two independent processes.

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Table of Contents

List of Figures

List of Tables

Endocrine Modulators and the Development of Sexually Dimorphic Characters in Weakly Electric Fish, *Mormyrus rume proboscirostris*

Introduction

Reproduction unifies all living organisms, and while the differences in mechanisms and structures among species are vast, the underlying principles are in many cases quite similar. Chromosomes provide the inherited directions for the first stages of development. In vertebrates, immediately following fertilization, chromosomes are responsible for the physiological events that occur during sexual differentiation. The chromosomal sex of an animal choreographs a cascade of events resulting in the development of gonads, and subsequently, gonadal steroids. In vertebrates, gonadal steroids direct the expression of both primary and secondary sex characteristics, completing the normal development of the reproductive system (Phoenix, Goy, Gerall, & Young, 1959). However, the delicate system that facilitates reproduction can be negatively influenced by aberrations in the environment, both naturally occurring and man-made (Orlando & Guillette, 2007).

A departure from the normal course of development, regardless of the source, results in a vast spectrum of abnormalities, from minor changes in morphology and behavior to complete genotypic sex reversal in invertebrates (Kime, 1995). Today's environment turns increasingly less stable, and therefore, the negative effects of pollution on human and wildlife populations are more complex and less well understood (Orlando & Guillette, 2007).

Despite early controversy, it is now widely accepted that exposure to toxins adversely impacts the development of animals and the homeostasis of their endocrine system (see reviews in Baker, 2001; Tanabe, 2002). In 1962, Carlson's book *Silent*

Spring was one of the first to raise issue with the use of pesticides, and demonstrate the negative effects toxins had on animal development. Due to the popularity of the book, some have attributed the start of the environmental movement to her research and publications (Kline, 2000). While some criticized her for fear-mongering, her supporters maintain that the goal was to show that interfering with natural processes often has unknown repercussions.

> "These sprays, dusts, and aerosols are now applied almost universally to farms, gardens, forests, and homes - nonselective chemicals that have the power to kill every insect, the *good* and the *bad* to still the song of birds and the leaping of fish in the streams, to coat the leaves with a deadly film, and to linger on in soil - all this though the intended target may be only a few weeds or insects. Can anyone believe it is possible to lay down such a barrage of poisons on the surface of the earth without making it unfit for all life? They should not be called *insecticides* but *biocides*." (Carlson, 1962, p. 7)

This is one of the more well known passages from *Silent Spring*, the dramatic portrayal of a legitimate argument.

Endocrine Disruptors – the Environmental Lesson

Any substance present in the environment, either naturally occurring or man-made, has the potential to be toxic. Humans are constantly subjected to toxins that, in large doses, can cause severe problems both physiologically and behaviorally (Segner et al., 2003). These toxins occur in a number of different capacities, from commonly used products such as coffee, chocolate, gasoline, nail polish remover, and cleaning supplies (Baker, 2001) to their presence in select food-chains and waterways (EPA, 2004; WHO, 1996). The immune system is designed to protect the body from the negative effects of toxins; however, there are limitations to how well these defenses can respond.

Exposure to a toxic substance can be transient and go unnoticed, or chronic and problematic. Similarly, the result can be as minor as a headache, or as severe as death. Most toxic exposures are self-inflicted; however, the unintentional toxic exposure of animals and plant life is due in large part to the activities of humans, mainly waste that is produced from commercial and agricultural activities (Baker, 2001; EPA, 2004).

While all substances have the potential to be toxic, endocrine disrupting chemicals (EDC's) are a class of toxins that cause abnormalities in the development and function of reproductive systems (Mendes, 2002). The Environmental Protection Agency (EPA) defines EDC's as "exogenous agents that interfere with the production, release, transport, binding action, metabolism, or elimination of natural ligands in the body responsible for maintaining homeostasis and regulating developmental processes" (EPA, 2004, p. 7). The definition most often cited in the literature is that EDC's are agents that can compromise the control of homeostasis, developmental processes, and reproductive behavior, and therefore adversely affect health and development of organisms (Baker, 2001; Sharpe, 2001; Mendes, 2002).

According to the EPA, there are 79 known EDC's. There are, however, approximately 87,000 chemicals, 900 pesticides, 75,000 industrial chemicals, and 10,000 food additives still to be tested (EPA, 2004). The EPA has listed endocrine disruption as number 6 on its list of most important fields of study. EDC's go by many names including hormone mimics, xenoestrogens, eco-estrogens, hormone related toxicants, phytoestrogens, and environmental estrogens; however, they all are similarly defined.

The long term goals set for endocrine disruptor screening include (1) to provide a better understanding of the science underlying the effects of exposure, assessment, and risk management of endocrine disruptors, (2) to determine the extent of the impact of

endocrine disruptors on humans, wildlife, and the environment, and (3) to support the EPA's screening and testing program (EPA, 2004). To achieve these goals, the EPA and Office of Research Development created the Endocrine Disruptor Screening Program to identify environmental contaminates that effect the endocrine systems of humans and wildlife.

A substance with endocrine disrupting properties can influence development in different ways. The three most common mechanisms for endocrine disruption are to mimic a naturally occurring hormone, antagonize the action of a naturally occurring hormone, or to act directly on the production of hormones and their metabolites (Baker, 2001; Tesfalidet, 2004). How a substance affects the endocrine system is ultimately determined by the nature and biology of the target. Characteristics that are affected by hormonal fluctuations (often environmentally induced) are at a high risk of EDC effects. Most well researched EDC's are estrogenic in their effects, resulting in the feminization of males, but androgenic EDC's have become of greater interest recently as we know less about them.

Hormone mimics, such as PCB's (polychlorinated biphenyls), appear to the body as hormones, and thereby affect (either by limiting or antagonizing) production of endogenous hormones (Mensink et al., 2002). Substances such as organotin compounds do not mimic hormones; rather, they hinder the metabolic processes required for optimal hormonal balance (Mendes, 2002). Hormones such as diethylstilbestrol (a synthetic hormone used to prevent miscarriage) act on the body the same way endogenous hormones do. These toxins make it impossible for the endocrine system to maintain homeostasis as the body regulates hormone production through negative feedback.

The ultimate result of exposure to EDC's in humans and non-human animals is compromised reproductive fitness, which is due to abnormalities in the endocrine system and associated phenotypic structures (Tanabe, 2002). If the reproductive system is damaged to the point where mating is no longer possible, members of that species can potentially become extinct. All species are potentially threatened by EDC's including reptiles, amphibians, fish, birds, and mammals (for review see Orlando & Guillette, 2007). Male alligators living in water polluted by environmental estrogens develop smaller gonads and decreased endogenous testosterone levels (Guillette, Pickford, Crain, Rooney, & Percival, 1996). Wild caught frogs exposed to pesticides including atrazine showed a significant increase in the frequency of intersex individuals as well as other estrogenic abnormalities (Hayes et al., 2002). Fish exposed to EDC's display a wide variety of abnormalities, often characterized as feminization of male phenotypes and masculinization of female phenotypes (for review see Kime, 1995). In studies on reproduction in brown trout (*Salmo trutta*) it has been demonstrated that chronic exposure to androgenic EDC's decreases egg to fry ratio in spawn clutches (Dumas, Bassenave, Jarry, Barriere, & Glise, 2007). In numerous avian species a decrease in the thickness of their eggshell has been identified as a result of exposure to the pesticide Dichlorodiphenyltrichloroethane (DDT) (Kavlock, 1999).

Mammals are gonochorists and their sexual differentiation is controlled by circulating sex hormones that are synthesized in the gonad (Wilson, 1994). Androgens and estrogens play a vital role in the differentiation process in both sexes. Unlike lower vertebrates such as fish, and invertebrates such as snails (Segner et al., 2003), exposure to androgens and estrogens in mammals and birds can masculinize and feminize their

phenotype, but cannot change their gonadal sex, and only interfere with the completeness in its expression (Orlando & Guillette, 2007).

Research on endocrine disruptors is crucial for the survival of animal populations; however, humans are also exposed to EDC's and therefore potentially at risk (Rogan & Ragan, 2007; Antizar-Ladislao, 2008). It has recently come to light that waste water contains high amounts of both androgens and estrogens (amongst other chemicals), resulting from the metabolites of pharmaceuticals (Kolpin et al., 2002; Cartinella et al., 2006). The aromatase inhibitor fadrozole is frequently prescribed to post-menopausal women as a treatment for estrogen-induced abnormalities (e.g. breast cancer), as it reduces available estrogen. Birth control pills contain high levels of synthetic forms of estrogen and progesterone. These pharmaceuticals are *designed* to be EDC's, and when excreted and released into the environment via wastewater, they pose a threat to human and non-human animals through groundwater, and possibly the food chain (Ankley et al., 2002; EPA 2004; WHO, 1996; Gabet, Miège, Bados, & Coquery, 2007). The developmental impact of human exposure to these EDC's is not well understood, however, recent literature has begun to address the problem (Rogan & Ragan, 2007; Antizar-Ladislao, 2008).

Hormone Manipulations – Understanding Normal Development

The Viennese endocrinologist Eugen Steinach (1907) could be credited as the first to scientifically address the role of sex steroids in the development and differentiation of the mammalian system. In his investigations, the sex glands of male guinea pigs (*Cavia porcellus*) were transplanted into the reproductive region of female guinea pigs, which resulted in females exhibiting masculine behaviors including mounting and dominance

displays. Other early investigations into sexual differentiation and development addressed the dimorphic behaviors affected by the sex steroids testosterone and estrogen (Dantchakoff, 1938; Raynaud, 1938). During this time researchers first suggested a bisexual potential in all animals, dependent on the hormones present during early stages of development, and again later in life as an adult (Dantchakoff, 1938). These hormones are responsible for the development of a functional reproductive system, but also for the expression of sexually appropriate secondary characteristics. The use of animals in investigating the role of hormones, timing of sexual differentiation, and the influence on sexual development has since become standard, allowing scientists to address issues that can not be carried out using human subjects (Baroiller, Guigen, & Fostier, 1999).

In 1959 Phoenix et al. proposed a model of sexual differentiation and development that changed our understanding of hormone action. By creating "hermaphroditic" animals they proposed that steroid hormones have different actions dependent on critical periods and the timing of exposure. Prior to sexual differentiation the *organization* of maleness and femaleness is affected and changed, following differentiation the *activation* of previously organized systems is affected.

> "For the neural tissues mediating mating behavior, corresponding relationships seem to exist. The embryonic and fetal periods are periods of organization or *differentiation* in the direction of masculinization or feminization. Adulthood, when gonadal hormones are being secreted, is a period of activation: neural tissues are the target organs and mating behavior is brought to expression" (Phoenix et al., 1959, p. 379).

Using the guinea pig (*Cavia porcellus*) they demonstrated that females exposed to testosterone prior to sexual differentiation developed male-typical behaviors including mounting and decreased female-typical behaviors such as lordosis when testosterone

levels are maintained as adults. These changes were permanent when animals were first exposed prior to differentiation, but temporary when first treated as adults.

Hormones given to lower vertebrates during adulthood results in masculinization or feminization of secondary sex characteristics (similar to mammals); in some cases, however, hormones given prior to differentiation can result in complete sex reversal, such as in tilapia, salmon, and bass (Uguz, Iscan, & Togan, 2003). This difference in the timing of hormone exposures in lower vertebrates, and the onset of critical periods, raises questions as to the universality of the discovery that hormones have different actions at different times. While hormones do have organizing effects, the organizational component can often be overturned by the activational component (Arnold & Breedlove, 1985). The development of the endocrine system is not controlled by two separate systems acting at different times; rather, one system is exerting its actions at two separate times.

This discovery has led to increased interest into the actions of hormones at different stages of development. Arnold and Breedlove (1985) have challenged the notion that hormones exert strictly organizational or activational effects. Their work shows that hormones may have different effects at different times, but the mechanisms are similar, and often the effects between organization and activation cannot be separated.

A Fish's Place in Endocrine Research

Manipulating the endocrine system of aquatic organisms through hormone exposures has applications in both the scientific and commercial domains, and has a long history, beginning with Turner (1942) and Grobstein (1940, 1948) who exposed teleost fish to different androgens and estrogens, addressing the role of exogenous hormones in

male and female development. Fish are of particular importance in determining the development of then endocrine system because they possess both major systems of sex determination, hermaphroditic (protandrous and protogynous) and gonochoristic (undifferentiated and differentiated), thereby representing a vast range of complex developmental systems (Yamamoto, 1969; Park, Kim, Cho, & Kim, 2004).

Hermaphroditic fish are simultaneously male and female, or switch from male to female based on external zeitgebers. Gonochoristic fish either immediately develop testes or ovaries (differentiated), or go through an ovary-like stage prior to gonadal differentiation (undifferentiated) at which time androgens or estrogens exert their influence on the system resulting in the two sexes (Yamamoto, 1969). These different systems for sex determination provide a novel opportunity to investigate both the organizational and activational affects of steroid hormones. Yamamoto (1959) viewed it like this: "Sex differentiation in differentiated species should be compared to a game of tug-of-war. The team that is even a little stronger wins the game so that a stationary state is seldom maintained" (p. 153).

Research has provided support to the notion that steroid hormones are critical for inducing sex differences in gonochoristic fish, in both males and females, thereby making them ideal subjects for investigating the mechanisms of sexual differentiation (Pickford & Atz, 1957; Yamamoto, 1969; Hunter & Donaldson, 1983; Landsman & Moller, 1988; Patino, 1997; Devlin & Nagahama, 2002). Gonochoristic fish have genetic sex determination, and display a wide variety of sex determining systems, including XX-XY and ZZ-ZW (Nagahama, 1994; Devlin & Nagahama, 2002).

Estrogens are responsible for ovarian growth, whereas androgens stimulate testicular development; however, the notion that these steroid hormones exist

independently in males and females has long been disproved (Arnold & Breedlove, 1985). In fact, testosterone is a major ovarian hormone and a precursor to estrogens, and estrogens are synthesized in the testis for sperm production. In teleost fish under normal conditions, the hypothalamo-pituitary-gonadal (HPG) axis is stimulated by environmental zeitgebers. The secretion of gonadotropin releasing hormone (GnRH) stimulates the pituitary to produce gonadotropins (function as FSH and LH in fish) (Prat, Sumpter, & Tyler, 1996). In males and females gonadotropins trigger testosterone production, which subsequently aromatizes to estrogen in females, and to a lesser extent in males (Nagahama, 1994).

In differentiated gonochorists, males and females exposed to their heterotypical hormones (estrogens or androgens) prior to the period of gonadal differentiation results in masculinization or feminization of the gonad phenotype, respectively (Yamamoto, 1969). In undifferentiated gonochorists estrogen is effective at producing female phenotypes if administered prior to sexual differentiation, however, androgen administration can block the development of an ovary, but cannot masculinize the gonadal phenotype (Yamamoto, 1969; Orlando & Guillette, 2007). To this end, andro-induced intersexed gonads (testis with ovarian tissue growth) are more likely to occur in undifferentiated gonochorists exposed to androgens (Yamamoto, 1969). In general, intersexed fish are likely the result of lower dosage levels and short exposure periods (Yamamoto, 1969).

Although the genotypic sex can not reverse in response to hormones or EDC's, the phenotypic characteristics of fish are perhaps the most labile in response to environmental changes (Orlando & Guillette, 2007). These differences include the expression of primary characteristics (e.g. testes and ovaries, sperm and oocyte

production), as well as secondary characteristics (e.g. courtship and social behaviors) (Baroiller et al., 1999).

The extent to which the primary or secondary sex characteristics are influenced depends largely on the hormone dosage, age of fish, and route of administration. Historically, research has focused on the feminization of males, and largely ignored the masculinization of females, with some exceptions. Steroid hormones, including androgens, estrogens, and their synthetic counterparts, can easily be administered to fish either through implant, ingestion, or immersion (Hunter & Donaldson, 1983, Krisfalusi & Cloud, 1999). The administration route chosen is based on the developmental stage of the organism, the properties of the hormone, and the feasibility of the technique with any given species (e.g. implants are not possible at the embryonic stage) (Hunter & Donaldson, 1983).

The non-aromatizable 17α-methyldihydrotestosterone (MDHT), a synthetic androgen, as well as the aromatizable 17α-methyltestosterone (MT), have both been shown to be effective masculinizing hormones for vertebrates and invertebrates, including many teleost species (Dulka & Maler, 1994; Kitano, Takamune, Nagahama, & Abe, 2000). The use of MDHT reduces the occurrence of paradoxical feminization that occurs when aromatizable androgens (like MT) are converted to estrogen, resulting in a noticeable feminization of the population (Davis, Simco, et al. 1990; Davis, Goudie, et al. 1992; Rinchard, Dabrowski, Abiado, & Ottobre, 1999). Increased levels of circulating androgens are converted into estrogen through aromatization, and the system is subsequently flooded with estrogen.

Using newly hatched fry of the channel catfish (*Ictalurus punctatus*), Davis et al. (1990, 1992) used MT and MDHT immersions to achieve masculinization. While a group

exposed to MDHT resulted in 90% males, a group exposed to MT resulted in less than 50% males. Fitzpatrick, Schreck, and Gale (1997) also compared the effects of MT versus those of MDHT, and found that MDHT was more effective at producing phenotypically all-male populations than was MT.

Nile tilapia (*Oreochromis niloticus*) is a commercially important species often exposed to androgens to increase the number of phenotypic males, which are larger, uniform in size, and generally more desirable (Guerrero, 1975; Wasserman & Alfonso, 2003; Ezaz, Myers, Powell, McAndrew, & Penman, 2004). Tilapia exposed to MDHT at 14 days post fertilization (DPF) resulted in a nearly 100% male-biased sex ratio after one immersion, whereas those fish exposed to a single immersion in MT resulted in a 90% male-biased sex ratio (due to paradoxical feminization) (Wasserman & Alfonso, 2003).

Integration – Endocrine Disruptors in Aquatic Environments

Water is the ideal medium for toxins to travel across large areas, affecting the maximum number of living organisms (Kavlock, 1999). Thus, research on the effects of toxins has largely been aimed at aquatic systems. The impact that humans have on the sustainability of aquatic environments has become a popular issue with increased awareness of environmental stressors including pollution. Fully understanding how the actions of humans affect the global biosphere and all its inhabitants is of utmost importance on the sustainability of ecosystems (Kime, 1995).

Fish are exposed to endocrine disrupting chemicals through three main systems; effluents from industrial sites, agricultural runoff, and human waste byproducts. Industrial effluents are the byproducts of production, and are routinely discharged into waterways. Alkalines and bisphenol are two of the most common, exerting an estrogenic

effect on males (EPA, 2004). Paper mill effluents exert an androgenic effect on exposed fish, and the effects are analogous to laboratory treatments using androgens (Bortone & Cody, 1996; Rosa-Molinar, Fritzsch, & Hendricks, 1996; Orlando & Guillette, 2007). When pesticides are given to crops, their final destination is water systems. DDT is a pesticide historically used to control mosquitoes and atrazine is a commonly used herbicide. Both have an estrogenic effect on male development (Ankley et al., 2002; Hayes et al., 2002; Bringolf, Belden, & Summerfelt, 2004). Waste water pollutes fish habitats with high amounts of both androgens and estrogens (amongst other chemicals), resulting from the metabolites of pharmaceuticals (Kolpin et al., 2002; Cartinella et al., 2006). Tributyltin (TBT) is another human-generated byproduct, not from waste, but from the unintentional pollution through commercial activities (Tesfalidet, 2004). Regardless of the source, these chemicals impact the health of all organisms.

Differential effects between adult and juvenile exposure to toxins is related to the theory of organization and activation (Phoenix et al., 1959). In the case of EDC's, during critical periods maleness and femaleness of an organism is still being *organized*, therefore, exposure to a chemical prior to the end of the critical period will result in anomalies in the expression of permanent sexual dimorphisms. In adults, early sexual differentiation is organized, but is indirectly *activated* by environmental cues (Rosa-Molinar et al., 1996). If these cues are from an endocrine disruptor, adverse effects may occur directly on the organism. The age of the organism, the chemical properties of a given toxin, the dose they are exposed to, and the mode of exposure influence the effects the EDC has on the organism's development and physiology (Segner et al., 2003).

Recent reports of high steroid hormone levels in water systems have shed light on the effects of human waste byproducts on fish development. Hormones are frequently

prescribed for a number of reasons, and though helpful to the patient, these predominately synthetic hormones reenter the environment through wastewater. For example, fadrozole, a pharmaceutical grade aromatase inhibitor, has been implicated in the masculinization of fathead minnows (Ankley et al., 2002). Similarly, estrogen and progesterone from birth control pills are released into the environment via wastewater, and have been shown to feminize male fish in a natural setting (Gabet, Miège, Bados, & Coquery, 2007).

In sexually mature organisms, EDC's can alter the reproductive fitness of an animal, however, this occurs only at high doses and usually subsides after the compound is no longer present (Segner et al., 2003). In developing organisms, a response is attained from low doses, and subsequent development is permanently impaired.

The Unusual Case of Tributyltin

Organotin compounds were first reported to have endocrine disrupting effects in France in the 1970's, where organotin-induced imposex was observed in Pacific oyster (*Crassostrea gigas*) (Mochida et al., 2007). Organotin compounds are present in many commercially available products including PVC, glass coating, and antifouling paint (Tesfalidet, 2004). While the di and mono forms of butyltin and phenyltin that are used as protective coatings for plastic, glass, and PVC are inorganic and have not been conclusively shown to be harmful, tri-organotin compounds, mainly tributyltin and its derivatives, are organic and pose more of a threat (Tesfalidet, 2004). It was originally thought that tri-organotin compounds could be used as fungicides without harm to the environment since they are not toxic at initial exposure. However, extended exposure to water breaks down the tin-carbon bonds, and the substance becomes more hazardous.

It was only after this discovery that the implications became clear; however, the toxic effects were already observed (Evans, Birchenough, & Brancato, 2000 a). Tri-organotin compounds are found in virtually all developed waterways, and although a world-wide ban is scheduled for 2008, problems associated with organotin exposure will persist long after the toxic source is removed (Evans et al., 2000 a; Evans, Birchenough, & Fletcher, 2000 b; Mochida et al., 2007).

Tributyltin (TBT) is an extremely effective antifouling biocide that has been used in marine paints since the 1960's (Evans et al., 2000 a, b). Trace amounts of TBT have been found in aquatic organisms in virtually all developed city ports. Estimated concentration of TBT in the environment range from 1.58 to 7.1 μg/L (EPA, 2004; Tesfalidet, 2004). It is one of, if not the most toxic substances to be introduced into bodies of water (Berge & Walday, 1999). Throughout the world TBT has been used as paint for boat bottoms and other marine structures, both commercially and privately. Since water is an ideal vehicle for substances to travel over large distances, aquatic endocrine disruptors, particularly TBT have become a great concern for human health and wildlife (Tesfalidet, 2004).

TBT is particularly toxic to aquatic organisms because of its stability in water, its long half-life in sediment, and its bioaccumulation in the food chain (EPA, 2004). It is degraded in water stepwise through its di and mono forms, and has a half-life of up to 30 days (EPA, 2004). However, accumulation in sediment accounts for its persistently negative effects (McAllister & Kime, 2003; EPA 2004). TBT is absorbed through sensory organs and carried to the brain via axonal transport; in fish this is primarily via the lateral line (Rouleau, Xiong, Pacepavicius, & Huang, 2003). It has been shown to have high toxicity at doses as low as 1-100 ng/l (McAllister & Kime, 2003), and a

comparatively low clearance rate between 0.08 and 0.18 ng/g (body weight) per day (IPCS, 1990; Berge & Walday, 1999). In teleost species the LC_{50} ranges from 1.5 to 240 µg/L, the NOEL (no observed effect level) is estimated as low as 0.01 ng/L in mollusks, and 0.01 µg/L in teleosts (IPCS, 1990). The $LOEC_{50}$ (lowest observed effective concentration) ranges from 0.01 to 0.5 µg/L (Berge & Walday, 1999).

Tributyltin is an *unusual* EDC because it is not a hormone mimic but rather a compound that prevents aromatization of testosterone to estrogen (Evans et al., 2000 a). Under normal conditions cytochrome P450 aromatase is responsible for the synthesis of testosterone into estrogen. The lack of estrogen due to aromatase inhibition affects both males and females negatively (Dimitriou, Catharios, & Miliou, 2003). Estrogen is commonly thought to be an exclusive female hormone; however, it is important in the development of male-typical structures and physiology as well (Arnold & Breedlove, 1995). It is responsible for the formation of Sertoli and Leydig cells, which are critical in testosterone production and active spermatogenesis (Haubruge, Petit, & Gage, 2000; McAllister & Kime, 2003). The most often noted effect of TBT exposure in males is a decreased sperm count and related anomalies; in females, the reduction of viable oocytes.

In gastropods, the increased androgen level most often results in the formation of a penis in females, known as imposex, which interferes with reproduction (Mensink et al., 2002). Whelks are directly affected by TBT, as its use as a paint additive is specifically designed to keep gastropods from attaching to man made objects. The common whelk (*Buccinum undatum*) is a snail like gastropod that lives in fresh- and salt-water habitats (Mensink et al., 2002). Exposure of female whelks to TBT leads to the expression of imposex; this effects reproduction because as the penis grows, it does so over the female's reproductive organs. Imposex is often reported following TBT

exposure, and the penis of a whelk is easily identified, therefore it has become a popular species in the study of endocrine disruptors.

Researchers in Spain have identified a natural population of dog whelks (*Nassarius reticulates*) that had been environmentally exposed to TBT, and subsequently developed morphological abnormalities (Barreiro, Gonzalez, Quintela, & Ruiz 2001). Over the course of one year, researchers collected animals and water samples from five different river sites in a populated port in Northwest Spain. A total of 74 female whelks were inspected for the occurrence of imposex, 52 of which had a discernable penis structure, and 32 of which had an abnormal female reproductive systems (sterility, lack of female structures) (Barreiro et al., 2001).

In vertebrates, exposing females to TBT prior to sexual differentiation results in masculinization of the reproductive system. TBT exposed salmon (*Oncorhynchus mykiss*), develop as functional males (Piferrer, Baker, & Donaldson, 1993; Piferrer, Zanuy, Carrillo, Solar, Devlin, & Donaldson, 1994). Flounder larvae exposed to TBT develop sex reversed males in 25% of the population (Shimasaki et al., 2003). Females exposed to TBT as adults show a decrease in the number of late stage oocytes, and inhibited ovarian development (Zhang et al., 2007). In behavioral analyses, three-spine sticklebacks exposed to TBT have been shown to reduce their anti-predator behaviors (Wibe, Nordtug, & Jenssen, 2001).

McAllister and Kime (2003) investigated the effects of TBT exposure on males using zebrafish (*Danio rerio*) and found that sperm count and morphology were negatively affected in fish exposed to TBT. TBT exposed males had a decrease in the amount of viable sperm between 24% and 60% and had morphological abnormalities including lack of flagella and conjoined sperm. A similar study done on guppies (*Poecilia*

reticulata) found a 40% to 70% decrease in sperm count occurred in fish that were exposed to TBT for 21 days (Haubruge et al., 2000). TBT inhibits the aromatization of testosterone to estrogen, thus males and females have abnormally high levels of testosterone following TBT exposure, and lack sufficient estrogen.

Electric Fish – a Novel Approach to Endocrine Research

Weakly electric African mormyriform and South American gymnotiform fishes produce electric discharges generated from myogenic or neurogenic electric organs, and perceive these discharges through specialized lateral line-derived receptors, tuberous and ampullary electroreceptors (Bass, 1986; Zakon, 1986). Both the production and perception aid in social communication as well as in navigation and spatial orientation (Heiligenberg, 1977; Bullock & Heiligenberg, 1986; Kramer, 1990; Moller, 1995; von der Emde, 1998; Moller, 2006). Mormyrid fish are found in freshwater rivers and lakes of Africa and make up the largest single group of electrogenic fish with approximately 200 species (Hopkins, 1986). The discovery of an electrosense in these fish was made well over 50 years ago (Lissmann, 1958), and in that time both the African and South American species have become valuable model systems to explore many physiological processes including electrogenesis, neurobiology, electroreception, taxonomy, and electrocommunication (Sullivan & Hopkins, 2004; Bass & Zakon, 2005; Moller, 2006).

Mormyrid fish exhibit sexually dimorphic characters in their electric organ discharge (EOD), anal-fin complex, and a unilateral gonad. The electric organ discharge waveform is sexually dimorphic (Hopkins, 1981; Landsman, Harding, Moller, & Thomas, 1990; Herfeld & Moller, 1998; Voustianiouk, 2003; Stell 2006), species-specific (Hopkins, 1981; Sullivan, Lavoue, & Hopkins, 2000), and under hormonal control (Bass

& Hopkins, 1983, 1985; Landsman, 1995). The electric organ itself is receptive to androgens, and generally in males, the length of the second phase (P2) is longer than in females (Figure 2.3). Androgens thicken the wall of the electrocytes in the electric organ, increasing the period of conductance, and the duration of the recorded output (Bass and Hopkins, 1983, 1985; Moller, 1995). This communication signal is discrete, important in conveying information during mating, and plays a critical role is the reproductive process (Hopkins, 1981; Moller, 2006).

The anal-fin complex plays a vital role in reproductive behavior. During spawning, the male uses its larger, indented "feathery" anal-fin to envelop the female's anal-fin, forming a spawning pouch (Kirschbaum, 1975, 1987, 1995; Schugardt, 1997; Kirschbaum & Schugardt, 2002). Female mormyrids exposed to androgens take on the male anal-fin bone morphology and musculature (Herfeld & Moller, 1998; Stell, 2006), including basal anal-fin ray expansions (Brown, Benveniste, & Moller, 1996), differences in the size of basal fin ray expansion and body wall indentation (Moller, Schugardt, & Kirschbaum, 2004), longer pterygiophores, and wider anal-fin rays (Greisman & Moller, 2005). These structural sexual dimorphisms can easily be observed using radiography.

The expression of sexual dimorphism in osteological as well as muscular characters is controlled by hormonal activity. The activational role of steroid hormones has been studied in sub-adult and adult mormyrid fish (Landsman et al., 1990; Herfeld & Moller, 1998; Voustianiouk, 2003; Stell, 2006). Little, however, is known about these effects on the early stages of development, or the effects of EDC's. It is suspected that early tests of TBT toxicity were carried out in South Africa, and it is well documented that African waterways are amongst the most polluted (Kishimba et al., 2004). Therefore, studying the effects of EDC's on electric fish endogenous to Africa serves to clarify both

the basic effects on electric fish communication as well as the applied toxicological implications.

Aims and Predictions

Experiment (1) provides background and procedures that were adopted in breeding *Mormyrus rume proboscirostris* under laboratory conditions. Experiment (2) explores the organizational role of MDHT, a non-aromatizable androgen, in the development of sexual dimorphisms in *M. r. proboscirostris*. The aim was to document the effects of this particular androgen, and to generate a control for comparison with data on the effects of endocrine disruptors on the organization of sexual dimorphisms. These effects were investigated in experiment (3) with fish exposed at early stages of development to TBT, an aromatase inhibitor. Experiment (4) investigates the activational effects of TBT on the expression of sexual dimorphisms in adult fish. The aim was to compare the effects of TBT administered to adult fish with those of androgen-treated adult mormyrid fish and other aquatic organisms reported in the literature.

The overall prediction is that *M. r. proboscirostris* are susceptible to the effects of androgens and endocrine disruptors, and these effects may lead to reduced reproductive capabilities. To this end we tested the following predictions:

1. *Early-life exposure to MDHT will result in androgen-specific organization of sexually dimorphic phenotypic characters in males and females at sub-adult and adult stages.*

   We predict that the anabolic effects of MDHT will masculinize, i.e. activate the male-typical expression of (1) the morphology of the electric organ and thus the emitted EOD, and (2) the expansion of basal anal-fin rays, both demonstrable 4 months post exposure. After 4 months, the gonad will not be developed, but at 24 months we predict a masculinized ovary in females, and a hyper-masculinized testis in males.

2. *Based on the results of experiment 2, early-life exposure to TBT will have an androgenic effect on the organization of sexually dimorphic characters in males and females at sub-adult and adult stages.*

   We predict that the aromatase inhibiting effects of TBT will masculinize, i.e. activate the male-typical expression of (1) the morphology of the electric organ and thus the emitted EOD, and (2) the expansion of basal anal-fin rays. At sexual maturity we predict a masculinized ovary in females, and a hyper-masculinized testis in males resulting in ovotestis.

3. *Adult males and females exposed to TBT will elicit androgen-specific activational transformations of sexually dimorphic secondary characters.*

   We predict that a single 27-day exposure to TBT at sexual maturity during gonadal recrudescence will (in the absence of aromatization of testosterone to estrogen) mimic the anabolic effects of MDHT and masculinize (1) the electric organ and thus phase 2 of the EOD, and (2) the expansion of basal anal-fin rays in males and females.

4. *The aromatase inhibiting effects of TBT will adversely affect the reproductive potential of males and females.*

   We predict that the lack of aromatization will result in a decrease in the number of viable oocytes and spermatozoa as their development is estrogen dependent.

EXPERIMENT 1

Breeding *Mormyrus rume proboscirostris*

A systematic study of the effects of androgens and endocrine disruptors (EDC's) on organizational processes required exact knowledge of the fish's age. To accomplish this, it was necessary to breed our own stocks of *M. r. proboscirostris* (Figure 1.1).

Figure 1.1 – Adult male *M. r. proboscirostris*, age 2, 14 cm standard length.

Adopting the methods of Kirschbaum (1987) and Schugardt (1997), ours is one of only a few laboratories able to breed mormyrid fish. Controlled, continuous addition of low-conductivity water to fish initially maintained in high-conductivity water mimics the transition from dry to rainy season in the fish's natural habitat and elicits gonadal recrudescence and spawning.

Methods

Subjects

Juvenile *M. r. proboscirostris* were initially obtained from Dr. Frank Kirschbaum (Humboldt University, Berlin, Germany) and raised to maturity in our former laboratory facilities at the American Museum of Natural History, New York City. At approximately

five years of age, one male (20 cm SL) and three females (*M* = 14 cm SL) were transferred to our laboratory at Hunter College. They were housed in a 300 L tank that was outfitted and arranged following Kirschbaum (1987) (Figure 1.2). Fish were maintained on a 12:12 L:D photoperiod; temperature ranged between 27º C and 29º C, pH between 7.2 and 7.6, and conductivity between 1200 µS/cm and 1500 µS/cm. Fish were fed blackworms (Neumans Fish Food, Hackensack, NJ) *ad libitum*.

Figure 1.2 – Tank arrangement adopted from Kirschbaum (1987) and Schugardt (1997) for breeding mormyrid fish. Grates along the bottom of the tank allow the eggs to drop down and be separated from fish.

Eliciting Gonadal Recrudescence

To begin the process, water from an overhead cistern (110 µS/cm, 28º C, pH 7.4) was *dripped* into the breeding tank at a rate of 1 *drop*/sec using 8 mm tubing; the drip rate was controlled with a hose clamp. Water conductivity decreased at a rate of 25 µS/cm per day which eventually resulted in gonadal recrudescence. Following a decrease to 107 µS/cm over 4 days, the first clutch of eggs was produced (described herein). Subsequent spawnings occurred at various intervals while conductivity was further decreased.

Raising Fry

A total of 107 fry were transferred to Pyrex glass dishes (25 per dish) and excess debris was removed. Fry were incubated (Quincy Labs, Model 12-140) at 29º C; water was aerated constantly and changed twice daily. To this end, eggs were siphoned into 8 mm tubing, and released into a clean dish of water from the breeding colony. Eggs that were not fertilized were removed as it is apparent by their white color (Figure 1.3).

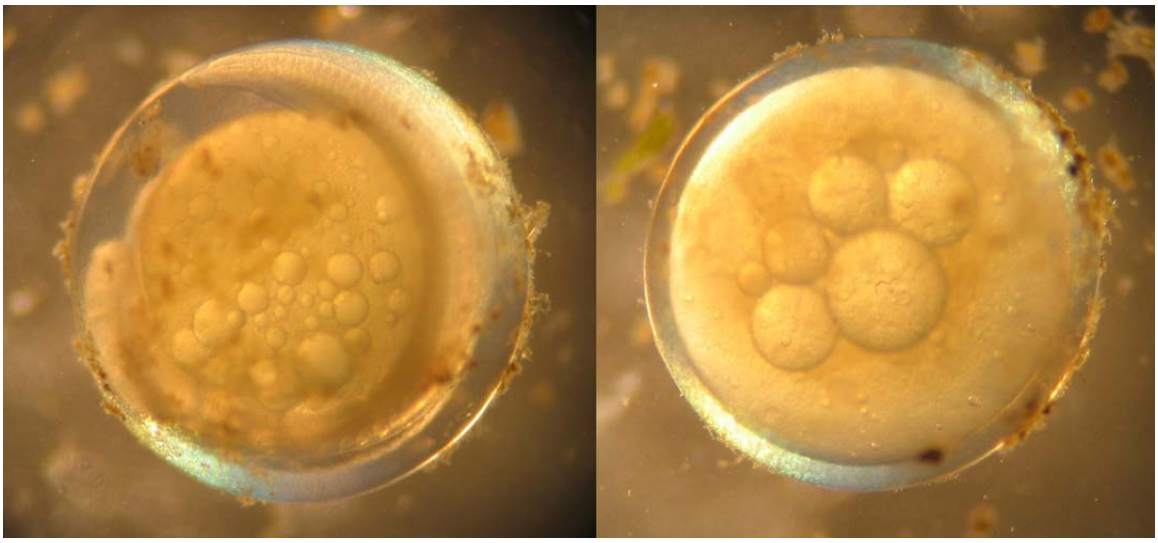

Figure 1.3 – On the left is an egg at 1 day post fertilization. The tail and eye are visible at 10x magnification. On the right is a non-fertilized egg. Each is approximately 2 mm in diameter.

After three days, fry began to hatch, and their casings were periodically removed to avoid fouling. Of the 107 collected eggs, 24 were fertilized and hatched. Fertilization success varied with some clutches yielding no hatching eggs, while others have a fertilization success rate of up to 80%. The hatching success rate in *M. r. proboscirostris* ranged from 40 to 70% in our experiments, with smaller clutches (<100) yielding more viable fry than large ones (>100). This is in accord with rates reported by Schugardt (personal communication, 8/06).

At seven days post fertilization (DPF) hatched fry were fed *Paramecium caudatum* until 12 DPF, when they were fed *Artemia* larvae. At 17 DPF fish were transferred to 8 L tanks, fed *Artemia* for 10 days, and then diced blackworms. At 30 DPF fish were transferred to 50 L tanks and raised under standard aquarium care for the duration of their development, (Figure 1.4).

Figure 1.4 – *M. r. proboscirostris* at (from top) at 2, 7, and 17 DPF.

IACUC Approval

All breeding experiments were carried out following IACUC regulations and approval (PM 2/08-01)

Comments

These procedures were repeated over a period of two years to produce fry for our experiments. In subsequent spawnings (11 to date) we have collected over 2,200 eggs, approximately 500 of which were fertilized and raised past their most vulnerable stage (17 DPF) for a survival rate of approximately 24%. The fish that were raised have been used in the experiments of this thesis, as well as other laboratory projects.

Although we have been successful with our breeding experiments, the ratio of fertilized to non-fertilized eggs is often lower than we would prefer (average of 1:4). Though this is common in mormyrid species, we continually strive to improve conditions thus facilitating a higher survival-rate. The male originally used in our breeding experiments is still active, along with one of the original females. Together we can assume that they spawned the majority of all fertilized clutches.

The complicated behavioral interactions and hierarchies that we have observed were extraordinary, and we feel this has a profound impact on success rates. These include subordinate female fighting, dominant female isolation within the tank, and male guarding of the dominant female and chasing of the subordinates. Future studies aimed at understanding this community will greatly impact conditions, and may be useful in improving hatch-rates.

EXPERIMENT 2

Early-life Exposure to 17α-methyldihydrotestosterone

Exposing fish to hormones early in life is a powerful tool to understanding of how sexual dimorphisms develop (Krisfalusi & Cloud, 1999; Sharpe, 2001; Devlin & Nagahama, 2002). In normal, i.e. non-treated fish, phenotypic analyses are used to assign sex when the genotype is unknown. In hormonally manipulated fish, the genetic sex may be obscured, but the expression of these same phenotypic characteristics can be used to (1) determine which characteristics are male or female-typical, (2) investigate the potential process in which gonadal hormones affect sexual differentiation, and (3) identify the critical periods for the development of sexual dimorphisms. Subsequently, hormone manipulations are useful in designing experiments whereby one can determine the effects of toxic exposures and identify potential risks to fish populations (Baker, 2001). As a number of pollutants have either androgenic or estrogenic effects on the organization and activation of phenotypic characters, hormone exposure studies are relevant to both basic and applied applications (for review see Kime, 1995).

Attempts to determine the genotype of our fish through genetic sex markers (Kirschbaum, personal communication, 3/06) and karyotyping (our own observations) were not successful. However, the use of secondary sex characteristics in determining the sex ratio in a population is standard practice when the genotypic sex is unknown (Pezzanite & Moller, 1998; Stell, 2006). *Mormyrus rume proboscirostris* show multiple dimorphisms which are easily assessed throughout development, and provide an excellent opportunity to identify the sex ratios in a population. Furthermore, by manipulating the fish' hormonal milieu, these characteristics allow us to investigate the expression of

sexually dimorphic (androgenic) traits (Bass & Hopkins, 1983, 1985; Landsman & Moller, 1988; Landsman et al., 1990; Landsman, 1995; Herfeld & Moller, 1998).

The present study investigates the effects of 17α-methyldihydrotestosterone (MDHT) exposure on the organization of sexual dimorphisms in *M. r. proboscirostris*. MDHT is a non-aromatizable androgen, thus it does not metabolize into estradiol. Endogenous testosterone is synthesized from cholesterol and androstenedione, which in turn can metabolize into MDHT and estradiol (Figure 2.1).

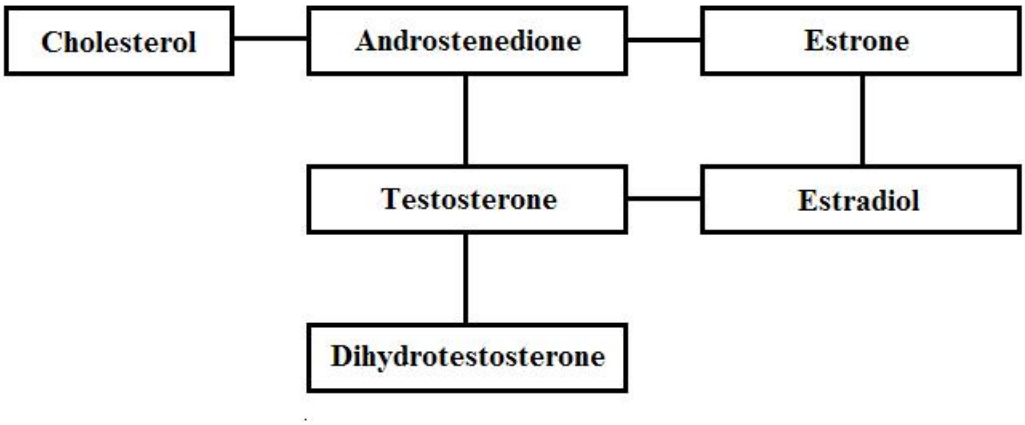

Figure 2.1 – The metabolism of cholesterol to androgen and estrogen. Testosterone is reduced to dihydrotestosterone or aromatized into estradiol.

The aims of this study were to enhance our understanding of the role androgens play in sexual differentiation, document the effects of MDHT, and generate a control for comparison with data on the effects of endocrine disruptors on the organization of sexual dimorphisms in mormyrid maturation and gonadal recrudescence.

Based on standard methods applied in aquaculture (e.g. Fitzpatrick et al., 1997), we predicted that early-life exposure to MDHT will result in an androgen-specific organization of sexually dimorphic characters in males and females in sub-adult and adult

stages of development. Specifically, that the anabolic effects of MDHT will masculinize the male-typical expression of (1) the morphology of the electric organ and thus the emitted EOD, and (2) the expansion of basal anal-fin rays, both demonstrable already at four months post exposure. At four months of age, the gonad will not have developed, but at 24 months, we predicted (3) a hyper-masculinized testis in males and a masculinized ovary (ovotestis) in females.

Methods

Subjects

A total of 200 *Mormyrus rume proboscirostris* were used in this experiment, all of which were bred in the laboratory. Larvae were selected at 17 days post fertilization (DPF) and randomly assigned to either treatment or control groups. Following hormone exposure, at 120 DPF (4 months) 130 fish were selected, 70 treated and 60 controls (group 1). The remaining fish (70 treated) were analyzed 2 years post-exposure (group 2). All fish were the 4th generation (F4) of one set of breeding animals (see experiment 1) and therefore shared reduced genetic variation.

Hormone Preparation and Exposure

Stock solutions of MDHT (17α-methyldihydrotestosterone, Sigma Chemicals, 521-11-9) were prepared prior to treatment. The solution was made by combining 3 mg of MDHT with 3 ml of ethanol, for a final concentration of 1 mg/ml. To achieve a final MDHT concentration of 3 mg/L, 3 ml of the stock solution was added to 1 L treatment tanks. (Note: a group of fry were exposed to 1.5 mg/L with similar results). Water was aerated for five minutes before fry were added to the tank. There were 23-25 fry per tank during the exposure period (Figure 2.2). They were left in the tank for 3 hours, after

which time they were rinsed, and transferred to a clean tank. This procedure was performed at 17, 20, 23, and 26 DPF in mid-afternoon. The control group was handled the same way as the treatment group; however, their treatment tank did not contain hormones. At the completion of the hormone exposures, fish were moved to 60 L aquaria and raised under standard aquarium care (see experiment 1). Waste water was collected, and turned over to the hazardous waste facility for disposal.

Figure 2.2 – Tank arrangement for hormone exposures On the left are treatment tanks, on the right are housing tanks immersed in a larger tank to help maintain temperature.

To asses the sexually dimorphic characteristics, EOD's were recorded from individual fish, they were then sacrificed, gonadectomized, radiographed, fixed in formalin and transferred to 70% ethanol.

Electric Organ Discharge

Adult *M. rume proboscirostris* exhibit a sexual dimorphism in the electric organ discharge (EOD) waveform during the rainy season when conductivity is low, and hormonal activity is at its peak (Figure 2.3).

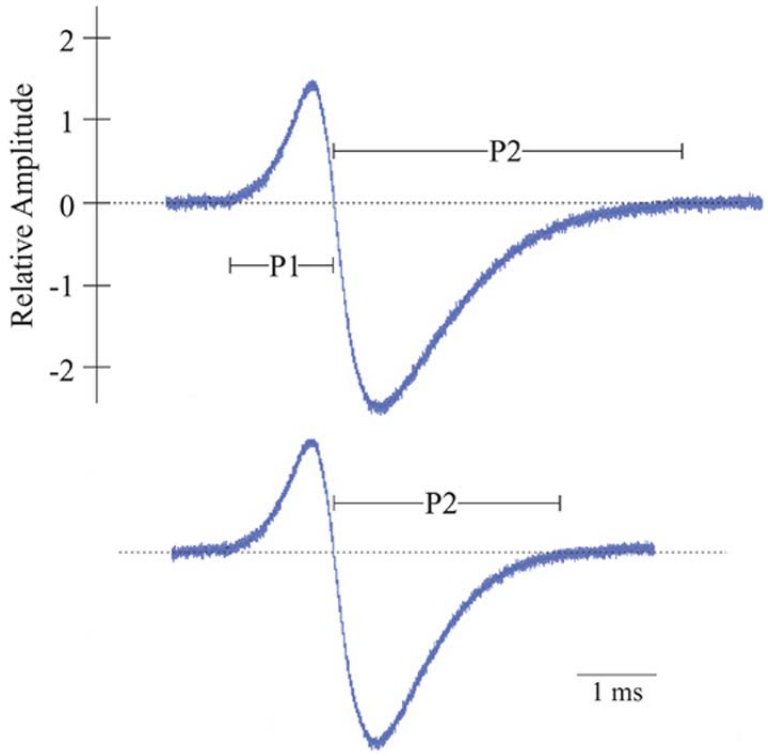

Figure 2.3 – The EOD is sexually dimorphic during the rainy season when hormonal activity is at its peak. The recording on top is from a male, the bottom from a female. P2 is longer in males than in females.

In group 1, recordings were obtained while fish were in rainy season conditions, between 250 µS/cm and 300 µS/cm. Fish were restrained in 15 mm mesh tubing, and Ag/AgCl electrodes were placed atop the tube, 15 cm apart. In group 2, recordings were obtained from fish with water conductivity ranging between 350 µS/cm and 450 µS/cm. Ag/AgCl electrodes were placed at opposite ends of an 8 L recording tank. EOD's recorded from fish in both groups were amplified and displayed on the screen of an oscilloscope (Tektronix TDS 340). The two phases (P1, P2) of selected EODs were directly measured from the screen and recorded in µsec. Data were obtained by two observers in succession, and the average readings were used for analysis.

Sacrifice and Gonad Removal

Fish were sacrificed (n = 200) by overdose with tricaine methanesulfonate (MS-222, Sigma Chemicals). Demographic data including length, weight, and anal-fin length were obtained. Fish were dissected and (in group 2) their gonad removed, sex identified, and measured (dimensions and weight). Fish and gonads were fixed separately in formalin (10%) for 24 hours, and subsequently transferred to 70% ethanol.

Anal-fin Ray Base Morphology

To assess the male-typical expansion of the anal-fin ray bases (Figure 2.4), fish were radiographed (Faxitron, model 43807 N) using Kodak radiography film. When screening live fish, a dose of 1 mg/L of MS-222 was used as an anesthetic and the gills were kept moist throughout the procedure. Yielding best results, fish were x-rayed for 45 to 100 sec (65 kilovolt peak). Live fish following radiography were transferred to aerated water and revived. Radiographs were processed using standard developing procedures.

In both groups 1 and 2, the presence of male-typical basal anal-fin expansions was judged independently by several observers. As the expansion is easily observed in x-rays, in both juveniles (group 1) and adults (group 2) there was little disagreement over its expression (see results) (Figure 2.4). Nominal data was collected (as yes or no) and used as a dependent variable.

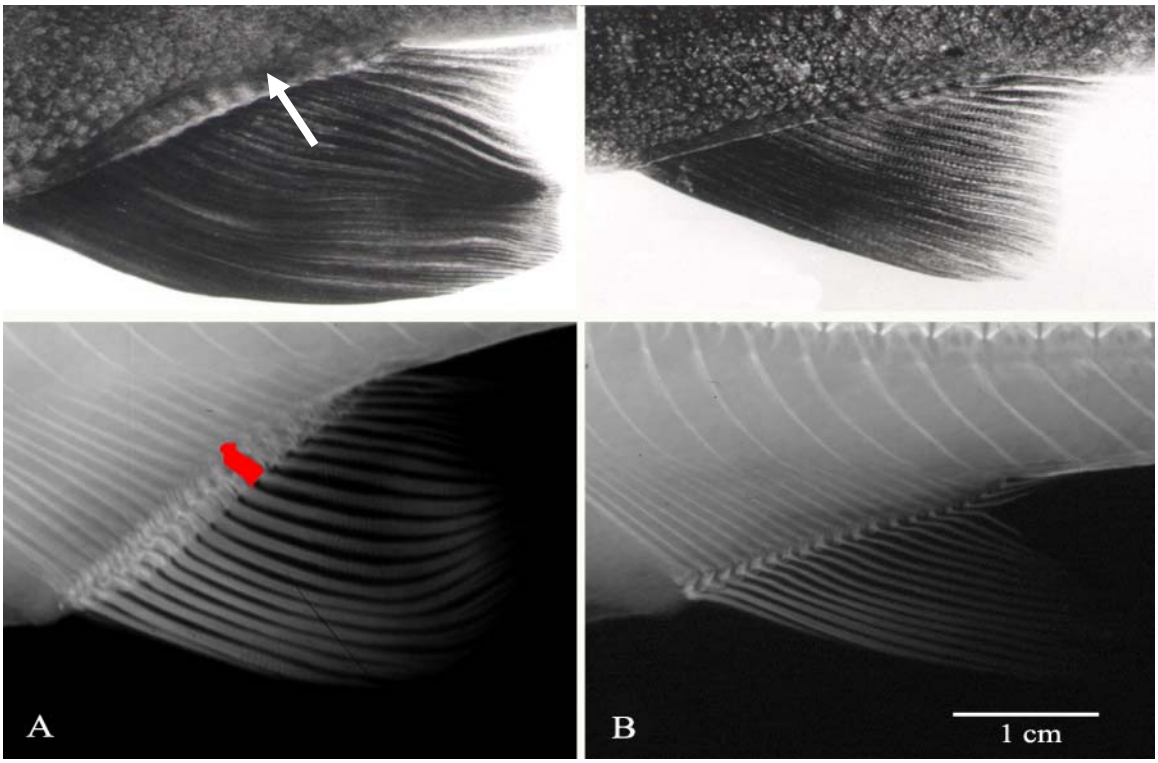

Figure 2.4 – The anal-fin complex in adult male (A) and female (B) *M. r. proboscirostris*: (top) viewed externally, white arrow indicates expansion area (from Schugardt, 1997), (bottom) in radiographs, red outline shows the distension of an expanded anal-fin ray base.

IACUC Approval

All hormone exposure experiments were carried out following IACUC regulations and approval (PM 12/05-01).

Results

Group 1

Electric Organ Discharge

The second phase of the EOD in *M. r. proboscirostris* is sexually dimorphic during the low conductivity rainy season (Figure 2.3), as it is longer in males than in females. The duration of P1 is not sexually dimorphic, and has not been shown to change seasonally. The duration of P1 and P2 was recorded twice for each fish prior to sacrifice, and the mean of these two data points (in μsec) was used for analysis.

There were significant correlations between the standard length of the fish and the duration of P1, $r(110) = .68$, $p < .01$, and P2, $r(110) = .62$, $p < .01$. This correlation does not occur in adults, and was attributed to ongoing development. It was therefore necessary to adjust for the influence of size in our data set. This was achieved by standardizing the duration of each phase to a unit length of the fish (1 cm). A standard duration index was calculated by dividing phase duration by standard length and multiplying the result by 100.

The duration of the P1 index was significantly different between groups. The duration index of P1 in treated fish ($M = 479$, $SD = 50.32$) was significantly larger than that in control fish ($M = 445$, $SD = 46.28$), $t(1, 109) = 13.42$, $p < .001$ (Figure 2.5). The duration of the P2 index in treated fish ($M = 1216$, $SD = 136.13$) was significantly larger than that in control fish ($M = 1169$, $SD = 96.77$), $t(1, 109) = 4.17$, $p = .044$ (Figure 2.6).

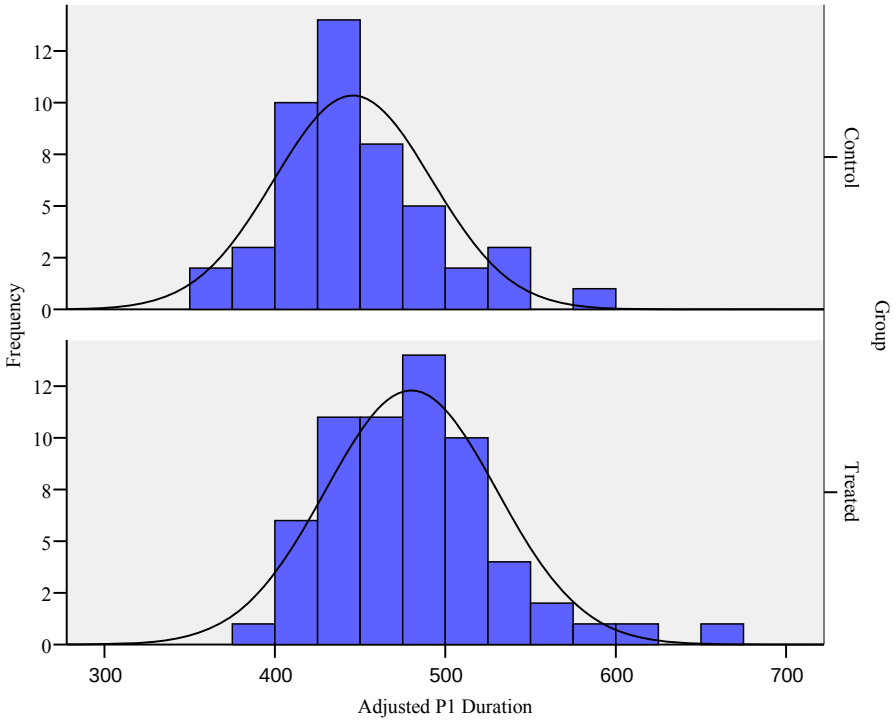

Figure 2.5 – Frequency of P1 duration indices in treated and control fish (n = 130) (group 1).

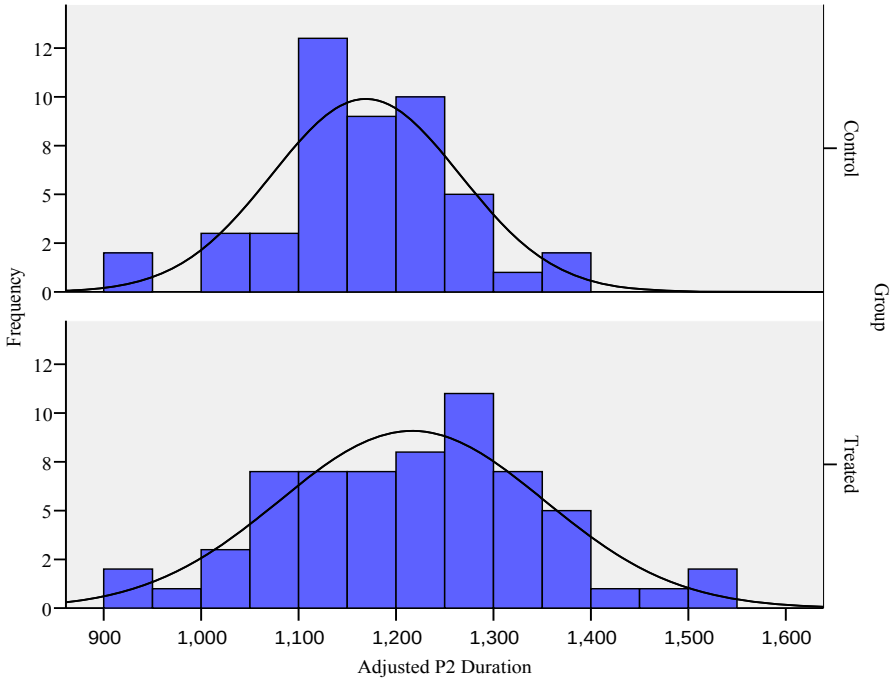

Figure 2.6 – Frequency of P2 duration indices in treated and control fish (n = 130) (group 1).

Anal-fin Ray Morphology

To identify the presence of expanded anal-fin ray bases, radiographs were analyzed by four observers, two of whom were blind to the prediction that more treated fish would have expanded anal-fin bases than untreated fish. Based on typical male and female morphology (Figure 2.4), each fish was classified as either *expanded* or *unexpanded*. In 91% of the cases, there was total agreement between the four raters, in the remaining 9%, majority agreement was accepted. A chi-square test revealed a significant difference in the number of fish with fin-ray base expansions in treated and control groups, χ^2 (4, N = 111) = 29.35, p < .001). Of the 63 treated fish, 53 had expansions (84%), compared to the 48 controls, of which 12 were judged to exhibit expansions (25%) (Figure 2.7).

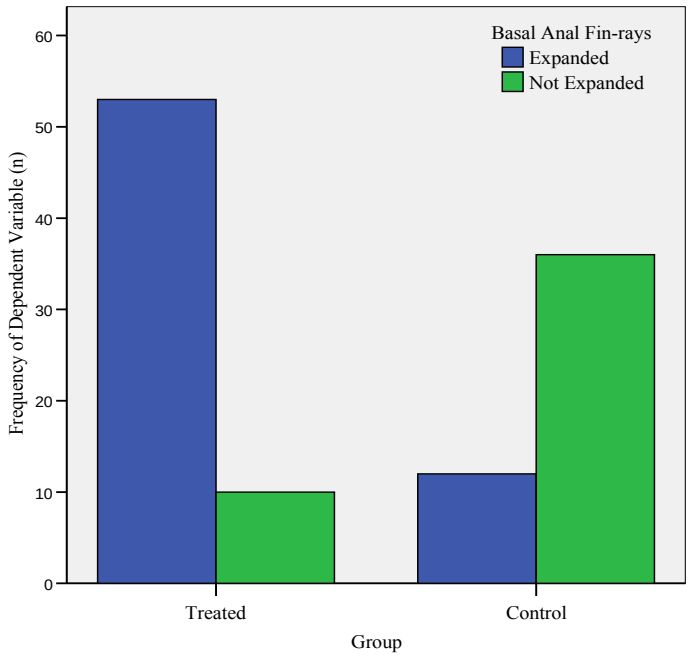

Figure 2.7 – Frequency of expanded anal-fin rays in treated and control fish (group 1).

Gonadal Status

Light microscopic examination showed an undifferentiated gonad. Fish normally mature at 12-15 month of age (135 mm SL); thus our specimens, at four months, were immature and the inability to distinguish gonads was not unexpected. For this thesis, a histological analysis was not performed. Separately, however, a histological study of the mormyrid gonad at 7, 17, 30, and 60 days of age is now under way in our laboratory in collaboration with colleagues at the University of Alberta, Canada. The results will help in understanding the outcome of our early-life exposure experiments, and resolve the question as to whether mormyrids are differentiated or undifferentiated gonochorists (see discussion below).

Group 2

The genotypic sex was unknown in these fish (N = 70), thus we are reporting here the effects of MDHT on the expression of three sexually dimorphic phenotypes: the EOD, the basal anal-fin ray expansions, and the gonad.

Electric Organ Discharge

The mean durations of P1 and P2 in treated fish were compared with corresponding data from a group of fish serving as breeding stock (3 females and 2 males). Control data were obtained at the same time and under the same water conditions as those recorded from the treated fish. In untreated males (n = 2) the average P1 duration was 306 μsec, and in females (n = 3) it was 282 μsec. The mean duration of P1 in treated fish (M = 343 μsec) was longer than that of both control males and females, and could thus be judged as male-typical.

In untreated males the average P2 duration was 880 μsec and in females 733 μsec. The mean duration of P2 in treated fish (M = 909.9 μsec) tended to be male-typical as it was longer than the male standard. There were 14 treated fish with a P2 duration shorter than 880 μsec, however, none had a P2 duration shorter than 733 μsec (the female average). Though statistical significance has not been established, it appears that both phases of the EOD were elongated in MDHT treated fish.

Anal-fin Ray Morphology

At 24 months (sexual maturity), expanded anal-fin ray bases were present in 52% of the fish. For comparison, the expression of basal anal-fin ray expansions was identified in a group of related control fish that were sexually mature and in breeding conditions. There were 7 males and 8 females, and of these, all males showed basal fin ray expansions, whereas none of the females did.

Gonadal Status

We predicted a masculinized ovary in females, and a hyper-masculinized testis in males. Treatment resulted in identifiable gonadal phenotypes: 35 fish showed an ovary, only one fish had normally developed testis, 25 were intersex (Figure 2.8), and 9 possessed a macroscopically unidentifiable gonad. Among the intersex, one was a true hermaphrodite with both testis and ovary on either side.

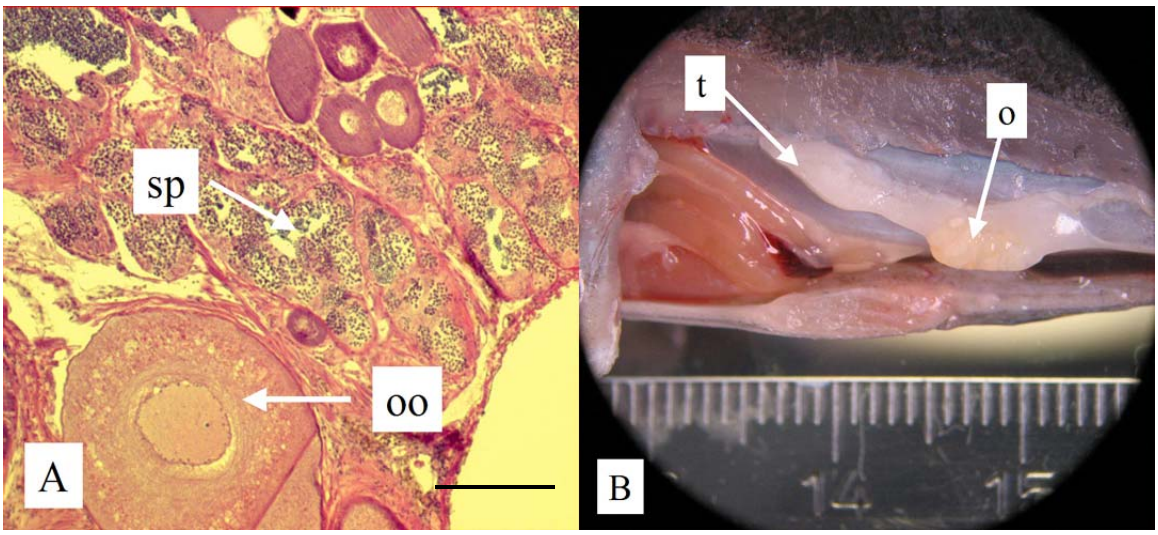

Figure 2.8 – Occurrence of ovotestis; (A) 10x magnification with spermatozoa (sp) and oocytes (oo) (bar = 100 µm); (B) macroscopic structure with testis (t) and ovary (o) (scale - mm). Micrographs, courtesy of F. Kirschbaum.

In this study there was no gonadal control group. Note, however, that none of the MDHT-induced anomalies have ever been observed in untreated fish in our laboratory (Moller, personal communication, 4/08). The genotype of the fish was unknown. The expressed ambiguous phenotypes (EOD, anal fin complex, and gonad) did not allow such a genotypic assignment. Before revisiting and resolving this ambiguity about the fish's genetic sex, we illustrate the occurrence of P1 and P2 duration and expanded fin-ray bases as a function of the expressed gonadal phenotype (Figures 2.9, 2.10, & 2.11). While the presence of expanded fin-ray bases varied from 50 to 100 % depending on the gonadal phenotype, the duration of P1 and P2 was male-typical in nearly all treated fish.

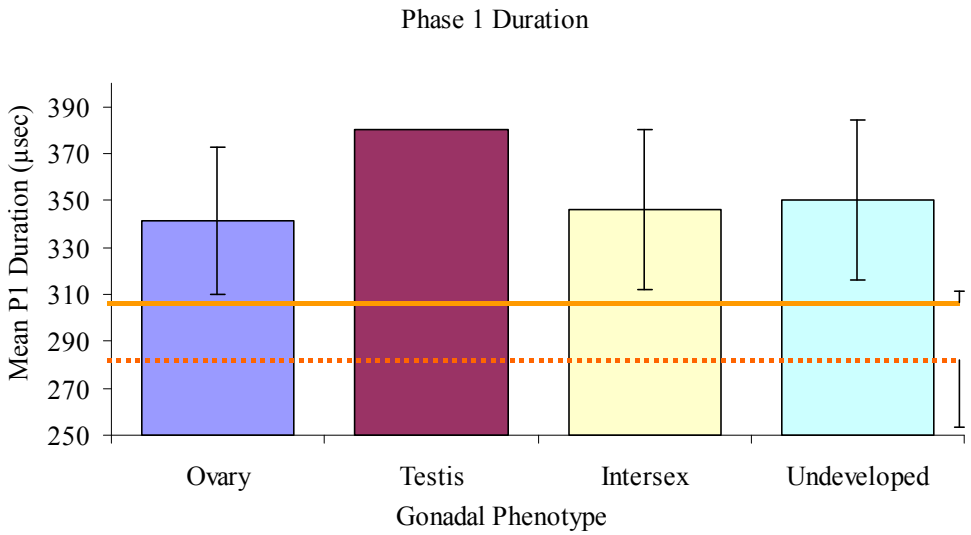

Figure 2.9 – Mean phase 1 duration as a function of gonadal phenotypes. Solid orange line indicates control males (M = 306 µsec), dashed line indicates control females (M = 282 µsec) (error bars +/- 1 SD).

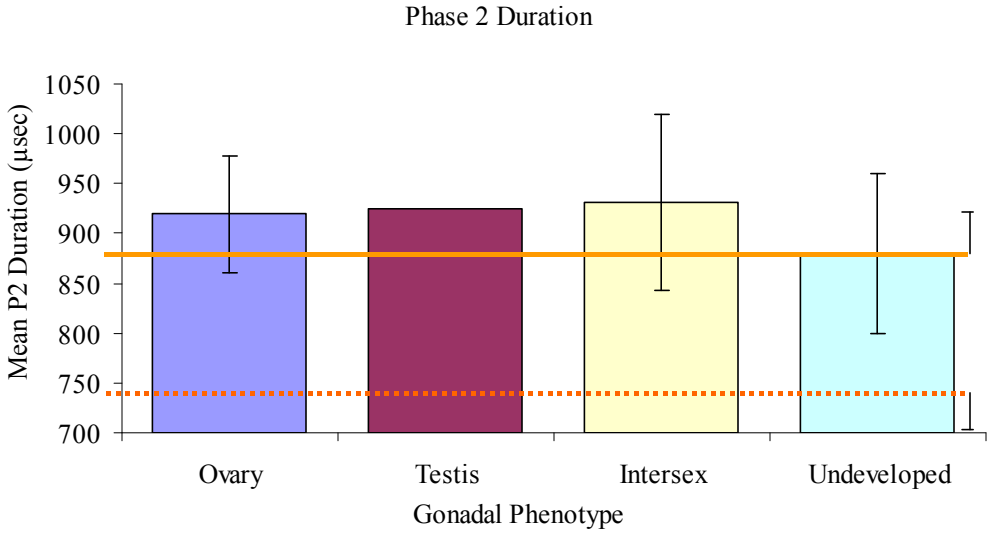

Figure 2.10 – Mean phase 2 duration as a function of gonadal phenotypes. Solid orange line indicates control males (M = 880 µsec), dashed line indicates control females (M = 733 µsec) (error bars +/- 1 SD).

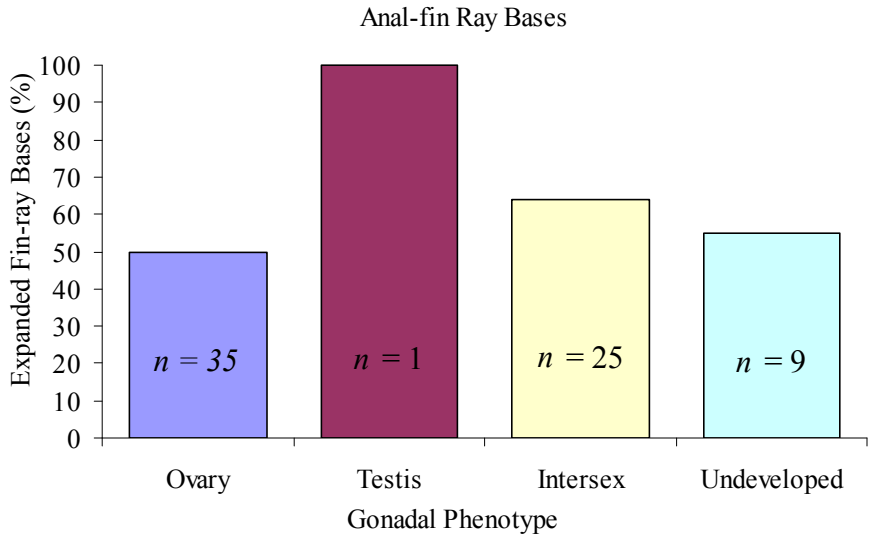

Figure 2.11 – Percentage of fish with expanded anal-fin ray-bases as a function of gonadal phenotype.

A chi-square test across the 4 observed gonadal phenotypes (Figure 2.11) showed a significant difference, χ^2 (3, N = 70) = 22.76, p < .001. A follow up analysis showed the difference was between the ovary and testis phenotypes.

Discussion

The results of this study have illustrated the complexity of organizational processes involved in the sexual differentiation of *M. r. proboscirostris*. The literature on androgen exposure in teleost fry reports nearly 100% success rates in masculinizing the adult phenotype (Guerrero, 1975; Fitzpatrick et al., 1997; Kitano et al., 2000). Our experiments, however, realized incomplete masculinization following exposure to MDHT, resulting in male-typical EOD durations and basal fin-ray expansions. The gonadal phenotype, however, contrary to our prediction and published data on other teleosts, showed both normally developed gonads and severe gonadal abnormalities.

Ambiguous Gonadal Phenotypes Provide Cues to Fish's Genetic Sex

These unexpected results highlight the ways in which male and female phenotypes could be difficult to discern following hormone exposure, but offer insight into the timing of sexual differentiation (Kitano et al., 2000). The complete masculinization of androgen-treated teleosts reported by Guerrero (1975), Dulka & Maler (1994), Wasserman & Alfonso (2003), and Ezaz et al., 2004, and the incomplete masculinization in our fish, was very likely due to differences in the fish's respective developmental critical periods and the timing of our hormone treatments. We will argue that both factors help explain the gonadal abnormalities and apparent discrepancies with other studies.

Sexual differentiation in gonochoristic fish follows one of two developmental paths. In differentiated gonochorists the primordial germ tissue develops into ovary and testis at about the same time early in development (critical period), whereas in undifferentiated gonochorists, ovarian tissue develops first, followed later by the differentiation of ovarian tissue into testes (Yamamoto, 1959, 1969). This difference in timing has noticeable consequences when assessing the organizational effects of exogenously administered gonadal steroids. If hormone treatment misses the critical period in differentiated gonochorists, i.e. occurs too late, gonads will develop normally in all fish with an expressed sex ratio of 1:1 (Baroiller, et al., 1999). As our results have clearly demonstrated, this was not the case in MDHT treated *M. r. proboscirostris*. We therefore must consider the alternative, namely that mormyrids are undifferentiated gonochorists, and propose that early ovarian differentiation from primordial germ tissue had occurred prior to the timing of MDHT treatment; thus females should develop a normal ovary. We further propose that MDHT treatment occurred within a time frame

during which testes normally differentiate, thus the normal development of the male gonad was disrupted. Our findings lent strong support to these assumptions.

The sex ratio in several species of mormyrid fish has been reported to be 1:1 (Moller et al., 2004; Stell, 2006). Thus our current cohort of 70 fish should consist of 35 genetic males and 35 genetic females. Following our argument that MDHT treatment missed the critical window for female organization, we predicted 35 fish with a normal ovary which was substantiated by our data. We noted 35 fish with developed ovaries. Resuming the argument that MDHT treatment interfered with the organization of the male gonad, we predicted abnormal gonadal development in the remaining fish. Again, the reported gonadal phenotypes substantiated our argument. Twenty-five fish exhibited different stages of arrested testis development, ranging from an early gyno-intersex stage showing the ovary with embedded testicular tissue to a later andro-intersex stage showing testis with embedded ovarian tissue, categorized as intersex. One fish possessed a fully developed testis, in which case MDHT treatment must have missed the organizational critical period for the male gonad. Nine fish did not develop an identifiable gonad.

The expression of these gonadal phenotypes was attributed to individual developmental differences. In one fish we found bilateral ovaries with testicular tissue, and in another, bilateral testes with oocytes. Two fish showed a hermaphroditic phenotype with a developed testis and an ovary on either side. Further studies should address the possible underlying mechanisms that led to these abnormalities. We conclude that our data strongly support the contention that *M. r. proboscirostris* is an undifferentiated gonochorist. In further support, Yamamoto (1975) suggested that in "differentiated species it is almost impossible to produce true intersexes after the onset of gonadal sex differentiation […] although it is possible to produce them in

undifferentiated species." Only a few species are known to be differentiated gonochorists, and among them sexual differentiation is fairly stable. Having screened over 50,000 specimens of *Xiphophorus maculates,* a differentiated gonochorist (Wolf, 1931), Bellamy and Queal (1951) reported never having come across a single sporadic intersex specimen. Likewise, in the Medaka, *Oryzias latipes,* another differentiated gonochorist, "not a single spontaneous true intersex was ever observed" (Yamamoto, 1953).

Having established (inferred) the genetic sex of *M .r. proboscirostris* in our study, we can revisit the results on EOD phases and basal fin-ray expansion (Figures 2.9, 2.10, and 2.11). The data demonstrated a masculinized phenotype in MDHT treated fish 24 months post exposure. While the EOD remains masculinized in nearly 100% of fish, the basal fin-ray expansions diminished in females. It is apparent that males almost all have expanded fin ray bases, which we attributed to their own endogenous testosterone production. In females, less than half showed expanded fin-ray bases, which we attributed to insufficient levels of androgens 24 months after treatment.

Electric Organ Discharge

In both groups, the EOD (seasonal and plastic) was masculinized following androgen exposure. Under normal conditions the male EOD in *M. r. proboscirostris* as in most mormyrids fluctuates with hormonal changes elicited by seasonal changes, most notably reflected in the length of P2 (Bass & Hopkins, 1983; Landsman et al., 1990). Therefore, the electric organ retains a high degree of plasticity throughout the fish's lifetime, and exposure to androgens at any time will result in an increase in the androgen-sensitive phase duration (Bass & Hopkins, 1983, 1985; Landsman, 1995). The effects of hormones on EOD waveforms are restricted to those which change seasonally, thus,

hormones do not affect EOD characteristics that are not seasonally expressed (Bass, 1986). By exposing fish to androgens during early stages of development, we activated the development of a male-typical electric organ, and thus also a male-typical EOD.

MDHT exposure resulted in the male-typical EOD transformation of P2 in both the 4-month old and adult fish. We also recorded a significant increase in the duration of P1. This lengthening of P1 in immature fish was unexpected as it is not observed in natural adult male populations, and may constitute a transitional stage. Comparable changes in multiple phase duration in response to exogenous androgen administration were reported for *Gnathonemus petersii* (Landsman et al., 1990; Voustianiouk, 2003) and *Brienomyrus niger* (Herfeld & Moller, 1998). As in other mormyrid fish a juvenile electric organ develops in *M. r. proboscirostris* prior to the later adult organ that becomes fully functional at day 90-100 (Kirschbaum, personal communication, 4/08). How the effects on the larval electric organ are transferred to the adult organ is unclear, and should be addressed in later studies.

Our findings demonstrated that early-life exposure to hormones can have both organizational and activational effects on morphology and behavior, and that the two processes can not be easily separated (Arnold & Breedlove, 1985).

Anal-fin Ray Morphology

In group 1, 84% of treated fish had male-typical expansions of the basal anal-fin rays. This is in accord with previous findings in *M. r. proboscirostris* showing that androgen-treated juveniles are able to express male-typical basal expansions (Kemen, personal communication, 4/08). Under normal conditions, the basal expansion of the anal-fin develops in sexually maturing males regardless of seasonal conditions, i.e.

irrespective of rainy or dry season. Exogenous androgen administration at 17 DPF mimicked this process and started activation of the development of a sexual dimorphism, i.e. the male typical anal-fin bone expansion in juvenile males and females. Such early expression of sexually dimorphic secondary characters in response to accidental or pathological exposure to gonadal steroids is well documented (Rosa-Molinar et al., 1996; Bortone & Cody, 1999).

In adult fish (group 2), about half the sampled population (52%) exhibited expansions of the basal fin rays. Stell (2006) and Voustianiouk (2003) have demonstrated developmental plasticity in adult mormyrids following exposure to androgens, including the expression of male-typical anal-fin ray bases. Rosa-Molinar et al. (1994) found related osteological expression in the anal-fin complex of mosquitofish, *Gambusia affinis affinis,* following androgen exposure. Male mosquitofish develop a gonopodium in the anal-fin. Females exposed to androgens within a critical period develop this male-typical structure, however, once the period has passed, the structure does not develop.

We began by asking whether or not androgen exposure at 17 DPF caused an immediate and permanent masculinization of the anal-fin (group 1), or organized the anal-fin to be masculinized upon reaching adulthood (group 2). Kemen (personal communication, 4/08) found that the anal-fin complex of sub-adult *M. r. proboscirostris* (4 months) was also masculinized by androgen exposure, despite the fact that the normal production and circulation of hormones does not begin until puberty, i.e. 7-11 months later. These findings can offer explanations as to why only partial masculinization occurred in fish in the current investigation. Based on the appearance of male-typical anal-fin ray bases in 4-month old fish, we propose that early-life androgen treatment resulted in an immediate expansion and enlargement of the fish's anal-fin ray bases.

Future studies should therefore examine the anal-fin complex in treated fish at even earlier stages of development.

More than 50% of treated fish in group 2 exhibited male-typical anal-fin bone expansions 24 months after treatment. There was a strong correlation between the presence of testicular tissue and expanded anal-fin ray bases; however, a number of fish possessed an ovary and expanded fin-ray bases. The current explanation is based on the organization and activation of the sexually dimorphic anal-fin. After 4 months, most fish had expanded fin ray bases, after 24 months most fish with ovaries had female-typical fin ray bases, and fish with testis had male-typical fin ray bases.

We predicted that early androgen exposure activated male-typical anal-fin expansions. After 12 hours of exposure to MDHT, we propose that the anal-fin was organized and activated to be male-typical. Twenty-four months later, we presume that most fish with ovaries, i.e. the designated genotypic females, lacked the androgen necessary to maintain the male-typical character. It has been shown that juvenile males exposed to androgens develop a male-typical EOD, and that this trait persists long after the androgen is removed (Choi, personal communication, 4/08). This may be a similar situation in bone morphology, where males retain androgen-induced changes whereas females revert to their original state once the hormone is removed. This may account for fish in groups 1 and 2 that did not show expanded fin-ray bases.

Although expansion of the anal-fin ray bases seems to be permanent, Herfeld and Moller (1998) did show a gradual decline in anal-fin ray expansions in genotypic females over 18 months following MT (methyltestosterone) exposure. No one has studied the effects of MDHT over as long a period as 24 months, and it is reasonable to propose that between 4 months and 24 months the lack of androgens resulted in a reduction in bone

mass in fish with normally developed ovaries. Estrogen provides maintenance to bone structures, but androgens are responsible for their initial expression.

Epilogue

Experiment 2 has provided the basis for the following experiments. It was necessary to understand how androgens affect sexually dimorphic characteristics before investigating the effects of toxins on the development of the anal-fin, EOD, and gonads. Now that we have identified the developmental plasticity in these animals, EDC research can commence. The presence of hormone mimics and other toxins in virtually all waterways has potentially lethal effects. *M. r. proboscirostris* are affected by hormone manipulations, thus we can base our experiments in part 4 on the assumption that their high degree of plasticity makes them susceptible to endocrine disruptors during the earliest stages of development.

EXPERIMENT 3

Effects of Tributyltin on the Organization of Sexually Dimorphic Characters in *Mormyrus rume proboscirostris*

Pollution of the environment has led to an increased awareness of the effects that human activities have on animal and human ecosystems (Baker, 2001). Fortunately, those outside the scientific community have begun to take note as well. Local periodicals routinely report on the effects of pollutants, using catchy titles like *Gender-Bending* and *Toxic Sludge* to grab the reader's interest and report a serious problem.

The paint additive tributyltin (TBT) has been in use for well over 30 years as a protective agent aimed at keeping invasive species, e.g. mollusks, from attaching to aquatic crafts and structures (Evans et al., 2000 a, b; Tesfalidet, 2004). Although effective in its intended use, its aromatase inhibiting actions have been shown to adversely impact organizational and activational processes, in juveniles and adults respectively, particularly those that are endocrine-dependent (e.g. IPCS, 1990).

As an endocrine disrupting chemical (EDC) TBT is highly toxic to most vertebrates, particularly so to aquatic species (Evans et al., 2000 a; EPA, 2004). Understanding the ways in which TBT interferes with all aspects of development is of great importance to both aquaculturists and behavioral ecologists. The use of *M. r. proboscirostris* provides us with a unique set of sexual characters that can be used to better understand the effects of early-life exposure to TBT and the processes affecting sexual differentiation and development. By assessing the same characters that were used in hormone manipulations (experiment 2), the anal-fin ray bases and the electric organ discharge (EOD), we can compare the effects of an EDC with those of hormone exposure. This will provide a better understanding of the ways in which EDC's,

particularly those that are androgenic in the expression, can mimic hormones and affect development.

Based on the results of experiment 2, we predicted that early-life exposure of *M. r. proboscirostris* to TBT will have an androgenic effect on the organization of sexually dimorphic characters in males and females at sub-adult and adult stages. Specifically, we predicted that the aromatase inhibiting effects of TBT will masculinize, i.e. activate the male-typical expression of (1) the morphology of the electric organ and thus the emitted EOD, and (2) the expansion of basal anal-fin rays. At sexual maturity we predicted a masculinized ovary in females, and a hyper-masculinized testis in males resulting in intersex stages of testicular development.

Methods

Subjects

Fry were raised following the procedures outlined in experiment 1. For this investigation 95 fry were used in total, beginning at 17 days post fertilization (DPF).

Tributyltin Exposure

Stock solutions of TBT were prepared prior to treatment. TBT (1 mg) was combined with acetone (1 ml) for a stock concentration of 1 mg/ml. To achieve a final concentration of 100 μg/L, 0.1 ml of stock solution was added to 1 L of water. In our first experiment, 50 fish at 10 DPF were placed in 2 tanks (25 each) with 1 L of water. TBT stock solution (0.1 ml) was added to the tank and aerated for five minutes before fish were added.

In a second experiment, 24 fish were exposed to a lower concentration of TBT, at a later developmental stage (17 DPF, as in experiment 2). To accomplish this, 0.01 ml of

TBT stock solution was added to 1 L of water for a final concentration of 10 µg/L. Water was aerated for 20 minutes before the 24 fish were added.

In a third experiment, 21 fish were exposed to yet a lower concentration of TBT, also at 18 DPH. To this end, 0.001 ml of stock solution was added to 1 L of water for a final concentration of 1 µg/L. Water was aerated for 1 hour before the 21 fish were added. This concentration has shown to be within an ecologically relevant range of 1.58 to 7.1 µg/L found in fresh water estuaries (EPA, 2004; Tesfalidet, 2004) For larval fish the LC_{50} ranges from 1.5 to 240 µg/L and the $LOEC_{50}$ ranges from 0.01 to 0.5 µg/L (IPCS, 1990; Berge & Walday, 1999). Experimental doses reported in the literature from 100 ng/L to 1000 µg/L with effects seen in all but those concentrations less than 0.01 µg/L (IPCS, 1990).

IACUC Approval

All TBT exposure experiments were carried out following IACUC regulations and approval (PM 4/08-T3) and EPA guidelines for disposal of waste water.

Results

In all three TBT exposed groups mortality was 100%. In the first two trials, 100 µg/L at 10 DPF and 10 µg/L at 17 DPF, fish died within a one-day period. In the third trial with 1 µg/L at 17 DPF, fish survived for four days before succumbing.

Comments

There is no literature to indicate that mormyrid fish are in any way more sensitive to toxic environments than other teleosts. The reason for the 100% mortality in our fish is not understood. At this time, the question as to whether it was the TBT or the acetone

vehicle that caused mortality cannot be answered conclusively, as a suitable vehicle control experiment was not performed.

It has been demonstrated that toxins are more lethal to fish in larval stages than adults (IPCS, 1900). We also now know (*post hoc*) that acetone is more toxic than other equally suitable vehicles, e.g. ethanol (Wibe et al., 2001; Zhang et al., 2007). However, our methodology was adapted from previous research using fish at similar developmental stages with acetone as a vehicle and no issues with mortality (Haubruge et al., 2000; Mensink et al., 2002; McAllister & Kime, 2003). While there is no way to be sure, based on subsequent research, we now suspect the acetone to have been the cause of mortality. Acetone was therefore no longer used as a vehicle.

For the reason outlined above, we cannot make any statements regarding the prediction that early-life exposure of *M. r. proboscirostris* to TBT will have an androgenic effect on the organization of sexually dimorphic characters in males and females. As we have shown in experiment 2, the MDHT-induced gonadal phenotype was not considered to be *masculinized*, thus a TBT exposed comparison group would have been helpful in interpreting our results. We predicted a masculinized ovary in females, and a hyper-masculinized testis in males. If TBT was androgenic, as we thought, then similar effects may have been seen here as in experiment 2.

There are a number of reasons why we ceased our experiment, all of which were logistic. We used the maximum amount of animals allowed at the time (IACUC approval), our breeding group then went into a period of dry-season conditions, and the full time care needed to adequately carry out further experiments was not available. Thus, we have provided a spring board for future research on the organizational effects of TBT on the sexual dimorphisms displayed in *M. r. proboscirostris*.

EXPERIMENT 4

Effects of Tributyltin on the Activation of Sexually Dimorphic Characters in *Mormyrus rume proboscirostris*

Tributyltin (TBT) is a topical antifouling agent used in marine infrastructure that has been shown to inhibit the aromatization of testosterone to estrogen in many aquatic species resulting in a variety of androgen-induced abnormalities (Evans et al., 2000 a). Since 1987 there has been a ban on the non-commercial application of TBT, but the toxic repercussions of its popularity throughout the past 30 years have a persistently negative effect on the environment (Evans et al., 2000 a; EPA, 2004). Currently, a world-wide ban is expected in September, 2008, however, it is estimated that it will take 20 years or more for TBT to be completely eradicated from the environment once it is no longer in use (EPA, 2004). Therefore the question of how TBT exposure affects aquatic organisms is important now, and will remain relevant for some time to come.

The main goal of this thesis was to acquire a comprehensive understanding of how hormones and toxins affect the development of *M. r. proboscirostris*. Previous data (e.g. Stell, 2006) and the results of our second experiment using MDHT provided the means to investigate the effects of the endocrine disruptor TBT on the activation of dimorphic traits. As we have developed an understanding of how hormones (androgens) affect the expression of sexual dimorphisms, we can now relate the effects of exposure to endocrine disruptors to those of similar hormone manipulations. This knowledge will help us to later make inferences as to how changes in the animal's phenotype might interfere with its social behavior and reproduction in particular. As reported in experiment three, TBT exposure of newly hatched fry turned out to be 100% lethal. The question remained as to how these toxins affect sexually mature animals. The

justification for our TBT investigations with adults is found in the *post factum* utility of understanding adult activation prior to testing juvenile organization.

We investigated the effects of chronic TBT exposure on the activation of sexual dimorphisms in adult *M. r. proboscirostris.* The aim was to compare the effects of TBT exposure in adult mormyrid fish with those observed in hormone manipulation studies using adult and sub-adult *M. r. proboscirostris* (Kemen, personal communication, 4/08) and related species, *B. niger* (Stell, 2006) and *G. petersii* (Voustianiouk, 2003). These studies have demonstrated that adult mormyrids exposed to androgens generally develop a male typical electric organ discharge (EOD), male-typical anal-fin ray base expansions, and exhibit gonadal abnormalities (Stell, 2006).

We predicted that adult males and females exposed to TBT will elicit androgen-specific activational transformations of sexually dimorphic secondary characters. Specifically, that a single 27-day exposure to TBT at sexual maturity during gonadal recrudescence will (in the absence of aromatization of testosterone to estrogen) mimic the anabolic effects of MDHT and masculinize (1) the electric organ and thus phase 2 of the EOD, and (2) the expansion of basal anal-fin rays in males and females.

In addition, we predicted that the aromatase inhibiting effects of TBT, affecting the aromatization of testosterone to estradiol, will adversely affect the reproductive potential of males and females. Specifically, that the lack of aromatization will result in a decrease in the number of viable oocytes and spermatozoa as their development is estrogen dependent.

Methods

Subjects

In this experiment there were 12 male and 9 female *M. r. proboscirostris,* all of which were bred in our laboratory. Fish ranged in age from 18 to 36 months, and were originally identified as sexually mature based on standard length (118 to 245 mm, M = 166.47) (Moller et al., 2004) (Figure 1). All fish were the 4th generation (F4) of one set of breeding animals (see experiment 1) and therefore shared low genetic variation.

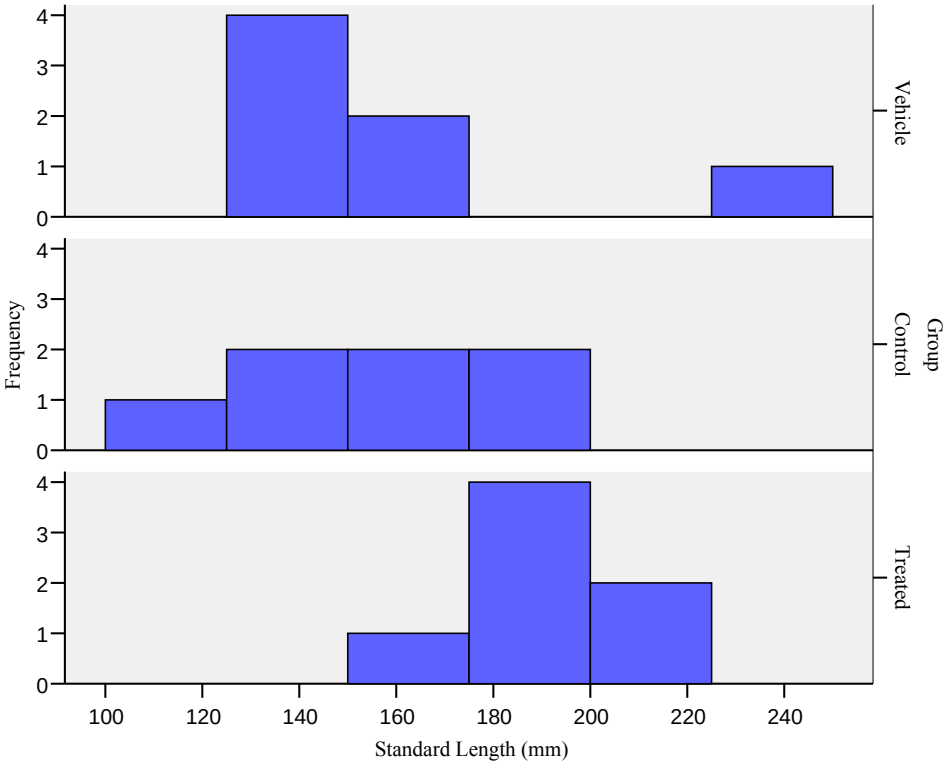

Figure 4.1 – Histogram of standard length (mm) in TBT treated, control, and vehicle fish.

Experimental Conditions

Three tanks were arranged two weeks prior for treated, control, and vehicle control fish, to achieve and stabilize optimal water conditions. Tanks were between 250 and 260 L, and outfitted with air stones and a number of hiding places. Temperature was

maintained at 27° C, and pH within a range of 6.8 to 7.4. To simulate the onset of rainy and thus breeding season, fish were selected from communal tanks that had experienced a conductivity drop of approximately 50 µS/cm a day over two weeks, i.e. a decrease from dry season conditions (>1000 µS/cm) to rainy season (<300 µS/cm).

Fish were randomly assigned to the three experimental tanks, all were maintained at 300 µS/cm. The daily 50 µS/cm conductivity drop continued until water had attained 100 µS/cm, a level that was maintained throughout the experiment in all tanks with small water changes made every other day. It was imperative that fish remained in low conductivity rainy season conditions to facilitate maximum hormonal activity, which was identified by the onset of gonadal recrudescence. The conditions were comparable to those of our hormone exposure experiments (experiment 2). Fish were maintained on a 12:12 L:D photoperiod and fed blackworms *ad libitum.*

Chemical Preparation

Bis(tributyltin) oxide (TBT) (96% purity) was obtained from Sigma Chemical (Aldrich; 56-35-9). It has been shown that acetone, which is a popular solvent in aquaculture, has a high toxicity, but that ethanol is a suitable and preferable alternative for dissolving TBT in water (Wibe et al., 2001; Zhang et al., 2007). The TBT stock solution was made by adding 1 ml of ethanol to 1 mg of TBT, for a concentration of 1mg/ml. To achieve a final water concentration of 10 µg/L we added 0.01 ml of stock solution to the treatment tank for every 1 L of water. For freshwater species the experimental doses for acute toxicity in adults ranges from 2 µg/L to 12 µg/L, with a LOEC of 100 ng/L (IPCS, 1990). The concentration of 10 µg/L has been shown to be

ecologically relevant and cause negative effects on the expression of sexual dimorphisms (McAllister & Kime, 2003; Nakayama et al., 2007).

Tributyltin Exposure

To achieve a final TBT concentration of 10 µg/L, 2.5 ml of stock was added to 250 L of water. TBT was initially introduced into the experimental tank two hours before the fish were. Subsequently, TBT and water were added as needed following water changes to maintain a concentration of 10 µg/L. Waste water was collected, and upon completion of the project, turned over to the hazardous waste facility. The conductivity of the control fish tank was maintained similar to that maintained in the tank housing the treated fish. Vehicle control fish were exposed to 10 µl/L of ethanol (2.5 ml in 250 L tank), and the level was maintained following water changes in the same fashion as that for the treated fish. The experiment lasted 27 days, after which EODs were recorded, fish were sacrificed, gonadectomized, fixed in formalin for 24 hours then 70% ethanol, and radiographed.

Electric Organ Discharge

To record the EOD, fish were placed in a 15 L holding tank that was fitted with a shelter inside of a net to keep the fish stationary, thus improving the accuracy of the recordings. Ag/AgCl electrodes were affixed to both ends of the tank, and connected to an amplifier and oscilloscope (Tektronix TDS 340). The duration of phases 1 (P1) and 2 (P2) was recorded in µsec twice by independent observers (Figure 4.2), and the mean duration of each phase was used for statistical analysis. The correlation between the length of P1 and P2 with fish size (standard length) was not significant. Therefore data were not adjusted to eliminate size as a potential confounding variable.

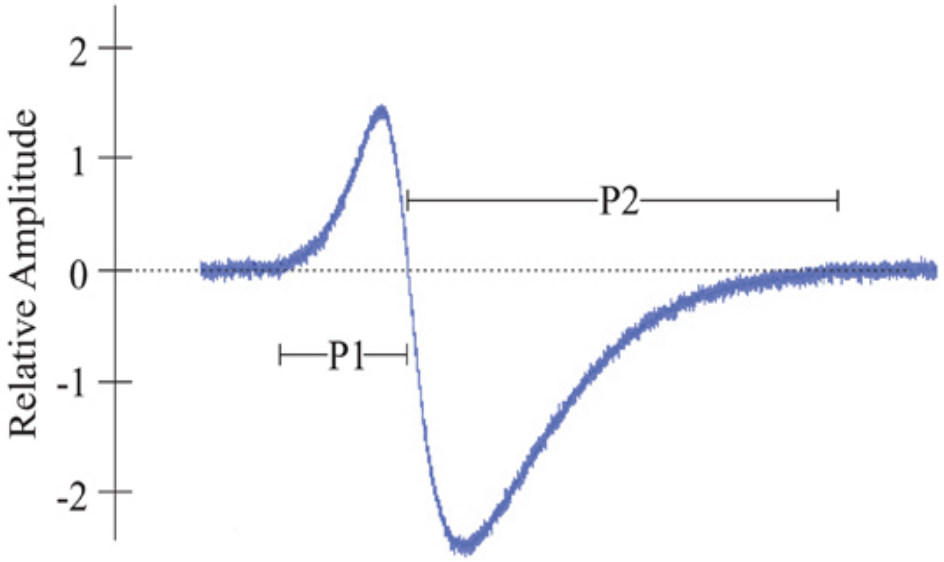

Figure 4.2 – Representative male EOD. P2 duration is sexually dimorphic.

Gonadectomy

Following EOD recording, fish were sacrificed by overdosing them with MS-222. Demographic data including standard length, weight, and anal-fin length were obtained. Fish were dissected and their gonad removed, sexed, and measured (dimensions and weight). Both fish and gonads were fixed separately in formaldehyde for 24 hours and subsequently transferred to 70% ethanol.

Radiography

Preserved fish were radiographed using a Kodak In-vivo Digital X-ray Image Station (400MM). Fish were exposed to 35 kilovolt peak (kVp) radiation for 2 minutes; radiographs were stored in the computer for later analysis. It has been demonstrated that in mature males, and hormone treated females, rays 10 through 13 of the anal-fin ray bases represent the widest section of the expansion area, with the longest basal anal-fin

rays (Brown et al., 1996; Moller et al., 2004). The length of the fin-ray bases 10 through 13 in females is generally small, as they do not, under normal conditions, possess the male-typical expansions.

We introduced a new measure to assess the area of expansion that would be comparable in *masculinized* males and females. The length of the most distended anal-fin ray base between 10 and 13 was measured (mm) from the body wall to the fin-ray margin (Figure 4.3). This measure was obtained from each fish using SigmaScan Pro™ software and served as a dependent variable in the data analysis.

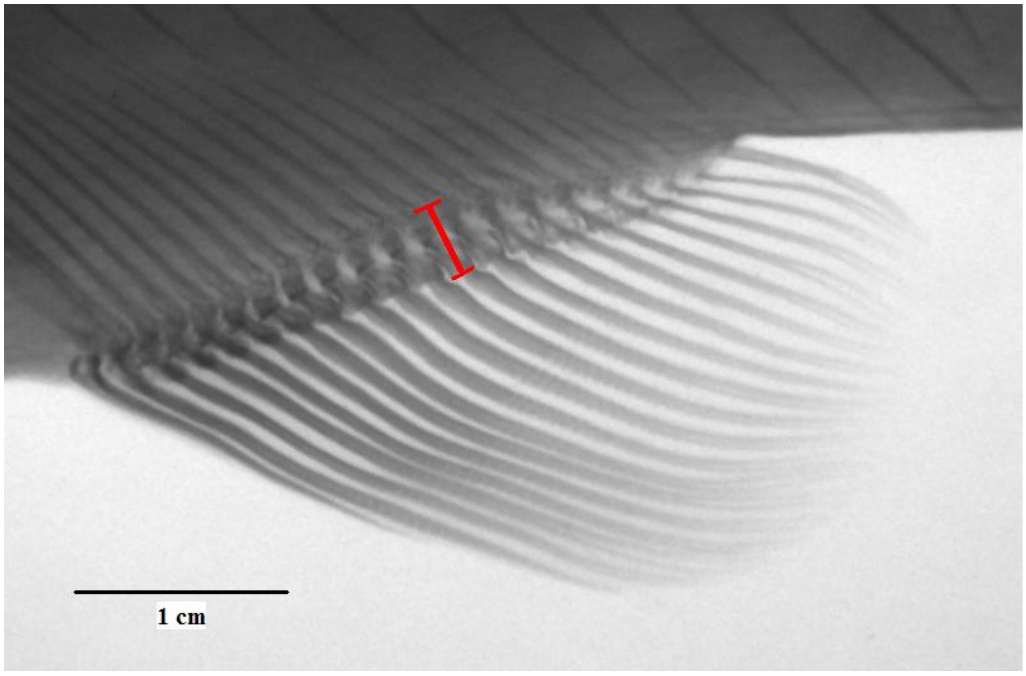

Figure 4.3 – Anal-fin complex of a treated male *M. r. proboscirostris* (T3). Red bar located at ray 12 represents length of the most distended fin-ray base.

Histology

Preserved gonads were prepared for histology following Presnell and Schreibman (1997). Tissue was initially rinsed twice daily in phosphate buffered solution (PBS) for 3 days, and then transferred into a 30% sucrose solution with PBS. After three days the gonads began sinking to the bottom, at which time they were saturated with sucrose. The tissue was embedded in embedding medium and frozen at –80° C. Gonads were sectioned (15 to 20 μm) using a Leica cryostat (CM3050-S) and sections placed on polylysine coated slides.

To stain the tissue we followed the standard directions in Romeis (1989) for Fast Red, Analin Blue, and Orange G staining, which is ideal for gonadal tissue. After a 24-hour drying period, the slides were covered in Permount™ and covered. Analysis of the tissue was done on a Nikon E400 Microscope and measured using SPOT™ software package.

Measurements of the Testis

Due to methodological errors related to fixation, sectioning, and/or staining of the testis, morphometrics turned out to be inaccurate. A quantitative assessment was therefore not possible.

Measurements of the Ovary

Similar methodological problems affected ovary histology as the tissue tended to spread out or splinter. In contrast to testicular tissue, we were able to clearly identify oocytes at different stages of development. The splintered tissue does not affect the ratios of earlier and later stages. Thus, we subjected the sections to meristic analyses and compared counts of oocytes at early and late stages of development.

Oocytic development is similar in most teleost species. The classification of developmental stages followed Coward, Bromage, Hibbitt, and Parrington (2002) and Tyler and Sumpter (1996). Accordingly, we identified four stages of oocyte development: (1) early perinucleolar, (2) late perinucleolar, (3) cortical alveoli, and (4) vitellogenesis. The operational definitions used were as follows:

Stage 1 - Early perinucleolar; oocyte is small and irregularly shaped, with a large nucleus and scant cytoplasm. The nucleus is stained dark, as is the cytoplasm as compared to later stages.

Stage 2 - Late perinucleolar; oocyte is about double in size from previous stage, and the nucleus has divided. Vacuoles appear in the cytoplasm.

Stage 3 - Cortical alveoli; spherical yolk bodies develop within the oocyte. Dark stained alveoli appear along the periphery, and grow inwards.

Stage 4 - Vitellogenic; yolk spheres accumulate, and the size of the cell is noticeably larger than previous stages. The yolk spheres accumulate in the center, and form a large mass.

At 10X magnification, the number of oocytes at each stage was counted for each fish in 20 different sections, yielding a total of 160 observations. The areas counted were distributed throughout the ovary, and care was taken that there was no repetition in which cells were counted due to overlap of sampling areas. Figure 4.4 shows the distribution of oocytes at different stages of development.

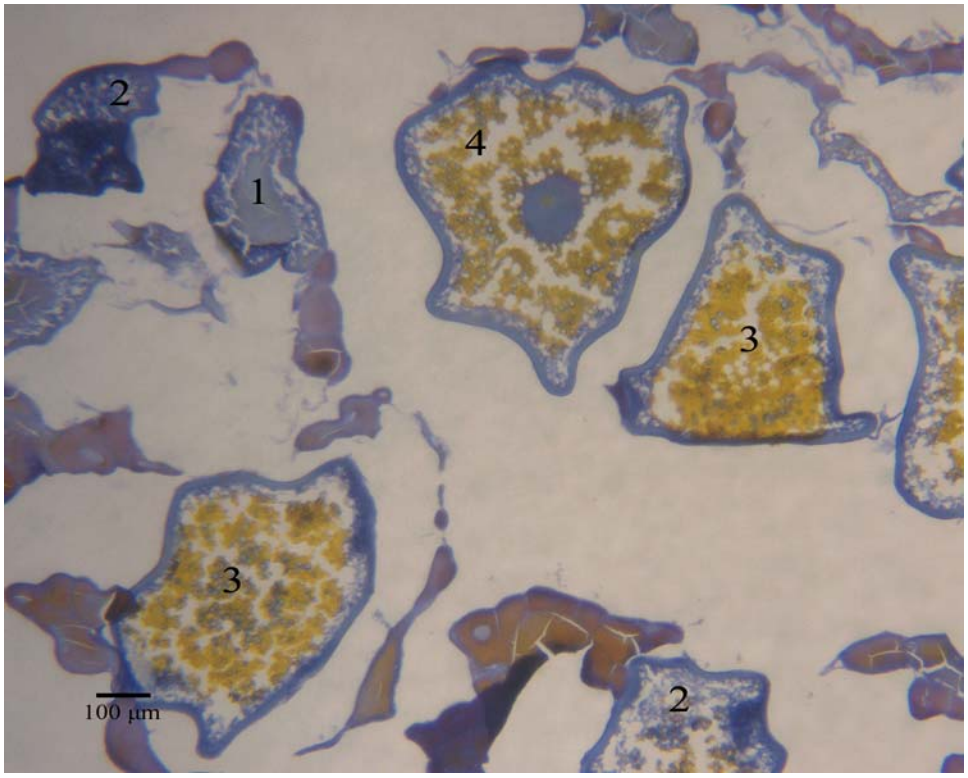

Figure 4.4 – Four oocyte stages were identified; (1) early perinucleolar, (2) late perinucleolar; (3) cortical alveoli; and (4) vitellogenic. Note: the tissue is splintered resulting in gaps among oocytes.

IACUC Approval

All TBT exposure experiments were carried out following Federal regulations and IACUC approval (4/08-T3).

Results

Table 4.1 provides a summary of the demographics for all subjects. In addition to standard measures, e.g. standard length, the anal-fin length (AFL) and the gonadosomatic index (GSI) are included.

Fish	Group	Sex	Standard Length (mm)	Weight (g)	AFL (mm)	Gonad			GSI	
						Length (mm)	Width (mm)	Weight (g)		
T1	Treated	F	180	61.96	25	11	6	0.13	*0.210*	*
T2	Treated	M	180	53.02	22	2	1	0.01	*0.019*	**
T3	Treated	M	209	100.42	22	32	5	0.19	**0.189**	
T4	Treated	F	162	41.22	13	19	10	1.44	**3.493**	
T5	Treated	M	205	92.36	26	41	4	0.3	**0.325**	
T6	Treated	M	178	74.32	20	29	8	0.25	**0.336**	
T7	Treated	F	183	63.73	21	22	15	2.98	**4.676**	
C1	Control	F	143	36.76	13	22	18	2.85	**7.753**	
C2	Control	M	184	74.99	22	27	5	0.25	**0.333**	
C3	Control	M	198	98.92	22	49	7	0.41	**0.414**	
C4	Control	F	144	32.35	14	17	8	0.63	*1.947*	*
C5	Control	F	166	49.74	19	21	14	1.67	**3.357**	
C6	Control	M	156	34.84	14	-	-	-	-	**
C7	Control	M	118	15.69	12	-	-	-	-	**
VC1	Vehicle	M	245	165.3	29	44	8	0.82	**0.496**	
VC2	Vehicle	M	152	35.84	21	22	2	0.02	*0.056*	**
VC3	Vehicle	M	130	18.8	13	10	1	0.01	*0.053*	**
VC4	Vehicle	M	168	52.32	13	36	4	0.13	**0.248**	
VC5	Vehicle	F	132	30.27	12	26	14	1.98	**6.541**	
VC6	Vehicle	F	138	32.9	14	21	14	1.47	**4.468**	
VC7	Vehicle	F	125	22.08	13	23	12	0.42	*1.902*	*

* Female not in breeding conditons, not used in analysis of EOD or gonad
** Male not in breeding conditons, not used in analysis of EOD or gonad

Table 4.1 – Demographics for each fish.

To assess effects of TBT exposure on gonadal recrudescence and thus the expression of sexually dimorphic characters, fish had to be in breeding conditions. Gonads with a GSI value below 2.0 in females and 0.15 in males signaled that fish were not fully in reproductive conditions (Schugardt, 1997). Recrudescence was not attained in

five males and three females which were therefore excluded from the analysis of EOD and gonadal histology (Table 4.1).

With one exception (male P1 duration), there was no significant difference between control and vehicle (ethanol) groups. As P1 is not reported to be sexually dimorphic in adult fish (see experiment 2), we combined data from control and vehicle control fish to increase the data base for comparison of untreated males and females.

Electric Organ Discharge

There were three males in the treated group, and two males each in the control and vehicle control groups. An ANOVA of the mean P1 duration (μsec) between control and vehicle control males (M = 477, SD = 116.62) and females (M = 467, SD = 186.26) revealed a mean difference of 10 μsec, which was not significant. Our predictions did not assume a significant difference in the duration of P1 between males and females. An ANOVA of the mean P2 duration (μsec) between control and vehicle control males (M = 1228.75, SD = 139.31) and females (M = 1114.5, SD = 97.73) revealed a mean difference of 114.25 μsec, which was not significant.

Our predictions that there is a sex difference were based on the assumption that there is a difference in P2 duration. While not significant, the mean difference of 114.25 μsec indicated a trend towards increased P2 duration in males.

An ANOVA of the mean P1 duration (μsec) between treated (M = 844, SD = 126.99), control (M = 310, SD = 8.48), and vehicle control (M = 624, SD = 73.53) males revealed a significant difference in the duration of P1 in males between groups, $F(2,6)$ = 18.139, p = .01 (Figure 4.5). The ANOVA of P2 duration in treated, control, and vehicle control males revealed nominal but non-significant mean differences (35 - 60 μsec).

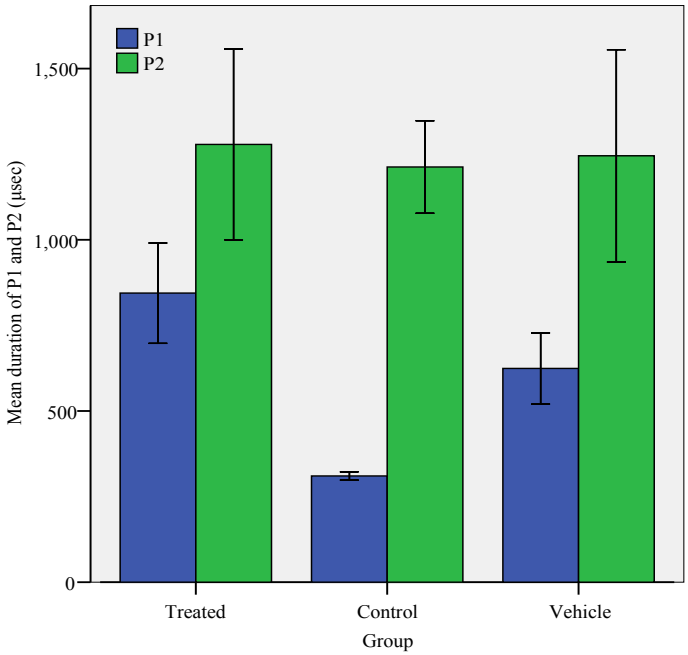

Figure 4.5 – Mean duration of EOD Phases 1 and 2 in TBT treated, control, and vehicle males (n = 7) (+/- 1 SD).

Post-hoc tests (LSD) revealed a significant, but unexpected difference in the mean P1 duration between treated and control fish (534 µsec, p = .004), but a non significant difference in the mean between treated and vehicle control (220 µsec, p = .068), and a significant difference in the mean between control and vehicle control (314 µsec, p = .032). Thus, treated males generated a longer P1 duration than control and vehicle males. There was no significant difference between treated and vehicle groups.

An ANOVA of the mean P1 duration (µsec) of females between treated, control, and vehicle control groups revealed no significant differences. An ANOVA of the mean P2 duration (µsec) between treated (M = 1540, SD = 69.3), control (M = 1106.5, SD = 111.72), and vehicle controls (M = 1122.5, SD = 9.9) revealed a significant difference in the mean duration of P2 between groups, $F(2,5) = 12.415$, p = .035 (Figure 4.6).

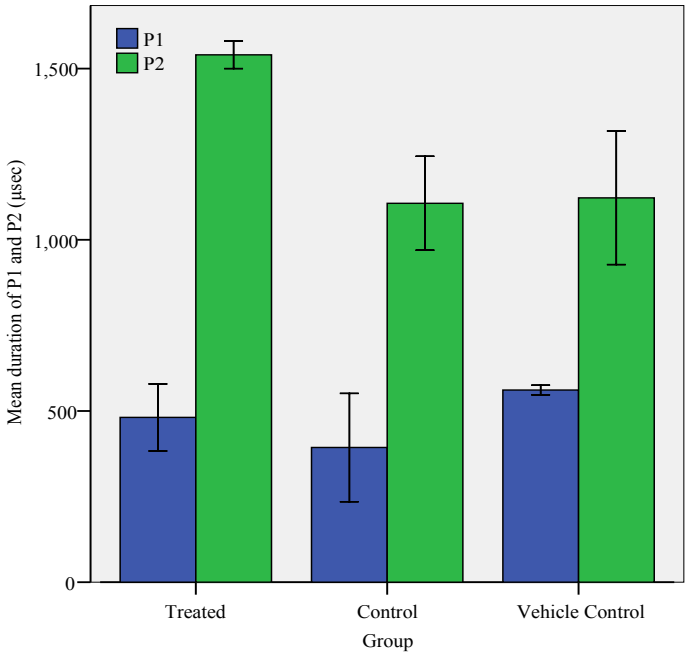

Figure 4.6 – Mean duration of EOD Phases 1 and 2 in TBT treated, control, and vehicle females (n = 6) (+/- 1 SD).

Post-hoc tests (LSD) revealed a significant difference in mean P2 duration between treated and control fish (433.5 µsec, p =.022), treated and vehicle control fish (417.5 µsec, p = .024), and no significant difference between control and vehicle controls. The duration of P2 was longer in treated females that it was in either control or vehicle control females.

While TBT affected an elongation of P1 in treated males but not in females, it had the opposite effect on P2, not causing any change in the male P2, but increasing its duration in females, mimicking the longer P2 characteristic of sexually mature males.

Radiography

Fish that were excluded from EOD and gonad data analysis are included here see Table 4.1). The justification to include fish that were not sexually mature or were not in rainy season conditions was based on previous work on *M. r. proboscirostris* which strongly suggested that the anabolic effects of testosterone on the anal fin ray complex is temporally separated from and independent of gonadal steroid driven gonadal recrudescence (Schugardt, 1997; Moller et al., 2004; Schugardt et al., in prep).

To demonstrate any differences in anal-fin morphology in males and females, we compared the length of their most distended anal-fin ray base (mm) in both control groups. There were four control and four vehicle control males; there were three control and three vehicle control females. An ANOVA revealed a significant difference in the length of the most distended fin ray base between males ($M = 1.7$, $SD = 0.91$) and females ($M = 0.60$, $SD = 0.12$), $F(1,13) = 8.529$, $p = .013$. Our measure is validated by the fact that control males had a significantly wider maximally-distended fin-ray base than females.

A comparison of the mean length (mm) of the most distended anal-fin ray base of males (n = 12) between treated ($M = 2.81$, $SD = 0.31$), control ($M = 1.81$, $SD = 1.08$), and vehicle controls ($M = 1.58$, $SD = 0.85$) revealed no significant differences in basal fin-ray length, $F(2,11) = 2.56$, $p = 1.32$. The length of the most distended anal-fin ray base in males was not different between treated, control, and vehicle control fish.

The same comparison in females (n = 9) between treated ($M = 1.64$, $SD = 0.8$), control ($M = 0.63$, $SD = 0.14$), and vehicle controls ($M = 0.57$, $SD = 0.13$) revealed a *nearly* significant difference, $F(2,8) = 4.77$, $p = .057$ (Figure 4.7). Treated female fish

had a longer fin ray base than either control group, and a number of treated females were observed to have a male-typical anal-fin complex based on gross morphology.

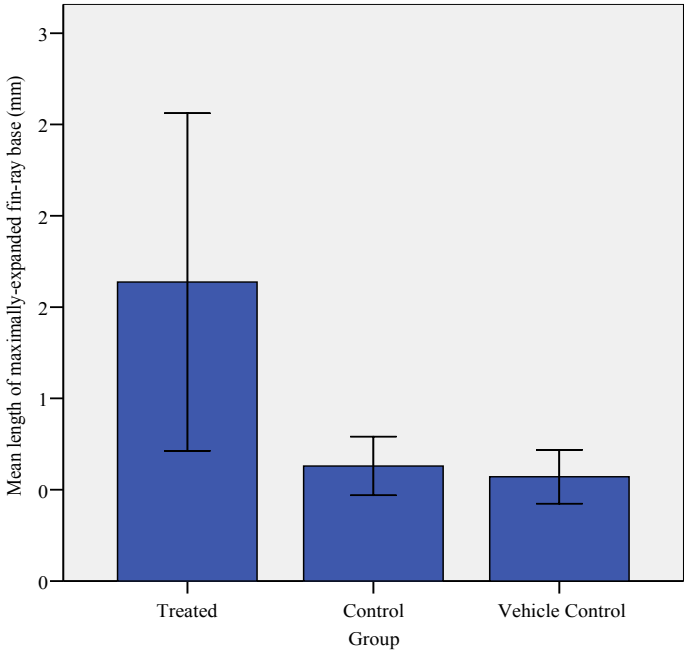

Figure 4.7 – Mean length of fin-ray base at the widest point in females (error bars +/- 1 SD).

Post-hoc tests (LSD) revealed a significant difference in the mean length of the most distended anal-fin ray base in females between treated and control fish (1.01 mm, $p = .041$), treated and vehicle control fish (1.06 mm, $p = .03$), and no significant difference between control and vehicle control (0.05 mm, $p = .89$). Thus, the length of the anal-fin ray base at its most distended point was longer in treated females that in either control or vehicle control females. Such a distended basal fin-ray area is typically associated with males and not in females.

Gonad Histology

Analysis of the gonads excluded fish that had not recrudesced (see Table 4.1). In the treated group there were three males and two females; in both the control and vehicle control groups there were two males and two females. Because of methodological limitations, testes measures were not considered. The number of developing oocytes in each developmental stage was counted in ovaries.

Testis Morphology

A noticeable qualitative difference between control/vehicle and TBT-treated males was manifest in an abundance of orange-stained areas in control and vehicle fish, and an apparent lack in treated fish. The orange-stained areas likely contain Sertoli and Leydig cells, which facilitate spermatogenesis and sperm maturation. They are clustered within the cell walls, and have a high affinity for the Orange G stain (Billard, Fostier, Weil, & Breton, 1982; Mochida et al., 2007). We found this marked area to be a possible indicator of active spermatogenesis, which was lacking in treated fish.

Ovary Morphology

For females, data were compared between groups (ANOVA) to assess the number of eggs in stage 1 (early perinucleolar) and stage 4 (vitellogenic); (see methods for detailed explanation). Means, standard deviations, and standard errors for these measures are presented in Table 4.2.

		N	Mean	Std. Deviation.	Std. Error
Stage 1	Treated	2	50.6	5.940	4.200
	Control	2	28.8	4.031	2.850
	Vehicle	2	30.5	6.647	4.700
Stage 2	Treated	2	5.65	.707	.500
	Control	2	5.35	.354	.250
	Vehicle	2	6.38	2.581	1.825
Stage 3	Treated	2	3.85	.919	.650
	Control	2	5.38	.742	.525
	Vehicle	2	4.85	.212	.150
Stage 4	Treated	2	.45	.354	.250
	Control	2	6.33	1.096	.775
	Vehicle	2	7.85	1.626	1.150

Table 4.2 – Means, standard deviations, and standard errors for measured characters.

The number of stage 1 oocytes was significantly higher in TBT exposed fish compared to control and vehicle controls, $F(2,5) = 9.22$, $p = .052$. In addition, the number of stage 4 oocytes was significantly lower in TBT exposed fish compared to control and vehicle controls, $F(2,5) = 23.066$, $p = .015$) (Figure 4.8). Post-hoc tests detail the relationship between conditions for each variable (Table 4.3). The ovary in TBT exposed fish have a large number of stage 1 oocytes, but only a small number of stage 4 oocytes. Figure 4.9 illustrates the difference in ovarian recrudescence in treated and control females at 4x magnification.

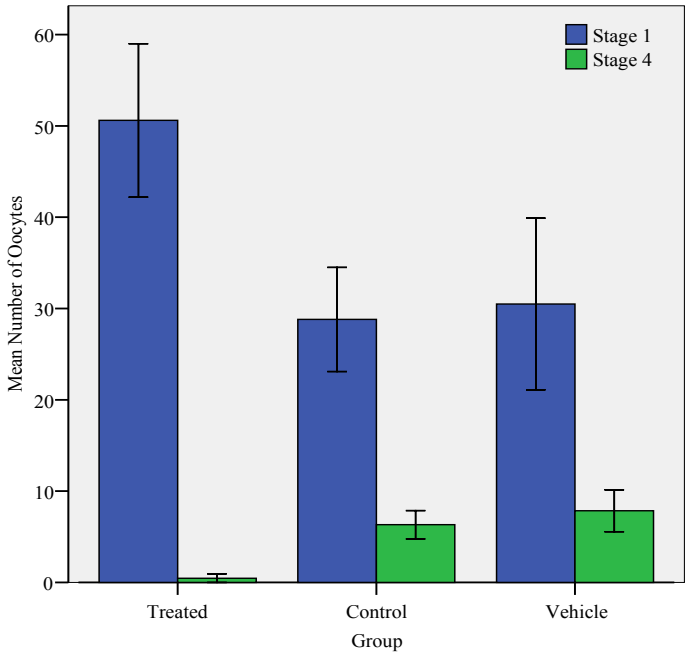

Figure 4.8 – Mean number of oocytes in each stage as a function of group in female fish (error bars +/- 1 SD).

Dependent Variable			Mean Difference	Std. Error	Sig.
Stage 1	Treated	Control	21.800	5.648	.031
		Vehicle	20.100	5.648	.038
	Control	Treated	-21.800	5.648	.031
		Vehicle	-1.700	5.648	.783
	Vehicle	Treated	-20.100	5.648	.038
		Control	1.700	5.648	.783
Stage 4	Treated	Control	-5.875	1.151	.015
		Vehicle	-7.400	1.151	.008
	Control	Treated	5.875	1.151	.015
		Vehicle	-1.525	1.151	.277
	Vehicle	Treated	7.400	1.151	.008
		Control	1.525	1.151	.277

Table 4.3 – LSD Post-hoc tests of significant differences in two dependent variables as a function of group.

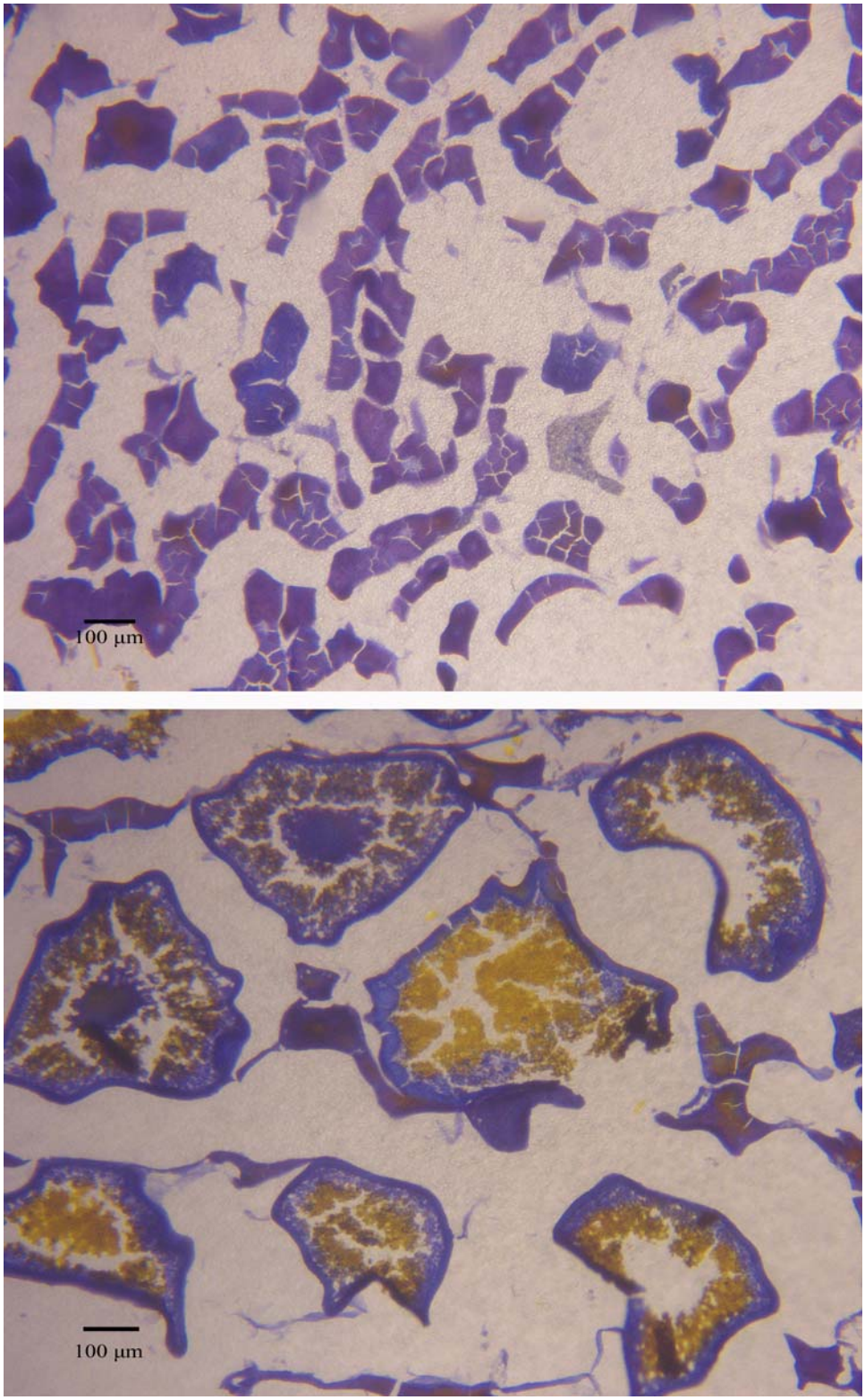

Figure 4.9 – Ovary at 4x magnification, (top) treated and (bottom) control. Apparent is the large number of stage 1 oocytes and small number of stage 4 oocytes in TBT exposed fish.

Discussion

We have demonstrated that chronic exposure to TBT has a masculinizing effect on the female EOD and anal-fin ray bases, and inhibits the development of stage 4 oocytes. Furthermore, the anal-fin ray bases in males were not affected by chronic TBT exposure, but TBT did affect EOD duration and may have affected spermatogenesis. The phenotypic transformations that occurred in *M. r. proboscirostris* following TBT exposure can be attributed to the inhibition of aromatase, resulting in an increase in testosterone and dihydrotestosterone, and a decrease in estrogen (Mochida et al., 2007). This lends support to the original prediction that (1) exposure of adult males and females to TBT elicits androgen-specific activational transformations of sexually dimorphic secondary characters, and (2) tentative support for the prediction that the aromatase inhibiting effects of TBT (lack of estrogen) adversely affect the reproductive potential of males and females.

Seasonal Conditions

The decision to *exclude* fish that were not fecund and in breeding conditions from the EOD analyses was based on Schugardt (1997) who demonstrated that *M. r. proboscirostris* males with a GSI less than 0.15 and females with a GSI less than 2.0 were not in reproductive conditions. To demonstrate that the selected fish *were* in reproductive, rainy season conditions, we demonstrated a trend in the mean duration of P2 in control fish that was longer in males than in females. Such an EOD-related sexual dimorphism occurs in mormyrids only occur under breeding conditions (Landsman et al., 1990; Herfeld & Moller, 1998; Voustianiouk, 2003; Stell 2006). There was no difference

in the mean duration of P1 between control males and females, which is to be expected, as it is not reported to be sexually dimorphic in *M. r. proboscirostris.*

The decision to *include* fish that were not fecund and in rainy season conditions in anal-fin morphology analyses was based on earlier investigations in *M. r. proboscirostris* that have provided strong evidence that males develop expanded anal-fin ray bases between 18-24 months of age when they measure about 135 mm regardless of whether they experienced seasonal changes in water conductivity, i.e. a change from low conductivity during the rainy, breeding season and high conductivity during the dry, non-breeding season (Moller et al., 2004; Schugardt et al., in prep.). We have shown in these fish a significant difference in the length of the most distended fin-ray base between male and female controls with males exhibiting a much wider fin ray extension than females.

Electric Organ Discharge in Males

A general characteristic of the mormyrid EOD is its sexual dimorphic display during breeding season with the phase 2 portion of the EOD elongated in male *M. r. proboscirostris* compared to females. We predicted an increase in the duration of P2 in males following TBT exposure which would have confirmed a hyper-masculinization of the EOD following androgen exposure (Choi, personal communication, 4/08). We did not observe such an effect in *M. r. proboscirostris*; on the contrary, however, TBT affected an increase in the duration of the non-sexually dimorphic phase 1 in males.

One possible explanation for the increase in P1 duration in males exposed to TBT is found in the stalk innervation of the electric organ. In *M. r. proboscirostris* the motor neurons do not penetrate the electrocytes of the electric organ, but synapse on the anterior face (Bass, 1986). Depolarization of this face makes the first phase of the EOD positive,

resulting in current flow through the electrocyte affecting depolarization (with a time delay) of the posterior face. “The anterior face of the electrocyte has a longer duration. This face also has many folds and surface innervations that greatly increase its surface area compared to the posterior face. The degree of surface proliferation may affect a membrane’s total resistance and capacitance and, in turn, its spike generating properties” (Luft, 1958, as cited by A. Bass in Bullock & Heiligenberg, 1986, p. 45). Thus, we attribute the characteristics of P1 to properties of the anterior face of the electrocyte, and propose that the effects of TBT exposure targeted this structure in males.

It has been demonstrated that TBT inhibits ATP production by targeting select ion channels (von Ballmoos, Brunner, & Dimroth, 2004), and increases the frequency of inhibitory post-synaptic currents (in rats) (Kishimoto et al., 2001). The maintenance of electrocyte membranes requires the constant presence of ATP in high concentration (Markham, personal communication, 4/5/08). If ATP was inhibited in the anterior face of the electrocyte and there was a delay in depolarization (increased resistance), P1 would have a longer duration. Why this effect was only seen in males, we can only speculate. Androgen exposure is generally associated with thickening of the electrocytes, which likely did not occur in TBT exposed males. Further, as TBT is not an androgen mimic, it affects membrane ion channels, whereas androgens exert genomic effects on the electrocyte resulting in structural transformation (thickening). The results in both cases are reflected in changes in timing of the EOD phases. Perhaps in males the influence of TBT exposure in the electric organ produced a secondary effect not previously reported.

Recent findings from our laboratory have shown P1 and not P2 duration in *M. r. proboscirostris* to vary under a controlled L:D (12:12) light-dark cycle (Moller, Berry, Napoli, & Pustilnikova, in prep.). As the results of these experiments become clear, they

may help to explain the unexpected increase in P1 duration following exposure to TBT. There was no prediction for a change in P1 duration; these results, however, represent unusual findings, and subsequent experiments should be designed to further explore this effect.

There was no difference in the duration of P2 in males between TBT exposed fish and controls. Although we expected an excess in circulating androgens to *hyper-masculinize* the length of P2, as reported for juvenile *Brienomyrus niger* (Moller, personal communication, 4/08), this was not observed. Androgenic changes in the electric organ and the EOD are related to a *thickening* of the electrocyte. As the fish were juveniles, androgens could still exert their anabolic effects on the electrocytes, whereas in adult breeding male *M. r. proboscirostris* such effects could perhaps have reached a limit, and in spite of excess androgens no further thickening and an increased P2 was possible.

Electric Organ Discharge in Females

Studies have shown that the male-typical increase in duration of P2 is hormone dependent, and can be elicited in females that are exposed to androgens (Bass & Hopkins, 1983, 1985; Landsman et al., 1990; Herfeld & Moller, 1998; Voustianiouk, 2003; Stell, 2006). TBT is an aromatase inhibitor, and thus fish exposed to it should have excess testosterone levels, as endogenous testosterone is not converted into estrogen (Spooner, Gibbs, Bryan, & Goad, 1991; Haubruge et al., 2000; McAllister & Kime, 2003). High levels of testosterone likely accounted for the male-typical P2 duration expressed in females, as androgens are known to thicken the electrocytes (Freedman, Olyarchuk, Marchaterre, & Bass, 1989; Stell, 2006). TBT exposure resulted, as predicted, in an increase in the duration of P2 in females mimicking the effect of androgen exposure.

TBT had no effect on P1. It is plausible that because of the difference in membrane morphology of the electrocytes' anterior face in males (see above) and females (no difference in anterior and posterior faces), the receptors response is sexually dimorphic.

Our results have demonstrated that adult females exposed to TBT for 27 days develop a male-typical P2 EOD duration. These results are similar to those in studies where female mormyrids exposed to androgens develop male-typical alterations in the major phases of the EOD (Bass & Hopkins, 1985; Landsman & Moller, 1988; Voustianiouk, 2003; Stell, 2006). It is important to note that *B. niger* (Stell 2006) and *G. petersii* (Voustianiouk, 2003) have a 4-phase EOD, and androgens specifically target those phases which are sexually dimorphic.

Bone Morphology

We predicted a change in the width of the most distended basal anal-fin ray in males following TBT exposure, which, however, was not borne out by our results. TBT exposed males did not have a longer most distended fin-ray base than untreated males. There is presumably a limit to the amount of expansion that is morphologically possible, as has been shown in females. Investigations into the effects of androgen exposure in mature males on the expansion of fin-ray bases would clarify this issue.

TBT prevents aromatization of androgens to estrogens, and thus results in an excess of circulating androgens (e.g. Evans et al., 2000 a). Females were expected to develop male-like expanded anal-fin ray bases. This was clearly demonstrated in our experiment, and lent support to the prediction that the effects of TBT exposure on anal-fin bone morphology in females mimic the effects of androgens (Landsman et al., 1990; Herfeld & Moller, 1998; Stell, 2006).

Testis Morphology

We predicted that TBT exposure reduces the fish's reproductive potential, specifically that this would be reflected in a decrease in spermatogenesis and thus available spermatozoa. A qualitative analysis revealed differences between treated and control fish affecting tissue containing Sertoli and Leydig cells. These findings are in line with our predictions and corroborated earlier reports that estrogen is vital in spermatogenesis (Billard et al., 1982; Haubruge et al., 2000; Sharpe, 2001; McAllister & Kime, 2003; Mochida et al., 2007).

Estrogen is responsible for the formation of Sertoli and Leydig cells, which are critical in spermatogenesis (Haubruge et al., 2000; Dimitriou et al., 2003; McAllister & Kime, 2003). We suggest that exposure to the aromatase inhibitor TBT impaired estrogen synthesis, thus impairing Sertoli and Leydig cell formation (Leino et al., 2005; Santos et al., 2006). The deleterious effects of TBT on spermatogenesis could be reversed by simultaneously administering estrogen and TBT which demonstrates the important role that estrogen has on sperm production (Santos et al., 2006).

ATP is essential in facilitating spermatogenesis (Rurangwa, Biegniewska, Slominska, Skorkowski, & Ollevier, 2002). As TBT inhibits ATP production (von Ballmoos et al., 2004); this could be one of the mechanisms through which spermatogenesis is decreased following TBT exposure. Our findings were in accord with our predictions, and therefore lent support to the prediction that TBT exposure reduced the male's reproductive potential.

Ovary Morphology

We predicted that TBT exposure would reduce the female's reproductive potential resulting from an impairment of oogenesis. This prediction was confirmed as TBT treated females produced significantly fewer stage 4 oocytes than controls. The ovaries of these females also contained more stage 1 cells than controls, which we attributed to a cessation or reduction of oogenesis. Gonadotropins are responsible for the production of testosterone in the ovary which is converted to estrogen via cytochrome P450 aromatase. In spite of an abundance of testosterone, TBT exposure limited aromatization of testosterone to estrogen and thus impaired normal vitellogenesis (Nagahama, 1994).

These results are comparable with data from studies where females exposed to TBT (e.g. salmon, *Oncorhynchus mykiss,* dog-whelks, *Nucella lapillus,* and cuvier, *Sebastiscus marmoratus*) showed a decrease in viable late-stage oocytes (Piferrer et al., 1993; Piferrer et al., 1994) and a decrease in testosterone levels in the ovary (Spooner et al., 1991; Zhang et al., 2007). Our prediction that TBT exposure results in ovarian abnormalities supported the prediction that TBT reduces female fecundity.

Epilogue

Exposure to TBT clearly had an adverse effect on the development of critical phenotypic characters in both male and female mormyrid fish. It is easy to understand why exposure to a toxin would interfere with development, regardless of the structures affected. What we were able to demonstrate by first developing a framework of how androgens affect the development of sexually dimorphic characters, was to show that the effects of an aromatase inhibitor are, in part, similar to those of androgens. The amount of

time fish are exposed to a toxin affects the severity of its reactions, and had our exposure period been longer there may have been additional effects.

Our results provide support for the hypotheses that TBT exposure has an androgenic effect on the expression of secondary sex characters and reduces fecundity in males and females. We tentatively conclude that exposure to TBT has a dual effect on adult *M. r. proboscirostris* in that secondary characters (EOD and bone expansion) are mostly due to the excess in androgens, while the primary characteristic (gonad) is mostly influenced by a lack of sufficient estrogen.

Mormyrids have a relatively long generation cycle (1-1 ½ years). A study such as the current one was therefore likely to encounter issues with the number of available recrudesced subjects. Thus, the number of subjects was small. However, given the observed dramatic effects of TBT on even a small number of subjects, led us to conclude that exposure to TBT as adults can result in serious endocrine disruptions in the form of phenotypic abnormalities.

The use of electric fish as a water quality monitor has been suggested for over 30 years (Geller, 1984). Grove and Moller (1979) tested the sensitivity of electric fish (*Brienomyrus niger*) to insecticides (dieldrin, malathion) and, based on changes in the fish's EOD discharge rate, it appeared they could detect these toxins. Similarly, Lewis, Kay, and Hanna (1995) investigated the effects of butyltin exposure by measuring the change in discharge rate in *G. petersii,* and demonstrated that these fish altered their EOD rate in response to water polluted with the endocrine disruptor tributyltin. While the potential use of *M. r. proboscirostris* as a sentinel species is intriguing given the number of easily observable phenotypic characters (i.e. EOD and anal-fin ray morphology), because of the fish's long generation cycle, mormyrids may not be the most ideal species.

However, the ecological relevance of understanding how EDC's affect electric fish physiology should not be diminished despite the obstacles their long generational cycle may cause. African waterways are amongst the most polluted and attempts to rectify this are often met with strong opposition to change (Kishimba et al., 2004).

While deleterious TBT effects have been documented, we can still only infer as to how these changes would impact on the survival of *M. r. proboscirostris,* or any other related species that may come in contact with TBT polluted waters. The impact of exposure to endocrine disruptors goes far beyond the scope of the current research. It is our hope that this work can lead to more applied situations and to also to an understanding how these phenotypic abnormalities can interfere with the animals' social behaviors and reproduction.

Concluding Remarks

Early-life treatment to androgens suggested the critical developmental windows for sexual differentiation in undifferentiated gonochoristic teleosts. Based on the extensive evaluation of the adult phenotypes, we concluded that the female gonad was organized prior to androgen treatment, whereas the male gonad was most likely organized within the timeframe of treatment (17-27 days post fertilization). In contrast, we proposed that the activation of secondary characters (EOD duration and basal fin-ray expansion) occurred immediately following treatment as demonstrated in 4-month old juveniles. The androgen treatment experiments provided an important and necessary background to continue investigating the effects of tributyltin. Exposure of adults to this endocrine disruptor had a dual effect. It mimicked androgenic effects as shown in the expression of male-typical EOD duration and anal-fin morphology, and adversely affected the reproductive potential in males and females.

This study has demonstrated the organizational and activational effects of exposure to both hormones and endocrine disruptors. To investigate these effects, the weakly electric fish *Mormyrus rume proboscirostris* has been an excellent preparation because of easily quantifiable sexually dimorphic characters, including the electric organ discharge, the anal-fin complex, and gonadal morphology. Studying both the effects of exogenous androgen (MDHT) at an early stage of development and an aromatase inhibiting endocrine disruptor, TBT, in a mormyrid fish with a relatively long maturational period did provide a unique opportunity to shed some light on the mechanisms underlying the expression of the fish's secondary sexual dimorphisms.

The information obtained through these studies is relevant both to fish biologists, and environmental scientists. Pollution is a real problem, and aquatic species are some of

the most susceptible to their impact as water is the endpoint for most environmental toxins. Thus, understanding how toxins affect the expression of reproductive traits is vital in maintaining fish populations. Our studies provided evidence that both exogenous hormones and toxins interfere with the normal expression of secondary sexual characters. Thus it is all too easy to infer the impact these compounds will have on reproduction. Future studies must be aimed at understanding this next step. If successful reproduction is inhibited by water borne toxins, than the problems related to pollution are potentially deadly.

Beyond their impact on fish physiology is the question of how exogenous steroids and other endocrine disruptors affect human well-being. There is increasing awareness of how consumption of fish can be potentially dangerous, as toxins collect in fatty tissue. Fish are a major dietary source, and as such are a highly valuable economic commodity.

Understanding how toxic substances affect aquatic organisms, better tools must be developed to reduce the risk of contamination of aquatic environments, and thus ultimately prevent human exposure. Our studies have raised more questions than we have answered. The results have strongly supported the notion that the endocrine system retains a high degree of plasticity into adulthood, and that organization and activation are not two independent processes.

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