2009

Effects of endocrine disruptors on biomarkers of reproductive function in the western mosquitofish, Gambusia affinis

Samiksha Ashok Raut
University of Alabama at Birmingham

EFFECTS OF ENDOCRINE DISRUPTORS ON BIOMARKERS OF REPRODUCTIVE FUNCTION IN THE WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS*

by

SAMIKSHA RAUT

ROBERT A. ANGUS, COMMITTEE CHAIR
LARRY R. BOOTS
WILLIAM M. HOWELL
R. DOUGLAS WATSON
STEPHEN A. WATTS

A DISSERTATION

Submitted to the faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

BIRMINGHAM, ALABAMA

2009

EFFECTS OF ENDOCRINE DISRUPTORS ON BIOMARKERS OF REPRODUCTIVE FUNCTION IN THE WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS*

SAMIKSHA RAUT

BIOLOGY

ABSTRACT

Chemicals introduced into the environment by anthropogenic activities have the capacity to interfere with the endocrine system of living organisms and act as endocrine disruptors. To date, most studies have focused on estrogenic endocrine disruptors but reports of androgenic endocrine disruptors continue to increase. In the current study, the western mosquitofish, *Gambusia affinis* was utilized as a model organism to assess the effects of some known and suspected androgenic and estrogenic endocrine disruptors on various reproductive biomarkers. The biomarkers utilized were: anal fin elongation and differentiation, expression of the vitellogenin gene, and sperm production in males. In the studies described in this dissertation, mosquitofish were exposed via the static renewal method to four known or suspected endocrine disruptors: progesterone, methyltestosterone, spironolactone and triclosan. In each case, dose-dependent effects on the biomarkers were observed, indicating that these chemicals have the potential to act as endocrine disruptors. We conclude that reproductive biomarkers in the western mosquitofish, *Gambusia affinis* used in these studies are suitable for future endocrine disruption studies.

ACKNOWLEDGMENTS

My deepest gratitude to my mentor, Dr. Robert Angus for his continued instruction, always being so kind to me, immense patience and above all for introducing me to this fascinating world of endocrine disruption. A heartfelt thank-you to Dr. Stephen Watts for giving me this life-time opportunity to pursue higher education in US, general advice and his willingness to help me at all times. I would like to thank Dr. Howell and (the late) Dr. Jenkins for getting me involved in the "steroid detective story of the paper mill effluent", kind help and great inspiration. I would like to thank Dr. Watson for being such a wonderful Endocrinology teacher which fuelled further interest in research and teaching, Dr. Larry Boots for his help and willingness to serve on my committee, Dr. Wibbels for his generous help on all days of the week and great inspiration which enabled me to work hard, Dr. Mickie Powell for her help and always being there to answer the millions of the questions I managed to ask her, members of the Angus and Watts lab *et al*., for their help and friendship, a special thank you to my dear undergrads the "unsung heroes of research" for helping me out on various fishing trips, extraordinary animal care and for spotless n' steroid less acid-cleaning! I am indebted to Dr. Cusic, Dr. Gilchrist and Raymond Odum for my teaching endeavors. I would like to thank the Department of Biology for their research facilities and teaching assistantship, Kathy Angus for encouraging experiments in the kitchen which kept me saner throughout my graduate career, my Family and friends for their unending love and support. Last but not the least, I would like to

thank my office buddies for their wonderful friendship, bad humor and completing my education on American college football.

TABLE OF CONTENTS

LIST OF TABLES

Table *Page*

LIST OF FIGURES

Figure *Page*

EFFECTS OF METHYTESTOSTERONE ON SECONDARY SEX CHARACTERISTICS AND VITELLOGENIN INDUCTION IN THE FEMALE WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS*

MASCULINIZATION OF FEMALE WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS* EXPOSED TO PROGESTERONE

LIST OF FIGURES (Continued)

Figure *Page*

EFFECTS OF SPIRONOLACTONE ON SECONDARY SEX CHARACTERISTICS AND VITELLOGENIN PRODUCTION IN FEMALE WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS*

EFFECTS OF TRICLOSAN ON VITELLOGENIN INDUCTION AND SPERM PRODUCTION IN MALE WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS*

LIST OF ABBREVIATIONS

AR	Androgen receptor
AED	Androstenedione
ADD	Androstadienedione
BPA	Bisphenol A
EE2	17α- ethinylestradiol
DDT	Dichloro-diphenyl-trichloroethane
EDC	Endocrine disrupting chemicals
MT	Methyltestosterone
PCB	Polychlorinated biphenyls
SPL	Spironolactone
TCS	Triclosan
VTG	Vitellogenin
WWTPs	Wastewater treatment plants

GENERAL INTRODUCTION

Over the last few decades, there has been a continuous increase in the reported incidence of disorders involving secondary sexual characters or the reproductive system, such as breast cancer [1], testicular cancer, reduced sperm counts and cryptorchid testes among human populations [2, 3]. Similar developmental, physiological, and reproductive disorders have been observed in wildlife as well [4]. Research over the past two decades has suggested a strong correlation between the introduction of massive amounts of hormonally active synthetic chemicals into the environment, because of industrialization, and increases in the rates of diverse developmental defects or pathological conditions in human and wildlife populations. For example, in his extensive literature review, W. H. James concluded that exposure to various environmental synthetic chemicals is associated with altered sex ratios in humans [5]. In wildlife, a strong correlation has been observed between exposure to synthetic chemicals and altered sex ratios in fish populations [6]. Such synthetic chemicals, when present in the environment, can end up in organisms and disrupt their endocrine systems in multiple ways. Such chemicals are commonly known as "Endocrine Disrupting Chemicals" (EDCs). An EDC has been defined as an "exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" [7].

Both scientists and many members of the general public today are well aware of the deleterious health effects of common pesticides, such as DDT (Dichloro-Diphenyl-Trichloroethane) and toxic products of industrial processes, such as dioxins and polychlorinated biphenyls (PCBs). Once in the body, DDT is metabolized into biologically active metabolites. These cause alterations in adrenal functions in humans [8]. Dioxins damage the immune system [9], and PCBs are associated with non-Hodgkin's lymphoma [10]. Another chemical, bisphenol A (BPA), a common component of polycarbonate plastics and epoxy resins, acts as a systemic estrogenic compound in the body [11] and has been reported in human urine[12], colostrum [13] and adipose tissue [14]. Further, a recent clinical study has shown that high urinary concentrations of BPA were associated with an increased incidence of cardiovascular disease, diabetes, and liver-enzyme abnormalities [15]. Although most EDCs act as estrogens or antiestrogens, several compounds with androgenic and/or anti-androgenic activity have been identified and have been implicated to cause reproductive abnormalities. For a detailed review, see [16]. Studies on humans have also shown an association between EDC exposure and increased incidence of hormone-dependent cancers and various reproductive abnormalities [17].

Similarly, EDCs have been reported to pose the greatest risk to aquatic organisms since water serves as the ultimate sink for all anthropogenic wastes of natural and synthetic origin. Although these compounds are present at relatively low concentrations in most aquatic environments, they may become bioconcentrated in the various tissues of the body and more so in the adipose tissues of aquatic animals due to their lipophilic nature. It is therefore quite possible that the mobilization of the adipose tissue during the

process of reproduction could release these lipophilic contaminants into the bloodstream causing various physiological and developmental abnormalities. Numerous studies have reported EDCs causing adverse effects on development, fecundity, and reproductive output of aquatic organisms. Some of the sources of EDCs in the aquatic environments include effluent from paper mills [18-20], sewage treatment works [21, 22], runoff from concentrated animal feeding operations [23] and effluents from drug manufacturing facilities [24].

EDCs exert their physiological and cellular effects via multiple mechanisms. There has been no consensus on classifying the different mechanisms of action of EDCs but it is recognized that they can function as either hormone agonists or antagonists by binding to intracellular receptor proteins and subsequently modulating intra-cellular signaling. Still other EDCs disrupt the process of transport or release of endogenous hormones [7]. Interestingly, EDCs such as 4-nonyphenol can exert their effects by more than one pathway [25]. Since many of the cellular signaling mechanisms are evolutionarily conserved, studies on endocrine disruption in aquatic organisms are also relevant to humans. Due to this increasing concern over EDCs present in the aquatic environment, there is a need for validated model test organisms and for effective and sensitive biomarkers to identify and evaluate the effects of EDCs on aquatic organisms for better risk assessment.

A biomarker is defined as a "xenobiotically induced variation in cellular or biochemical components, processes, structures or functions that are measurable in a biological system or samples"[26]. Various biomarkers have been developed in both inverte-

brates and vertebrates. Among the vertebrates, fish have been popular models since they lead a complete aquatic existence as compared to amphibians and reptiles. Most of the reproductive events in fish are regulated by endogenous hormones [27]. EDCs are known to alter the effects of endogenous hormones by acting as agonists, antagonists, or steroidogenic enzyme disruptors. Therefore, reproductive processes, which are affected due to EDC exposure, can be used as sensitive biomarkers to study endocrine disruption. For example, the normally female-limited production of vitellogenin (VTG), an egg yolk protein precursor, can be induced in male fish on exposure to a compound with estrogenic activity, such as the synthetic estrogen, 17α-ethynylestradiol [28]. Similarly, several reproductive characteristics in fish such as expression of the egg zona radiata proteins [29], secondary sexual characteristics, such as elongated anal fins [30] and nuptial tubercles [31, 32], plasma sex-steroid hormones [33], steroidogenic enzymes [34], and altered reproductive behavior [35-37] are used as biomarkers of exposure to both estrogenic and androgenic compounds.

The model organism for the experiments that comprise this dissertation was the western mosquitofish, *Gambusia affinis*. This fish belongs to the livebearing family Poeciliidae which consists of guppies and their relatives [38]. These fish are small in size; the adults are about 2 - 8 cm long. Their life-cycle is reasonably short and they can be easily bred and raised in aquaria. Young mature in 2 - 6 months (depending on species and environmental conditions) and reproductively active females will produce a brood of 1 - 50 or more fry approximately once a month [38]. Poeciliid fishes have been popular models for scientific studies and have been used in a wide variety of disciplines, including repro-

duction, development, genetics, teratology, cancer, behavioral, and evolutionary studies. The eastern (*Gambusia holbrooki*) and western (*G. affinis*) mosquitofish occur naturally or as introduced populations throughout the southern, eastern and the central United States and also in Hawaii, the Philippines, Italy, Spain, New Zealand and other countries. *Gambusia affinis* is the world's most widespread species of freshwater fish, having been introduced worldwide for the biological control of mosquitoes. Therefore, these fish not only serve as an important laboratory test organism but can also serve as a sentinel species for field studies in many parts of the world.

Mosquitofish have numerous characteristics that make them an effective and sensitive *in-vivo* screening system for testing both known and suspected estrogenic and androgenic endocrine disruptors. Male mosquitofish are smaller than the females and have an important secondary sex characteristic, a highly modified anal fin called the gonopodium. The gonopodium is used to transfer sperm into the reproductive tract of the female during copulation [39]. The development of the anal fin into a gonopodium is androgen dependent and occurs during sexual maturation in males. Although the gonopodium is normally limited to males only, the anal fin of a female mosquitofish can be induced to develop into a gonopodium-like structure when exposed to a chemical, or chemicals, with androgenic activity [40, 41]. Therefore, masculinization of the female anal fin is regarded as a useful morphological biomarker of androgen exposure. It has also been shown that, if males are exposed to estrogens during the period of sexual maturation, development of the gonopodium will be inhibited [28]. Expression of female-limited VTG

production and reduction in sperm count are two important biomarkers of estrogen exposure in male mosquitofish [42].

The objectives of this study were to examine the effects of different known and suspected endocrine disruptors on biomarkers of reproductive function in female and male mosquitofish.

- In one study, we followed up on a previous study that indicated that the hormone precursor progesterone, which has been isolated from pine trees [43] and also from the river water and sediment receiving the paper mill effluent [44] can be modified by bacteria into biologically active androgenic compounds capable of masculinizing the female western mosquitofish, *Gambusia affinis*.
- We characterized the effects of different concentrations of the known potent masculinizing agent, methyltestosterone on two reproductive traits of female western mosquitofish: masculinization of the anal fin and inhibition of the estrogen-regulated process of VTG production.
- We investigated the paradoxical capacity of a diuretic with antiandrogenic activity in humans, spironolactone, to act as an androgen in female mosquitofish. The traits investigated were anal fin masculinization and inhibition of VTG production.
- Finally, we investigated the estrogenic effects of a widely used antibacterial agent, triclosan, in male mosquitofish using inhibition of sperm production and stimulation of normally female-limited VTG production as biomarkers.

EFFECTS OF METHYTESTOSTERONE ON SECONDARY SEX CHARACTERISTICS AND VITELLOGENIN INDUCTION IN THE FEMALE WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS*

by

SAMIKSHA A. RAUT AND ROBERT A. ANGUS

In preparation for submission to *Comparative Biochemistry and Physiology*

Format adapted for dissertation

ABSTRACT

Numerous field and laboratory studies have demonstrated that a variety of anthropogenic compounds can act as endocrine disruptors. Most of the studies to date have focused on estrogens. However, reports of androgenic endocrine disruptors continue to increase. It is therefore important to understand the effects of androgenic endocrine disruptors on aquatic organisms. In this study, we examined the effects of the synthetic model androgen methyltestosterone (MT). Females were exposed for five weeks to MT at four concentrations: nominal 10, 100, 1000, and 10000 ng/L, via water using the static renewal method. Morphological masculinization, as evidenced by development of an elongated and modified anal fin, was observed in the three highest exposure groups. The expression of vitellogenin mRNA was significantly inhibited in the three highest exposure groups as compared to the controls. These results support the use of anal fin ray elongation and vitellogenin mRNA expression as useful biomarkers for androgen exposure in mosquitofish. Future studies will investigate and compare the response of these biomarkers to other androgens by utilizing MT as a positive control.

INTRODUCTION

There is substantial evidence that anthropogenic compounds have the potential to disrupt the endocrine system of living organisms (Colborn et al. 1993; Tyler et al. 1998; Guillette et al. 2006). Several studies have shown the deleterious effects exerted by these endocrine disrupting compounds in both vertebrates as well as invertebrates. Most studies to date have focused on investigating the effects of environmental estrogens and anti-

estrogens. However, reports of environmental androgens continue to increase. Androgenic activity from sewage treatment works has been reported by Thomas *et al.,* 2002. Androstenedione has been detected up to >100 ng/L from river water receiving effluent from paper mills (Durhan et al. 2002; Jenkins et al. 2003). Additionally, androstenedione and testosterone have been detected in surface waters as a result of inputs from diverse sources such as dairy wastewater, aquaculture effluents and even spawning fish (Kolodziej et al. 2004). Metabolites of the extensively used growth promoter trenbolone acetate have been identified from beef feedlot discharges and have been shown to elicit a significant androgenic response in CV-1 cells (Durhan et al. 2006). Furthermore, the model androgen methyltestosterone (MT) which has been extensively used to investigate the effects of xenoandrogens (Zerulla et al. 2002; Hornung et al. 2004; Pawlowski et al. 2004; Kang et al. 2008) has been detected from the wastewater from a chemical company at a concentration of 1.3 ng/L (Blankvoort et al. 2005). Since reports of androgenic endocrine disruptors continue to increase, there is a need to develop sensitive and reliable biomarkers of androgen exposure in model organisms used in endocrine disruptor studies.

Fish have been by far the most preferred aquatic model organism for investigating the effects of endocrine disrupting compounds in the laboratory. Various morphological and molecular biomarkers have been developed in various fish models to provide cost effective assessments of the effects of estrogenic and androgenic endocrine disruptors (Scholz et al. 2008). Poeciliid fishes possess morphological characteristics which make them a useful model organisms for investigating the effects of potential endocrine disrupting chemicals (Bortone et al. 1994). A mature male Poeciliid fish possess an elon-

gated and modified anal fin, called a gonopodium. The gonopodium functions in the transfer of sperm to the reproductive tract of the female during the process of copulation (Peden 1972). The development of the anal fin into a gonopodium is androgen dependent and normally occurs during sexual maturation in males. The anal fin of a female mosquitofish, *Gambusia affinis* will be induced to develop into a male-like gonopodium when exposed to androgens (Turner 1942; Angus et al. 2001). Although the sequence of events involved in the development of a gonopodium in an androgen exposed female mosquitofish is similar to the development of a male gonopodium, the extent of masculinization is dependent upon the nature of the androgen. Based on this morphological characteristic, the western mosquitofish, *Gambusia affinis,* serves as an important model organism for field as well as laboratory studies on endocrine disruption.

In this study, we selected MT as a test chemical as it is a recommended reference chemical for endocrine disruption studies in fish models (OECD 1999). Earlier studies have characterized a dose-response relationship between exposure to MT via diet and female anal fin masculinization (Stanko 2005). However, there is a need to characterize a dose-dependent relationship between anal fin masculinization in female mosquitofish when exposed to MT via water-route. Additionally, MT is an aromatizable androgen (Ankley et al. 2001; Zerulla et al. 2002; Hornung et al. 2004) and it is therefore important to investigate the effects of MT on the estrogen-regulated process of vitellogenin (VTG) production. Previous studies have shown the use of anal fin masculinization and VTG inhibition in female mosquitofish as effective biomarkers of androgen exposure (Stanko et al. 2007). Therefore, this study aims at characterizing the dose-dependent responses of

MT on anal fin masculinization and VTG production in female mosquitofish, *Gambusia affinis*. Furthermore, the data generated from this study can then be used to compare the biomarker responses with other androgens when MT is used as a positive control for endocrine disruption studies in female mosquitofish, *Gambusia affinis*.

MATERIALS AND METHODS

Animals

Mature female western mosquitofish, *Gambusia affinis* were collected from Thomas Spring Pond, a spring-fed pond in Bessemer, Alabama. After collection these fish were returned to the University of Alabama at Birmingham and allowed to acclimate in the laboratory for two weeks. The acclimated fish were then randomly assigned to the treatments, (n = 10 per treatment).

Methyltestosterone treatment – static renewal exposure

Fish were exposed to methyltestosterone (4-androsten-17α-methyl-17β-ol-3-one, MT) via water by the static renewal method. The fish were kept individually in 1-L unaerated model 14005 Kimax beakers (Kimble Glass, Inc. Vineland, NJ, USA), which hold 1L with an additional 2.5 cm air space. The water was dechlorinated tap water with 0.25 ppt Instant Ocean Sea Salt (Spectrum Brands, Inc., Atlanta, GA, USA) added to provide trace minerals and buffering capacity. Water temperature was 24 $\pm$ 2 ^{o}C. Fish were maintained under a 14:10 light: dark cycle. The water was changed every other day and MT

re-added. Water chemistry parameters including pH, dissolved oxygen, ammonia and nitrate were monitored once a week and maintained at appropriate levels.

A 10^6 nM stock solution was made by dissolving MT (Steraloids, Newport, RI, USA) in 5 ml of dehydrated absolute ethanol (Pharmaco, Brookfield, CT, USA) and 95 ml propylene glycol (Fischer Chemicals, USA) to produce a final volume of 100 mL. Final working concentrations of 10, 100, 1000 and 10000 ng/L were prepared by 10X serial dilution of the stock solution to 1L of water in each beaker. These concentrations were selected on the basis of a preliminary toxicity range-finding study. The solvent control group received ethanol and propylene glycol equivalent to that received by the highest concentration experimental group. Fish were fed twice a day with Silver Cup granulated trout starter food (Nelson & Sons, Inc., Murray, UT, USA). Food was stored at 4 ^{o}C.

Anal fin and physiological measurements

On day zero before the start of the exposure, fish were anesthetized by immersion in 300 mg/L MS-222 (tricaine methanesulfonate, Argent Labs, Redmond, WA, USA) and their standard length and weight was recorded. Standard length was measured from the tip of the snout to the end of the caudal peduncle and recorded to the nearest 0.2 mm. Fish were pat dried on a tissue paper and weighed on a digital scale to the nearest 0.1 mg. The anal fins were photographed with a Polaroid DMC Ie digital camera (Polaroid, Waltham, MA, USA) mounted on a Leica MZ6 stereomicroscope (Leica Microsystems Inc., Bannockburn, IL, USA). Images captured by the camera were transported to a computer running Adobe Photoshop (Adobe Systems, San Jose, CA, USA) and saved as jpeg files.

Anal fin ray lengths were measured in pixels using Image Tool software (University of Texas Health Science Center, San Antonio, TX, USA). At the magnification used to photograph most of the fins, each pixel represented 0.003 mm. Anal fin ray measurements were measured at one-week intervals for five weeks. Anal fin ray elongation was quantified as the ratio of length of ray 4, which elongates during gonopodial development, to that of ray 6, which does not elongate (Angus et al. 2001). After the completion of the five week exposure period, the fish were sacrificed by immersion in 120 mg/L MS-222. The standard length and the mass of the fish were again recorded. The livers were removed and cryopreserved in liquid nitrogen (-160 °C) and stored at -80 °C.

Hepatic Vitellogenin: Quantitative Real Time Polymerase Chain Reaction (q- RTPCR)

Total RNA was extracted from frozen liver tissue using the Trizol method protocol as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The cDNA was amplified in an Opticon Continous Fluorescent Detector (MJ Research, Waltham, MA) using IQTM SYBR Green supermix (Bio-Rad Laboratories). The primers were designed specifically for VTG using the mRNA sequence from GenBank (Accession number DQ190844) and using Primer3 software (http://frodo.wi.mit.edu/). The primers for the 18S reference housekeeping gene were designed based on *Oncorhynchus mykiss* mRNA sequence (Genbank Accession Number: AF243428) because the 18S mRNA sequence for *Gambusia affinis* has not been determined (Leusch et al. 2005). The sequences of the primers are shown in Table 1.

Primer	Sequence
Forward VTG	5' - ACCAGGGACCTGAACAACTG - 3'
Reverse VTG	5' - GATGGCATTAGCGACTGGTT - 3'
Forward 18S	5' - CCTGCGGCTTAATTTGACTC - 3'
Reverse 18S	5' - AACTAAGAACGGCCATGCAC - 3'

Table 1. Primer sequences used for RT-PCR of a *Gambusia affinis* vitellogenin gene transcript.

Real-time PCR was performed in triplicate for each liver sample, averaged, and normalized to endogenous 18S RNA reference transcripts. At least five fish per treatment group were analyzed for VTG gene expression. Quantification of the hepatic VTG mRNA expression was carried out by using the $2^{-\Delta\Delta CT}$ method (Livak et al. 2001).

Statistical Methods

All the summary statistics are expressed as mean $\pm$ standard error. The cutoff for statistical significance was $p < 0.05$. Ratios are not normally distributed but can be rendered approximately so by an appropriate transformation. Anal fin 4:6 length ratios were log transformed based on the findings of a previous study that transformation successfully normalizes the data (Angus et al. 2001). Since time 0 data were available for each fish, the average (end-start) difference in the transformed 4:6 ratio was compared to 0 using a paired t-test. VTG mRNA expression levels were compared between treatment groups and the control using the nonparametric Kruskal-Wallis test, followed by Dunn's test

(Dunn 1964). All the other physiological variables were analyzed using ANOVA followed by Dunnett's post hoc test was used to compare the treatment group means to that of the control.

RESULTS

Fin Ray Elongation

The 4:6 anal fin ray elongation ratio of the female mosquitofish exhibited a dose-dependent response to MT exposure. Figure 1 shows week 0 and week 5 mean anal fin ray 4:6 ratios for fish exposed to MT by the static renewal method. Fish exposed to all but the lowest concentration of MT (10 ng/L) showed significant elongation at the end of the five week exposure period. Figure 2 shows the 4:6 anal fin ray elongation ratios of MT exposed female mosquitofish over a five week exposure period. Figure 3 shows elongated anal fins from representative females exposed to different concentrations of MT via water.

Hepatic Vitellogenin Expression

The fin-ray elongation ratios in the female mosquitofish exposed to MT suggested the masculinization of the females. Therefore, we decided to investigate the effects of MT on the expression of VTG mRNA, a gene that is induced by estrogen and normally expressed only in females. To do so, we collected livers from female mosquitofish exposed to MT for five weeks, isolated the mRNA, and determined VTG expression relative to 18S ribosomal RNA using real time PCR. The assay and the amplification efficiency of both the primer sets were determined in another experiment (see Triclosan ex-

periment Fig.2). The amplification efficiency was always greater than 90% for both the primer sets. Tests of homogeneity of slope indicated that the linear regression lines of primer sets for the target and reference genes were parallel to each other, suggesting that their amplification efficiencies are approximately equal. We also verified amplification specificity by performing melting curve analysis after PCR amplification. This showed a single peak, indicating the specificity of PCR amplification (data not shown). Moreover, the variability among C_t values for 18S from all samples was negligible, supporting the rationale for the use of 18S as the reference gene.

The hepatic VTG mRNA expression was significantly decreased, as compared to the solvent control, when exposed to MT at 100ng/L and above concentrations (Fig. 4). The VTG mRNA expression was almost non-detectable at 1000ng/L and 10,000 ng/L MT concentrations.

Physiological Variables

The mean changes in the mass of the treatment groups over a period of five weeks are shown in Figure 5. The mean mass of the fish in the 10, 100, 1000 and 10000 ng/L MT treatment group increased significantly as compared to the solvent control. The effect of the MT treatment on the standard length is shown in Figure 6. The standard length of the female mosquitofish exposed to 100, 1000 and 10000 ng/L MT increased significantly as compared to the solvent control.

DISCUSSION

In this study we have investigated the masculinization of female western mosquitofish when exposed to MT for a period of five weeks by the static renewal method. Anal fin masculinization, as evidenced by significant anal fin ray elongation, was observed in all the treatment groups except the lowest concentration of MT (10 ng/L). VTG mRNA expression was inhibited in the treatment groups that showed anal fin masculinization. Furthermore, there was a significant increase in the mass of the exposed fish over the five week exposure period in all the treatment groups as compared to the control. A significant increase in standard length was observed in all the treatment groups except the lowest MT concentration (10 ng/L).

In Poeciliid fish, the metamorphosis of a gonopodium is characterized by an initial phase of rapid growth and segmentation of anal fin rays three and four, accompanied with some thickening of the rays along with fusion of their branches. The initial elongation phase is followed by a later phase of differentiation which includes development of spines, hooks and serrae at the tip (called the tip apparatus). After the onset of the phase of differentiation, there is no further growth of the anal fin rays. A quantitative study by Turner (1942) showed that female mosquitofish when exposed to a MT concentration greater than 1 mg in 2,000,000 cc water (1664 nM) results in a precocious differentiation of the anal fin ray tip apparatus and prematurely terminates their further growth. Turner hypothesized that minute quantities of androgenic hormone secreted by the developing testis in the male fish is required to initiate the early phase of elongation in gonopodial development. Further, a greater output is required for the later phase of differentiation of

the gonopodium. Results of the present study support this hypothesis. The maximum elongation response was not seen in the fish exposed to the highest MT concentration (10000 ng/L), but rather in the group exposed to 1000 ng/L MT. Further, in the 10000 ng/L MT treatment group, differentiation of the tip apparatus occurred rapidly (after the first week of exposure); so that elongation ceased before much had occurred. This can be seen in Figure 2 by a constant value of the 4:6 anal fin ray ratio over an exposure period of five weeks. On the other hand, in the 1000ng/L MT treatment group, elongation, as indicated by the 4:6 anal fin ray ratio, continued until the second week of exposure before the onset of the phase of differentiation leading to the complete development of the tip apparatus and cessation of elongation.

MT exerts its mechanism of action by binding with high affinity to fish androgen receptors (AR) (Sperry et al. 1999). In mosquitofish, the AR isoforms, ARα and ARβ are predominantly expressed in the distal region of the outgrowing anal fin rays (Ogino et al. 2004). These receptors are active and have been shown to regulate mosquitofish gonopodial development through sonic hedgehog *(Shh)* pathway (Ogino et al. 2004). In this study, we did not observe any signs of masculinization in the lowest MT (10 ng/L) treatment group suggesting that this concentration may not have been sufficient to induce the robust response from ARs required to induce masculinization. The preceding MT concentrations (100 ng/L and 1000 ng/L) showed a masculinization response with the formation of hooks and spines, suggesting a dose dependent activation of ARs. The highest MT concentration group (10000 ng/L), however, showed decreased masculinization as compared to lower concentration of 1000 ng/L suggesting decreased saturation of ARs or

downregulation of ARs in response to excessive ligand (MT). This would terminate the further elongation of the anal fin rays with the onset of the phase of differentiation. These results suggest that the lowest no observed effect concentration for MT in adult female mosquitofish is >10ng/L and that development of a gonopodium in female mosquitofish can be regarded as a reliable biomarker of androgen exposure.

In this study, we also noted a dose-dependent decrease in the hepatic VTG mRNA expression in all the masculinized MT treatment groups. Similar results have been demonstrated in many other fish model systems. For example, plasma VTG inhibition was observed in adult female mumichog (*Fundulus heteroclitus*) when exposed to 1000 ng/L and 100 ng/L of MT for 7 and 14 days respectively (Sharpe et al. 2004). An intraperitoneal injection of MT (20 mg/kg) administered to mature female tilapia (*Oreochromis niloticus*) resulted in a distinct decline of serum VTG protein and VTG mRNA levels (Lazier et al. 1996). Female eelpout (*Zoarces viviparous*) when exposed to MT for 20 days exhibited a decline in VTG protein levels but only significantly in the 100 ng/L MT treatment group (Korsgaard 2006). Similarly, spawning fathead minnows (*Pimephales promelas*) when exposed to the synthetic non-aromatizable androgen 17-β-trenbolone for 21 days showed significant reductions in the circulating VTG levels (Ankley et al. 2003).

In contrast, several other studies have shown VTG induction, in both male and female fish, when exposed to MT. Hornung *et al*. showed that MT can be aromatized into methylestradiol which has a relative binding affinity to the estrogen receptor of 68.3% (Hornung et al. 2004). This binding of methylestradiol to estrogen receptor stimulates the estrogen-dependent increase in VTG induction. Increase in VTG expression has been re-

ported in MT (10, 50, 100μg/L) exposed juvenile fathead minnows for 14 days (Zerulla et al. 2002). A significant increase in the plasma VTG concentration has been demonstrated in male (>1 μg/L) and female (>50 μg/L) fathead minnows (Pawlowski et al. 2004). Further, MT exposure resulted in VTG induction at a low concentration of 4.5 ng/L in adult male zebrafish (*Danio rerio*) (Andersen et al. 2006) and also has been demonstrated in response to many other androgens including MT in primary cultures of immature male rainbow trout hepatocytes (Mori et al. 1998). In the present study, there was no significant decrease in VTG mRNA expression in the lowest MT treatment group (10 ng/L). On the other hand, at concentrations >10 ng/L, there was a decrease in VTG expression. There could have been many different mechanisms responsible for the decrease in VTG expression. First, it is possible that exposure to high concentrations of MT may have resulted in reduced estrogen levels due to an inhibitory effect on gonadotrophins production by a negative feedback effect at the hypothalamo-pituitary axis. This has been the proposed mechanism hypothesized by Lazier *et al.,* for a decline in serum estrogen levels and also VTG levels (Lazier et al. 1996) in Tilapia (*Oreochromis niloticus*) when injected intraperitoneally with methyltestosterone. Alternatively, it is also possible that MT at a higher concentration may be aromatized into methylestradiol. However, the concentration of methylestradiol formed probably is not sufficient to induce VTG production as MT was renewed every other day in the present static renewal treatment. Secondly, MT at higher concentration could bind the estrogen receptor and thus, could potentially act as estrogen antagonist, finally contributing to decreased VTG production. MT as well as tamoxifen have been demonstrated to be antagonistic to VTG production (Lazier et al.

1996). Thus, it is possible that at high concentrations, MT is acting as an estrogen antagonist inhibiting VTG production and also as an androgen agonist inducing masculinization.

MT is commonly used in aquaculture industry around the world to sex reverse female fish and produce all-male populations (Pandian T.J 1995). Many studies have also reported a significant increase in fish growth when exposed to MT, consistent with its anabolic effects (Kuhn 2002). Our results are in agreement with these studies as there was a significant increase in mass in all the MT treatment groups as compared to the solvent control. It appears that MT has anabolic effects in fish as well as in mammals. The MT treatment groups also exhibited a significant increase in standard length except for the lowest 10 ng/L MT treatment. However, we still need to determine why such a response was seen only in this particular exposure concentration.

In conclusion, the present study clearly indicates that exposure to MT in western female mosquitofish; *Gambusia affinis* induces a dose-dependent masculinization of the anal fin and inhibition of VTG mRNA expression. These results support the use of these traits as useful biomarkers of androgen exposure in western mosquitofish. Now that the effects of MT on these traits have been characterized, MT may be usefully employed as a positive control treatment in other studies of endocrine disrupting chemicals.

REFERENCES

Andersen, L., Goto-Kazeto, R., Trant, J.M., Nash, J.P., Korsgaard, B., Bjerregaard, P., 2006. Short-term exposure to low concentrations of the synthetic androgen methyltestosterone affects vitellogenin and steroid levels in adult male zebrafish (*Danio rerio*). Aquat Toxicol 76, 343-352.

Angus, R.A., McNatt, H.B., Howell, W.M., Peoples, S.D., 2001. Gonopodium development in normal male and 11-ketotestosterone-treated female mosquitofish (*Gambusia affinis*): a quantitative study using computer image analysis. Gen Comp Endocrinol 123, 222-234.

Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J., Makynen, E.A., 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). Environ Toxicol Chem 20, 1276-1290.

Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., Henry, T.R., Denny, J.S., Leino, R.L., Wilson, V.S., Cardon, M.C., Hartig, P.C., Gray, L.E., 2003. Effects of the androgenic growth promoter 17-beta-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environ Toxicol Chem 22, 1350-1360.

Blankvoort, B.M.G., Rodenburg, R.J.T., Murk, A.J., Koeman, J.H., Schilt, R., Aarts, J.M.M.J.G., 2005. Androgenic activity in surface water samples detected using the AR-LUX assay: indication for mixture effect. Environ Toxicol Pharmacol 19, 263-272.

Bortone, S.A., Davis, W.P., 1994. Fish intersexuality as indicator of environmental stress. Bioscience 44, 165-172.

Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101, 378-384.

Dunn, O.J., 1964. Multiple comparisons using rank sums. Technometrics 6, 241-252.

Durhan, E.J., Lambright, C., Wilson, V., Butterworth, B.C., Kuehl, O.W., Orlando, E.F., Guillette, L.J., Jr., Gray, L.E., Ankley, G.T., 2002. Evaluation of androstenedione as an androgenic component of river water downstream of a pulp and paper mill effluent. Environ Toxicol Chem 21, 1973-1976.

Durhan, E.J., Lambright, C.S., Makynen, E.A., Lazorchak, J., Hartig, P.C., Wilson, V.S., Gray, L.E., Ankley, G.T., 2006. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. Environ Health Perspect 114 Suppl 1, 65-68.

Guillette, L.J., Jr., Moore, B.C., 2006. Environmental contaminants, fertility, and multioocytic follicles: a lesson from wildlife? Semin Reprod Med 24, 134-141.

Hornung, M.W., Jensen, K.M., Korte, J.J., Kahl, M.D., Durhan, E.J., Denny, J.S., Henry, T.R., Ankley, G.T., 2004. Mechanistic basis for estrogenic effects in fathead minnow (*Pimephales promelas*) following exposure to the androgen 17alpha-methyltestosterone: conversion of 17alpha-methyltestosterone to 17alpha-methylestradiol. Aquat Toxicol 66, 15-23.

Jenkins, R.L., Wilson, E.M., Angus, R.A., Howell, W.M., Kirk, M., 2003. Androstenedione and progesterone in the sediment of a river receiving paper mill effluent. Toxicol Sci 73, 53-59.

Kang, I.J., Yokota, H., Oshima, Y., Tsuruda, Y., Shimasaki, Y., Honjo, T., 2008. The effects of methyltestosterone on the sexual development and reproduction of adult medaka (*Oryzias latipes*). Aquat Toxicol 87, 37-46.

Kolodziej, E.P., Harter, T., Sedlak, D.L., 2004. Dairy wastewater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment. Environ Sci Technol 38, 6377-6384.

Korsgaard, B., 2006. Effects of the model androgen methyltestosterone on vitellogenin in male and female eelpout, *Zoarces viviparus* (L). Mar Environ Res 62 Suppl, S205-210.

Kuhn, C.M., 2002. Anabolic steroids. Recent Prog. Horm.Res. 57, 411-434.

Lazier, C.B., Langley, S., Ramsey, N.B., Wright, J.M., 1996. Androgen inhibition of vitellogenin gene expression in tilapia (*Oreochromis niloticus*). Gen Comp Endocrinol 104, 321-329.

Leusch, F.D., Van den Heuvel, M.R., Laurie, A.D., Chapman, H.F., Gooneratne, S.R., Tremblay, L.A., 2005. Quantification of vitellogenin mRNA induction in mosquitofish (*Gambusia affinis*) by reverse transcription real-time polymerase chain reaction (RT-PCR). Biomarkers 10, 429-438.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Mori, T., Matsumoto, H., Yokota, H., 1998. Androgen-induced vitellogenin gene expression in primary cultures of rainbow trout hepatocytes. J Steroid Biochem Mol Biol 67, 133-141.

OECD, 1999. Final Report from the Organization for Economic Cooperation and Development expert consulting meeting. In: Report 9906, Environmental Health and Safety Division, Paris, France, London, UK, 28-29thOctober1998

Ogino, Y., Katoh, H., Yamada, G., 2004. Androgen dependent development of a modified anal fin, gonopodium, as a model to understand the mechanism of secondary sexual character expression in vertebrates. FEBS Lett 575, 119-126.

Pandian T.J, S.S.G., 1995. Hormonal induction of sex reversal in fish Aquaculture 1-22.

Pawlowski, S., Sauer, A., Shears, J.A., Tyler, C.R., Braunbeck, T., 2004. Androgenic and estrogenic effects of the synthetic androgen 17alpha-methyltestosterone on sexual development and reproductive performance in the fathead minnow (*Pimephales promelas*) determined using the gonadal recrudescence assay. Aquat Toxicol 68, 277-291.

Peden, A.E., 1972. The function of gonopodial parts and behavioral pattern during copulation by Gambusia (Poecillidae). Can J Zool 50, 955-968.

Scholz, S., Mayer, I., 2008. Molecular biomarkers of endocrine disruption in small model fish. Mol Cell Endocrinol 293, 57-70.

Sharpe, R.L., MacLatchy, D.L., Courtenay, S.C., Van Der Kraak, G.J., 2004. Effects of a model androgen (methyl testosterone) and a model anti-androgen (cyproterone acetate) on reproductive endocrine endpoints in a short-term adult mummichog (*Fundulus heteroclitus*) bioassay. Aquat Toxicol 67, 203-215.

Sperry, T.S., Thomas, P., 1999. Identification of two nuclear androgen receptors in kelp bass (*Paralabrax clathratus*) and their binding affinities for xenobiotics: comparison with Atlantic croaker (*Micropogonias undulatus*) androgen receptors. Biol Reprod 61, 1152-1161.

Stanko, J.P., 2005. Reproductive and developmental effects of bioactive constituents of pulp mill effluent on female mosquitofish, *Gambusia affinis*. In: Biology, vol. Ph.D. University of Alabama at Birmingham, Birmingham,

Stanko, J.P., Angus, R.A., 2007. In vivo assessment of the capacity of androstenedione to masculinize female mosquitofish (*Gambusia affinis*) exposed through dietary and static renewal methods. Environ Toxicol Chem 26, 920-926.

Thomas, K.V., Hurst, M.R., Matthiessen, P., McHugh, M., Smith, A., Waldock, M.J., 2002. An assessment of in vitro androgenic activity and the identification of environmental androgens in United Kingdom estuaries. Environ Toxicol Chem 21, 1456-1461.

Turner, C.L., 1942. A quantitative study of the effects of different concentrations of ethynltestosterone and methyl testosterone in the production of gonopodia in females of *Gambusia affinis*. Physiol Zool 15, 263-234.

Tyler, C.R., Jobling, S., Sumpter, J.P., 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit Rev Toxicol 28, 319-361.

Zerulla, M., Lange, R., Steger-Hartmann, T., Panter, G., Hutchinson, T., Dietrich, D.R., 2002. Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (*Pimephales promelas*). Toxicol Lett 131, 51-63.

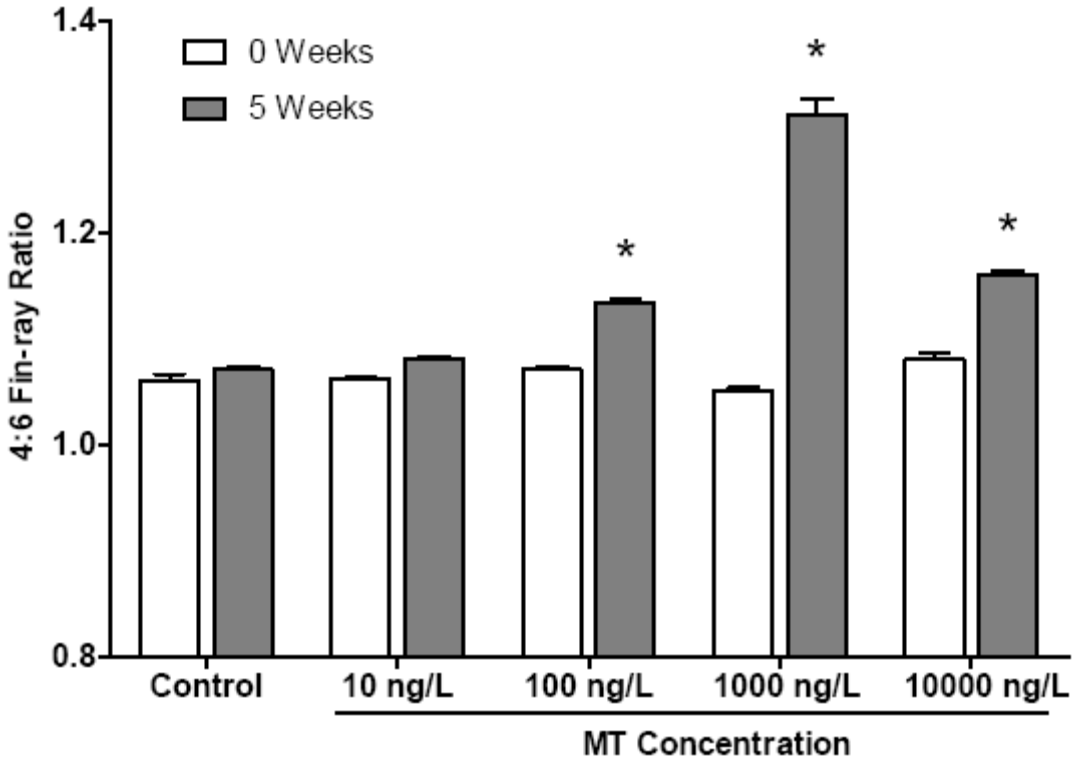

Fig. 1. The 4:6 anal fin ray elongation ratios of static renewal methyltestosterone (MT) treatment groups. Female mosquitofish were exposed to varying concentrations of MT for five weeks via static renewal method and masculinization was assessed by the elongation of anal fin ray, measured as 4:6 ratio. Data are presented as mean + S.E. (n = 10). An asterisk (*) indicates the mean at 5 weeks differs significantly ($p < 0.05$) from the mean at 0 weeks (paired t-test).

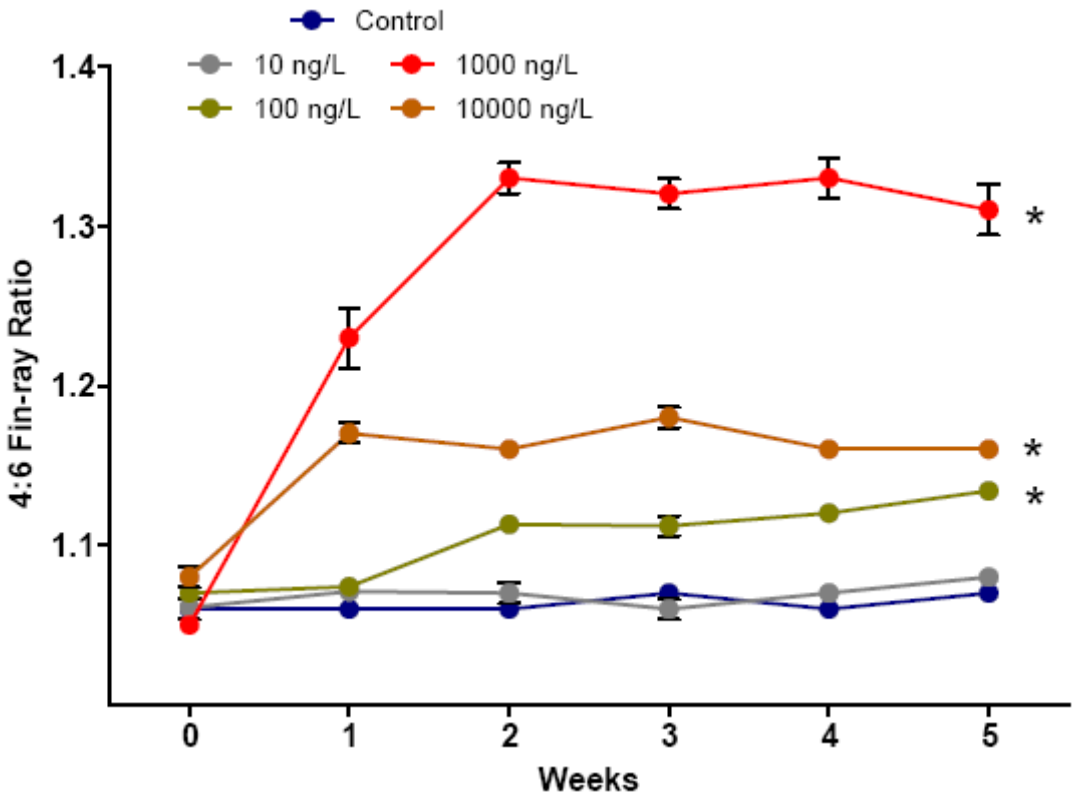

Fig. 2. Concentration response of anal fin elongation ratios. Methyltestosterone (MT) exposure induces masculinization in female mosquitofish after two weeks of treatment. Female mosquitofish were exposed to varying concentrations of MT for up to five weeks via static renewal method. Masculinization was assessed at the end of each week by measurement of the anal fin ray measured as 4:6 ratio. Data are presented as mean ± S.E (n = 10). An asterisk (*) indicates values significantly different ($p < 0.05$) from the control. Standard error bars are not visible on some measurements because they were small and covered by plotting symbols.

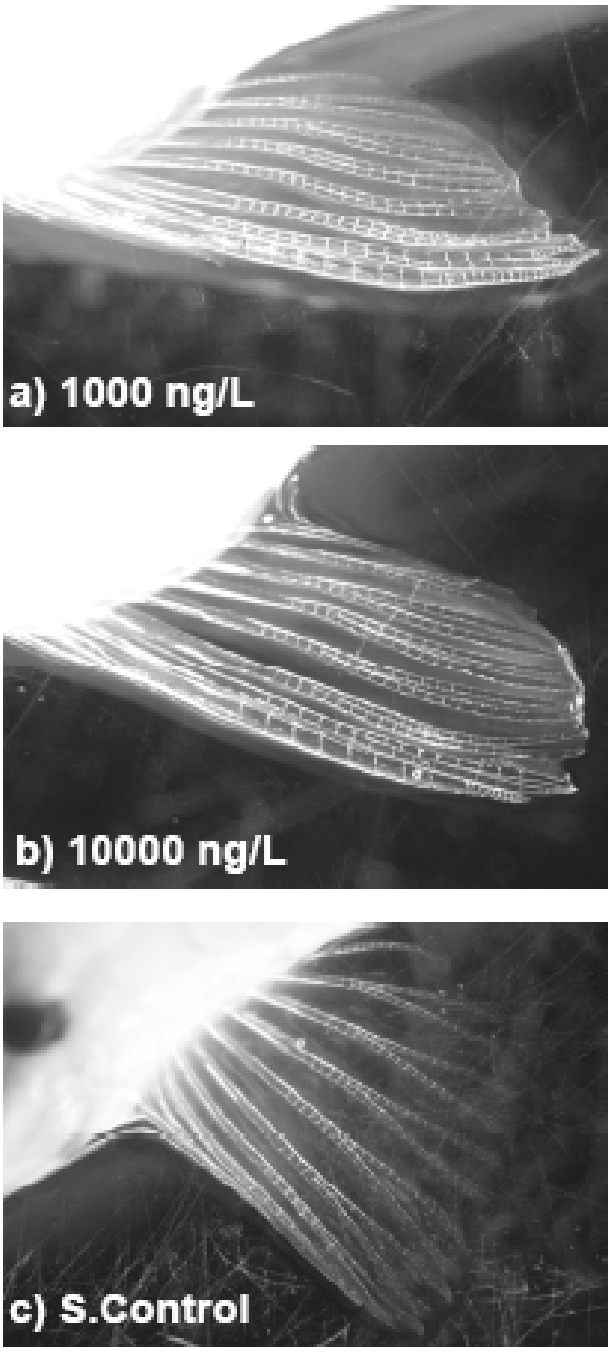

Fig. 3. *Gambusia affinis* anal fins. Anal fins of representative female mosquitofish exposed for five weeks to 1000 ng/L MT (a), 10000 ng/L MT (b), and solvent control (c) by the static renewal method.

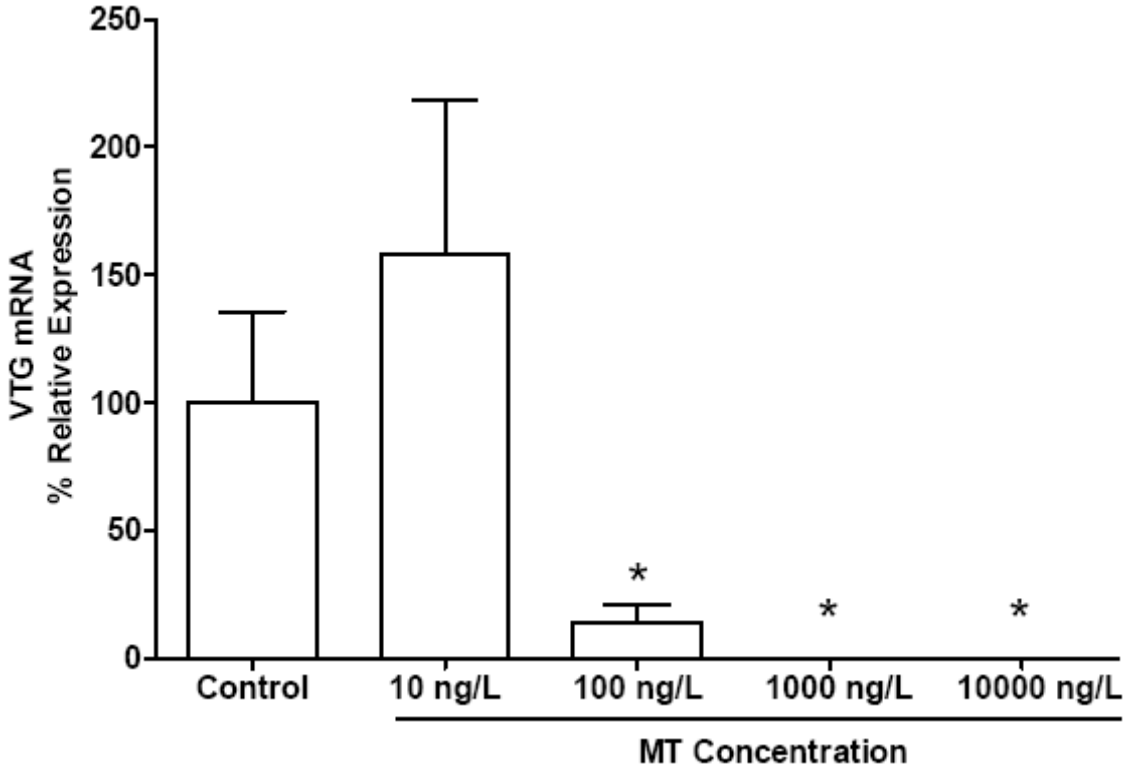

Fig. 4. Vitellogenin expression in methyltestosterone treatment groups. Methyltestosterone (MT) exposure inhibits vitellogenin (VTG) mRNA expression in female mosquitofish. Female mosquitofish were exposed to varying concentrations of MT for five weeks via the static renewal method. Livers were extracted at the end of five weeks after sacrificing fish and hepatic RNA was isolated. Hepatic VTG mRNA expression was quantified using real-time PCR and is presented here as % relative expression to the control. Data are presented as mean + S.E. ($n \geq 5$). An asterisk (*) indicates values significantly different from control ($p < 0.05$).

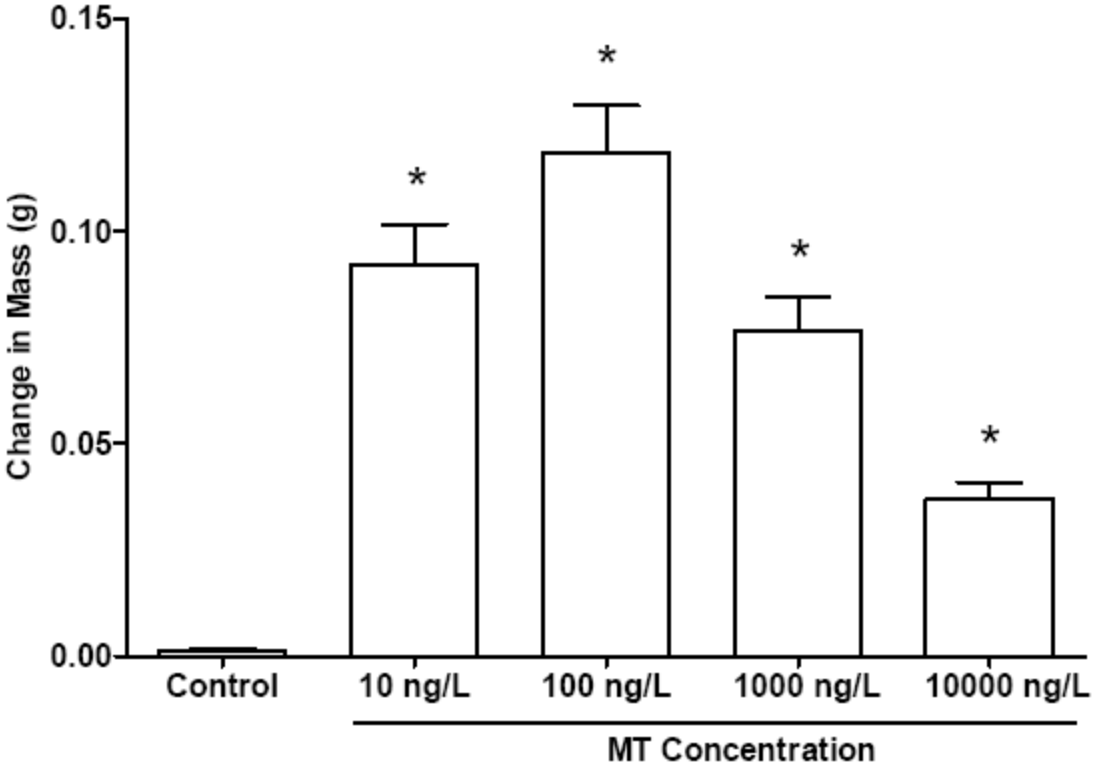

Fig. 5. Changes in mass of fish in the static renewal treatment: Methyltestosterone (MT) exposure increases mass in female mosquitofish. Female mosquitofish were exposed to varying concentrations of MT for five weeks via static renewal method. Total body weights were measured at the beginning and end of the experiment and change in mass ($Weight_{week5} - Weight_{week0}$) was calculated. Data are presented as mean + S.E. (n = 10). An asterisk (*) indicates values significantly different from control ($p < 0.05$).

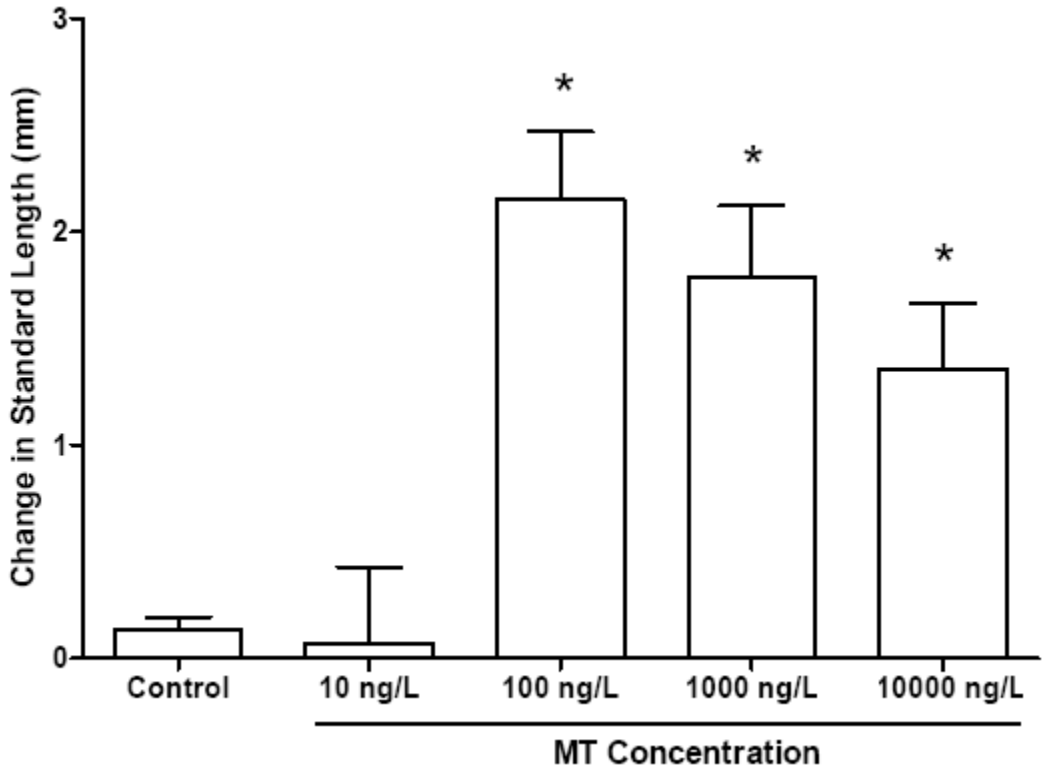

Fig. 6. Changes in the standard length of fish in the static renewal treatment. Methyltestosterone (MT) exposure increases body length in female mosquitofish. Female mosquitofish were exposed to varying concentrations of MT for five weeks via static renewal method. Total body lengths were measured at the beginning and end of the experiment and change in length ($Length_{week5} - Length_{week0}$) was calculated. Data are presented as mean + S.E. (n = 10). An asterisk (*) indicates values significantly different from control ($p < 0.05$).

MASCULINIZATION OF FEMALE WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS,* EXPOSED TO PROGESTERONE

by

SAMIKSHA A. RAUT, W. MIKE HOWELL, RONALD L. JENKINS AND ROBERT A. ANGUS

In preparation for submission to *Environmental Toxicology and Chemistry*

Format adapted for dissertation

ABSTRACT

Previous studies have reported the presence of progesterone in the sediment and water column of the Fenholloway River, a coastal Florida river receiving effluent from a paper mill and containing populations of masculinized female mosquitofish, *Gambusia holbrooki*. It was earlier speculated that microbial degradation of pine pulp-derived phytosteroids results in the formation of progesterone. Recently, progesterone has been detected in pine trees and has been hypothesized to serve as the primary steroid precursor for the production of androgens responsible for masculinizing the mosquitofish in the Fenholloway River. We therefore investigated the effects of progesterone exposure on the secondary sex characteristics of female western mosquitofish, *Gambusia affinis*. In this study, adult and immature female mosquitofish were exposed to 100, 200 and 500 nM progesterone concentrations via the water by the static and static renewal methods (renewed every 7 days) for eight weeks respectively. The objective was to allow microbial transformation of the progesterone to take place potentially resulting in the formation of bioactive androgens. A visible biomarker of masculinization in female mosquitofish is the elongation and modification of the anal fin into a gonopodium-like structure, which is normally a male-limited secondary sex characteristic. Morphological masculinization of females was not observed in any of the progesterone static treatment groups. In contrast, static renewal treatment resulted in masculinization in the 200 and 500 nM progesterone treatment groups. These results suggest that *in-vivo* exposure to progesterone via the static renewal method in immature mosquitofish, *Gambusia affinis* is sufficient to induce masculinization.

INTRODUCTION

It has become evident in the last two decades that compounds of anthropogenic origin have the ability to act as endocrine disruptors. The effects of these compounds have been detected in both animals and human beings (Colborn 2004; Guillette and Moore 2006; Kavlock et al. 1996; Sumpter and Johnson 2008). Estrogens are among the most widely studied endocrine disruptors. However, reports of environmental androgens continue to increase. The first evidence of endocrine disruption due to androgens was reported by Howell *et al.,* (1980) when masculinized female mosquitofish, *Gambusia holbrooki,* were found in a river receiving paper mill effluent. Mosquitofish belong to the livebearer family Poeciliidae, and are sexually dimorphic. Male mosquitofish have an elongated and modified anal fin, called a gonopodium which is used to transfer sperm to the reproductive tract of the female during copulation. The development of the gonopodium in the males is androgen-dependent and begins after the onset of sexual maturity (Turner 1941). On the contrary, the anal fin of a female mosquitofish does not normally change shape at sexual maturity. However, the genes responsible for gonopodial development occur in both sexes. When exposed to an androgen, the anal fin in a female begins to develop into a gonopodium-like structure (Angus et al. 2001; Turner 1942). Masculinization of the female mosquitofish anal fin has been used as a morphological biomarker of androgen exposure in both field and laboratory studies (Howell *et al.,*1980; Stanko and Angus 2007). Masculinization, as indicated by other biomarkers, has also

been reported in other fish exposed to effluent from paper mills. For example, male-biased sex ratios were seen in populations of eelpout (*Zoarces viviparous*) from the vicinity of a Kraft pulp mill near the Baltic coast in Sweden (Larsson and Forlin 2002). This finding was validated when laboratory exposure of zebrafish (*Danio rerio*) to the paper mill effluent caused male biased sex ratios (Orn et al. 2006). Other physiological effects of paper mill effluent on aquatic organisms include altered social behavior and sexual characteristics (Toft et al. 2004), altered sex steroid levels and oxidative stress (Oakes et al. 2005), decreased gill ATPase activity (Parvez et al. 2006), decreased serum vitellogenin (Fentress et al. 2006), reduced gonadal size, delayed sexual maturity, reduced secondary sexual characteristics, and reduced circulating steroid levels (Van der Kraak et al. 1992).

The demonstrated capacity of paper mill effluent to masculinize females as well as to cause other physiological alterations stimulated an interest to discover the causative agents present in the effluent. A study utilizing solid-phase extraction followed by reverse-phase high-performance-liquid chromatography separation and using the fractions in cell-based androgen receptor activity assays demonstrated that some components of Fenholloway River water were androgenic (Parks et al. 2001). However, the androgens were not initially identified. In a subsequent study, Jenkins *et al.,* (2001) confirmed the presence of androstenedione in Fenholloway River water. Durhan *et al.,* (2002) confirmed this result. Jenkins *et al.,* 2003 reported the presence of progesterone and androstenedione in Fenholloway River sediment. In the same study they also detected the presence of progesterone in the river water column (6.5 nM), river sediment (155 nM) and

also from the water column of a reference site (0.3 nM), which does not receive paper mill effluent.

Progesterone has also been reported in a primary paper mill effluent in Sweden (Larsson DGJ 2000). It was earlier believed that the source of the progesterone in a river dominated by paper mill effluent was the degradation of phytosterols by bacteria present in the paper mill settling ponds and/or water and sediment of the receiving river. However, recently Carson *et al.,* (2008) have shown that progesterone occurs naturally in the pine wood pulp utilized in the paper manufacturing process. They hypothesized that progesterone may serve as the predominant steroid precursor for the production of environmental androgens by microbial transformation or as a precursor for endogenous androgen production by fish (Carson et al. 2008). In this study, we have examined the effect of progesterone exposure on the secondary sex characteristic of female mosquitofish. For this study, adult and immature female mosquitofish were exposed to varying concentrations of progesterone by the static and static renewal method for a period of eight weeks. The extent of masculinization was determined by measuring the lengths of anal fin rays that elongate during masculinization.

MATERIALS AND METHODS

Animals

Two experiments were conducted. The animals used in these two experiments were adult and immature female mosquitofish *Gambusia affinis.* These fish were collected from Thomas Spring Pond, a spring-fed pond in Bessemer, Alabama, USA. The

adult and immature female fish were segregated after collection. An immature mosquitofish develops 10 segments on the third anal fin ray at about 50 days of age and they can be sexed at that time by an experienced observer with almost no error (Turner, 1941). In immature females, the rays are all about the same thickness and many basal segments have ankylosed. In contrast, in males, rays 3 and 4 are much thicker than the others and ankylosis of basal segments is much reduced compared to females. After collection these fish were allowed to acclimate in the laboratory for two weeks before the experiment. The acclimated female fish were then randomly distributed into the treatment groups. There were five animals per group including the vehicle control group which was exposed to ethanol and propylene glycol equivalent to the highest progesterone concentration.

Progesterone treatment – static and static renewal exposure

Fish were exposed to progesterone for eight weeks via water by the static method in one experiment and by the static renewal method in the other experiment. In both experiments, the fish were kept individually in unaerated 1L beakers containing dechlorinated tap water with 0.25 parts per thousand Instant Ocean Sea Salt (Marineland Aquarium Products, Moorpark, California, USA) added to provide trace minerals and buffering capacity. Water temperature was $24 \pm 2\ ^{o}C$. Fish were maintained under a 14:10 h light: dark cycle. In the static renewal experiment, the water was changed and progesterone added every seven days. In the static experiment, water was not changed and progesterone was not re-added for the duration of the eight week experiment.

For both experiments, a 10^6 nM stock solution of progesterone (Sigma Chemicals, Saint Louis, USA) was made in ethanol (31.45 ml). Final working dilutions of 100, 200, and 500 nM were prepared in 1 L beakers by diluting the stock solution with propylene glycol (68.55 ml) which was used as a carrier. These concentrations were selected on the basis of a range-finding study to determine toxicity. A separate 10^6 nM stock solution of trenbolone acetate (Steraloids, Inc., Newport, RI, USA) was made by dissolving trenbolone acetate in ethanol and propylene glycol. A concentration of 50 nM was used as a positive control. This concentration of trenbolone acetate was selected on the basis of an independent range-finding study carried out in our laboratory. Progesterone and trenbolone acetate were added only on day zero of the exposure and water was not replaced throughout the eight weeks in the static exposure method. In the static renewal experiment, progesterone and trenbolone acetate were added after every seven days and water was replaced throughout the eight weeks of exposure. Fish were fed once a day with Silver Cup granulated trout starter food (Nelson & Sons, Inc., Murray, UT, USA). Food was stored at 4 ^{o}C.

Anal fin measurements

On day zero before the start of the exposure, fish were anesthetized by immersion in MS-222 (300 mg/L tricaine methanesulfonate, Argent Labs, Redmond, WA, USA) and anal fins were photographed. The anal fins were photographed with a Polaroid DMC Ie digital camera (Polaroid, Waltham, MA, USA) mounted on a Leica MZ6 stereomicroscope (Leica Microsystems Inc., Bannockburn, IL, USA). Images captured by the camera

were transported to a computer running Adobe Photoshop (Adobe Systems, San Jose, CA, USA). Image Tool Software (University of Texas Health Science Center, San Antonio, TX, USA) was used to measure the fin ray lengths in pixels and ratios were rounded to the nearest 0.01 (Stanko and Angus 2007). Anal fin ray elongation was quantified as the ratio of the length of ray 4, which elongates during gonopodial development, to that of ray 6, which does not elongate. After the completion of the eight weeks of exposure, anal fin ray pictures were taken again.

Statistical Methods

All summary statistics are expressed as mean + standard error. The significance level for means comparisons was set at $p < 0.05$. Ratios are not normally distributed but can be rendered approximately so by an appropriate transformation. Anal fin 4:6 length ratios were log transformed based on the findings of (Angus et al. 2001). Since time 0 data were available for each fish, the average (end-start) difference in the transformed 4:6 ratio was compared to 0 using a paired t-test.

RESULTS

Figure 1 shows the week 0 and week 8 mean anal fin ray 4:6 ratios for female mosquitofish exposed to progesterone by the static exposure method. None of the progesterone-exposed fish in this treatment exhibited significant anal fin elongation at the end of eight-week exposure period. However, there was significant anal fin elongation in the TA treated fish. Figure 2 shows the week 0 and week 8 mean anal fin ray 4:6 ratios for

fish exposed to progesterone by the static renewal exposure method. There was a significant anal fin elongation in the 200 and 500 nM progesterone treatment groups at the end of eight-week exposure period. Figure 3 (a) and (b) shows a representative masculinized anal fin from the 500 nM and 200 nM static renewal progesterone treatment groups and (c) shows a representative modified anal fin from the trenbolone acetate treatment group.

DISCUSSION

The present study shows that *in vivo* static renewal exposure to progesterone results in the masculinization of immature female mosquitofish, *Gambusia affinis*. There was a significant increase in the 4:6 anal fin elongation ratios in the 200 and 500 nM PGT treatment groups. On the other hand, static exposure treatment did not induce any signs of masculinization in the progesterone treatment groups.

Commonly found phytosteroids in paper mill effluent include, β-sitosterol (72%), stigmastanol (11%) and campesterol (8%) (Conner 1976). It was initially hypothesized that these phytosteroids undergo microbial transformation into progesterone which is further converted into androgens capable of masculinizing female mosquitofish. The recent discovery of progesterone in pine trees has led to a new hypothesis that progesterone from the wood pulp may also serve as a steroid precursor for the production of environmental and/or endogenous androgens (Carson et al. 2008). Anal fin masculinization as observed in the 200 and 500 nM progesterone treatment groups in the current study is consistent with this hypothesis.

Several genera of microorganisms such as *Arthrobacter*, *Nocardia*, *Protaminobacter*, *Serratia*, *Streptomyces*, *Mycobacterium* and *Microbacterium* produce enzymes that can convert cholesterol and other C-17 sterols into androstenedione (AED) and androstadienedione (ADD) (Nagasawa M 1969). Similarly, several fungi in the genus *Basidiomycetes* are also known to transform progesterone (Schuytema E 1963) . By using an *in-vitro* model, Jenkins *et al.,* have shown the ability of *Mycobacterium smegmatis* to biotransform progesterone into androgens such as AED and ADD (Jenkins et al. 2004). In a previous study, the same group isolated ADD from the water column (0.14 nM) and sediment (2.4 nM) of a river receiving the paper mill effluent (Jenkins et al. 2003). ADD has been isolated as a principal product formed by the microbial transformation of cholesterol and phytosteroids and also from tall (pine) oil sterols (Marsheck WJ 1972; Roy et al. 1991). Therefore, it is possible that, in the present static renewal exposure study, progesterone was transformed by some naturally occurring bacteria present in the environment into an androgenic compound such as ADD capable of masculinizing female mosquitofish. Further, ADD at a concentration of 50 and 500 nM has been shown to induce masculinization in female mosquitofish after a period of six weeks (Stanko 2005).In this static renewal study, water and progesterone were exchanged every seven days. We believe that this exposure time interval was sufficient for the microbial breakdown of progesterone into androgens capable of masculinizing the fish. However, in the 200 and 500 nM progesterone treatment groups we did not observe the later phase of differentiation: formation of hooks, spines and serrae. These findings suggest that the concentration of androgens formed was sufficient to initiate an earlier phase of elongation and a pro-

longed exposure is required for the onset of the later phase of differentiation. These results are consistent with Turner's hypothesis that small quantities of androgens from developing testis are required to induce an early phase of elongation and subsequent higher concentrations are required to induce the later phase of differentiation (Turner 1942). There is a need for future studies to isolate the bacteria involved in the breakdown of progesterone along with their transformed androgenic products. On the other hand, in our static exposure study, we did not observe any signs of masculinization. We believe that since progesterone was added once, the concentration of androgens generated due to the microbial breakdown probably was not sufficient to induce masculinization.

Alternatively, it is also possible that native bacterial flora in the gut of fish could have transformed the progesterone into ADD resulting in masculinization. ADD has been reported from the feces of the cattle who were given a subcutaneous injection of progesterone, suggesting the involvement of native microbial flora in the breakdown of progesterone (Miller et al. 1956). A third possibility is that the progesterone could have been utilized as a precursor for the synthesis of endogenous androgens by the fish, ultimately leading to masculinization. Further, based on our preliminary findings, we also found that masculinization was more effectively induced in an immature female fish than adults. Perhaps immature fish more readily utilize progesterone as a precursor to synthesize androgens leading to masculinization as observed in the present study. It would be also interesting to investigate whether there is any effect of progesterone exposure on fecundity when the masculinized fish reach maturity.

In conclusion, results from the present study show that static renewal progesterone exposure induces masculinization response in immature female mosquitofish, *Gambusia affinis*. Also, these results are consistent with the hypothesis that progesterone from the pine trees acts as a predominant steroid precursor for the production of environmental androgens by microbial transformation or as a precursor for endogenous androgen production by fish.

REFERENCES

Angus RA, McNatt HB, Howell WM, Peoples SD. 2001. Gonopodium development in normal male and 11-ketotestosterone-treated female mosquitofish (*Gambusia affinis*): a quantitative study using computer image analysis. Gen Comp Endocrinol 123(2):222-34.

Carson J, Jenkins R, Wilson E, Howell W, Moore R. 2008. Naturally Occurring Progesterone in Loblolly Pine: a Major Steroid Precursor of Environmental Androgens. Environ Toxicol Chem:1.

Colborn T. 2004. Neurodevelopment and endocrine disruption. Environ Health Perspect 112(9):944-9.

Conner A, Nagaoka, M.,Rowe, J.W., and Perlman, D. 1976. Microbial conversion of tall oil sterols into C_{19} steroids. Appl.Environ Microbiol. 32:310-311.

Durhan EJ, Lambright C, Wilson V, Butterworth BC, Kuehl OW, Orlando EF, Guillette LJ, Jr., Gray LE, Ankley GT. 2002. Evaluation of androstenedione as an androgenic component of river water downstream of a pulp and paper mill effluent. Environ Toxicol Chem 21(9):1973-6.

Fentress JA, Steele SL, Bart HL, Jr., Cheek AO. 2006. Reproductive disruption in wild longear sunfish (*Lepomis megalotis*) exposed to kraft mill effluent. Environ Health Perspect 114(1):40-5.

Guillette LJ, Jr., Moore BC. 2006. Environmental contaminants, fertility, and multioocytic follicles: a lesson from wildlife? Semin Reprod Med 24(3):134-41.

Howell BD, Bortone SA 1980. Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia affinis holbrooki*: evidence for environmentally-induced masqulinization. Copeia:676-681.

Jenkins RL, Wilson EM, Angus RA, Howell WM, Kirk M. 2003. Androstenedione and progesterone in the sediment of a river receiving paper mill effluent. Toxicol Sci 73(1):53-9.

Jenkins RL, Wilson EM, Angus RA, Howell WM, Kirk M, Moore R, Nance M, Brown A. 2004. Production of androgens by microbial transformation of progesterone in vitro: a model for androgen production in rivers. Environ Health Perspect 112(15):1508-11.

Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, Lucier G, Luster M, Mac MJ, Maczka C and others. 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ Health Perspect 104 Suppl 4:715-40.

Larsson DG, Forlin L. 2002. Male-biased sex ratios of fish embryos near a pulp mill: temporary recovery after a short-term shutdown. Environ Health Perspect 110(8):739-42.

Larsson DGJ HH, Förlin L. 2000. More male fish embryos near a pulp mill. Environ Toxicol Chem 19:2911–2917.

Marsheck WJ KS, Muir RD. 1972. Microbial degradation of sterols. Appl Microbiol 23:72-77.

Miller WR, Turner CW, Fukushima DK, Salamon, II. 1956. The identification of C19 steroids in bovine feces. J Biol Chem 220(1):221-5.

Nagasawa M BM, Tamura G, Arima K. 1969. Microbial transformation of sterols : part III. Cleavage of sterol side chains by microrganims. Agric.Biol Chem 33:1644-1650.

Oakes KD, Tremblay LA, van der Kraak GJ. 2005. Short-term lab exposures of immature rainbow trout (*Oncorhynchus mykiss*) to sulfite and kraft pulp-mill effluents: effects on oxidative stress and circulating sex steroids. Environ Toxicol Chem 24(6):1451-61.

Orn S, Svenson A, Viktor T, Holbech H, Norrgren L. 2006. Male-biased sex ratios and vitellogenin induction in zebrafish exposed to effluent water from a Swedish pulp mill. Arch Environ Contam Toxicol 51(3):445-51.

Parks LG, Lambright CS, Orlando EF, Guillette LJ, Jr., Ankley GT, Gray LE, Jr. 2001. Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. Toxicol Sci 62(2):257-67.

Parvez S, Sayeed I, Raisuddin S. 2006. Decreased gill ATPase activities in the freshwater fish *Channa punctata* (Bloch) exposed to a diluted paper mill effluent. Ecotoxicol Environ Saf 65(1):62-6.

Roy PK, Khan AW, Basu SK. 1991. Transformation of sitosterol to androsta-1, 4-diene-3, 17-dione by immobilized Mycobacterium cells. Indian J Biochem Biophys 28(2):150-4.

Schuytema E HM, Sieher D, Merits I, Schenck J, Smith M, Varner E. 1963. Tranformations of progesterone by Basidiomycetes. Appl Microbiol:256-259.

Stanko JP. 2005. Reproductive and developmental effects of bioactive constituents of pulp mill effluent on female mosquitofish, *Gambusia affinis*. Birmingham: University of Alabama at Birmingham.

Stanko JP, Angus RA. 2007. In vivo assessment of the capacity of androstenedione to masculinize female mosquitofish (*Gambusia affinis*) exposed through dietary and static renewal methods. Environ Toxicol Chem 26(5):920-6.

Sumpter JP, Johnson AC. 2008. 10th Anniversary Perspective: Reflections on endocrine disruption in the aquatic environment: from known knowns to unknown unknowns (and many things in between). J Environ Monit 10(12):1476-85.

Toft G, Baatrup E, Guillette LJ, Jr. 2004. Altered social behavior and sexual characteristics in mosquitofish (*Gambusia holbrooki*) living downstream of a paper mill. Aquat Toxicol 70(3):213-22.

Turner CL. 1941. Morphogenesis of the gonopodium in *Gambusia affinis affinis*. J.Morphol 69:161-185.

Turner CL. 1942. A quantitative study of the effects of different concentrations of ethynl-testosterone and methyl testosterone in the production of gonopodia in females of *Gambusia affinis*. Physiol Zool 15:263-234.

Van der Kraak GJ, Munkittrick KR, McMaster ME, Portt CB, Chang JP. 1992. Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. Toxicol Appl Pharmacol 115(2):224-33.

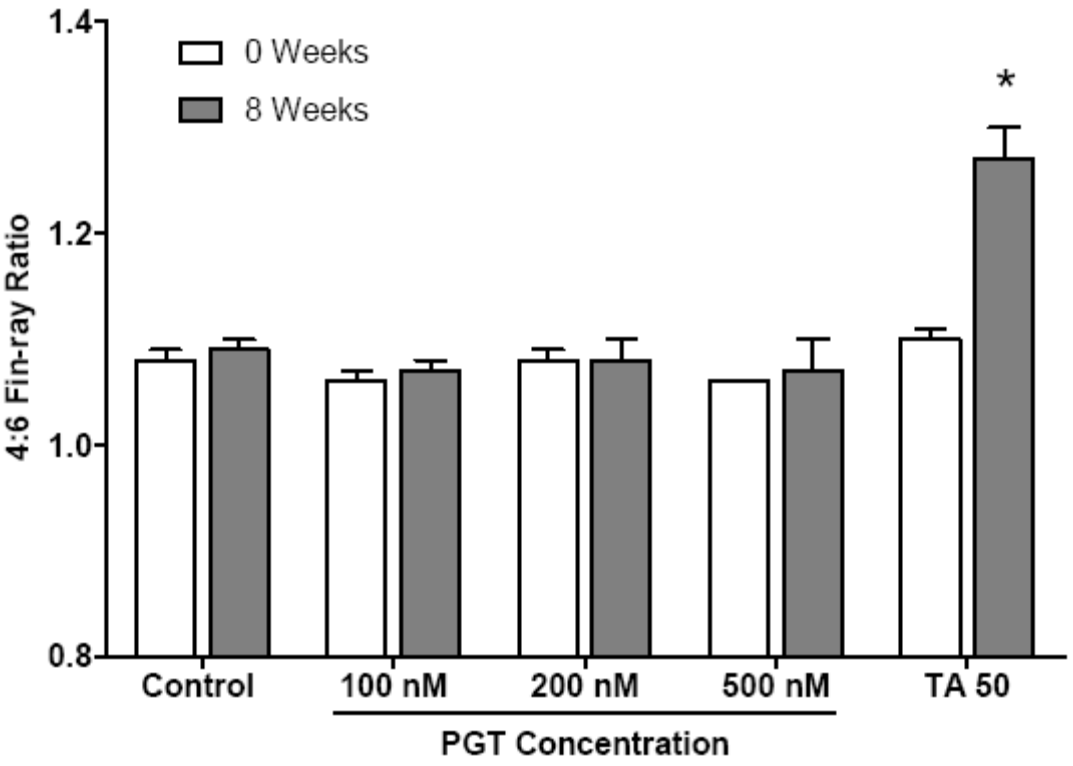

Fig. 1. The 4:6 anal fin ray elongation ratios of static exposure progesterone treatment groups. Female mosquitofish were exposed to varying concentrations of progesterone (PGT) for eight weeks via static exposure method and masculinization was assessed by the elongation of anal fin ray, measured as 4:6 ratio. Data are presented as mean + S.E. (n = 5 per group). An asterisk (*), indicates the mean at eight weeks differs significantly ($p < 0.05$) from the mean at 0 weeks (paired t-test).

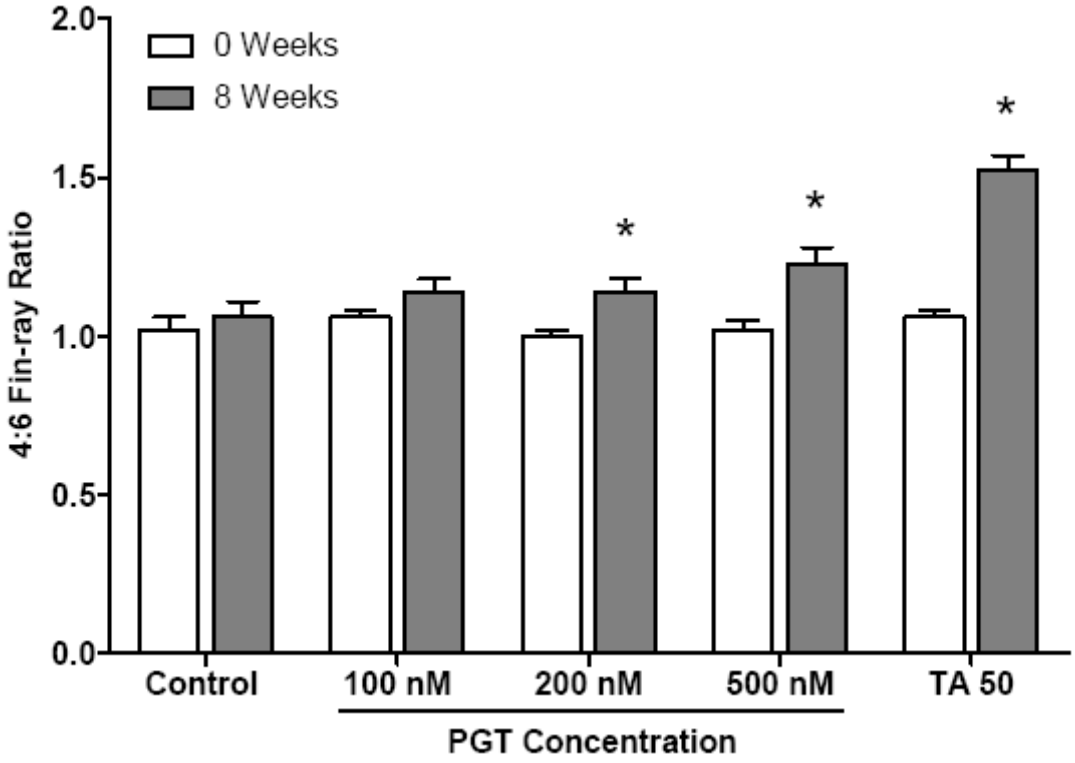

Fig. 2. The 4:6 anal fin ray elongation ratios of static renewal exposure progesterone treatment groups: Female mosquitofish were exposed to varying concentrations of progesterone (PGT) for eight weeks via static renewal method and masculinization was assessed by the elongation of anal fin ray, measured as 4:6 ratio. Data are presented as mean + S.E. (n = 5 per group). An asterisk (*), indicates the mean at eight weeks differs significantly ($p < 0.05$) from the mean at 0 weeks (paired t-test).

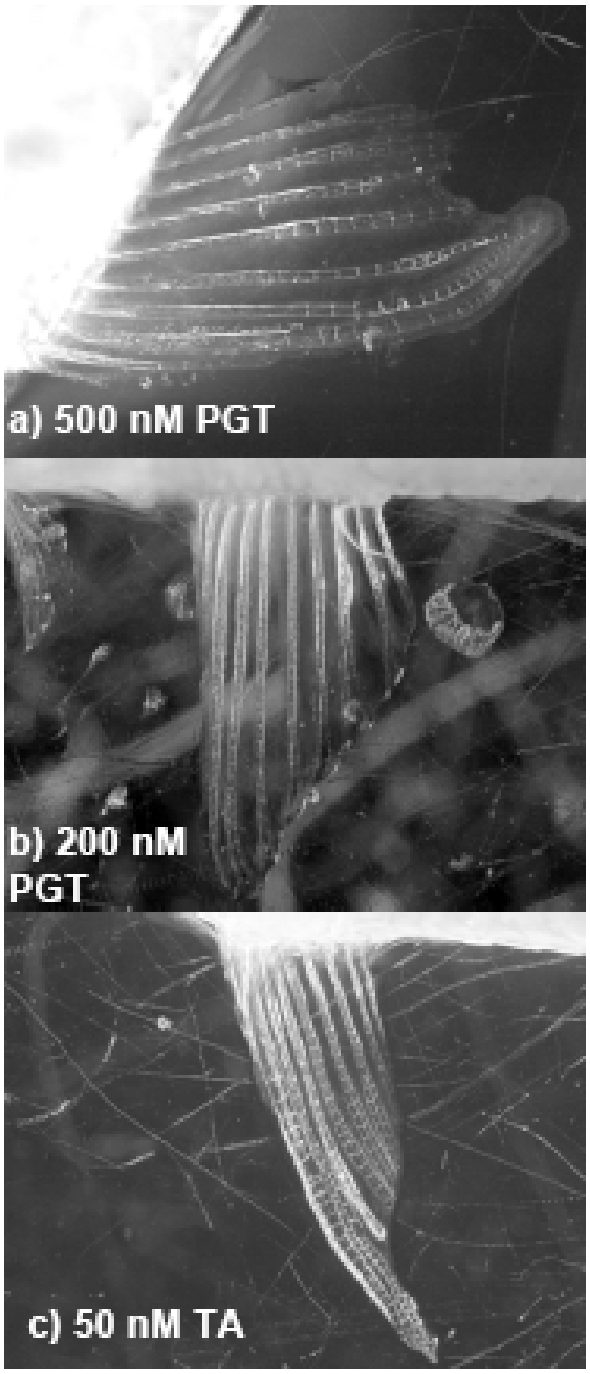

Fig. 3. *Gambusia affinis* anal fins: Anal fins of representative female mosquitofish exposed for eight weeks to (a) 500 nM progesterone (PGT) (b) 200M PGT and (c) 50 nM TA (Trenbolone acetate) by the static renewal method.

EFFECTS OF SPIRONOLACTONE ON SECONDARY SEX CHARACTERISTICS AND VITELLOGENIN PRODUCTION IN FEMALE WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS*

by

SAMIKSHA A. RAUT, W. MIKE HOWELL AND ROBERT A. ANGUS

In preparation for submission to *Comparative Biochemistry and Physiology*

Format adapted for dissertation

ABSTRACT

Numerous studies, both in the field and laboratory, have demonstrated that a variety of anthropogenic compounds can act as endocrine disruptors. Recently, discovery of pharmaceuticals in wastewater treatment plant effluent is raising a new environmental concern. Hence, there is a need to investigate the impacts of these compounds on aquatic organisms. Additionally, for accurate risk assessment it is necessary to determine whether these pharmaceuticals have similar effects in humans. Spironolactone (SPL) is an aldosterone antagonist intended for human use, generally as a potassium-sparing diuretic. It also exhibits anti-androgenic effects in humans. In this study, we examined the effects of SPL on secondary sex traits and vitellogenin gene expression in female western mosquitofish, *Gambusia affinis*. Fish were exposed for five weeks to SPL at four concentrations: 10, 100, 250, and 500 nM, via water, using the static renewal method. Morphological masculinization of females, as evidenced by development of an elongated and modified (with serrae and hooks at the tip) anal fin was observed in the three highest concentrations. Significant elongation of anal fins was observed in the lowest exposure group, but without the development of serrae and hooks. A dose-dependent inhibition of the estrogen-regulated vitellogenin gene expression was observed in the SPL-treated fish. These results thus demonstrate that SPL has androgenic and/or anti-estrogenic activity in fish, in contrast to the anti-androgenic effects seen in humans.

INTRODUCTION

Some chemicals introduced into the aquatic environment by human activities have the capacity to act as endocrine disruptors in both humans and wildlife (Colborn et al. 1993; Tyler et al. 1998; Colborn 2004; Guillette et al. 2006). Recently, pharmaceuticals are being recognized as an important class of emerging contaminants. They are used in human and veterinary medicine and end up in the wastewater stream via excretion or by being discarded into the wastewater stream. These drugs are designed to have specific biological activities at low concentrations in living organisms. After ingestion by a patient, they are excreted, usually as conjugated or unconjugated metabolites. Even if they are excreted as inactive conjugated metabolites; they may be transformed back into their parent compound by the activity of microbes in a wastewater treatment plant. Some of the commonly recognized sources of environmental pharmaceuticals include effluents from public wastewater treatment plants (Watkinson et al. 2009), hospitals (Holm et al. 1995; Brown et al. 2006) landfill leachates (Holm et al. 1995), and drug manufacturing facilities (Larsson et al. 2007). Pharmaceuticals have been measured in wastewater effluent from pharmaceutical manufacturing plants in concentrations ranging from less than 1 ng/L to as high as 31,000 μg/L (Larsson et al. 2007). Unused or expired drugs frequently enter the wastewater stream by being disposed down the drain. These may end up in receiving waters if they are not completely eliminated during the sewage treatment process.

Pharmaceuticals that have been detected from the aquatic environment include a wide range of drugs such as anti-inflammatory, beta-blockers, sympathomimetics, anti-

epileptics, lipid regulators, and antibiotics (Fent et al. 2006). Despite the ubiquity of these compounds in our surface waters, there exists a serious gap in our knowledge of the effects they are exerting on wildlife. Among the few studies done to date, effects include the feminization of male fish exposed to effluent from sewage treatment plants due to the presence of 17α-ethinylestradiol, a synthetic estrogen widely used as a human contraceptive (Desbrow et al. 1998). The rapid decline of vulture populations in India and Pakistan has been attributed to the consumption of carcasses of livestock treated with the non-steroidal anti-inflammatory drug, diclofenac (Shultz et al. 2004; Green et al. 2007; Taggart et al. 2007). Laboratory studies have investigated the effects of drugs such as fluoxetine (Brooks et al. 2003), clofibric acid (Nunes et al. 2004; Runnalls et al. 2007), and ibuprofen (Gravel et al. 2007) on aquatic organisms. However, the endocrine disrupting potential of many drugs has not been determined in aquatic organisms such as fish. It is therefore, important to investigate the effects of these drugs on fish for better overall environmental risk assessment. Data generated from such studies would also contribute information on whether these drugs elicit similar effects in lower organisms as those observed in humans.

Spironolactone (SPL) is a synthetic 17-lactone drug commonly used as a diuretic in the management of hypertension and hyperaldosteronism in humans. It acts as an aldosterone antagonist and also exhibits anti-androgenic effects. Due to its anti-androgenic properties, it is used to treat hirsutism, acne and hair loss in women with the male pattern baldness gene. In contrast to its effects in humans, it has been reported that female western mosquitofish (*Gambusia affinis*) become masculinized when exposed to SPL (Howell

et al. 1994). However, the endocrine disrupting capacity of this compound has not been investigated in detail. The objective of the present study was to characterize the effects of SPL exposure on two biomarkers of endocrine disruption, anal fin masculinization and vitellogenin (VTG) inhibition in the western female mosquitofish, *Gambusia affinis.*

G. affinis is a member of the livebearing family Poecillidae and is native to the southeastern United States. It is a useful biomonitor species for endocrine disrupting compounds in aquatic environments because of its widespread occurrence around the world, large environmental tolerances, and ease of use in the laboratory. A mature male mosquitofish possesses an elongated and modified anal fin, called a gonopodium, which is used to transfer sperm to the female during copulation. Development of the gonopodium is androgen dependent and normally occurs during sexual maturation in males. However, when a female is exposed to androgens, the anal fin becomes modified into a gonopodium-like structure (Turner 1942; Angus et al. 2001). Because of its sensitivity to androgens and visibility, the anal-fin masculinization of female mosquitofish is regarded as a useful morphological biomarker of androgen exposure. Additionally, previous studies carried out in our laboratory have shown that inhibition of VTG gene expression is another biomarker of androgen exposure in female mosquitofish (Stanko et al. 2007). In this study, female mosquitofish were exposed to various concentrations of SPL and the effects on anal fin masculinization and VTG gene expression were investigated.

MATERIALS AND METHODS

Animals

Mature female western mosquitofish, *Gambusia affinis* were collected from Thomas Spring, a spring-fed pond in Bessemer, Alabama. After collection, these fish were returned to the University of Alabama at Birmingham and allowed to acclimate in the laboratory for two weeks. The acclimated fish were then randomly distributed among the treatments (n =10 per treatment).

Spironolactone treatment – static renewal exposure

Fish were exposed to SPL via water by the static renewal method. The fish were kept individually in 1 L unaerated model 14005 Kimax beakers (Kimble Glass, Inc. Vineland, NJ, USA), which hold 1 L with an additional 2.5 cm air space. The water was dechlorinated tap water with 0.25 ppt Instant Ocean Sea Salt (Spectrum Brands, Inc., Atlanta, GA, USA) added to provide trace minerals and buffering capacity. Water temperature was 24 $\pm$ 2 ^{o}C. Fish were maintained under a 14:10 light: dark cycle. The water was changed every other day and SPL re-added. Water chemistry parameters including pH, dissolved oxygen, ammonia and nitrate were monitored once a week and maintained at appropriate levels.

A 10^{6} nM stock solution was made by dissolving SPL (Sigma chemicals, Saint Louis, USA) in 5 ml of dehydrated absolute ethanol (Pharmaco, Brookfield, CT, USA) and 95 ml propylene glycol (Fischer Chemicals, USA) to get a final volume of 100 mL. Final working concentrations of 10, 100, 250 and 500 nM were prepared by adding the

stock solution to 1 L of water in each beaker. These concentrations were selected on the basis of a preliminary toxicity range-finding study. A separate stock solution of 10^6 nM methyltestosterone (MT) (Steraloids, Newport, RI, USA) was made by dissolving MT in absolute ethanol and propylene glycol. A concentration of 3.32 nM was used as a positive control based on an independent study carried out in our laboratory. The solvent control group received ethanol and propylene glycol equivalent to that received by the highest concentration experimental group. Fish were fed twice a day with Silver Cup granulated trout starter food (Nelson & Sons, Inc., Murray, UT, USA). Food was stored at 4 ^{o}C.

Anal fin and physiological measurements

On day zero before the start of the exposure, fish were anesthetized by immersion in 300 mg/L MS-222 (tricaine methanesulfonate, Argent Labs, Redmond, WA, USA) and their standard length and weight was recorded. Standard length was measured from the tip of the snout to the end of the caudal peduncle and recorded to the nearest 0.2mm. Fish were pat dried on a tissue paper and weighed on a digital scale to the nearest 0.1 mg. The anal fins were photographed with a Polaroid DMC Ie digital camera (Polaroid, Waltham, MA, USA) mounted on a Leica MZ6 stereomicroscope (Leica Microsystems Inc., Bannockburn, IL, USA). Images captured by the camera were transferred to a computer running Adobe Photoshop (Adobe Systems, San Jose, CA, USA) and saved as JPEG files. Anal fin ray lengths were measured in pixels using Image Tool software (University of Texas Health Science Center, San Antonio, TX, USA). At the magnification used to photograph most of the fins, each pixel represented 0.003 mm. Anal fin rays were measured

at one-week intervals for five weeks. Anal fin ray elongation was quantified as the ratio of length of ray 4, which elongates during gonopodial development, to that of ray 6, which does not elongate (Angus et al. 2001). After the completion of the five week exposure period, standard length and the mass of the fish were again measured.

Hepatic Vitellogenin: Quantitative Real Time Polymerase Chain Reaction (q- RTPCR)

Total RNA was extracted from frozen liver tissue using the Trizol method protocol as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The cDNA was amplified in an Opticon Continous Fluorescent Detector (MJ Research, Waltham, MA) using IQTM SYBR Green supermix (Bio-Rad Laboratories). The primers were designed specifically for VTG using the mRNA sequence from GenBank (Accession number DQ190844) and using Primer3 software (http://frodo.wi.mit.edu/). The primers for the 18S reference housekeeping gene were designed based on *Oncorhynchus mykiss* mRNA sequence (Genbank Accession Number: AF243428) because the 18S mRNA sequence for *Gambusia affinis* has not been determined (Leusch et al. 2005). The sequences of the primers are shown in Table 1.

Primer	Sequence
Forward VTG	5' - ACCAGGGACCTGAACAACTG - 3'
Reverse VTG	5' - GATGGCATTAGCGACTGGTT - 3'
Forward 18S	5' - CCTGCGGCTTAATTTGACTC - 3'
Reverse 18S	5' - AACTAAGAACGGCCATGCAC - 3'

Table 1. Primer sequences used for RT-PCR of a *Gambusia affinis* vitellogenin gene transcript.

Real-time PCR was performed in triplicate for each liver sample, averaged, and normalized to endogenous 18S RNA reference transcripts. At least five fish per treatment group were analyzed for VTG gene expression. Quantification of the hepatic VTG mRNA expression was carried out by using the $2^{-\Delta\Delta CT}$ method (Livak et al. 2001).

Statistical Methods

All the summary statistics are expressed as mean $\pm$ standard error. The cutoff for statistical significance was $p < 0.05$. Ratios are not normally distributed but can be rendered approximately so by an appropriate transformation. Anal fin 4:6 length ratios were log transformed based on the findings of a previous study that transformation successfully normalizes the data (Angus et al. 2001). Since time 0 data were available for each fish, the average (end-start) difference in the transformed 4:6 ratio was compared to 0 using a paired t-test. VTG mRNA expression levels were compared between treatment groups and the control using the nonparametric Kruskal-Wallis test, followed by Dunn's test

(Dunn 1964). All the other physiological variables were analyzed using ANOVA followed by Dunnett's post hoc test to compare the treatment group means to that of the control.

RESULTS

Fin Ray Elongation

The 4:6 anal fin ray elongation ratio of the female mosquitofish exhibited a dose-dependent response to SPL exposure. Figure 1 shows anal fin ray 4:6 ratios for fish exposed to SPL by the static renewal method over a period of five weeks. Fish exposed to all the SPL treatments showed significant elongation at the end of the five-week exposure period. Figure 2 shows the 4:6 anal fin ray elongation ratios of SPL-exposed female mosquitofish at weekly intervals over the five week exposure period. After two weeks, the anal fins of the fish in the 100 nM and higher concentrations of SPL and the MT group had ceased elongation. Anal fins in the 10 nM SPL treatment group appear not to have completed elongation at 5 weeks exposure. Figure 3 shows elongated anal fins from representative females exposed to different concentrations of SPL.

Hepatic Vitellogenin Expression

The fin-ray elongation ratios in the female mosquitofish exposed to SPL suggested the masculinization of the females. Therefore, we decided to investigate the effects of SPL on the expression of VTG mRNA, a gene that is induced by estrogen and normally expressed only in females. To do so, we collected livers from female mosquitofish exposed to SPL for five weeks, isolated the mRNA, and determined VTG expression rela-

tive to 18S ribosomal RNA using real time PCR. The assay and the amplification efficiency of both the primer sets were determined previously (See triclosan experiment, Fig.2). The amplification efficiency was always greater than 90% for both the primer sets. Tests of homogeneity of slope indicated that the linear regression lines of primer sets for the target and reference genes were parallel to each other, suggesting that their amplification efficiencies are approximately equal. We have also verified primer specificity by performing melting curve analysis after PCR amplification. This showed a single peak, indicating the specificity of PCR amplification (data not shown). Moreover, the variability among C_t values for 18S from all samples was negligible, supporting the rationale for the use of 18S as the reference gene.

The hepatic VTG mRNA expression was significantly decreased, as compared to the solvent control, in fish exposed to SPL at 100nM and greater (Fig. 4). The VTG mRNA expression was almost non-detectable at 250 nM and 500 nM concentrations.

Morphological Variables

The mean changes in the mass of the treatment groups over a period of five week are shown in Fig. 5. The mean mass of the fish in the 250 and 500 nM SPL treatment group was decreased significantly as compared to the solvent control. The effect of the SPL treatment on the standard length is shown in Fig. 5. There were no significant differences in standard length in any of the treatment groups as compared to the solvent control Fig. 6.

DISCUSSION

In this study we have investigated the paradoxical masculinization of female mosquitofish when exposed to SPL. Results were consistent with those of an earlier study (Howell et al. 1994). In contrast to humans where SPL has anti-androgenic effects, SPL shows androgenic effects in mosquitofish. Anal fin masculinization, as evidenced by a significant anal fin ray elongation, was observed in all the treatment groups. SPL also shows apparent anti-estrogenic effects in mosquitofish. VTG mRNA expression, which is normally induced by estrogen, was inhibited, as determined by q-RTPCR, in the same treatment groups that showed anal fin masculinization. Furthermore, there was a significant decrease in the mass of the exposed fish over the five week exposure period in 250 and 500 nM SPL treatment groups as compared to the solvent control. There were no significant differences in standard length in any of the treatment groups as compared to the solvent control.

Previous studies have characterized the development of a gonopodium in mosquitofish in response to androgen exposure (Turner 1941; Turner 1942). Normal gonopodial development can be divided into an initial stage of growth and production of new bone segments followed by a stage of differentiation of the terminal tip apparatus such as hooks, spines and serrae. After the development of the tip apparatus begins, there is no further growth of the anal fin rays or addition of segments. In the present study, we observed anal fin elongation in all the SPL treatment groups (Fig 1). At all concentrations of SPL, except the lowest, development of the gonopodium proceeded to the formation of serrae and hooks (Fig 2). In contrast, at the lowest concentration of SPL (10 nM), elonga-

tion occurred, but less rapidly than the higher concentrations, and without any development of hooks and spines during the five week period of this study. This result suggests that the lowest exposure concentration was not sufficient to induce the genes necessary for development of the tip apparatus during the later phase of differentiation. This response is consistent with Turner's hypothesis that, as males begin to mature sexually; minute quantities of androgenic hormone are initially secreted by the developing testis. At a low concentration, the androgen induces expression of the genes for elongation of the anal rays and production of new segments. Higher concentrations of androgen, which would normally occur later in development, inhibit the gene responsible for elongation and induce other genes which produce the structures of the tip apparatus. This hypothesis is supported by observation that MT, a "potent" androgen, induces the tip apparatus rapidly, giving the gonopodium little time to elongate. Similarly, the higher concentration of SPL all induced a tip apparatus and stopped elongating well short of the extent seen in a normal gonopodium. However, in the lowest SPL concentration, elongation appeared to still be underway at the end of the five week exposure period and differentiation of the tip apparatus had not yet begun.

A recent study has shown that the androgen receptor (AR) isoforms, ARα and ARβ are predominantly expressed in the distal region of the outgrowing anal fin rays (Ogino et al. 2004). These receptors have been shown to regulate mosquitofish gonopodial development through the sonic hedgehog *(Shh)* pathway. Since the development of a gonopodium takes place in two distinct phases: elongation and differentiation, there are likely to be two or more signaling pathways responsible for these phases. We are not

aware of any studies that have explored these signaling pathways in mosquitofish. It is possible that there is a dose-dependent upregulation of AR expression via these signaling pathways at higher androgen concentrations. Since the lowest exposure concentration of SPL did not induce the later phase of differentiation, it is possible that this concentration was not sufficient to induce a robust response of ARs and, perhaps, would never have induced the next signaling pathway necessary for differentiation of the structures in the tip apparatus. Future studies are necessary to characterize the dose-dependent AR expression and its correlation with the signaling pathways in response to an androgen or androgen-like compounds in female mosquitofish.

A few studies in fish model systems have utilized spironolactone to investigate the effects on mineralocorticoid functions in Atlantic salmon (McCormick et al. 2008) and also on renal function in spiny dogfish shark (*Squalus acanthias*) (Churchill et al. 1985). However, none of the studies to date have reported androgenic effects in response to SPL treatment. Spironolactone therapy in humans has utilized its effect either as a diuretic (aldosterone antagonist) or as an anti-androgen for the treatment of hirsutism. Sexual side effects, such as gynecomastia in men (possibly an anti-androgenic effect) and menstrual disturbances in women have been reported (Corvol et al. 1976). Therefore, the masculinization results in mosquitofish obtained in a previous study by Howell *et al*. (1994) and the present study are surprising. It has been shown that 11β-substituted spirolactones are potent human AR agonists *in-vitro* (Nirde et al. 2001). It is possible that, whereas SPL binds to androgen receptors in humans and does not induce a response (anti-androgen), it binds to androgen receptors in fish and does induce a response. Further,

synthetic derivatives of SPL have been shown to inhibit the activity of 17β-hydroxylases (Tremblay et al. 1999), a family of steroidogenic enzymes which play a crucial role in the regulation of testosterone and estradiol. It is possible that SPL may be inhibiting the activity of 17β-hydroxylase, resulting in the formation of androstenedione which is a naturally occurring steroid in teleost steroid pathway (Fig 7). Androstenedione is a precursor to testosterone and is a precursor to 11-ketotestosterone, an androgen responsible for the development of secondary sex characteristics and estrogen, a primary hormone in female fish (Kime 2001). The inhibition of 17β-hydroxylase would terminate the further synthesis of steroid hormones as shown in the pathway, resulting in the formation of androstenedione. Androstenedione thus formed, would activate the AR to masculinize female mosquitofish. Previous studies have demonstrated the ability of androstenedione to activate AR (Jenkins et al. 2003) and also to masculinize female mosquitofish (Stanko et al. 2007). However, future studies are required to determine if SPL has the capacity to inhibit 17β-hydroxylase. Also, it would be of interest to explore the capacity of SPL to activate the AR in mosquitofish by using competitive binding assays.

VTG inhibition has been reported in many fish model systems on exposure to aromatizable androgens such as MT (Lazier et al. 1996; Sharpe et al. 2004; Korsgaard 2006) and non-aromatizable androgens such as trenbolone acetate (Ankley et al. 2003). On the other hand, VTG expression has been found to remain unaffected in female mummichog (*Fundulus heteroclitus*) when exposed to anti-androgens such as cyproterone acetate (Sharpe et al. 2004) or female medaka (*Oryzias latipes*) exposed to flutamide (Kang et al. 2006). In the present study, we noted a dose-dependent decrease in the hepatic VTG

mRNA expression in all SPL treatment groups. These results are similar to our earlier study on MT in which we have correlated dose-dependent masculinization and vitellogenin inhibition (S. Raut and R. Angus, unpublished data). There could be many different mechanisms responsible for the reduction in vitellogenin production. First, it is possible that concentrations of estradiol are sufficiently reduced due to an inhibitory effect exerted by SPL on 17β-hydroxylase as discussed earlier. Alternatively, the SPL could also act as an estrogen receptor antagonist. Thus, this anti-estrogenic response of SPL could have contributed to the VTG inhibition as noted in this study. The findings of the present study, suggest that SPL could possibly exert a dual mode of action; it could apparently be acting as an androgen agonist inducing masculinization and also as an anti-estrogen causing decreased VTG expression. Future studies are warranted to investigate the mechanism of SPL which is responsible for VTG inhibition and also masculinization.

We observed a significant decrease in mass, compared to the solvent control, of fish exposed to 250 and 500 nM SPL. Decrease in the fish mass can be due to loss of appetite which is a sign of toxicity induced stress response. There were no significant differences in any of the treatment groups in the standard length when compared to the solvent control.

In conclusion, the results of the present study indicate that spironolactone has the capacity to induce an androgen-dependent trait (gonopodium) and to inhibit an estrogen-dependent trait (vitellogenin gene expression). Further research should be aimed at investigating the molecular mechanisms responsible for these effects in fish, which are quite different than those observed in humans. Finally, based on these findings we suggest that

for better aquatic environmental risk assessment for pharmaceuticals, comprehensive studies on aquatic organisms should be implemented.

REFERENCES

Angus, R.A., McNatt, H.B., Howell, W.M., Peoples, S.D., 2001. Gonopodium development in normal male and 11-ketotestosterone-treated female mosquitofish (Gambusia affinis): a quantitative study using computer image analysis. Gen Comp Endocrinol 123, 222-234.

Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., Henry, T.R., Denny, J.S., Leino, R.L., Wilson, V.S., Cardon, M.C., Hartig, P.C., Gray, L.E., 2003. Effects of the androgenic growth promoter 17-beta-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environ Toxicol Chem 22, 1350-1360.

Brooks, B.W., Foran, C.M., Richards, S.M., Weston, J., Turner, P.K., Stanley, J.K., Solomon, K.R., Slattery, M., La Point, T.W., 2003. Aquatic ecotoxicology of fluoxetine. Toxicol Lett 142, 169-183.

Brown, K.D., Kulis, J., Thomson, B., Chapman, T.H., Mawhinney, D.B., 2006. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. Sci Total Environ 366, 772-783.

Churchill, P.C., Malvin, R.L., Churchill, M.C., 1985. Lack of renal effects of DOCA, ACTH, spironolactone, and angiotensin II in *Squalus acanthias*. J Exp Zool 234, 17-22.

Colborn, T., 2004. Neurodevelopment and endocrine disruption. Environ Health Perspect 112, 944-949.

Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101, 378-384.

Corvol, P., Mahoudeau, J.A., Valcke, J.C., Menard, J., Bricaire, H., 1976. [Sexual side-effects of spironolactones. Possible mechanisms of their anti-androgen action]. Nouv Presse Med 5, 691-694.

Desbrow, C., Rutledge, E.J., Brighty, G.C., Sumpter, J.P., Waldock, M., 1998. Identification of estrogenic chemicals in STW effluent: 1. Chemical fractionazation and in vitro biological screening. Environ Sci Technol 32, 1549-1558.

Dunn, O.J., 1964. Multiple comparisons using rank sums. Technometrics 6, 241-252.

Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat Toxicol 76, 122-159.

Gravel, A., Vijayan, M.M., 2007. Non-steroidal anti-inflammatory drugs disrupt the heat shock response in rainbow trout. Aquat Toxicol 81, 197-206.

Green, R.E., Taggart, M.A., Senacha, K.R., Raghavan, B., Pain, D.J., Jhala, Y., Cuthbert, R., 2007. Rate of decline of the oriental white-backed vulture population in India estimated from a survey of diclofenac residues in carcasses of ungulates. PLoS ONE 2, e686.

Guillette, L.J., Jr., Moore, B.C., 2006. Environmental contaminants, fertility, and multioocytic follicles: a lesson from wildlife? Semin Reprod Med 24, 134-141.

Holm, J.V., Rugge, K., Bjerg, P.L., Christensen, T.H., 1995. Occurence and distribution of pharmaceutical organic-compounds in the groundwater downgradient of a landfill (Grindsted, Denmark). Environ Sci Technol 29, 1415-1420.

Howell, W.M., Hunsinger, R.N., Blanchard, P.D., 1994. Paradoxical masculinization of female western mosquitofish during exposure to spironolactone. Progressive Fish-Culturist 56, 51-55.

Jenkins, R.L., Wilson, E.M., Angus, R.A., Howell, W.M., Kirk, M., 2003. Androstenedione and progesterone in the sediment of a river receiving paper mill effluent. Toxicol Sci 73, 53-59.

Kang, I.J., Hano, T., Oshima, Y., Yokota, H., Tsuruda, Y., Shimasaki, Y., Honjo, T., 2006. Anti-androgen flutamide affects gonadal development and reproduction in medaka (*Oryzias latipes*). Mar Environ Res 62 Suppl, S253-257.

Kime, D.E., 2001. *Endocrine disruption in fish*. Kluwer Academic, Norwell, MA, USA.

Korsgaard, B., 2006. Effects of the model androgen methyltestosterone on vitellogenin in male and female eelpout, *Zoarces viviparus* (L). Mar Environ Res 62 Suppl, S205-210.

Larsson, D.G., de Pedro, C., Paxeus, N., 2007. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. J Hazard Mater 148, 751-755.

Lazier, C.B., Langley, S., Ramsey, N.B., Wright, J.M., 1996. Androgen inhibition of vitellogenin gene expression in tilapia (*Oreochromis niloticus*). Gen Comp Endocrinol 104, 321-329.

Leusch, F.D., Van den Heuvel, M.R., Laurie, A.D., Chapman, H.F., Gooneratne, S.R., Tremblay, L.A., 2005. Quantification of vitellogenin mRNA induction in mosquitofish (*Gambusia affinis*) by reverse transcription real-time polymerase chain reaction (RT-PCR). Biomarkers 10, 429-438.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

McCormick, S.D., Regish, A., O'Dea, M.F., Shrimpton, J.M., 2008. Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxycorticosterone and aldosterone

on osmoregulation, gill Na+,K+ -ATPase activity and isoform mRNA levels in Atlantic salmon. Gen Comp Endocrinol 157, 35-40.

Nirde, P., Terouanne, B., Gallais, N., Sultan, C., Auzou, G., 2001. Antimineralocorticoid 11beta-substituted spirolactones exhibit androgen receptor agonistic activity: a structure function study. Mol Pharmacol 59, 1307-1313.

Nunes, B., Carvalho, F., Guilhermino, L., 2004. Acute and chronic effects of clofibrate and clofibric acid on the enzymes acetylcholinesterase, lactate dehydrogenase and catalase of the mosquitofish, *Gambusia holbrooki.* Chemosphere 57, 1581-1589.

Ogino, Y., Katoh, H., Yamada, G., 2004. Androgen dependent development of a modified anal fin, gonopodium, as a model to understand the mechanism of secondary sexual character expression in vertebrates. FEBS Lett 575, 119-126.

Runnalls, T.J., Hala, D.N., Sumpter, J.P., 2007. Preliminary studies into the effects of the human pharmaceutical Clofibric acid on sperm parameters in adult Fathead minnow. Aquat Toxicol 84, 111-118.

Sharpe, R.L., MacLatchy, D.L., Courtenay, S.C., Van Der Kraak, G.J., 2004. Effects of a model androgen (methyl testosterone) and a model anti-androgen (cyproterone acetate) on reproductive endocrine endpoints in a short-term adult mummichog (*Fundulus heteroclitus*) bioassay. Aquat Toxicol 67, 203-215.

Shultz, S., Baral, H.S., Charman, S., Cunningham, A.A., Das, D., Ghalsasi, G.R., Goudar, M.S., Green, R.E., Jones, A., Nighot, P., Pain, D.J., Prakash, V., 2004. Diclofenac poisoning is widespread in declining vulture populations across the Indian subcontinent. Proc Biol Sci 271 Suppl 6, S458-460.

Stanko, J.P., Angus, R.A., 2007. In vivo assessment of the capacity of androstenedione to masculinize female mosquitofish (*Gambusia affinis*) exposed through dietary and static renewal methods. Environ Toxicol Chem 26, 920-926.

Taggart, M.A., Senacha, K.R., Green, R.E., Jhala, Y.V., Raghavan, B., Rahmani, A.R., Cuthbert, R., Pain, D.J., Meharg, A.A., 2007. Diclofenac residues in carcasses of domestic ungulates available to vultures in India. Environ Int 33, 759-765.

Tremblay, M.R., Luu-The, V., Leblanc, G., Noel, P., Breton, E., Labrie, F., Poirier, D., 1999. Spironolactone-related inhibitors of type II 17beta-hydroxysteroid dehydrogenase: chemical synthesis, receptor binding affinities, and proliferative/antiproliferative activities. Bioorg Med Chem 7, 1013-1023.

Turner, C.L., 1941. Morphogenesis of the gonopodium in *Gambusia affinis affinis*. J.Morphol 69, 161-185.

Turner, C.L., 1942. A quantitative study of the effects of different concentrations of ethynltestosterone and methyl testosterone in the production of gonopodia in females of *Gambusia affinis*. Physiol Zool 15, 263-234.

Tyler, C.R., Jobling, S., Sumpter, J.P., 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit Rev Toxicol 28, 319-361.

Watkinson, A.J., Murby, E.J., Kolpin, D.W., Costanzo, S.D., 2009. The occurrence of antibiotics in an urban watershed: From wastewater to drinking water. Sci Total Environ.

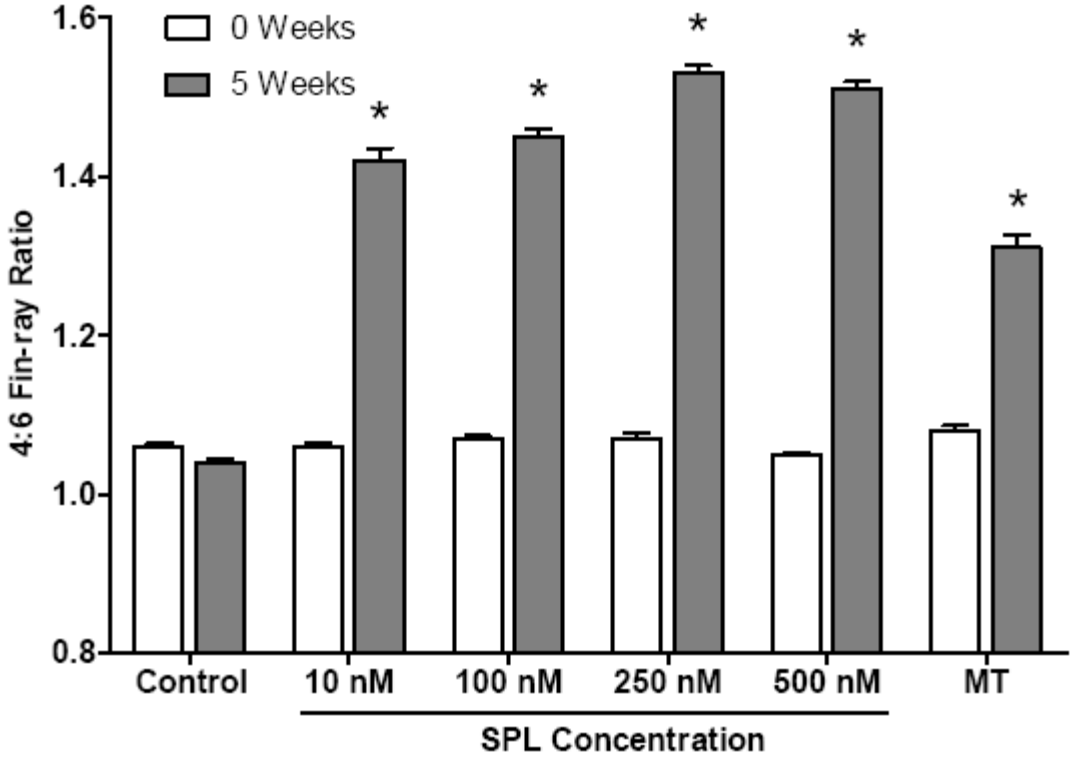

Fig. 1. The 4:6 anal fin ray elongation ratios of static renewal Spironolactone (SPL) treatment groups: SPL exposure induces masculinization in female mosquitofish. Female mosquitofish were exposed to varying concentrations of SPL for five weeks via static renewal method and masculinization was assessed by elongation of anal fin ray measured as 4:6 ratio. Data are presented as mean + S.E. (n = 10). An asterisk (*), indicates the mean at 5 weeks differs significantly ($p < 0.05$) from the mean at 0 weeks (paired t-test).

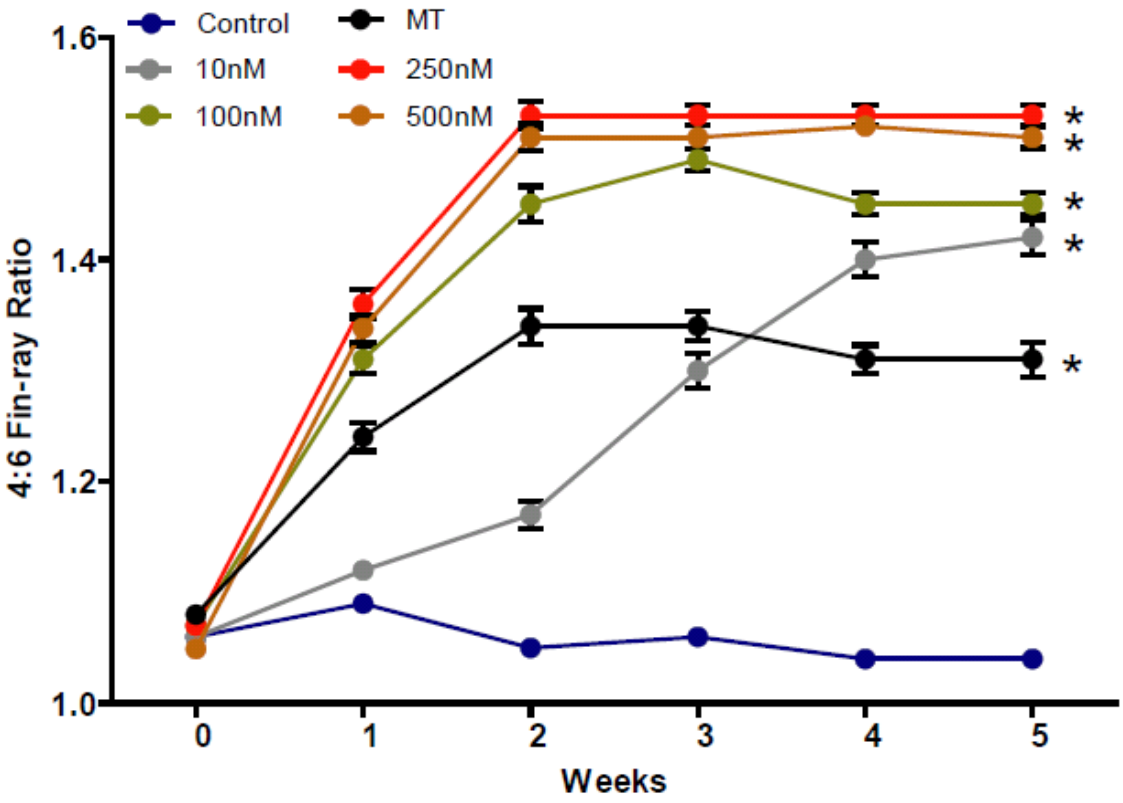

Fig. 2. Concentration response of anal fin elongation ratios. Spironolactone (SPL) exposure induces masculinization in female mosquitofish after five weeks of treatment. Female mosquitofish were exposed to varying concentrations of SPL for five weeks via static renewal method. Masculinization was assessed at the end of each week by elongation of anal fin ray measured as 4:6 ratio. Data are presented as mean ± S.E (n = 10). An asterisk (*), indicates values significantly different ($p < 0.05$).

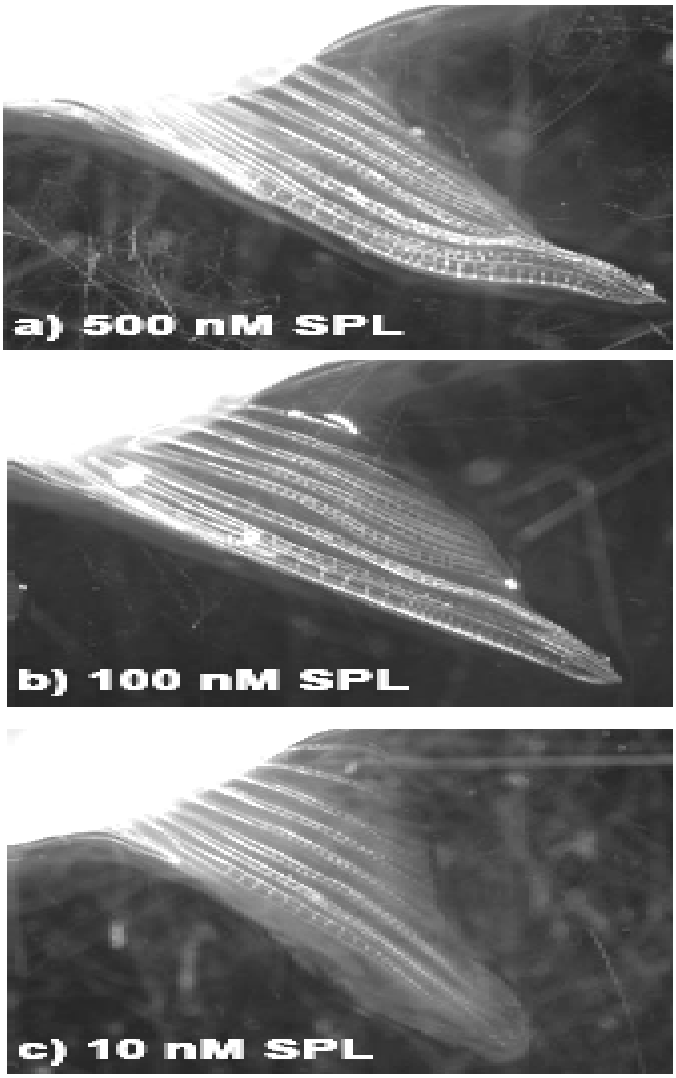

Fig. 3. *Gambusia affinis* anal fins. Anal fins of representative female mosquitofish exposed for five weeks to 500 nM SPL (a), 100 nM SPL (b), and 10 nM SPL (c) by the static renewal method.

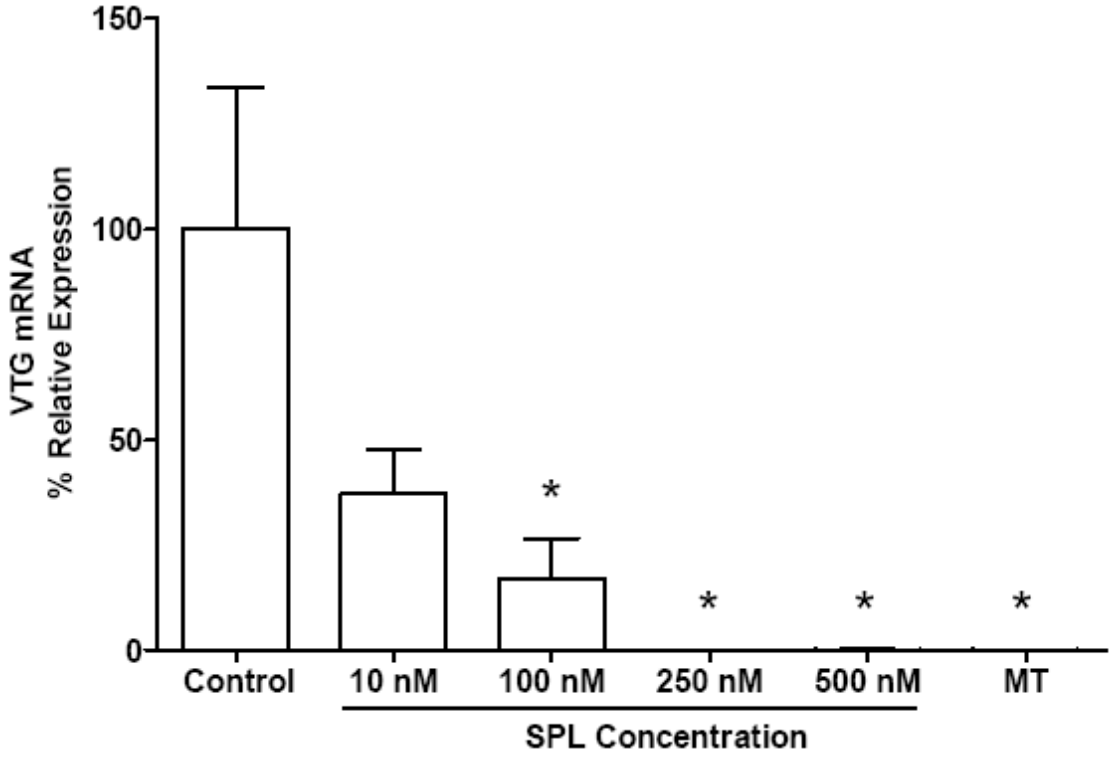

Fig. 4. Vitellogenin expression in spironolactone treatment groups: Spironolactone (SPL) exposure inhibits vitellogenin (VTG) mRNA expression in female mosquitofish. Female mosquitofish were exposed to varying concentrations of SPL for five weeks via static renewal method. Livers were extracted at the end of five weeks after sacrificing fish and hepatic RNA was isolated. Hepatic VTG mRNA expression was quantified using real-time PCR and is presented here as % relative expression to the housekeeping gene. Data are presented as mean + S.E. ($n \geq 5$). An asterisk (*), indicates means significantly different than control ($p < 0.05$).

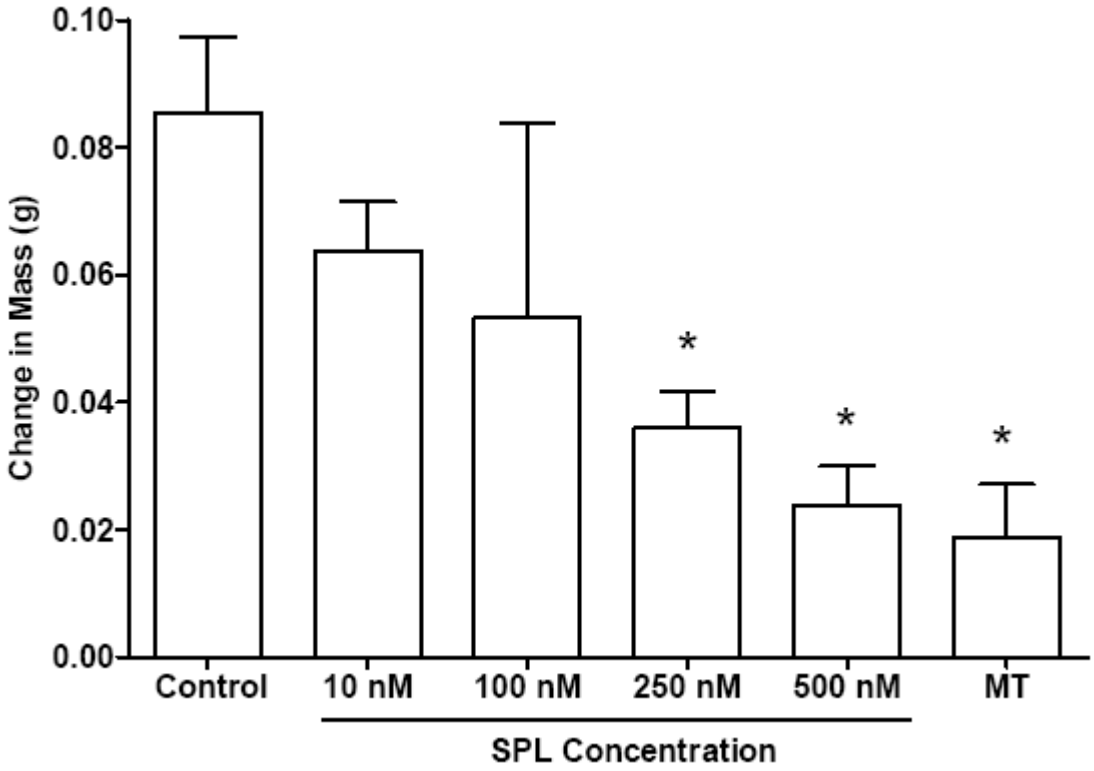

Fig. 5. Changes in mass of fish in the static renewal treatment. Spironolactone (SPL) exposure decreases mass in female mosquitofish. Female mosquitofish were exposed to varying concentrations of SPL for five weeks via the static renewal method. Total body weights were measured at the beginning and end of the experiment and change in mass ($Weight_{week5} - Weight_{week0}$) was calculated. Data are presented as mean + S.E. (n = 10). An asterisk (*), indicates values significantly ($p < 0.05$) different than the control.

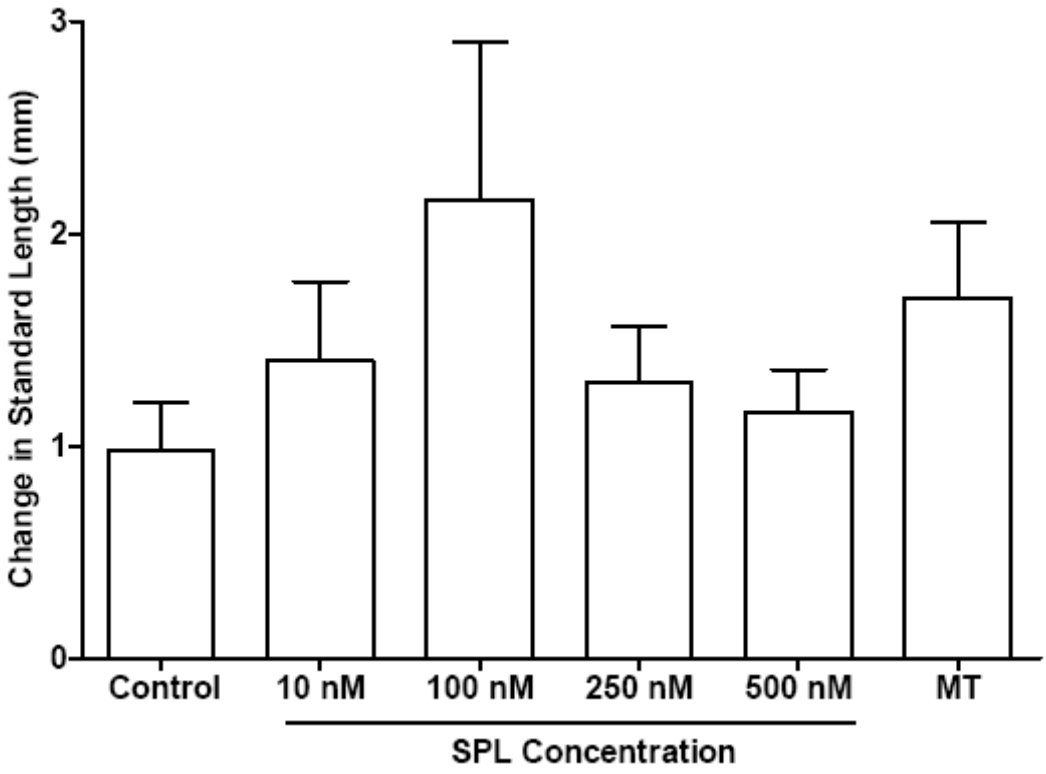

Fig. 6. Changes in the standard length of fish in the static renewal treatment: Spironolactone (SPL) exposure does not affect standard length in female mosquitofish. Fish were exposed to varying concentrations of SPL for five weeks via the static renewal method. Total body lengths were measured at the beginning and end of the experiment and change in length ($Length_{week5} - Length_{week0}$) was calculated. Data are presented as mean + S.E. (n = 10). An asterisk (*), indicates means significantly ($p < 0.05$) different than the control.

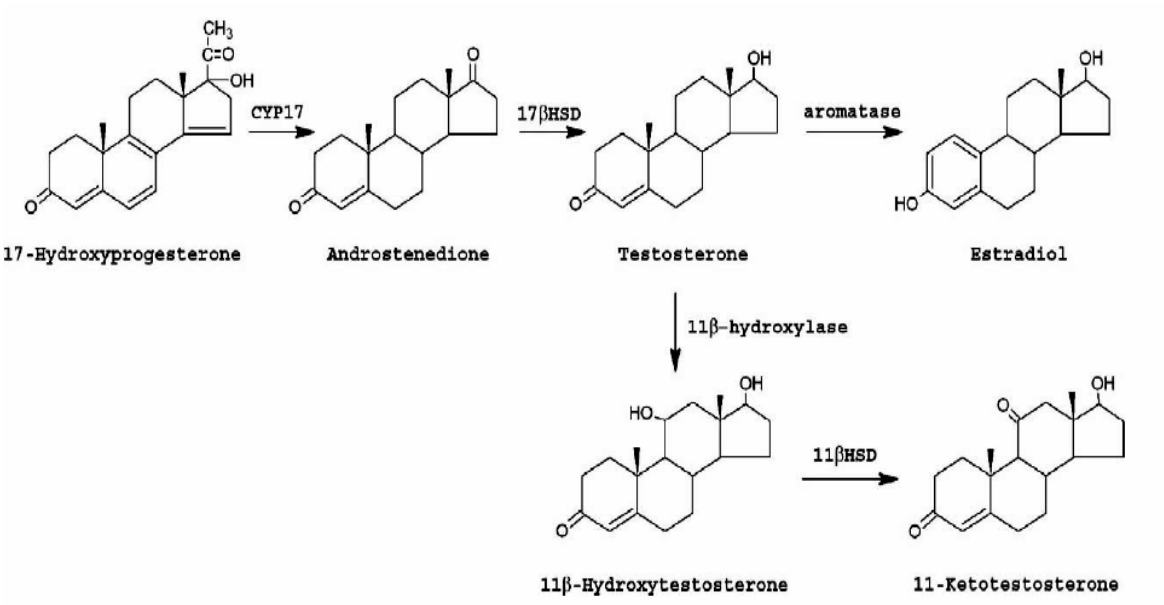

Fig. 7. Steroid metabolism pathway in teleosts.

EFFECTS OF TRICLOSAN ON VITELLOGENIN INDUCTION AND SPERM PRODUCTION IN MALE WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS*

by

SAMIKSHA A. RAUT AND ROBERT A. ANGUS

In preparation for submission to *Environmental Toxicology and Chemistry*

Format adapted for dissertation

ABSTRACT

Triclosan (TCS) is an antibacterial agent used in a variety of personal care and industrial products such as soaps, shampoos, shaving creams, toothpastes, mouthwashes, deodorants, cleaning supplies and textile goods. TCS and its environmentally transformed derivative, methyl-TCS have been detected in waters receiving effluent from public wastewater treatment plants. Previous studies have demonstrated that TCS has the potential to act as an endocrine disruptor. We tested the hypothesis that TCS acts as an endocrine disrupting agent in fish at environmentally relevant concentrations. Mature male western mosquitofish, *Gambusia affinis* were exposed to TCS concentrations of 100, 250, and 350 nM for 35 days by the static renewal method. Induction of the normally female-limited vitellogenin gene expression and changes in sperm counts were quantified as biomarkers of endocrine disruption. Vitellogenin mRNA expression, determined by real time-PCR analysis, was found to be significantly higher in the 350 nM TCS treatment group as compared to the control group. Additionally, sperm counts in the 350 nM TCS treatment group were found to be significantly lower than the control group. We also measured weight, hepatosomatic and gonadosomatic indices. The mean hepatosomatic indexes in the 200 nM and 350 nM treatment groups were significantly greater than the controls. The present study shows that TCS has the potential to act as an endocrine disruptor in male mosquitofish. These results also support the use of vitellogenin induction and reduction in sperm counts as reliable biomarkers of endocrine disruption in western male mosquitofish, *Gambusia affinis*.

INTRODUCTION

Triclosan (5-chloro-2-[2, 4-dichlorophenoxy]-phenol) (TCS) is a broad spectrum antibacterial agent which is extensively used in a variety of personal care and industrial products such as soap, shampoo, and textile goods. TCS is not removed completely from wastewater by public wastewater treatment plants (WWTPs) and, therefore, is frequently detected in waste and surface waters receiving effluent from WWTPs (Hua et al. 2005; Thomas and Foster 2004). Samples collected from the wastewater outfall of a specialty chemicals manufacturing plant in Rhode Island contained 10-20 μg/L of TCS in effluent water and 80-100 μg/L of TCS in the sediment (Lopez-Avila and Hites 1980). A recent study carried out in Japan detected TCS in canal water receiving domestic and agricultural wastewater in the range of 11-31 ng/L downstream of, and 55-134 ng/L at the outfall of a domestic WWTP (Nishi et al. 2008). TCS is a relatively stable lipophilic compound and therefore is readily bioavailable to aquatic organisms. For example, caged snails (*Helisoma trivolvis*) exposed at the outfall of a WWTP showed rapid bioaccumulation of TCS (Coogan and La Point 2008). TCS has been detected in bile samples of both caged fish and wild caught fish collected below outfalls of WWTPs (Adolfsson-Erici et al. 2002). Additionally, methyl-TCS, an environmentally transformed product of TCS has been detected in fish collected from various lakes in Switzerland (Balmer et al. 2004). At concentrations more than 1,000 times higher than have been detected in environmental samples TCS is toxic. The 48 h median effective concentration is 390 μg/L in *Daphnia*

magna, is 260 μg /L in *Pimephales promelas* and 70 μg /L in *Lepomis macrochirus* (Orvos et al. 2002).

The molecular structure of TCS resembles several non-steroidal estrogens, such as diethylstilbestrol and bisphenol A. This suggests that it has the potential to act as an endocrine disrupting agent. Indeed, in *in vitro* assays TCS displaced [^{3}H]-estradiol from estrogen receptors in MCF7 human breast cancer cells and from recombinant human ERα/ERβ (Gee et al. 2008), suggesting a possible disruption of normal endocrine functions. In rat Leydig cells, TCS reduces testosterone production by inhibiting adenylyl cyclase enzyme activity and disrupting the steroidogenic cascade of events (Kumar et al. 2008). Alarmingly, TCS has been detected in human milk, plasma (Adolfsson-Erici et al. 2002; Allmyr et al. 2006) and urine (Calafat et al. 2008), indicating that human beings are accumulating this potential endocrine disruptor. The endocrine-disrupting capacity of TCS has been demonstrated in some aquatic organisms. A 14 day exposure of medaka (*Oryzias latipes*) fry to TCS (100, 10, 1 μg/L) resulted in changes in fin length but did not significantly affect the sex ratio. Based on these results it was suggested that TCS is potentially weakly androgenic (Foran et al. 2000). Conversely, a 21 day exposure of TCS (20, 100 μg/L) resulted in increased vitellogenin (VTG) induction and it was concluded that a metabolite of TCS may be a weak estrogenic compound (Ishibashi et al. 2004). Premetamorphic tadpoles of the North American bullfrog, *Rana catesbeiana* when exposed to TCS at environmentally relevant concentrations (0.15+/-0.03 μg/L) have been shown to disrupt thyroid hormone-associated gene expression and also alter the rate of thyroid-hormone-mediated postembryonic development (Veldhoen et al. 2006). Given its

occurrence in the environment, it is important to investigate the potential of TCS to act as an endocrine disruptor in a variety of aquatic organisms.

The objective of this study was to investigate the effects of TCS on two measures of reproductive health in male western mosquitofish, *Gambusia affinis*. *G. affinis* is a member of the livebearing family Poecillidae and is native to the southeastern United States. It is a useful biomonitor species for endocrine disrupting compounds in aquatic environments because of its widespread occurrence around the world, large environmental tolerances, and ease of use in the laboratory. Previous studies have shown VTG induction and decreased sperm counts in male mosquitofish as reliable biomarkers of exposure to xenoestrogens in male mosquitofish (Melvin 2007). In this study, male mosquitofish were exposed to various environmentally relevant concentrations of TCS and the effects on vitellogenin induction and sperm count were investigated.

MATERIALS AND METHODS

Animals

Mature western mosquitofish, *Gambusia affinis* were collected by seine from the Cahaba River in Trussville, Alabama. After collection, the fish were returned to the University of Alabama at Birmingham, placed in aerated aquaria and allowed to acclimate for two weeks. The acclimated fish were then randomly distributed among the treatments (n = 10 per treatment).

Triclosan treatment – static renewal exposure

Fish were exposed to TCS via water by the static renewal method. The fish were kept individually in 1 L unaerated Kimax 14005 beakers, (Kimble Glass, Inc. Vineland, NJ, USA), which hold 1 L with an additional 2.5 cm air space. The water was dechlorinated Birmingham, AL tap water with 0.25 g/L Instant Ocean Sea Salt (Spectrum Brands, Inc., Atlanta, GA, USA) added to provide trace minerals and buffering capacity. Water temperature was 24 $\pm$ 2 ^{o}C. Fish were maintained under a 14:10 light: dark cycle. The water was changed every other day and TCS re-added. Water chemistry parameters including pH, dissolved oxygen, ammonia and nitrate were monitored once a week and maintained at appropriate levels.

A 10^{6} nM stock solution was made by dissolving TCS (Sigma-Aldrich Chemicals, Saint Louis, USA) in dehydrated absolute ethanol (Pharmaco, Brookfield, CT, USA). Final working concentrations of 100, 200 and 350 nM were prepared by adding appropriate volumes of the stock solution to 1 L of water in each beaker. These concentrations were selected on the basis of a preliminary toxicity range-finding study. A separate stock solution of 10^{6} nM 17α- ethinylestradiol (EE2) (Steraloids, Newport, RI, USA) was made by dissolving EE2 in absolute ethanol. A working concentration of 5 nM was used as a positive control for vitellogenin (VTG) induction. The solvent control group received ethanol equivalent to that received by the highest concentration experimental group, (350 μl/L). Fish were fed twice a day with Silver Cup granulated trout starter food (Nelson & Sons, Inc., Murray, UT, USA). Food was stored at 4 ^{o}C.

Morphological measurements

On day zero before the start of the exposure, fish were anesthetized by immersion in 300 mg/L MS-222 (tricaine methanesulfonate, Argent Laboratories, Redmond, WA, USA), dried by patting with Kim Wipe tissue paper and weighed to the nearest 0.1 mg. After the completion of the 35 day exposure period, each fish was again weighed, mobilized sperm were stripped for sperm count and the fish was then terminally anesthetized in 120 mg/L MS-222.The liver and testis were removed and weighed. Gonadosomatic (GSI) and Hepatosomatic indices (HSI) were calculated as organ weight as a percentage of the body weight.

Sperm Counts

Mature sperm in Poeciliid fish are bundled into packets called spermatophores. The protocol used for obtaining sperm from the spermatophores in *Gambusia affinis* was modified from a technique originally used for guppies (*Poecilia latipinna)* (Toft and Baatrup 2001). Briefly, fish were anesthetized by immersion in MS-222 and placed onto a sperm collecting plate containing a 20 µL droplet of 175 mM KCl. Spermatophores were stripped into the KCl droplet by gently pressing the abdomen of the fish just above the origin of the anal fin with a blunt-tipped glass stirring rod. The fish was then removed and the spermatophores were washed into a 0.6 ml microcentrifuge tube with two rinses of 80 µL of 175 mM KCl. The spermatophores were made to dissociate in the KCl by drawing into and out of a pipette 2-3 times and incubating for a minimum of 10 minutes.

A 10 µL aliquot of the sperm suspension was placed onto a Neubauer counting chamber (0.02 mm deep, Hausser Scientific, Horsham, PA, USA) and allowed to settle for 10 minute in a humidifier. The number of total sperm in the ejaculate was calculated by the following procedure. A compound microscope and digital camera were utilized to produce 10 digital images of each sperm sample at random locations in the chamber. The sperm in each image were counted. Since the volume of suspension included in the image was known (from the depth of the chamber and the area covered by the photograph), it was possible to calculate the number of sperm per volume of suspension. This was averaged over the 10 images taken per sample, and then, by back calculating to the total volume of the suspension, the estimate of the total number of sperm in the ejaculate was obtained.

Hepatic Vitellogenin: Quantitative Real Time Polymerase Chain Reaction (q-RTPCR)

After 35 days of exposure, fish were sacrificed by immersion in MS-222. Livers were removed, weighed, flash-frozen in liquid nitrogen (-160°C) and then stored at -80 °C. Total RNA was extracted using the Trizol method protocol as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The cDNA was amplified in an Opticon Continous Fluorescent Detector (MJ Research, Waltham, MA) using IQTM SYBR Green supermix (Bio-Rad Laboratories). The primers were designed specifically for VTG using the mRNA sequence from GenBank (Accession number DQ190844) and using Primer3 software (http://frodo.wi.mit.edu/). The primers for 18S

reference housekeeping gene were designed based on *Oncorhynchus mykiss* mRNA sequence (Genbank Accession Number: AF243428) because the 18S mRNA sequence for *Gambusia affinis* has not been determined (Leusch et al. 2005). The sequences of the primers are shown in Table 1.

Primer	Sequence
Forward VTG	5' - ACCAGGGACCTGAACAACTG - 3'
Reverse VTG	5' - GATGGCATTAGCGACTGGTT - 3'
Forward 18S	5' - CCTGCGGCTTAATTTGACTC - 3'
Reverse 18S	5' - AACTAAGAACGGCCATGCAC - 3'

Table 1. Primer sequences used for RT-PCR of a *Gambusia affinis* vitellogenin gene transcript.

Real-time PCR was performed in triplicate for each liver sample, averaged, and normalized to endogenous 18S RNA reference transcripts. At least five fish per treatment group were analyzed for VTG gene expression. Quantification of the hepatic VTG mRNA expression relative to the 18S RNA gene was carried out by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

PCR efficiency and specificity were validated for both VTG and 18S primers. To calculate the PCR efficiency, cDNA samples from positive control group (EE2) were serially diluted ten folds three times (10^{0}, 10^{-1}, 10^{-2}, and 10^{-3}) and PCR-amplified using the primer pair for VTG and 18S. The resulting Ct values for each primer set were plotted as

a function of the log concentration of cDNA and a linear trend-line was fit to the data. The resulting slope of the line fit to the data was used to determine the PCR efficiency as reported previously (Ginzinger 2002). To ensure amplification specificity, melting curve analysis was performed from 65 to 94 ^{o}C at the rate of 0.1 ^{o}C/s after PCR.

Statistical Methods

All the summary statistics are expressed as mean + standard error. The cutoff for statistical significance was $p < 0.05$. GSI and HSI values were log transformed prior to analysis of variance. Dunnett's post hoc test was used to compare the treatment group means to that of the control. VTG mRNA expression levels were compared between treatment groups and the control using the nonparametric Kruskal-Wallis test, followed by Dunn's nonparametric post hoc test for pair-wise comparisons (Dunn 1964).

RESULTS

Sperm Counts

The mean sperm counts after 35 days of exposure to TCS are shown in Fig. 1. Sperm counts were found to be significantly reduced in the 350 nM TCS and 5 nM EE2 treatment groups as compared to the solvent control.

Hepatic Vitellogenin Expression

In order to determine hepatic VTG expression, we collected livers from male mosquitofish exposed to TCS after the completion of 35 day exposure period. VTG ex-

pression in reference to 18S ribosomal RNA was determined using real time PCR protocol as described in methods. We first validated the assay by determining the amplification efficiency of both the primer sets. The samples from EE2 were serially diluted and PCR amplified. The resulting threshold cycle numbers were plotted against log dilution factors for each primer and a linear trend-line was fitted to the data (Fig. 2). The slope of this linear-fit was determined and amplification efficiency was calculated according to Ginzinger (2002). The amplification efficiency was always greater than 90% for both the primer sets. Tests of homogeneity of slope indicated that the linear regression lines of primer sets for the target and reference genes were parallel to each other, suggesting that their amplification efficiencies are approximately equal (Fig. 2). We also verified amplification specificity by performing melting curve analysis after PCR amplification which showed a single peak indicating the specificity of PCR amplification (data not shown). Moreover, the variability among C_t values for 18S from all samples was negligible supporting the rationale for the use of 18S as the reference gene.

In accordance with the hypothesis that TCS has estrogenic effects in male mosquitofish, the hepatic VTG mRNA expression was significantly increased (30-fold) in male mosquitofish exposed to TCS at 350 nM as compared to the solvent control (Fig. 3). As expected, the EE2 treatment group also showed significantly increased Vtg expression (~1500 fold) as compared to the solvent control (Fig. 3).

Physiological Variables

The mean changes in the mass of the treatment groups over a period of 35 days are shown in Fig. 4. The mean mass of the fish in the 200 nM TCS and EE2 groups decreased significantly as compared to the solvent control. None of the mean GSI values for the TCS treatment groups differed significantly from that of the solvent control (data not shown). The effect of the TCS treatment on the mean HSI is shown in Fig. 5. The mean HSI values of the male mosquitofish exposed to 350 nM and 200 nM TCS treatment groups differed significantly from the solvent control.

DISCUSSION

The results of the present study demonstrate that TCS induces VTG production and inhibits sperm production in male mosquitofish at environmentally relevant concentrations in a period of 35 days. There was a significant induction of VTG mRNA expression in the 350 nM TCS treatment group as compared to the solvent control. Additionally; the mean sperm count in this treatment group was significantly lower than the solvent control. No significant changes were observed in the GSI in any of the TCS treatment groups when compared to the solvent control. On the other hand, the mean HSI was found to be significantly higher in the 200 nM and 350 nM TCS treatment group than the solvent control. These results suggest that TCS acts as an endocrine disrupting agent in western male mosquitofish, *Gambusia affinis*.

Expression of vitellogenin in male fish is a reliable biomarker of exposure to environmental estrogens. We observed significant induction of VTG mRNA expression in

male mosquitofish exposed to TCS at a concentration of 350 nM for a period of 35 days. These results are similar to those of (Ishibashi et al. 2004) who observed induction of VTG in male medaka exposed to TCS at 100 and 200 μg/L (345.4 and 690.8 nM) for 21 days. However, in our study we observed VTG induction after 35 days of exposure as compared to the 21 day period as reported by (Ishibashi et al. 2004). The difference in the species and its sensitivity might account for the somewhat slower response seen in our study.

Previous studies have shown an association between reduced sperm counts and xenoestrogen exposure. This has been demonstrated in guppies (*Poecilia reticulata*) exposed to the synthetic estrogen EE2 at 112ng/L (Kristensen et al. 2005) and also in goldfish (*Carassius auratus*) at 50 ng/L (Schoenfuss et al. 2002). Reduced sperm counts in eastern mosquitofish (*Gambusia holbrooki*) have been related to disrupted sexual behavior (Toft and Guillette 2005) and in guppies (*Poecilia reticulata*) to reduced reproductive fitness (Kristensen et al. 2005). Therefore, it is important to investigate the effects of an endocrine disrupting compound on sperm counts. To our knowledge this is the first study investigating the effects of TCS exposure on sperm counts in fish or other aquatic organisms. Our results show that TCS significantly reduced mean sperm count in male mosquitofish after 35 days of static renewal exposure to 350 nM TCS. There could be several mechanisms involved in reducing the sperm count in the exposed fish. One possible explanation is that exposure to estrogens might disturb steroid feedback mechanisms, resulting in a reduction in androgen production and altered reproductive function (Trudeau 1993). Indeed, spermatogenesis in goldfish was suppressed by hypophysectomy and

restored by treatment with methyl testosterone (Pandey 1969). A recent study by Kumar *et al.* (2008), on rat Leydig cells suggested that TCS causes disruption of adenylyl cyclase activity which further causes the disruption of the intermediate steroidogenic cascade leading to depressed testosterone production. It would be of interest to investigate whether a similar mechanism is involved in reducing the sperm counts in fish.

We did not measure testosterone concentrations in the current study and therefore, its possible association with the reduced sperm counts cannot be determined. However, results of another study indicate that sperm count and testosterone levels may not be tightly correlated in mosquitofish. Male *G. holbrooki* exposed for a month to water from pesticide-polluted Lake Apopka had reduced sperm counts, as expected from exposure to estrogens, but did not have reduced testosterone levels (Toft and Guillette 2005). Further studies are necessary to elucidate the mechanism by which TCS causes reduced sperm counts in male mosquitofish.

One of the major functions of fish liver includes the metabolism of xenobiotics. The liver tends to increase in size when exposed to a toxic substance and an increase in HSI is regarded as a sensitive indicator of exposure to toxic substances. In our study, we have shown a significantly elevated mean HSI in the 200 and 350 nM TCS treatment groups as compared to the solvent control. Adult rare minnows (*Gobiocypris rarus*) when exposed to EE2 at a concentration of 25 ng/L also showed an increased HSI values, characterized by hypertrophy of the hepatocytes (Zha et al. 2007). Hypertrophy of the hepatocytes may be a possible reason for elevated HSI as observed in our study. Among the other physiological variables, we have observed a significant decrease in the fish mass in

the 200 nM TCS treatment group as compared to the solvent control. Decrease in the fish mass can be due to loss of appetite which is a sign of toxicity induced stress response. However, we still need to determine why such a response was seen only in this particular exposure concentration and not in the group exposed to the highest TCS concentration.

In conclusion, this study demonstrates that exposure to TCS can induce expression of the VTG in male mosquitofish – a common biomarker of estrogenic effect. It also caused reduced sperm counts in male mosquitofish – an indication of potential reproductive impairment. Further studies are warranted to investigate the mechanism of endocrine disruption by TCS. It is important to understand the effects of this compound on aquatic organisms due to its widespread domestic and industrial use and documented occurrence in treated wastewater. Results obtained from this study also support the utility of VTG mRNA induction and sperm counts as effective biomarkers of endocrine disruption in male mosquitofish.

REFERENCES

Adolfsson-Erici M, Pettersson M, Parkkonen J, Sturve J. 2002. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. Chemosphere 46(9-10):1485-9.

Allmyr M, Adolfsson-Erici M, McLachlan MS, Sandborgh-Englund G. 2006. Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. Sci Total Environ 372(1):87-93.

Balmer ME, Poiger T, Droz C, Romanin K, Bergqvist PA, Muller MD, Buser HR. 2004. Occurrence of methyl triclosan, a transformation product of the bactericide triclosan, in fish from various lakes in Switzerland. Environ Sci Technol 38(2):390-5.

Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. 2008. Urinary concentrations of triclosan in the U.S. population: 2003-2004. Environ Health Perspect 116(3):303-7.

Coogan MA, La Point TW. 2008. Snail bioaccumulation of triclocarban, triclosan, and methyltriclosan in a North Texas, USA, stream affected by wastewater treatment plant runoff. Environ Toxicol Chem 27(8):1788-93.

Dunn OJ. 1964. Multiple comparisons using rank sums. Technometrics 6:241-252.

Foran CM, Bennett ER, Benson WH. 2000. Developmental evaluation of a potential non-steroidal estrogen: triclosan. Mar Environ Res 50(1-5):153-6.

Gee RH, Charles A, Taylor N, Darbre PD. 2008. Oestrogenic and androgenic activity of triclosan in breast cancer cells. J Appl Toxicol 28(1):78-91.

Ginzinger DG. 2002. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol 30(6):503-12.

Hua W, Bennett ER, Letcher RJ. 2005. Triclosan in waste and surface waters from the upper Detroit River by liquid chromatography-electrospray-tandem quadrupole mass spectrometry. Environ Int 31(5):621-30.

Ishibashi H, Matsumura N, Hirano M, Matsuoka M, Shiratsuchi H, Ishibashi Y, Takao Y, Arizono K. 2004. Effects of triclosan on the early life stages and reproduction of medaka Oryzias latipes and induction of hepatic vitellogenin. Aquat Toxicol 67(2):167-79.

Kristensen T, Baatrup E, Bayley M. 2005. 17alpha-ethinylestradiol reduces the competitive reproductive fitness of the male guppy (Poecilia reticulata). Biol Reprod 72(1):150-6.

Kumar V, Balomajumder C, Roy P. 2008. Disruption of LH-induced testosterone biosynthesis in testicular Leydig cells by triclosan: probable mechanism of action. Toxicology 250(2-3):124-31.

Leusch FD, Van den Heuvel MR, Laurie AD, Chapman HF, Gooneratne SR, Tremblay LA. 2005. Quantification of vitellogenin mRNA induction in mosquitofish (*Gambusia affinis*) by reverse transcription real-time polymerase chain reaction (RT-PCR). Biomarkers 10(6):429-38.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25(4):402-8.

Lopez-Avila V, Hites RA. 1980. Organic compounds in an industrial wastewater. Their transport into sediments. Environ Sci Technol 14:1382-1390.

Melvin P. 2007. Sperm Production and Vitellogenesis as biomarkers of endocrine disruption in the male western mosquitofish, *Gambusia affinis*. Birmingham: University of Alabama at Birmingham.

Nishi I, Kawakami T, Onodera S. 2008. Monitoring of triclosan in the surface water of the Tone Canal, Japan. Bull Environ Contam Toxicol 80(2):163-6.

Orvos DR, Versteeg DJ, Inauen J, Capdevielle M, Rothenstein A, Cunningham V. 2002. Aquatic toxicity of triclosan. Environ Toxicol Chem 21(7):1338-49.

Pandey S. 1969. Effects of hypophysectomy on the testis and secondary sex characters of the adult guppy *Poecilia reticulata*. Can J Zool 47:775-81.

Schoenfuss HL, Levitt JT, Van der Kraak G, Sorensen PW. 2002. Ten-week exposure to treated sewage discharge has relatively minor, variable effects on reproductive behavior and sperm production in goldfish. Environ Toxicol Chem 21(10):2185-90.

Thomas PM, Foster GD. 2004. Determination of nonsteroidal anti-inflammatory drugs, caffeine, and triclosan in wastewater by gas chromatography-mass spectrometry. J Environ Sci Health A Tox Hazard Subst Environ Eng 39(8):1969-78.

Toft G, Baatrup E. 2001. Sexual characteristics are altered by 4-tert-octylphenol and 17beta-estradiol in the adult male guppy (*Poecilia reticulata*). Ecotoxicol Environ Saf 48(1):76-84.

Toft G, Guillette LJ, Jr. 2005. Decreased sperm count and sexual behavior in mosquitofish exposed to water from a pesticide-contaminated lake. Ecotoxicol Environ Saf 60(1):15-20.

Trudeau VL, Wade, M. G., Van Der Kraak, G., and Peter, R. E. 1993. Effects of 17B-estradiol on pituitary and testicular funcition in male goldfish. Can J Zool 71:1131-1135.

Veldhoen N, Skirrow RC, Osachoff H, Wigmore H, Clapson DJ, Gunderson MP, Van Aggelen G, Helbing CC. 2006. The bactericidal agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. Aquat Toxicol 80(3):217-27.

Zha J, Wang Z, Wang N, Ingersoll C. 2007. Histological alternation and vitellogenin induction in adult rare minnow (*Gobiocypris rarus*) after exposure to ethynylestradiol and nonylphenol. Chemosphere 66(3):488-95.

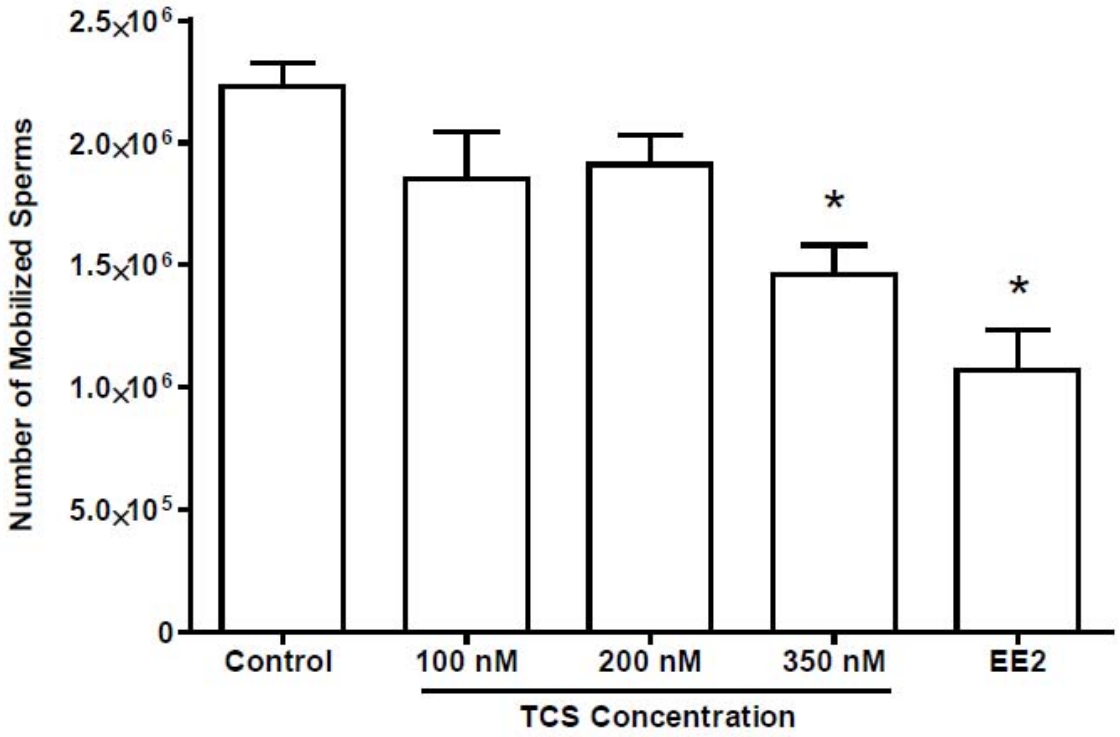

Fig. 1. Mean sperm counts (+SEM) of adult male mosquitofish, *Gambusia affinis* after exposure to Triclosan (TCS) for 35 days via static renewal treatment. Concentration refers to TCS treatment groups, except for the positive control group 17α-ethynylestradiol (EE2). The solvent control, ethanol, has been labeled "Control." An asterisk (*) indicates a mean significantly different from the solvent control ($p < 0.05$).

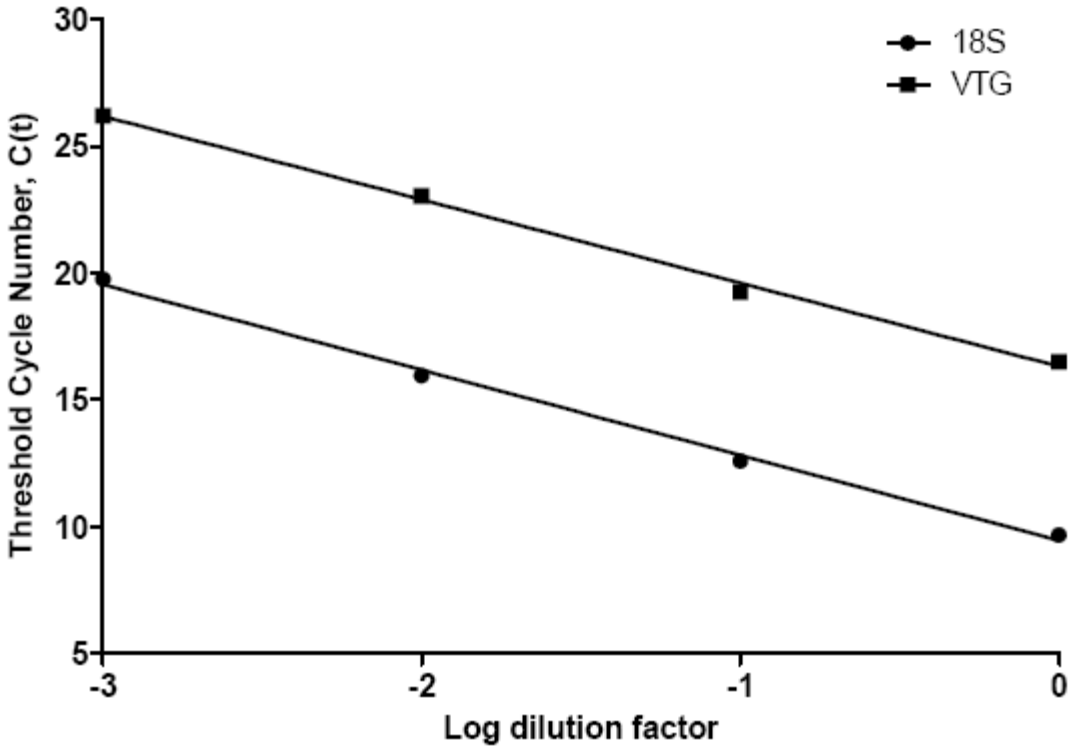

Fig. 2. Efficiency of VTG and 18S primers. 17α-ethynylestradiol (EE2) cDNA samples were serially diluted (10^{0},10^{-1},10^{-2}, and 10^{-3}) and amplified by the real-time PCR with forward and reverse primers VTG and 18S. The resulting Ct values for each primer template are plotted as a function of the log concentration of cDNA and a linear trend-line is fit to the data. The resulting slope of the line fit to the data is used to determine the PCR efficiency. No significant difference is observed in the slopes of the titration curves between target (VTG) and reference (18S) ($P > 0.05$).

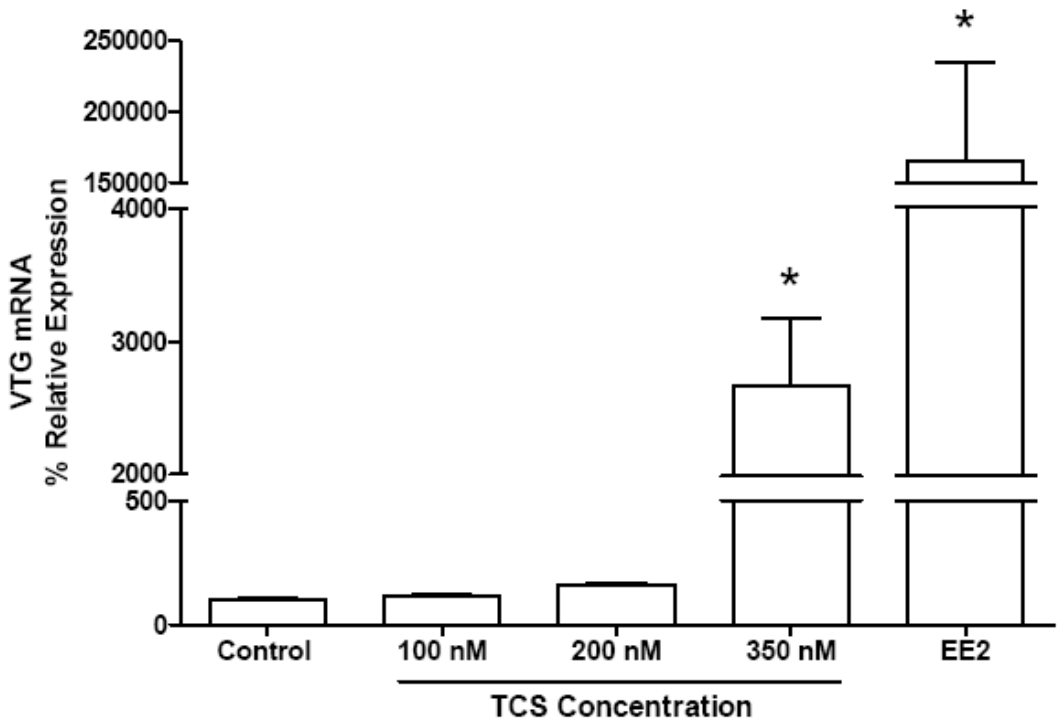

Fig. 3. Mean hepatic vitellogenin (VTG) mRNA % relative expression of adult male mosquitofish after exposure to TCS for 35 days via static renewal treatment: Concentration refers to Triclosan (TCS), except for the positive control group 17α-ethynylestradiol (EE2). The solvent control ethanol has been labeled as "Control." Values reported are means + standard error. An asterisk (*) indicates values significantly different from the solvent control ($p < 0.05$).

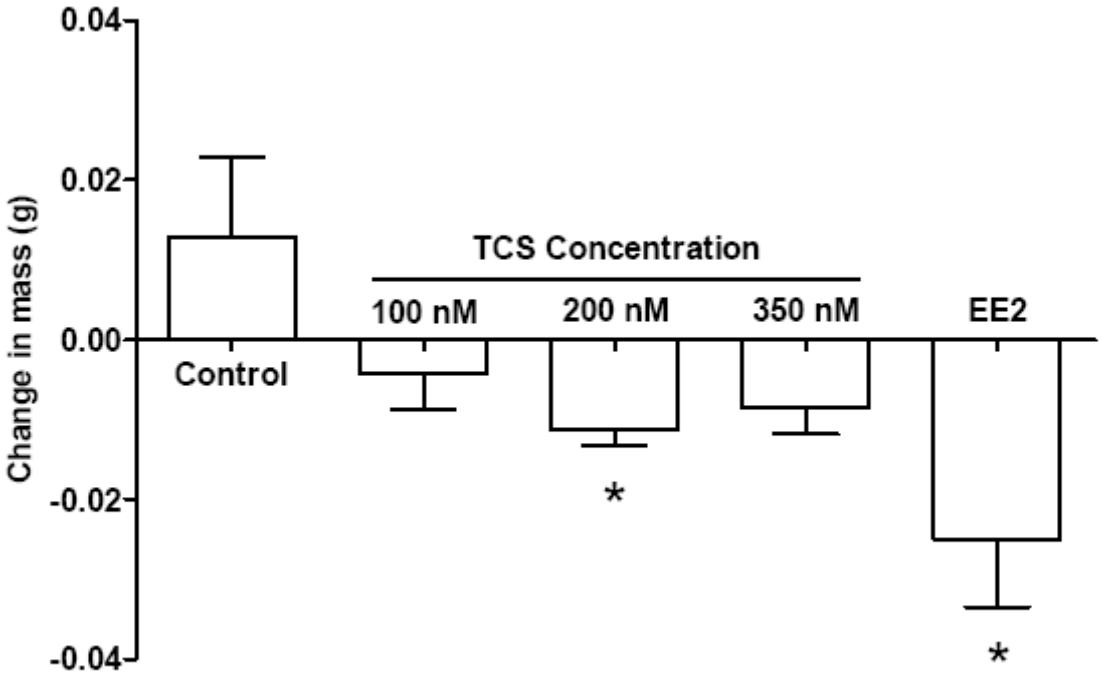

Fig. 4. Mean changes (+ standard error) over the 35 day exposure period in mass of the fish in TCS static exposure treatment groups: Concentration refers to Triclosan (TCS), except for the positive control group 17α-ethynylestradiol (EE2). The solvent control ethanol has been labeled as "Control." Values reported are means + standard error. An asterisk (*) indicates values significantly different from the solvent control ($p < 0.05$).

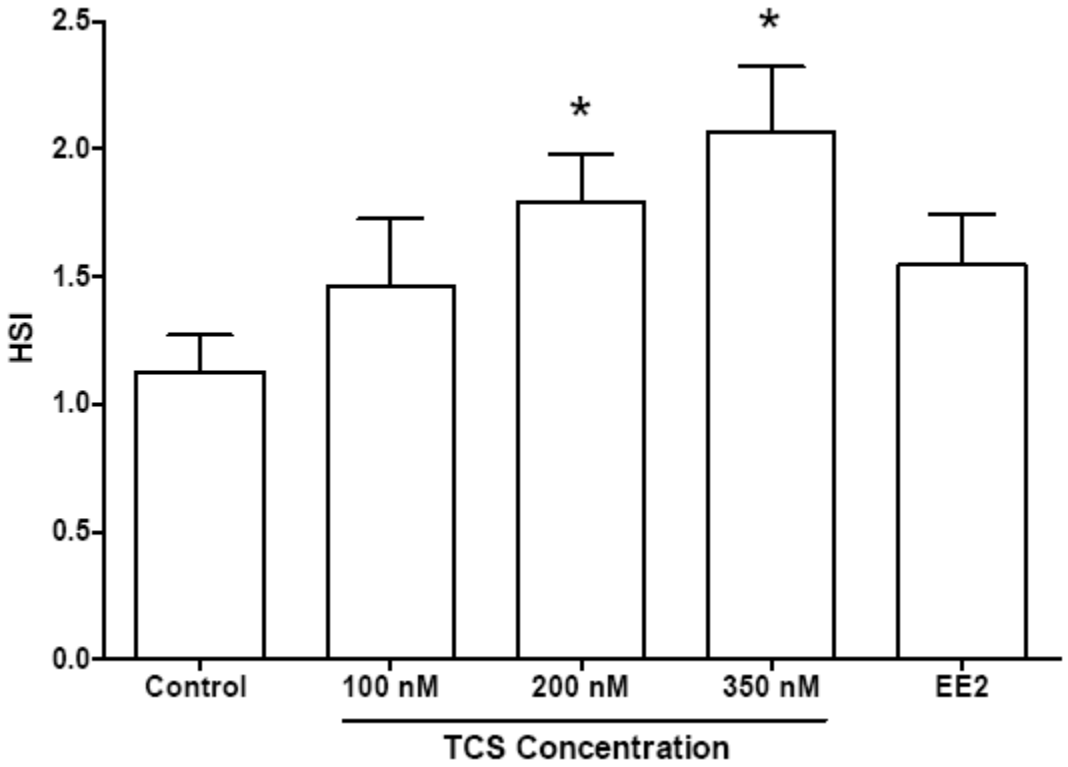

Fig. 5. Changes over the 35 day exposure period in HSI of the fish in TCS static renewal exposure treatment: Concentration refers to Triclosan (TCS), except for the positive control group 17α-ethinylestradiol (EE2). The solvent ethanol has been labeled as "Control." Values reported are means + standard error. An asterisk (*) indicates values significantly different from the solvent control ($p < 0.05$).

GENERAL DISCUSSION

There is considerable evidence that chemicals introduced into the environment have the capacity to disrupt the endocrine system of both wildlife and humans. Numerous studies have reported adverse effects of these chemicals on development, fecundity and reproductive characteristics of aquatic organisms. The major focus to date has been on environmental estrogens and anti-estrogens. However, androgens have also been detected. Therefore, there is a need for sensitive, effective and reliable biomarkers of exposure in aquatic organisms. Hence, the major objective of this study was to establish the use of various reproductive biomarkers of endocrine disruption in the western mosquitofish, *Gambusia affinis*. We have examined the effects of androgenic endocrine disruptors on anal fin masculinization and the estrogen regulated process of vitellogenin production in female mosquitofish. We also assessed the effects of estrogenic endocrine disruptors on vitellogenin induction and sperm production in male mosquitofish.

A detailed review of the literature indicated that previous studies had investigated the anal fin masculinization in female mosquitofish in response to androgens. However, to date very few studies have investigated a dose-dependent response on the extent of masculinization [40, 45]. From a toxicological perspective, it is important to investigate a dose-dependent response of an endocrine disruptor to obtain an estimate of environmental concentration or potency for better risk assessment. For our first two studies, we used

the static renewal exposure method. In this method, fish were exposed to the test chemical in pulses each time the chemical is re-added. The static renewal method is considered to be as suitable an exposure tool for evaluating the effects of endocrine disruptors as the flow-through method [46, 47]. The results from our methyltestosterone study clearly demonstrate dose-dependent relationships between MT exposure and anal fin masculinization and vitellogenin inhibition. This study also showed that methyltestosterone at a concentration of 1000 ng/L induces a maximum masculinization response after a 5-week exposure and therefore, this concentration can be utilized when using MT as a positive control when investigating other potential androgenic endocrine disruptors using female mosquitofish.

With the establishment of anal fin masculinization and vitellogenin inhibition as two effective biomarkers of androgen exposure, we then proceeded to assess the paradoxical masculinizing capacity of, spironolactone (an anti-androgen in humans). Although the paradoxical masculinizing activity of spironolactone in mosquitofish has been reported [48], a dose-dependent relationship between spironolactone exposure and anal fin masculinization had not been established. Results from our *in vivo* spironolactone exposure study have clearly established a dose-dependent relationship between spironolactone exposure and anal fin masculinization (an androgenic effect). In the same study, we have also shown a dose-dependent inhibition of vitellogenin production (an anti-estrogenic effect). These results also emphasize the importance of investigating the effects of a pharmaceutical with effects that would not have been predicted in an aquatic organism based on its effects in human beings. We do recognize that since this compound has not yet

been detected in the aquatic environment, these results could be regarded as environmentally important at this time. However, these data clearly suggest that there is greater need to investigate the effects of pharmaceuticals, which are being recognized as an emerging class of contaminants, on wildlife. Also, studies of this nature would be useful for better overall environmental risk assessment. Future studies are required to investigate the mechanism of action of this compound.

We then utilized male mosquitofish to assess the endocrine disrupting capacity of a widely used anti-bacterial agent, triclosan. The data from our *in vivo* static renewal exposure study indicate that, at an environmentally relevant concentration, triclosan induces vitellogenin gene expression (an estrogenic effect) and causes a decline in the sperm count (an anti-androgenic effect). A previous study reported vitellogenin induction in male fish (medaka) when exposed to triclosan [46]. However, to the best of our knowledge this is the first study investigating the effect of triclosan exposure on sperm production. These data suggest that triclosan is an endocrine disrupting agent in male mosquitofish. Triclosan has been reported to be environmentally transformed into a persistent compound, methyl-triclosan. Therefore, future experiments should investigate the endocrine-disrupting effects of methyl-triclosan and/or of various mixtures of these two compounds to see whether they exhibit additive or synergistic effects.

Finally, results from our static renewal progesterone exposure study have shown that exposure to progesterone can cause the masculinization of female mosquitofish. However, in these experiments, it was not determined whether progesterone per se was the masculinizing agent or whether it was biotransformed into other androgens capable of

masculinizing female mosquitofish, by either the fish or by microbes in the water. Nevertheless, this preliminary investigation of progesterone exposure to female mosquitofish demonstrated that progesterone, previously shown to be present in water and sediments of a paper mill effluent-dominated stream [44], may likely play a role in the observed masculinization of fish living in the stream.

In conclusion, we have quantified the effects of androgenic and estrogenic endocrine disruptors on various biomarkers in male and female mosquitofish. These results confirm that western mosquitofish, *Gambusia affinis* is a suitable model organism for endocrine disruption studies.

LIST OF GENERAL REFERENCES

1. Maffini, M.V., et al., Endocrine disruptors and reproductive health: the case of bisphenol-A. Mol Cell Endocrinol, 2006. 254-255: p. 179-86.

2. Giwercman, A., et al., Evidence for increasing incidence of abnormalities of the human testis: a review. Environ Health Perspect, 1993. 101 Suppl 2: p. 65-71.

3. Toppari, J., et al., Male reproductive health and environmental xenoestrogens. Environ Health Perspect, 1996. 104 Suppl 4: p. 741-803.

4. Colborn, T., F.S. vom Saal, and A.M. Soto, Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect, 1993. 101(5): p. 378-84.

5. James, W.H., Offspring sex ratios at birth as markers of paternal endocrine disruption. Environ Res, 2006. 100(1): p. 77-85.

6. Larsson, D.G. and L. Forlin, Male-biased sex ratios of fish embryos near a pulp mill: temporary recovery after a short-term shutdown. Environ Health Perspect, 2002. 110(8): p. 739-42.

7. Kavlock, R.J., et al., Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ Health Perspect, 1996. 104 Suppl 4: p. 715-40.

8. Lindhe, O., et al., Irreversible binding and adrenocorticolytic activity of the DDT metabolite 3-methylsulfonyl-DDE examined in tissue-slice culture. Environ Health Perspect, 2001. 109(2): p. 105-10.

9. Baccarelli, A., et al., Immunologic effects of dioxin: new results from Seveso and comparison with other studies. Environ Health Perspect, 2002. 110(12): p. 1169-73.

10. Engel, L.S., et al., Polychlorinated biphenyl levels in peripheral blood and non-Hodgkin's lymphoma: a report from three cohorts. Cancer Res, 2007. 67(11): p. 5545-52.

11. Kang, J.H., F. Kondo, and Y. Katayama, Human exposure to bisphenol A. Toxicology, 2006. 226(2-3): p. 79-89.

12. Calafat, A.M., et al., Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. Environ Health Perspect, 2005. 113(4): p. 391-5.

13. Kuruto-Niwa, R., et al., Measurement of bisphenol A concentrations in human colostrum. Chemosphere, 2007. 66(6): p. 1160-4.

14. Fernandez, M.F., et al., Bisphenol-A and chlorinated derivatives in adipose tissue of women. Reprod Toxicol, 2007.

15. Lang, I.A., et al., Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. Jama, 2008. 300(11): p. 1303-10.

16. Gray, L.E., et al., Effects of environmental antiandrogens on reproductive development in experimental animals. Hum Reprod Update, 2001. 7(3): p. 248-64.

17. Ejaz, S., et al., Endocrine disrupting pesticides: a leading cause of cancer among rural people in Pakistan. Exp Oncol, 2004. 26(2): p. 98-105.

18. Orlando, E.F., et al., Altered development and reproduction in mosquitofish exposed to pulp and paper mill effluent in the Fenholloway River, Florida USA. Aquat Toxicol, 2007.

19. Orn, S., et al., Male-biased sex ratios and vitellogenin induction in zebrafish exposed to effluent water from a Swedish pulp mill. Arch Environ Contam Toxicol, 2006. 51(3): p. 445-51.

20. Parrott, J.L., et al., Changes in growth and secondary sex characteristics of fathead minnows exposed to bleached sulfite mill effluent. Environ Toxicol Chem, 2003. 22(12): p. 2908-15.

21. Tyler, C.R., S. Jobling, and J.P. Sumpter, Endocrine disruption in wildlife: a critical review of the evidence. Crit Rev Toxicol, 1998. 28(4): p. 319-61.

22. Liney, K.E., et al., Health effects in fish of long-term exposure to effluents from wastewater treatment works. Environ Health Perspect, 2006. 114 Suppl 1: p. 81-9.

23. Orlando, E.F., et al., Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. Environ Health Perspect, 2004. 112(3): p. 353-8.

24. Larsson, D.G., C. de Pedro, and N. Paxeus, Effluent from drug manufactures contains extremely high levels of pharmaceuticals. J Hazard Mater, 2007. 148(3): p. 751-5.

25. Goksoyr, A., Endocrine disruptors in the marine environment: mechanisms of toxicity and their influence on reproductive processes in fish. J Toxicol Environ Health A, 2006. 69(1-2): p. 175-84.

26. Biological markers in environmental health research. Committee on Biological Markers of the National Research Council. Environ Health Perspect, 1987. 74: p. 3-9.

27. Matty, A.J., Fish Endocrinology. 1985, Portland, OR: Timber Press.

28. Angus, R.A., et al., Effects of 17alpha-ethynylestradiol on sexual development of male western mosquitofish (Gambusia affinis). Comp Biochem Physiol C Toxicol Pharmacol, 2005. 140(3-4): p. 330-9.

29. Arukwe, A., F.R. Knudsen, and A. Goksoyr, Fish zona radiata (eggshell) protein: a sensitive biomarker for environmental estrogens. Environ Health Perspect, 1997. 105(4): p. 418-22.

30. Howell , B.D., Bortone SA Abnormal expression of secondary sex characters in a population of mosquitofish, Gambusia affinis holbrooki: evidence for environmentally-induced masqulinization. Copeia, 1980: p. 676-681.

31. Jensen, K.M., et al., Effects of the feedlot contaminant 17alpha-trenbolone on reproductive endocrinology of the fathead minnow. Environ Sci Technol, 2006. 40(9): p. 3112-7.

32. Ankley, G.T., et al., Effects of the androgenic growth promoter 17-beta-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environ Toxicol Chem, 2003. 22(6): p. 1350-60.

33. Salierno, J.D. and A.S. Kane, 17alpha-Ethinylestradiol Alters Reproductive Behaviors, Circulating Hormones, and Sexual Morphology in Male Fathead Minnows (Pimephales promelas). Environ Toxicol Chem, 2008: p. 1.

34. Halm, S., et al., Exposure to exogenous 17beta-oestradiol disrupts p450aromB mRNA expression in the brain and gonad of adult fathead minnows (Pimephales promelas). Aquat Toxicol, 2002. 60(3-4): p. 285-99.

35. Toft, G., E. Baatrup, and L.J. Guillette, Jr., Altered social behavior and sexual characteristics in mosquitofish (Gambusia holbrooki) living downstream of a paper mill. Aquat Toxicol, 2004. 70(3): p. 213-22.

36. Toft, G. and L.J. Guillette, Jr., Decreased sperm count and sexual behavior in mosquitofish exposed to water from a pesticide-contaminated lake. Ecotoxicol Environ Saf, 2005. 60(1): p. 15-20.

37. Wibe, A.E., G. Rosenqvist, and B.M. Jenssen, Disruption of male reproductive behavior in threespine stickleback Gasterosteus aculeatus exposed to 17 beta-estradiol. Environ Res, 2002. 90(2): p. 136-41.

38. Constantz, G.D., Reproductive biology of poeciliid fishes, ed. J.r. G.K. Meffe and F.F. Snelson. 1989, Englewood Cliffs, NJ.: Prentice Hall. 33-50.

39. Peden, A.E., The function of gonopodial parts and behavioral pattern during copulation by Gambusia (Poecillidae). Can J Zool, 1972. 50: p. 955-968.

40. Turner, C.L., A quantitative study of the effects of different concentrations of ethynltestosterone and methyl testosterone in the production of gonopodia in females of Gambusia affinis. Physiol Zool, 1942. 15: p. 263-234.

41. Angus, R.A., et al., Gonopodium development in normal male and 11-ketotestosterone-treated female mosquitofish (Gambusia affinis): a quantitative study using computer image analysis. Gen Comp Endocrinol, 2001. 123(2): p. 222-34.

42. Melvin, P., Sperm Production and Vitellogenesis as biomarkers of endocrine disruption in the male western mosquitofish, Gambusia affinis, in Biology. 2007, University of Alabama at Birmingham: Birmingham.

43. Carson, J., et al., Naturally Occurring Progesterone in Loblolly Pine: a Major Steroid Precursor of Environmental Androgens. Environ Toxicol Chem, 2008: p. 1.

44. Jenkins, R.L., et al., Androstenedione and progesterone in the sediment of a river receiving paper mill effluent. Toxicol Sci, 2003. 73(1): p. 53-9.

45. Turner, C.L., Morphogenesis of the gonopodium in Gambusia affinis affinis. J.Morphol, 1941. 69: p. 161-185.

46. Ishibashi, H., et al., Effects of triclosan on the early life stages and reproduction of medaka Oryzias latipes and induction of hepatic vitellogenin. Aquat Toxicol, 2004. 67(2): p. 167-79.

47. Seki, M., et al., Effect of ethinylestradiol on the reproduction and induction of vitellogenin and testis-ova in medaka (Oryzias latipes). Environ Toxicol Chem, 2002. 21(8): p. 1692-8.

48. Howell, W.M., R.N. Hunsinger, and P.D. Blanchard, Paradoxical masculinization of female western mosquitofish during exposure to spironolactone. Progressive Fish-Culturist, 1994. 56: p. 51-55.

APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: February 20, 2009

TO: Robert A. Angus, Ph.D.
CH-378A 1170
FAX: 975-6097

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Endocrine Disruption and Population Studies with Mosquitofish
Sponsor: Internal
Animal Project Number: 090208346

On February 20, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Fish	A	450

Animal use is scheduled for review one year from February 2009. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 090208346 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
1670 University Boulevard
205.934.7692
FAX 205.934.1188

Mailing Address:
VH B10
1530 3RD AVE S
BIRMINGHAM AL 35294-0019