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ENDOCRINE-DISRUPTING EFFECTS OF SPIRONOLACTONE IN FEMALE WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS*

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Abstract—The discovery of pharmaceuticals in effluent from wastewater treatment plants and drug manufacturing facilities and in receiving waters has raised environmental concern. Because these compounds are ending up in the environment, it is important to investigate the effects of these compounds on wildlife as well as humans. The present study used a fish model to investigate the endocrine-disrupting effects of spironolactone (SPL), an aldosterone antagonist used as a diuretic, but which also exhibits antiandrogenic effects in humans. A dose–response study measured the effects of SPL on anal fin ray elongation, an androgen-dependent secondary sex trait, and expression of the vitellogenin gene, an estrogen-dependent trait, in female western mosquitofish, *Gambusia affinis*. Fish were exposed to SPL in the water for 35 d at four nominal concentrations: 10, 100, 250, and 500 nM (4.2, 41.7, 104.1, and 208.3 μg/L, respectively) via the static renewal method. Masculinization of females, as evidenced by development of an elongated and modified anal fin, was observed in the fish exposed to the three highest concentrations. Anal fin elongation was observed in the group exposed to the lowest SPL concentration, but without the development of a tip apparatus. These results confirm the results of a preliminary study that, in contrast to antiandrogenic effects seen in humans, SPL has androgenic and/or antiestrogenic activity in a fish. Environ. Toxicol. Chem. 2011;30:1376–1382. © 2011 SETAC

Keywords—Endocrine disruptor Androgen Antiandrogen Masculinization Diuretic

INTRODUCTION

Numerous chemicals introduced into the aquatic environment by humans have the capacity to act as endocrine disruptors in both humans and wildlife [1]. Recently, pharmaceuticals have been recognized as an important class of emerging contaminants [2]. Sources of environmental pharmaceuticals include effluents from public wastewater treatment plants [3], hospitals [4], drug manufacturing facilities [5], and landfill leachates [6]. Pharmaceuticals, by design, have specific biological activities at low concentrations in living organisms, usually far below toxic concentrations [7]. After ingestion by humans, 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 compounds by the activity of microbes in a wastewater treatment plant [8]. Wastewater treatment technologies currently in use remove pharmaceuticals and endocrine disruptors with efficiencies varying from low to >90% [9]. Alarmingly, treatment of wastewater from pharmaceutical manufacturing plants does not always appear to effectively remove their products from the effluent. 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 [5].

A veritable drugstore of pharmaceuticals has been detected in the aquatic environment; these include antiinflammatory drugs, beta blockers, sympathetomimetics, antiepileptics, lipid regulators, and antibiotics [10]. Despite the ubiquity of these compounds in our surface waters, a serious gap exists in our knowledge of the effects they are exerting on wildlife. From the few studies done to date, recognized effects include the feminization of male fish exposed to effluent from wastewater treatment plants due to the presence of various estrogens including 17α-ethynylestradiol, a synthetic estrogen widely used as a human contraceptive [11,12]. The rapid decline of vulture populations in India and Pakistan has been attributed to the consumption of carcasses of livestock treated with the nonsteroidal antiinflammatory drug, diclofenac [13–15]. Laboratory studies have investigated the effects of drugs such as fluoxetine [16], clofibric acid [17], and ibuprofen [18] on aquatic organisms. However, the endocrine-disrupting potential in aquatic organisms has not been investigated for most drugs. It is therefore important to understand the effects of these drugs on fish and other aquatic organisms if we are to make realistic environmental risk assessments. Data from such studies would also help us to determine whether these drugs elicit similar effects in aquatic organisms as those observed in humans.

Spironolactone (SPL) is a 17-lactone drug that acts as an aldosterone receptor antagonist. It is commonly used as a diuretic in the management of hypertension and hyperaldosteronism. It also exhibits antiandrogenic effects and for this reason is used to treat hirsutism, acne, and hair loss in women with the male pattern baldness gene and in hormone therapy of male-to-female transsexuals (http://www.nlm.nih.gov/medlineplus/druginfo/meds/a682627.html). In contrast to its effects in humans, it has been reported that female western mosquitofish (*Gambusia affinis*) became masculinized after exposure to SPL [19]. However, the previous study by Howell et al. [19] was preliminary; it did not investigate the endocrine-disrupting capacity of this compound in fish over a range of exposure concentrations, it used a single biomarker and did not quantify the changes observed. The objective of the present study was to determine whether the paradoxical results of Howell et al. [19]

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are repeatable and to quantify the concentration-dependent effects of SPL exposure on two biomarkers of endocrine disruption in female western mosquitofish, *G. affinis*: anal fin masculinization and inhibition of vitellogenin (VTG) gene expression.

Gambusia affinis is a member of the livebearing family Poeciliidae and is native to the southeastern United States. It has been a popular model species for the study of endocrine-disrupting compounds in aquatic environments because of its widespread geographic distribution, wide environmental tolerances, and ease of use in the laboratory. Mosquitofish are sexually dimorphic. A mature male possesses an elongated and modified anal fin, the 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 only. However, if a female is exposed to an androgen the anal fin transforms into a gonopodium-like structure [20,21]. Because of its sensitivity to androgens and visibility, the presence of a modified anal fin on a female mosquitofish is regarded as a useful morphological biomarker of androgen exposure. Additionally, previous studies carried out in our laboratory and others have shown that inhibition of VTG gene expression is another biomarker of androgen exposure in female mosquitofish [22,23]. In the present study, female mosquitofish were exposed to various concentrations of SPL and the effects on anal fin masculinization and VTG gene expression were evaluated.

MATERIALS AND METHODS

Animals

Mature female western mosquitofish, *G. affinis,* were collected from Thomas Spring Pond, a spring-fed pond in Bessemer, Alabama, USA. The pond is in a residential neighborhood with no point sources of pollution in the vicinity. The fact that it is spring-fed minimizes the amount of potential pollution from nonpoint surface runoff. Fish from this pond have been used for numerous studies in this laboratory and we have never observed biomarkers of endocrine disruption in control fish. After collection, the fish were returned to the University of Alabama at Birmingham, placed in filtered, aerated aquaria, and allowed to acclimate in the laboratory for two weeks. The acclimated fish were then randomly assigned to the treatment levels ($n = 10$ per treatment). The fish were not pregnant, as evidenced by lack of embryos when they were dissected at the end of the experiment.

Spironolactone treatment

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), which contain 1 L with an additional 2 cm air space. The water was dechlorinated (with sodium metabisulfite) Birmingham, AL tap water with 0.25 g/L Instant Ocean artificial sea salt (Spectrum Brands) added to provide trace minerals and buffering capacity. Water temperature was $24 \pm 2^{\circ}$C. Fish were maintained under a 14:10 light:-dark cycle. The water was changed every other day and SPL readded. Water pH, dissolved oxygen, ammonia, and nitrate were monitored once a week (pH 7 ± 0.5 units, DO >5 mg/L, ammonia and nitrate <0.25 mg/L).

A 1 mM stock solution was made by dissolving SPL (Sigma Chemicals) in 5 ml of dehydrated absolute ethanol (Pharmaco) and sufficient propylene glycol (Fisher Chemicals) to obtain a final volume of 100 ml. The stock solution was made at the beginning of the study and was stored at 4°C. Previous studies have shown that spironolactone is stable in aqueous solution, degrading by less than 2% over a period of 90 d [24]. Final working concentrations of 10, 100, 250, and 500 nM (4.2, 41.7, 104.1, and 208.3 μg/L, respectively) were prepared by adding an appropriate volume of the stock solution to a beaker and bringing the volume up to 1 L. These concentrations were selected on the basis of a preliminary toxicity range-finding study. A separate stock solution of 1 mM methyltestosterone (MT) (Steraloids) was made by dissolving MT in absolute ethanol and propylene glycol. A concentration of 3.32 nM (1.0 μg/L) 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 (350 μL/L). Fish were fed twice a day with Silver Cup granulated trout starter food (Nelson & Sons). Food was stored at 4°C. The experimental protocols used in the present study were approved by the University of Alabama at Birmingham's Institutional Animal Use and Care Committee.

Morphological measurements

On day 0, before the start of the exposure, fish were anesthetized by brief immersion in 300 mg/L MS-222 (tricaine methanesulfonate, Argent Labs) and their standard length and weight were recorded. Standard length was measured from the tip of the snout to the end of the caudal peduncle with a dial calipers and recorded to the nearest 0.2 mm. Fish were pat-dried with tissue paper and weighed on a digital scale to the nearest 0.1 mg. Mean weights and standard lengths of the different treatment groups at the beginning of the experiment are shown in Table 1. Fulton condition factors were calculated for each fish using the formula $K = 100 \times \text{weight/length}^3$ where weight is in grams and length is in centimeters. Because length was measured as standard length, condition factors were converted to those which would have been obtained using total length using the formula $K_{TL} = r^3 \times K_{SL}$ where r is the ratio of standard length to total length (which was determined in *G. affinis* by measuring $n = 4$ photographs) [25].

The anal fins were photographed with a Polaroid DMC Ie digital camera mounted on a Leica MZ6 stereomicroscope (Leica Microsystems). Images (1,600 × 1,200 pixels) captured by the camera were transferred to a computer running Adobe Photoshop (Adobe Systems) 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). 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 (35 days). Anal fin ray elongation was quantified as the ratio of length of ray 4, which

Table 1. Mean weights, standard lengths, and condition factors (± standard error) of the fish in the various treatment groups at the beginning of the experiment

Treatment (nM)	Weight (g)	Standard length (mm)	Condition factor
Control	0.496 ± 0.028	27.750 ± 0.287	1.344 ± 0.058
SPL10	0.315 ± 0.036	23.372 ± 1.036	1.405 ± 0.052
SPL100	0.347 ± 0.031	24.742 ± 0.730	1.306 ± 0.029
SPL250	0.286 ± 0.030	23.236 ± 0.821	1.294 ± 0.045
SPL500	0.319 ± 0.047	23.572 ± 0.957	1.349 ± 0.055
MT3.32	0.378 ± 0.045	24.706 ± 0.787	1.409 ± 0.065

SPL = spironolactone, MT = methyltestosterone (positive control).

elongates during gonopodial development, to that of ray 6, which does not elongate [21]. After the completion of the 35-d exposure period, standard length and the mass of the fish were again measured.

Hepatic vitellogenin

We collected livers from the female mosquitofish from the SPL and control treatments at the end of the 35-d exposure period, isolated the mRNA, and determined VTG gene expression relative to 18S ribosomal RNA using real-time polymerase chain reaction (PCR). Total RNA was extracted from frozen liver tissue using the Trizol protocol as described by the manufacturer (Invitrogen). First-strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The cDNA was amplified in an Opticon Continous Fluorescence Detector (MJ Research) using IQ 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 the *Oncorhynchus mykiss* mRNA sequence (GenBank Accession number: AF243428) because the 18S mRNA sequence for *G. affinis* has not been determined. The sequences of the primers are shown in Raut and Angus [26]. The assay had been validated and the amplification efficiency of both the primer sets determined previously [26].

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 [27].

Statistical methods

All of the summary statistics are expressed as mean ± standard error. 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 log transformation successfully renders the data suitable for analysis using parametric statistical procedures [21]. Changes in 4:6 anal fin ratios over time were analyzed using a repeated measures analysis of variance (RMANOVA). Paired *t* tests were used to compare means at each week with the time 0 mean to determine when significant differences first occurred. Because anal fin elongation was hypothesized, those tests were done one-tailed. Vitellogenin mRNA expression levels were compared between treatment groups and the control using the nonparametric Kruskal–Wallis test, followed by Dunn's tests to compare each of the treatments with the control. Differences between the group means of other variables (i.e., length, weight and condition factor) were analyzed by ANOVA using log-transformed data, followed by Dunnett's post-hoc tests to compare the means of each of the treatment groups to that of the control. For all statistical tests, the cutoff for significance was $p \leq 0.05$.

RESULTS

Fin ray elongation

Figure 1 shows the 4:6 anal fin ray elongation ratios of SPL-exposed female mosquitofish at weekly intervals over the 35-d exposure period. The time course of the change in 4:6 ratio differed significantly between treatments (RMANOVA, $F = 14.93$, $p < 0.001$) with a linear model explaining more

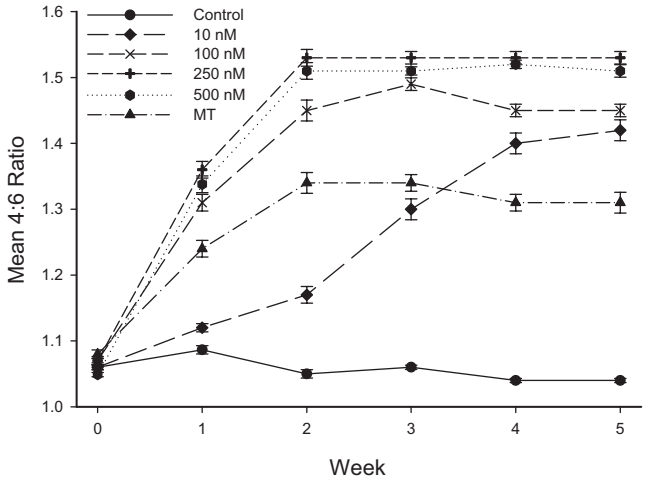

Fig. 1. Changes in anal fin length ratios over time. Female mosquitofish were exposed to different concentrations of spironolactone (10, 100, 250, and 500 nM) for 35 d. Control = vehicle negative control; MT = methyltestosterone positive control (3.32 nM). Anal fin rays 4 and 6 were measured weekly and elongation quantified as the 4:6 length ratio. Data are presented as mean ± standard error ($n = 10$ per treatment). At the end of the 35-d exposure, the means of all treatment groups were significantly greater than that of the negative control group.

of the variance than a polynomial model of any order. After one week of exposure the mean 4:6 ratios of all the treatment groups differed significantly from their respective means at time zero (paired *t* tests; for the 10 nM spironolactone treatment, $p = 0.023$; for the 100, 250, and 500 nM spironolactone treatments, $p < 0.001$; for the MT treatment, $p = 0.004$). From weeks 2 through 5, all of the mean 4:6 ratios were significantly greater than their time 0 means (paired *t*-tests, $p < 0.001$). Although the mean 4:6 ratios of the 100, 250, and 500 nM treatment groups increased rapidly during the first two weeks of the experiment, growth of anal ray 4 ceased at about that time in both groups and no further increase in the 4:6 ratio was observed over the next three weeks of the study. In contrast, although ray 4 grew more slowly in the 10 nM exposure group, growth continued throughout the 35 d of the study. Figure 2 shows elongated anal fins from representative females exposed to different concentrations of SPL.

Hepatic vitellogenin gene expression

The hepatic VTG mRNA expression tended to decrease with increasing SPL concentration. The mean VTG mRNA expression level was significantly less than that of the solvent control in fish exposed to SPL at 250 and 500 nM (Dunn's tests, $p < 0.05$, Fig. 3). In fact, mRNA levels in those groups were barely detectable.

Morphological variables

Changes in the mean mass, length, and condition factor of the treatment groups over a period of 35 d are shown in Figure 4. Fish in the 500 nM SPL and MT treatment groups gained significantly less mass over the 35 d of the experiment as compared to the solvent control (Dunnett's tests, $p = 0.010$ and $p = 0.004$, respectively, power = 0.906). Most groups did not grow significantly in length during the course of the experiment. The exception was the 100 nM SPL group. The mean change in length was significantly greater than that of the control (Dunnett's test, $p = 0.036$, power = 0.554). This may imply that a low concentration of spironolactone stimulates growth, or it may represent a type I error. Replication of the

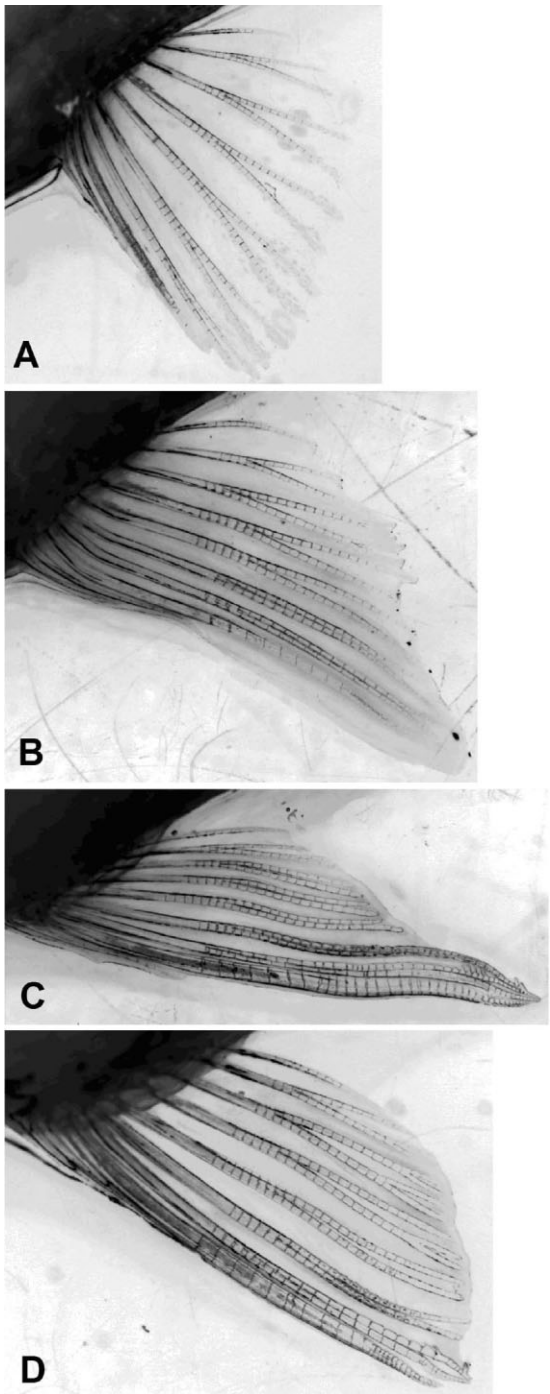

Fig. 2. *Gambusia affinis* anal fins. Anal fins of representative female mosquitofish (**A**) control, and exposed for 35 d to (**B**) 10 nM spironolactone, (**C**) 250 nM spironolactone and (**D**) 3.32 nM methyltestosterone.

experiment is needed to distinguish between these possibilities. Two of the treatment groups, 100 nM SPL and MT, showed significant changes in condition factor during the experiment compared to the control group (Dunnett's tests, $p = 0.004$ and 0.001, respectively, power = 0.969). Both showed increased condition, whereas the condition factor of the control group declined during the present study.

DISCUSSION

In the present study we investigated the paradoxical masculinization of female mosquitofish exposed to SPL. The results

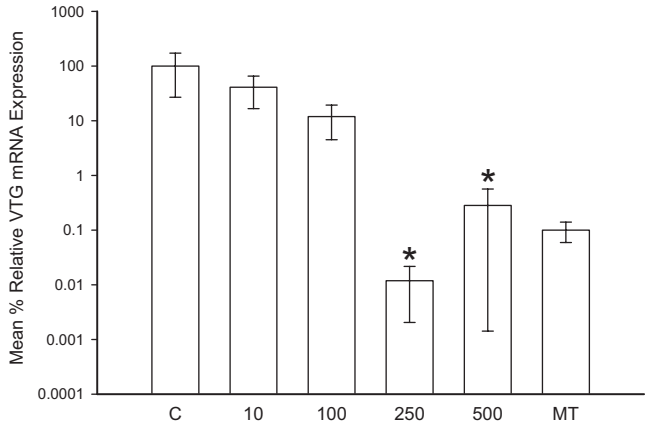

Fig. 3. Vitellogenin expression in spironolactone treatment groups: Female mosquitofish were exposed to different concentrations of spironolactone for 35 d (see legend of Fig. 1 for concentrations) and hepatic vitellogenin mRNA expression was quantified using real-time PCR. It is presented here as percent expression relative to the 18S rRNA housekeeping gene. Data are presented as mean ± standard error ($n \geq 5$ for each group). Asterisk (*) = mean significantly different than that of the control ($p \leq 0.05$); C = control; MT = methyltestosterone.

confirm and expand on those of an earlier study that first described this phenomenon [19]. In contrast to humans, where SPL has antiandrogenic effects, SPL has androgenic and possibly antiestrogenic effects in mosquitofish.

Anal fin masculinization, as evidenced by significant anal ray elongation, was observed in all the treatment groups (Fig. 1). Previous studies have characterized the development of a gonopodium in mosquitofish in response to androgen exposure [20,21,28]. 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, no further growth of the anal fin rays or addition of segments occurs. 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), elongation occurred, but less rapidly than the higher concentrations, and without any development of hooks and spines during the 35-d period of the present 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 [20] 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 genes 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. As a result, anal fins masculinized by exposure to MT show little anal ray elongation, but well-developed tip apparatus structures (Fig. 2D). Similarly, the higher concentrations of SPL (100, 250, and 500 nM) all induced a tip apparatus and fin elongation ceased well short of the extent seen in a normal gonopodium (4:6 length ratio of ≈2.7, Angus, R.A., unpublished data). However, in the lowest SPL concentration elongation appeared

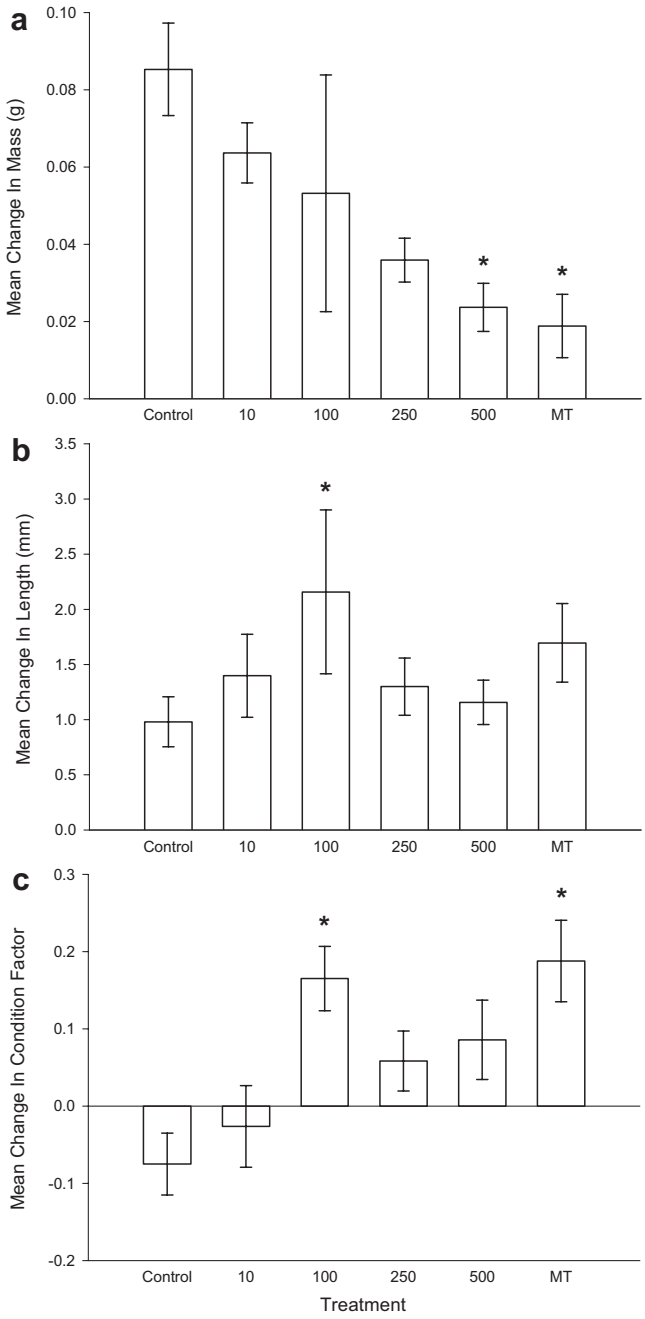

Fig. 4. Changes in mass (**a**), length (**b**), and condition factor (**c**) of fish over the 35-d exposure period (see legend of Fig. 1 for concentrations). Total body weights and standard lengths were measured at the beginning and end of the experiment and change in mass, length, and condition factor were calculated as the differences. Data are presented as mean + standard error ($n = 10$ per group). Asterisk (*) = mean significantly different than that of the control ($p \leq 0.05$); MT = methyltestosterone.

to still be continuing at the end of the 35-d exposure period and differentiation of the tip apparatus had not yet begun.

A recent study has shown that, in *G. affinis*, the androgen receptor (AR) isoforms ARα and ARβ are predominantly expressed in the distal region of the outgrowing anal fin rays [29]. These receptors have been shown to regulate gonopodial development through the sonic hedgehog pathway. Because 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 dose-dependent up-regulation of AR expression occurs by these signaling pathways at higher androgen concentrations. Because the lowest exposure concentration of SPL did not induce the later phase of differentiation, this concentration may not have been 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. Further studies are necessary to characterize the dose-dependent effect of SPL on AR expression and its correlation with the signaling pathways in response to an androgen or androgen-like compounds in female mosquitofish.

The observation that anal fin rays in female mosquitofish elongated when they were exposed to SPL indicates that they have been masculinized (they express an androgen-dependent trait normally only seen in mature males). In previous studies it has been observed that compounds that masculinize female mosquitofish also tend to inhibit estrogen-dependent processes [22,23]. Therefore, not unexpectedly, SPL also showed an apparent antiestrogenic effect in mosquitofish. Vitellogenin mRNA expression, which is normally induced by estrogen, was significantly inhibited, as determined by quantitative reverse-transcription PCR (q-RTPCR), in the same treatment groups (250 and 500 nM) that showed anal fin masculinization (Fig. 3).

A few other studies have investigated effects of SPL on fish model systems. McCormick et al. [30] investigated the effects of spironolactone on mineralocorticoid functions in Atlantic salmon. Churchill et al. [31] investigated its effects on renal function in spiny dogfish shark (*Squalus acanthias*). However, none of the studies to date have reported masculinization effects in response to SPL treatment in fish. Spironolactone therapy in humans has used its effect either as a diuretic (aldosterone antagonist) or as an antiandrogen. Sexual side effects, such as gynecomastia in men (possibly an antiandrogenic effect), have been reported [32]. Therefore, the masculinization results in mosquitofish obtained in a previous study by Howell et al. [19] and the present study are somewhat paradoxical. It has been shown that 11β-substituted spirolactones are potent human AR agonists in vitro [33]. It is possible that, whereas SPL binds to androgen receptors in humans and does not induce a response (antagonist), it binds to androgen receptors in fish and does induce a response (agonist). Furthermore, synthetic derivatives of SPL have been shown to inhibit the activity of 17β-hydroxysteroid dehydrogenase (HSD) [34], a steroidogenic enzyme. If SPL inhibits 17β-HSD, this would cause a buildup of androstenedione, a naturally occurring precursor to testosterone and estradiol in the teleost steroid synthesis pathway. Androstenedione, especially if present in abnormally high amounts, could bind to AR sufficiently to activate a response and cause masculinization of female mosquitofish. Previous studies have demonstrated the ability of androstenedione to activate AR [35] and also to masculinize female mosquitofish [36]. However, studies are required to determine if SPL inhibits 17β-HSD.

Vitellogenin gene inhibition has been reported in many fish model systems on exposure to aromatizable androgens such as MT [23,37,38] and nonaromatizable androgens such as trenbolone acetate [39]. On the other hand, VTG gene expression is unaffected in female fish exposed to antiandrogens, e.g., mummichog (*Fundulus heteroclitus*) exposed to cyproterone acetate [37] and medaka (*Oryzias latipes*) exposed to flutamide [40]. In the present study we noted a trend of decreasing hepatic VTG mRNA expression in fish exposed to increasing concentrations of SPL, with an almost complete shutdown of VTG mRNA

expression in the two highest SPL treatment groups. These results are similar to an earlier study on MT in which we correlated dose-dependent masculinization and vitellogenin inhibition [41]. Many different mechanisms could be responsible for the reduction in vitellogenin production. First, it is possible that estradiol concentrations are reduced due to an inhibitory effect exerted by SPL on 17β-HSD, as discussed earlier. Alternatively, SPL could act as an estrogen receptor antagonist. The findings of the present study suggest that SPL could exert a dual mode of action—as an androgen agonist inducing masculinization and also as an antiestrogen inhibiting VTG gene expression. Future studies are warranted to investigate the mechanisms by which SPL exerts its effects.

Drugs, both human and veterinary, are increasingly being detected in environmental samples [42,43]. Presently, little is known about the ecological effects of most of these chemicals [44]. There is reason to be concerned. Pharmaceuticals are designed to affect specific targets in the species for which they were developed. Because all vertebrate species are related at some level, targets tend not to be exclusive to the intended species. For example, fish share about 65 to 75% genetic homology with humans at over a thousand different drug receptors [45]. Thus, drugs intended for treating humans may potentially affect fish as well. In addition, because drugs are designed to be pharmacologically active at far lower concentrations than their toxic levels [7], traditional toxicity tests will not be effective in revealing potential effects on the health and reproductive fitness of wildlife at typical environmental levels [44,46]. Helpfully, the mode of action in mammals is known for most pharmaceuticals and therapeutic doses have been established. Mammalian acute toxicity to therapeutic efficacy ratio values are good predictors of acute to chronic effect ratios in fish and can be used to identify pharmaceuticals that are likely to affect fish at environmental concentrations, especially if the chronic response (e.g., expression of a biomarker) used in acute to chronic calculation is plausibly linked to the therapeutic mode of action of a particular pharmaceutical [7]. This has led to the proposed use of adverse outcome pathways [47]. An adverse outcome pathway links the molecular initiating event caused by a pharmaceutical to an adverse outcome at a biological level of organization relevant to risk assessment. Adverse outcome pathways facilitate the use of molecular or biochemical biomarkers for predicting the impacts of environmental chemicals on individuals and populations. Results of the present study provide another example that drugs intended for humans can affect other vertebrates, in this case a fish, at low concentrations. Knowledge of the mode of action, namely, binding to the cytosolic androgen receptor [48], leads to the prediction of possible endocrine-disrupting effects on vertebrates. But, in the case of fish, knowing the mode of action does not necessarily lead to an accurate prediction of the type of effect (antiandrogenic in humans but androgenic in fish).

CONCLUSION

The results of the present study indicate that SPL, which has antiandrogenic effects in humans, affects fish differently. It induces an androgen-dependent trait (gonopodium) and inhibits an estrogen-dependent trait (vitellogenin gene expression) in female *G. affinis*. Further studies are necessary to elucidate the molecular mechanisms responsible for these effects and the reasons why fish respond to SPL differently than humans. Finally, based on these findings we reiterate previous recommendations that, for adequate assessment of the aquatic environmental risk of pharmaceuticals, comprehensive studies on aquatic organisms should be implemented [45,47].

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