Investigating the impact of chronic atrazine exposure on sexual development in zebrafish

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ABSTRACT

Atrazine (ATZ) is a selective triazine herbicide used primarily for pre-emergent weed control in corn, sorghum and sugar cane production. It is one of the most widely used herbicides in North America. Some research published over the last decade suggests that chronic exposure to environmentally relevant ATZ concentrations can adversely impact gonadal development and/or sexual differentiation in amphibians and fish, while other studies report no effect, or moderate effects. As a result, contrasting conclusions have been published regarding the potential effects of the herbicide ATZ on aquatic species. Two near-identical four-month studies in 2009 (Study I) and 2010 (Study II), were performed investigating the potential for chronic ATZ exposure to affect zebrafish (*Danio rerio*) sexual development and differentiation. Zebrafish, were chronically exposed to 0, 0.1, 1, 10 µM ATZ or 1 nM 17ß-estradiol (E2). Fish were histologically examined to assign gender and to evaluate potential impacts of E2 or ATZ on gonadal development. Exposure to E2 consistently resulted in a significantly higher proportion of female fish to normal male fish when compared to unexposed fish (both studies). In both studies, ATZ exposure did not significantly influence the percentage of female or male fish when compared to unexposed fish. A greater percentage of abnormally developed male fish and fish lacking differentiated gonadal tissue was observed in Study II E2 exposures but not in ATZ exposures. Together, these studies indicate that long-term exposure to ATZ at or above environmentally relevant concentrations does not significantly impact zebrafish gonadal development or sexual differentiation.

Keywords: *atrazine; zebrafish; chronic exposure; sexual development; sex differentiation*

INTRODUCTION

Atrazine (ATZ) is a selective triazine herbicide widely used in the United States for annual broadleaf control. Recently, application of ATZ in the United States was estimated to be between 66 and 82 million pounds per year (Thelin and Stone, 2010). Application is primarily to corn, sorghum and sugarcane fields (99%), with minor, non-agricultural uses such as, golf courses, residential lawns, grasses grown for seed, landscape maintenance sites, forests, and recreational areas (1%) (USEPA, 2011a). Atrazine concentrations in surface waters are generally in the low parts-per-billion range (<10-20 µg/L), although, in some cases, higher short-term (peak) concentrations have been measured (>100 µg/L) in adjacent agricultural streams following significant runoff events (Huber, 1993; Solomon et al., 2008; Solomon, 1996). Over 150 site-years of monitoring program data for atrazine in Midwestern U.S. watersheds representing worst-case potential ATZ runoff concentrations (upper 20th percentile) indicate that for small watersheds (typically 9 to 40 sq. mi.), the median duration of peaks greater than 15 µg/L is 2 days (derived from data shown in (USEPA, 2011b). It's widespread use and physical properties can potentially result in exposure of ATZ to non-target individuals and ecosystems, including aquatic species after applications. Contrasting reports from researchers exist concerning the herbicide's effects to non-target species, even at high exposures.

Recent research has focused on the potential for ATZ to act as an endocrine disruptor in amphibians and fish. Studies have reported that environmentally relevant concentrations of atrazine ($\geq$ 0.1 µg/L to 25 µg/L) can change gonadal development in the amphibian test model *Xenopus laevis* (Hayes et al., 2006) and the native North American leopard frog species, *Rana pipiens* (Hayes et al., 2002a). Contrasting publications have reported that exposure to similar and higher concentrations of ATZ (0.1 -200 µg/L) do not influence gonadal development in aquatic species including studies of *X. laevis* and other North American and Australian amphibian species (Coady et al., 2004; Kloas et al., 2009; LaFiandra et al., 2008; Oka et al., 2008; Spolyarich et al., 2010; Storrs and Semlitsch, 2008). An independent review of the literature by the Environmental Protection Agency (EPA) Scientific Advisory Panel in 2007 concluded that there was sufficient evidence to refute the hypothesis that ATZ adversely affect gonadal development in *X. laevis* (USEPA, 2007; 2008)

In vivo experiments with fish have also produced varying results. Spano et al. reported adult male goldfish exposed to 1000 µg/L formulated atrazine had different plasma androgen and estradiol levels than their control counterparts; while, no effect on gonad sex steroids, gonadal somatic index (GSI) or vitellogenin concentration was observed for male goldfish at 100 or 1000 µg/L and no

significant hormone changes were observed in the plasma of males exposed to 100 µg/L or females (both concentrations) (Spanò et al., 2004). Several reproductive exposure studies have been published. Bringolf et al. found that a 21-day exposure of fathead minnows to ATZ concentrations of 5 and 50 µg/L did not significantly affect fecundity, fertilization success, gonadosomatic index, or vitellogenin levels (Bringolf et al., 2004). Another study reported a decrease in egg production by fathead minnow following 17-20 days of exposure to 0.5 to 50 µg/L attributed by the authors to a reduced number of total spawning events in these treatments and potentially abnormal gonadal histology (Tillitt et al., 2010). No effects however were reported for sex steroid gonadosomatic index, or breeding tubercles. The Tillitt (2010) study conflicts with the results of a third fathead minnow study of a similar design by the USEPA (2005) which reported no statistically significant differences in any of the reproduction parameters measured, including egg production.

Some molecular studies have measured aromatase induction, the enzyme responsible for converting androgens to estrogens, in fish following exposure to ATZ. Of these studies, some have not observed an induction of aromatase, including one with zebrafish (Kazeto et al., 2004) and one with goldfish (Nadzialek et al., 2008). However, a conflicting report in 2008 (Suzawa and Ingraham, 2008) observed a concentration-dependent increase in *Cyp19A1*, a gene responsible for promoting aromatase production found in the zebrafish ovary, but not *Cyp19A2* (expressed in the brain) following three day static exposure to 0.01, 0.1, 1, and 10 µM ATZ. In addition, Suzawa and Ingraham exposed 17 days post fertilization (dpf) zebrafish (*Danio rerio*) to 0.1 , 1, and 10 µM ATZ for 6 months and reported that ATZ induced a concentration-dependent increase in the percentage of females relative to the solvent, dimethyl sulfoxide, control; however, ATZ did not induce "intersex" in zebrafish at any of the treatments tested (Suzawa and Ingraham, 2008). Prior to 2008, no atrazine-associated effects on reproduction (Bringolf et al., 2004; Dionne, 1992; Macek et al., 1976) or sex ratio (Dionne, 1992) were reported in studies with fish.

Interpretation of how ATZ affects aquatic species is compounded by conflicting accounts of ATZ effects reported within the peer-reviewed literature. The objective of the present studies was to evaluate the potential chronic effects of ATZ on zebrafish gonad differentiation and sexual development. Estradiol was used as a positive control to ensure that the wild-type (5D) zebrafish strain utilized was sensitive to estrogenic effects under each study's test conditions. We aimed to control experimental variables and maximize statistical power through replication. The experiments were in part modeled from Suzawa and Ingraham (2008), and similarly zebrafish were exposed from 17 dpf through gonad

maturation. These chronic exposure studies targeted sensitive gonadal developmental windows and continued throughout sexual maturity to investigate the potential for atrazine exposure – 0, 0.1, 1, 10 μM (0, 21.6, 216 2160 μg/L respectively) – to impact gonadal development or sexual differentiation in wild-type zebrafish.

MATERIALS AND METHODS

Fish care and husbandry

Adult wild-type zebrafish, *Danio rerio*, strain tropical (5D) were reared at the Sinnhuber Aquatic Research Laboratory at Oregon State University. Adult fish were housed in 2.8-L polycarbonate tanks filled with reverse osmosis water containing 0.6% Instant Ocean (Aquarium Systems, Inc., Mentor, Ohio), fish water, in a re-circulating system. The water temperature was maintained at 28°C (±1°C) with a pH of 7.2 (±0.4), and fish were kept on a 14-hour light / 10-hour dark photoperiod. Fish were group-spawned and embryos were collected at 2 to 3 hours post fertilization, bleached with ~0.0023% bleach, rinsed with water and incubated in 0.1 % methylene blue in petri dishes until 5 dpf. Embryos were then acclimated in re-circulating fish water system and raised until 17 dpf. At 17 dpf, a total of 400 fish were randomly transferred to either forty 6.0 L black polycarbonate tanks (Study I) or forty 6.0-L stainless steel tanks (Study II). The tank interiors were nested containers that had mesh bottoms and acted as inserts that were used to minimize stress during the movement of fish into static-renewal solutions. The inserts were polycarbonate with nylon mesh (Study I) or stainless steel inserts with stainless steel mesh (Study II) bottoms for the 113 days of exposure (Supplementary Figure 1). Each tank was equipped with an oxygen bubbler in order to maintain optimum dissolved oxygen concentrations. Dissolved oxygen, temperature and pH were measured just prior to water changes. An Exstik® DO600 Dissolved Oxygen Meter (Extech Instruments Corporation, Waltham, MA) was used to measure dissolved oxygen, and a PH370 American Marine pH Meter (Aquatic Eco-Systems, Inc., Apopka, FL) was used to measure pH. Ammonia and nitrite were measured every 3 days throughout the study using colorimetric water test kits (Aquarium Pharmaceuticals, Chalfont, PA). Water quality measurements were only made in negative control replicates to prevent cross-contamination of 17ß-estradiol or atrazine solutions. All zebrafish care met Institutional Animal Care and Use Committee standards. Humane euthanasia was performed when necessary with tricaine mesylate solution (0.48 mg/mL) (w/v).

Chemicals and solution preparations

Technical-grade atrazine was obtained from Syngenta Crop Protection, LLC. (97.6% purity; Batch ID 428162, Design Code G30027H, Greensboro, NC). Technical-grade 17ß-estradiol was purchased from Sigma Aldrich (100 ± 3% purity; Product No. E1024-1G, St. Louis, MO). A 20-mg/L stock solution of ATZ was prepared every three days by dissolving ATZ, without a co-solvent, in fish water (4L). The stock solution was blended for 5 minutes and then stirred overnight. A 1 mg/mL E2 ethanol stock solution was prepared by dissolving 10 mg into 10 mL of 100% ethanol. After the E2 stock solution was stirred for 3 to 4 hours, a working stock solution was prepared in fish water (1-L) and stirred overnight. Working stocks were diluted further in fish water to prepare the final exposure solutions on solution renewal days. A solvent (ethanol) control was not used in this study as ethanol comprised only ≤0.00005% v/v of the final 17ß-estradiol exposure solution, a concentration well below any known developmental effects of ethanol on zebrafish (Reimers et al., 2004).

Acute toxicity study

Ten zebrafish (16 dpf) were randomly distributed to 21 5.0-L tanks with fish water. Starting on 17 dpf, fish were exposed to 0, 0.1, 0.5, 1, 5, 10, and 50 µM (0, 21.6, 108, 216 1080, 2160 and 10,800 µg/L respectively) until 31 dpf. Each treatment included three tank replicates and solutions were freshly prepared and renewed every three days (3-L solution per tank). Fish were observed on a daily basis during the entire 14-day exposure. Mortality and any unusual abnormalities in appearance or behavior of surviving fish were recorded. Fish were fed newly-hatched *Artemia* three times daily.

Two 113-Day chronic toxicity studies (Study I and Study II)

ATZ exposure concentrations for the chronic toxicity studies were selected based on concentrations tested by Suzawa and Ingraham (2008) and the acute toxicity test performed in our laboratory. For each study, ten zebrafish (17 dpf) per tank were randomly distributed to forty 6.0-L tanks containing ~3 L of fish water. Treatments were then designated to all tanks and exposure commenced. Fish were continuously exposed for 113 days to nominal concentrations of 0, 0.1, 1, and 10 µM ATZ (0, 21.6, 216 and 2160 µg/L) or 1 nM E2 (0.27 µg/L). Each treatment included eight replicate tanks and solutions were freshly prepared and renewed every three days. Renewal frequency was based upon the expected relatively rapid uptake and metabolism of E2 under test conditions—despite the relative

stability of ATZ (mean half-life in field water of 159 days (Solomon et al., 2008)). Experimental conditions, such as, pH, temperature and light conditions also favored stability of atrazine (Giddings et al., 2005). Water replacement frequency ensured favorable water quality conditions and tank environment for the health of the fish. The 0 µM ATZ treatment consisted of fish water only and acted as the unexposed control. Mortality and any abnormalities in appearance of surviving fish were observed and recorded daily over the 113-day exposure period. Dead fish were removed and discarded according to animal care protocols or fixed for histological analysis. Fish within each treatment group were fed newly-hatched *Artemia* 1 to 4 times daily and flake Zeigler® Adult Zebrafish Diet (Zeigler Bros, Inc., Gardners, PA) was supplemented 1 to 3 times daily. Feedings were reduced mid-study to twice a day and typically consisted of one *Artemia* feeding and one flake feeding. Starting at exposure day 90 (Study II only) and concluding on exposure day 108, viable embryos (6-78 hours post fertilization) were collected every third day from all treatment tanks, examined using a microscope and euthanized with tricaine solution. The age, number and quality of offspring was estimated and recorded for each tank.

At study termination, all fish were euthanized (tricaine) and wet weight and total body length were recorded. Surgical scissors were used to open the coelomic cavity, permitting fixative to penetrate internal organs. Fish were placed in blind-labeled tissue cassettes and all cassettes were added to 5-L buckets containing 4 L of Dietrich's fixative (≥20 mL of Dietrich's per fish). Fish were fixed for 60 hours under continuous agitation using a magnetic stir plate. For decalcification of bones, fish were transferred to 5-L buckets containing 4 L of Dietrich's fixative and 5% trichloroacetic acid (≥20 mL of Dietrich's and 5% trichloroacetic acid per fish) and the buckets were continuously agitated on a magnetic stir plate for 18 hours. Fish were then rinsed three times with 70% ethanol (20 mL of 70% ethanol per fish per rinse) and the solutions were stirred on a magnetic stir plate for at least 45 minutes for each ethanol rinse. Fish were stored in 70% ethanol until histologic preparation.

Chemical analysis

Chemical concentrations were analyzed, for the 113-day chronic toxicity studies, to verify that fish were exposed to near-nominal concentrations of ATZ or E2. Every three days during solution renewals, samples of freshly-prepared control fish water (38 total), E2 (38 total), and ATZ (114 total) exposure solutions were collected directly from 5 gallon Nalgene carboys containing exposure solutions immediately after mixing. A polystyrene serological pipette was used to transfer the solutions to 40-mL amber glass vials, and frozen at -20ºC until analysis. All amber glass collection vials were baked at ≥

350° C for 12 hours prior to sample collection or in a few cases where baking was not possible, rinsed with methanol prior to sample collection. Concentrations of parent ATZ and three major metabolites were not measured from aged, 3-day-old solutions, as degradation of parent ATZ was not expected due to its stability over this timeframe and the test conditions. Study I samples were analyzed at Oregon State University using Beacon Atrazine Plate Kits (Item No. 20-0002, Beacon Analytical Systems, Inc., Portland, ME) following dilution of ATZ exposure solutions. Nominal samples of 0.1, 1, and 10 µM atrazine were diluted by 10-, 100-, and 1000-fold, respectively, to fall within the range of the standard calibration curve (0.00023 to 0.023 µM, or 0.05 to 5 µg/L). To improve analytical accuracy over the enzyme immunoassay method, fish water (0 µM ATZ) and ATZ exposure solution samples (un-blinded and a blinded subset) from Study II were analyzed by LC-MS/MS at Syngenta Crop Protection, Greensboro, NC using method GRM014.02A. All samples of frozen E2 test solutions (Study I and Study II) were analyzed using a 96-well Estradiol Enzyme Immunoassay (EIA) Kit (Cat. No.: 582251 Cayman Chemical, Ann Arbor, MI). Concentrations of samples were determined using a BioTek Synergy MX plate reader (BioTek Instruments Inc., Winooski, VT) or Molecular Devices SpectraMax 190 plate reader equipped with SoftMax Pro 5.2 Software (Molecular Devices, Sunnyvale, CA). The plate reader wavelength was set 450 nm for ATZ measurements and 405 or 420 nm for E2 measurements. Sample concentrations were calculated using a 4-parameter logistic regression based on the respective standard calibration curve.

Histopathology

Following fixation, 130 dpf whole fish were sagittally bisected with a sharp razor blade. Fish were processed overnight using a Tissue-Tex VIP 5 tissue processor (Sakura Finetek, USA) or a Leica Biosystems TP1020 (Bannockburn, IL) and embedded in paraffin. Fifteen step sections (5-µm thick sections with a 50 µm interval between every three sections) containing representative gonadal planes for each fish were cut using a Leica Biosystems microtome (Bannockburn, IL), placed on glass slides, and stained with hematoxylin and eosin.

Gonadal sections were blindly evaluated, to determine histological assessments, by two analysts, Michael Kent and Tracy Peterson (Oregon State University). For the second experiment, S.W.F (Centre for Environment, Fisheries and Aquaculture Science Weymouth Laboratory U.K) evaluated a random selection of slides. The evaluations were equally divided among the pathologists, except in the case of questionable sections, which were evaluated by at least two of the pathologists. Gonads were

categorized as "Female" (100% ovarian tissue), "Male" (100% testicular tissue), "Testicular oocyte(s)" (TO) (phenotype: one or two primary oocytes observed in a male fish, or mixed sex gonad), "Undefined gonad" (gonadal tissue could not be defined) or "Abnormal male," a male fish with immature testicular development (e.g., the presence of spermatocytes but no spermatids), testicular degeneration, or reduced amount of testicular tissue or in one instance an ectopic oocyte in a male fish (Study II). The gonadal developmental abnormalities were defined as any deviation from mature male or female gonadal tissue. When chronic inflammatory changes were observed, suggestive of mycobacteriosis, additional sections were prepared and stained with Fite's acid fast technique for confirmation.

Statistical analysis

Data was analyzed using either Fisher's exact test or analysis of variance (ANOVA) in StatView version 5.0.1 (Sas Institute Inc, Cary, NC 1998). Study overall weights and lengths were analyzed by a Fisher's positive least significant difference ANOVA. Male and female weights were compared for Study I or Study II by Fisher's positive least significant difference ANOVA. Weights or lengths of treated male (or female) fish were compared to control by ANOVA (Tukey-Kramer). Male and female fish counts per tank were compared within the unexposed group to confirm that sex distribution was normal for the untreated fish (Study I and Study II were run separately). Male and female fish counts per treatment for ATZ and E2 treatments (Figure 2) were compared to control counts for Study (and Study II) using a Fisher's Exact Test. The total number of normally developed fish and abnormally developed fish (see Histopathology section) for any treatment was compared to control by Fisher's exact test.

RESULTS

No mortality occurred across all treatments (≤50 µM) over the course of the 14-day acute toxicity study. One fish in the 50-µM ATZ exposure group was smaller in size than the other nine fish and, on exposure day 7, one fish in the 1.0-µM ATZ exposure group appeared lethargic. However, these two fish survived the 14-day exposure period.

Water quality was measured over the course of both chronic studies; averages and standard deviations are presented in Table 1. Dissolved oxygen was always >5.70 mg/L, and on most days was >6.5 mg/L. Water pH averaged 7.3 and 7.6 for Study I and II respectively. Temperature was maintained at 27 °C for both studies. Ammonia and nitrite concentrations averaged 0.24 mg/L and 0.50 mg/L,

respectively for Study I. For Study II, ammonia and nitrite concentrations averaged 0.63 mg/L and 0.85 mg/L, respectively.

A summary of analytical data is also presented in Table 1. Stock solutions were sampled prior to renewal of exposure solutions and frozen until analysis. Study I ATZ concentrations across all sampling time-points for the 0.1, 1, and 10 µM nominal ATZ treatments averaged 0.088, 0.88, and 8.9 µM, respectively. Study II 0.1, 1, and 10 µM nominal ATZ treatments averaged 0.096, 0.92, and 9.4 µM, respectively. For the 1 nM E2 treatment, measured concentrations across all sampling time-points averaged 0.72 nM for Study I and 0.32 nM for Study II. Atrazine was detected in some Study I fish water, 0 µM ATZ (control) samples. ATZ was detected above the limit of detection (LOD) on Study I sampling days 3, 6, 21, 87, 90, 102, 111 (0.00093, 0.0011, 0.00028, 0.022, 0.016, 0.0071, and 0.00094 µM, or 0.20, 0.23, 0.063, 4.7, 3.4, 1.5, 0.20 µg/L respectively) and on days 78, 81, and 84 above the upper range of the calibration curve (>0.023 µM or > 5.0 µg/L). ATZ was not detected above the lower limit of the calibration curve (< 0.00023 µM or 0.05 µg/L) in most control samples (~75%). Because stock solutions were sampled prior to renewal exposure solutions, these occurrences of ATZ contamination likely resulted from contaminated glassware during sampling rather than contamination of the exposure solutions themselves. None the less, due to the sporadic contamination observed in Study I it was decided to repeat the study. In Study II, ATZ was not detected above the limit of quantitation (LOQ) (0.00023 µM) in any of the 0 µM ATZ control samples. Three samples vials broke when frozen or defrosted and were not analyzed (Study II) (two 1 µM, one 0 µM ATZ).

Mortality was low for both studies. Survivorship is presented as mean of eight replicates ± SEM. Percent survival for 0 µM ATZ tanks in Study I was high (90 ± 3.3%). No significant decreases in percent survival occurred following 113 days of exposure to 1 nM E2 (93 ± 4.1%) or 0.1, 1.0, and 10 µM ATZ (85 ± 3.3 %, 90 ± 4.2%, and 94 ± 2.6% respectively). High survivorship was also seen in Study II 0 µM ATZ tanks (99 ± 1.3%). Additionally, Study II treatment tanks E2 (96± 2.6%) and ATZ tanks 0.1, 1.0, and 10 µM, maintained high survivorship (100 ± 0%, 99 ± 3.5%, and 99 ± 1.3%, respectively).

Zebrafish body weight (wet weight) and total body lengths were recorded at the termination of the study for all treatments. Study I overall weights and lengths were significantly different than Study II weights and lengths (see axes Fig 1). Untreated female weight was significantly higher than untreated male weight for either study. After sex was histologically determined, mean weights and lengths (± standard error) for unexposed males and females were compared to their respective sex in the E2 and

ATZ exposures (Fig 1). "Abnormal male" fish (defined in Histopathology Section) in both studies were grouped with normal mature male fish because there was no statistical difference in weight or length between abnormal males and mature males within the same treatment.

Study I observed a significant decrease in male body weight in the 1.0 and 10 µM ATZ treatments (0.25 ± 0.035g and 0.28 ± 0.012) relative to unexposed males (0.32 ± 0.012g) and female body weight in the 10µM ATZ treatment (0.33 ± 0.011g) relative to unexposed females (0.42 ± 0.025g) (Fig. 1). Treatment did not affect length in Study I. Study II average male E2 weight (0.54 ± 0.0081g) was greater than unexposed males (0.43 ± 0.0079g); however, average E2 female weight (0.49 ± 0.020g) was similar to the unexposed control (0.53 ± 0.016 g) (Fig. 1). Average male body weights in Study II were significantly less than unexposed for 1 and 10 µM ATZ treatments (0.38 ± 0.015 and 0.36 ± 0.017 g respectively) (Fig. 1). Male weight for 0.1 µM ATZ exposed fish (0.40 ± 0.012 g) was comparable to unexposed control (Fig. 1). Female body weight averages for 10 µM ATZ were significantly less (0.43 ± 0.026 g respectively), while 0.1 and 1 µM ATZ (0.45 ± 0.019 and 0.47 ± 0.015 g) was not different than untreated females (Fig. 1).

Study I average untreated female length (27 ± 0.49 mm) was not significantly different than 0.1, 1, 10 µM ATZ (27 ± 0.41, 26 ± 0.38, 26 ± 0.20 mm) or E2 exposed fish (27 ± 0.55 mm). Similarly, average untreated male lengths in Study I (27 ± 0.32 mm) were not significantly different than 0.1, 1, 10 µM ATZ (27 ± 0.26, 26 ± 0.24, 26 ± 0.33 mm) or E2 exposed fish (27 ± 0.64mm)(Fig. 1). Study II male and female 10 µM ATZ- exposed zebrafish lengths (35 ± 0.36 and 36 ± 0.57 respectively) were significantly less than unexposed male and female fish (37 ± 0.19 and 38 ± 0.32). Study II males exposed to 0.1 µM and 1 µM ATZ (36 ± 0.24 and 36 ± 0.36) were not shorter than control males. Similarly, Study II unexposed female fish length was not different than 0.1 µM and 1 µM ATZ (37 ± 0.31 and 37 ± 0.23) exposed females. E2-exposed males (39 ± 0.28) were longer while females exposed to E2 (36 ± 0.43) were significantly shorter than unexposed in Study II (Fig 1).

The average percentage of male or female fish out of the total number zebrafish per treatment tank $\pm$ SEM (n=8) is depicted in Figure 2. Fish with gonad abnormalities (see Histopathology Section) were not included to calculate the percentage of male and female within any treatment (Fig. 2) and were not included in statistical analysis. Fish exposed to 1 nM E2 in Study I had a significantly more females (77 ± 4.7%), and less of males (23%) compared to untreated male and female counts (54% and 46 ± 4.8% respectively) (Fig. 2). Similarly, Study II fish exposed to 1 nM E2 had a significantly more females (86 ± 3.6%) and less normally developed males (14 %) compared to unexposed tanks (males,

41± 4.5% and females, 59 %). The number of males or females exposed to any of the ATZ treatments was not significantly different from the respective male or female numbers in unexposed fish tanks for either study (Fig 2).

For the purposes of these studies gonadal developmental abnormalities were histologically characterized and categorized as either abnormal male or undefined gonad or testicular oocyte(s) (terms defined Histopathology Section). Within the 1 nM E2 and 10 μM ATZ treatments (Study I), three and one fish, respectively, could not be identified as male or female since gonadal tissue was not observed within any of the 15 histologic sections available. The percentage of fish, in either treatment, with unidentifiable gonads was not significantly different from control (Fig 2b). Study II fish exposed to E2 had a significantly more abnormal males (twelve fish) and fish with indistinguishable gonad (six fish) (Fig 2b). Abnormal males were observed at a low frequency in other treatments with no statistically significant differences measured. Additionally, the undefined gonad phenotype was only present in one fish exposed to ATZ (10 μM) in Study I and was not seen in any fish exposed to ATZ in Study II.

Examination of gonadal tissues from 369 zebrafish from Study I across all treatments revealed five male fish with the testicular oocyte(s) TO phenotype (Fig 2b). The primordial oocytes were present in mature testes; fish with TO(s) were defined for the purposes of this study as the presence of 1 or more oocytes in a male fish or mixed gonad types. These five fish were identified in the ATZ treatments only (one, three, and one fish from 0.1, 1.0, and 10 μM ATZ respectively); however, no concentration-dependent response was observed. In all cases, histopathologic features were characterized by the presence of one or two primordial oocytes within mature testes displaying normal sequential development of spermatogonia, spermatocytes, and spermatids (Fig 3). Examination of gonadal tissues from 392 zebrafish from Study II across all treatments showed that five fish had the TO(s) phenotype. The previtellogenic oocytes were observed in three treatments, 0.1 and 1 μM ATZ and E2. Two zebrafish in the 0.1 μM ATZ tanks and one fish in 1 μM ATZ treatments were identified with a single primary oocyte (Fig 2b). However, in the E2 treatment two TO fish were observed, and described by mixed gonad features including testes-ova and ova-testes (Fig 3).The ova-testes gonadal tissue observed was characterized by the presence of spermatocytes, absence of spermatids, cystic degeneration in addition to vitellogenic follicles and follicular maturational progression. There were no significant differences found in the incidence of TO for Study I or II when compared to unexposed fish.

Three fish in Study I exhibited moderate, chronic inflammatory lesions in the viscera (one fish 0 μM ATZ, 1 nM E2, and 10 μM ATZ). One of the three fish (1 nM E2) displayed lesions consistent with

mycobacterial aerocystitis, which was subsequently confirmed by acid fast staining. Eight fish in Study II exhibited moderate, chronic inflammation or aerocystitis, peritonitis, or diffuse coelomitis (one fish in the 1 nM E2, and two fish per control, 1 µM and 10 µM ATZ). Two of these fish (one fish 0 µM and 1 µM ATZ treatments) were subsequently confirmed with mycobacteriosis by acid fast staining.

Limited spawning data was captured for Study II. Embryos (6-78 hours post fertilization) were collected from treatment tanks starting at exposure day 90 and concluding exposure day 108. No viable embryos were observed in any of the estradiol tanks. The age, number and quality of offspring were estimated. Spawning data is summarized (excluding age) in Table 2. No statistics were performed with this data, the record only serves as an observation that spawning occurred regularly for ATZ exposed and unexposed zebrafish.

DISCUSSION

Experimental Design

The potential impacts of ATZ exposure on aquatic organisms are still debated in the scientific literature. These studies aimed to further investigate the hypothesis that chronic ATZ exposure affects gonad development or the percentage of mature female and male zebrafish when compared to controls. In an effort to capture normal distribution for weight, length, sex ratios and sexual development variables were stringently controlled for this research. This included optimizing sample size, stocking density, volume of exposure solution, treatment concentrations and water quality. Additionally, unexposed controls served to account for quality measures such as water temperature, dissolved oxygen, and number of fish per tank which may have been shown to affect fish health or sex ratio outcome (Maack and Segner, 2003). Positive controls were employed to confirm that the fish strain was sensitive to targeted endocrine disruption and that the exposure duration and parameters were sufficient for endocrine-mediated effects. Concentrations of ATZ were chosen based on acute exposures and to match a published study with zebrafish (Suzawa and Ingraham, 2008). Importantly, exposure concentrations were at or markedly above environmentally relevant concentrations and at 10-fold dilutions to capture a large range of exposure scenarios. Exposure concentrations were verified by LC-MS or immunoassay to confirm that actual exposure concentrations were near nominal concentrations. The LC-MS analysis (un-blinded) was followed by a blinded analysis of a sample subset to verify results. These chronic studies aimed to expose zebrafish during important stages in sexual development throughout sexual

maturation. Additionally, exposure duration and timing for this study was modeled after the Suzawa 2008 publication that reported chronic ATZ increases the proportion of female zebrafish population relative to control (Suzawa and Ingraham, 2008). While samples indicate contamination of 0 µM ATZ tanks in Study I for some study days, the agreement of the biological results for Study I and Study II support that this was most likely analytical sample contamination rather than tank contamination. No ATZ was detected in Study II control exposure fish water (0 µM ATZ).

Zebrafish Health

Fish survival for all treatments exceeded acceptable control survival rates (minimum of 70%) for fish early life-stage toxicity tests routinely conducted for pesticides (USEPA, 1996) and, consequently, effects on zebrafish proportions was not attributed to sex-specific mortality for either study.

Mycobacteriosis a background infection in zebrafish (Murray et al., (in press); Whipps et al., 2008), and was confirmed by acid fast staining in one fish from Study I and two fish from Study II. This was consistent with low levels of this infection consistently observed at this laboratory (Kent et al., 2011). Mycobacteriosis was not treatment-specific. Both incidence and mortality were low and these infections were not thought to have interfered with other aspects of the studies.

Study II body weights and lengths (all treatments) were greater than Study I (Fig 1a and 1b). This is most likely due to changes that were made in the feeding protocol and tanks design to increase survivorship in Study II. Small nesting tanks (3 L) were employed at the beginning to Study I and later, the fish were transferred to a large nesting tank (6 L) identical to Study II tanks except made with polycarbonate material. While the material of the tank is not likely to have affected the size of the fish, the change in tank size may have affected the fish growth rate and thus size. Feeding protocols differed between studies and likely contributed to fish size differences observed. Feeding protocol changes consisted of frequency and quantity of meals. Study I fish were feed 2-3 times for the first few months, whereas Study II fish were feed 3-4 times for the first two months. Despite increasing the frequency of feeding later in Study I, fish size was not comparable to Study II. Both studies fed *Artermia* at least once daily throughout the study. Diet components were identical. It is not known what diet protocol is superior. Feeding protocols should be balanced to maximize fish health (growth rates) and water quality. Notably, while feeding protocols were different, the outcomes of both studies are markedly similar. Average weights for unexposed male fish were less than their female control counterparts for both studies. In Study II, the average male weight in the E2 treatment was significantly greater than the

control, while the average E2 female weight was slightly lower than females from control tanks (not significant). The E2 male weight increase may have been treatment related; however, a mechanism for this effect is unknown and similar differences were not observed in Study I.

Average wet weights and lengths of male zebrafish were not consistently reduced following chronic ATZ exposure; however, male length and weight in Study II was reduced in the 1 and 10 µM ATZ treatments. Average body weights and lengths for the female zebrafish for all ATZ treatments were not significantly different when compared to unexposed controls with one exception – 10 µM ATZ reduced female body weights (both studies) and body lengths (Study II only) when compared to controls. No concentration-dependent effects were seen across sexes; however, it is possible that chronic exposure to ATZ concentrations ≥ 10 µM (2160 µg/L) is responsible for this reduction in zebrafish size. Concentrations in this range are above environmentally realistic exposures and exceed reported growth NOECs (No Observable Effect Concentration) for several other fish species (Giddings et al., 2005). Histologic examination of all fish showed no overt explanation for the reduction in body weight; for example, there was no evidence of systemic toxicity.

Sex Ratios

To assess gonadal development of adult zebrafish, a four-month timeline was established to allow for full maturation of the gonad. Attempting to interpret the feminizing effects of exogenous estrogens by histological examination of zebrafish gonads during protogynic or transitional zebrafish development could lead to inaccurate conclusions (Maack and Segner, 2003). Histological evaluation of gonadal tissues verified that fish were developmentally mature and, in Study II, spawning had commenced indicating completed sexual development at study terminations. Fish were exposed from 17 to 130 dpf covering critical time points in development through maturity. Segner (2003) reported that early life stage exogenous estrogen exposure effects (reproductive performance, vitellogenin levels and gonadal differentiation) could be reversed if fish were raised through adulthood following an early exposure period of <42 dpf ; however, exposure through day 75 or a later life stage exposure (42-75 dpf) reduced fertilization capability, delayed start of spawning, elevated vitellogenin levels and altered gonad differentiation (Segner et al., 2003). Zebrafish do not have sex chromosomes. Sexual differentiation is thought to be a multigenic process that most likely begins with sex determination, followed by the formation of a juvenile ovary (a bipotential state) that then transforms into sex-specific gonadal tissue (Orban et al., 2009). The length of time necessary for transformation can vary and the

mechanisms or genes involved have not been identified (Orban et al., 2009). While these studies focused on exposure to ATZ from 17 dpf through adulthood, it is not known if perhaps other windows of exposure could alter the outcome we observed.

E2 disrupted gonadal development in male fish, demonstrating that exposure occurred over sensitive time points in sexual development. E2 induced abnormalities in male gonadal development, primarily in reduced sequential differentiation from spermatogonia to spermatocytes and spermatids. Estradiol also significantly reduced the number of mature males, thus affecting the proportion of female fish in a mature population. This chronic study confirmed that the zebrafish strain (5D) was sensitive to exogenous estrogen exposure. Water quality and stocking densities have been reported to contribute to skewing a population's sex ratio (Maack and Segner, 2003). The number of unexposed females in Study I was not significantly different from the percentage observed in Study II; however there were more untreated females in Study II. The feeding regime followed in Study II may have affected the number of females in the control as it has been reported that faster growing fish are more likely to be female (Lawrence et al., 2008). For fish in Study II, wet weight measurements increased on average by ~0.1 g and lengths increased by ~10 mm (Fig 1) when compared to Study I.

When reviewing the literature, studies report combinations of effects or null effects on either growth or sexual development which are often inconsistent with other publications. The published effects of ATZ on sex development of aquatic organisms are mixed. While some studies report that ATZ exposure does not skew sex ratios at low concentrations (Kloas et al., 2009; LaFiandra et al., 2008; Oka et al., 2008; Spiteri et al., 1999), one publication reported hemaphroditism (Hayes et al., 2002b) after ATZ exposure, and another study reported complete sex reversal (Hayes et al., 2010). Suzawa and Ingraham (2008) reported a significant increase in the proportion of females that was concentration-dependent after atrazine exposure. The present studies do not support these findings in zebrafish. In fact, they contradict the Suzawa (2008) chronic exposure study; however, sample size was not included the Suzawa publication. For both of the present studies, the number of fish and tank replicates used was maximized to identify significant changes in sex percentages. Taken together, these studies demonstrate that ATZ exposure at or above environmental concentrations do not significantly alter differentiation or gonadal development (17-130 dpf) in zebrafish.

Abnormal Males

The number of abnormal males in Study I was not increased for any treatment. In Study II, the estradiol treatment induced a variety of gonadal developmental abnormalities, testicular degeneration, reduced testicular tissue and immature testicular tissue, primarily. The percentage of males with mature gonads in the E2 treatment was significantly less than control. One consideration for the differences in the number of abnormal males between the two experiments is that the growth measurements differed, i.e., Study II weights were increased compared to Study I (Fig 2). The difference in growth may have been because the fish feeding was optimized in Study II to avoid mortality. Because of rapid growth male fish in Study II may have reached maturity earlier than Study I, then with continued exposure to E2 no basal hormonal state could be attained resulting in abnormally developed males. Another possibility is that lower E2 water concentrations seen in Study II (Table 1) in combination with rapid growth resulted in a departure from normal gonad development in the E2 males rather than a higher percentage of females.

One male fish (1 μM ATZ), in Study II, was observed with an "ectopic" oocyte trapped between the sclera and outer connective tissue of the eye and eosinophils associated with the liver. No literature was found on incidence of ectopic oocytes in male zebrafish. ATZ exposures did not produce significant differences seen in the number of abnormal males when compared to unexposed fish for either study.

Testicular oocytes

For the purposes of these studies the TO phenotype was defined as the presence of one or more oocytes in male fish, or the presence of mixed gonadal tissues. Five and three male zebrafish exposed to different concentrations of ATZ were observed with testicular primordial oocytes in Study I and II, respectively. The TO phenotype observed in ATZ fish (one or two oocytes present in mature testicular) was markedly different than the E2 TO(s) phenotype where mixed gonadal tissue was observed (Fig 3). Two fish in Study II estradiol tanks observed with the TO(s), mixed gonad, phenotype (Fig 3).

The background incidence of testicular oocytes has not been well established in zebrafish. It has been published that the frequency of spontaneous "intersex" gonads (contain oocytes and sperm cells) in adult zebrafish is thought to be low (Dietrich and Krieger, 2008; Segner et al., 2003); however, the background incidence of this phenotype in laboratory-reared colonies of wild-type (5D) zebrafish is unknown. Although previtellogenic oocytes were seen at a very low frequency in both studies, this did not provide evidence that frequency of TO phenotype is different from unexposed fish. Additional primordial oocytes may not have been detected based on the sectioning intervals (50 μm) used in this

study; however, sections were likely representative of the treatment groups. There was no relationship between concentration and increased incidence of oocytes observed in male gonad or the incidence of mixed-gonad.

Primary oocyte incidence was not consistent for either study, i.e. phenotypes were observed in different treatments. For instance examination TO sections were observed in Study I (10 µM ATZ) but no sections of 10 µM ATZ exposed fish were found with TO in Study II. The TO phenotypes observed in ATZ tanks could have resulted from residual tissues that did not complete transition as zebrafish sexual development is characterized by a juvenile ovary as previously discussed. In females, immature ovaries pass through a gonadal transition stage, resulting in normal development of mature ovarian tissue; however, in males, oocytes undergo apoptosis, resulting in testicular development followed by sexual differentiation until complete. The exact timing of each part of the sexual development processes is not fully understood nor is it agreed upon in the literature (Maack and Segner, 2003; Orban et al., 2009). Because of the bipotenial for sex determination in zebrafish sexual development, the observation of one or two primordial oocytes in mature male testes could represent a normal transition from ovary to testis; however, because the TO phenotype observed in ATZ exposures for both studies was limited to only one or two previtellogenic oocytes (in mature testicular tissue) it is unlikely that the phenotype was characteristic of rudimentary hermaphroditism or delayed gonadal transition. Study I reported similar primary oocyte phenotype frequencies to Study II.

As previously mentioned, some studies have reported that ATZ induces mixed sex or testicular oocytes in aquatic organisms. Concentration, frequency, nomenclature, and phenotype (hermaphroditism, intersex, or testicular oocytes) are not consistent across reports. Additionally some studies have not observed the primary oocyte phenotype (Hayes et al., 2010; Langlois et al., 2009; Oka et al., 2008), including one with zebrafish (Suzawa and Ingraham, 2008). Histologic analysis of unexposed zebrafish from various strains would help establish a background incidence of TOs in adult male zebrafish.

Spawning (Study II only)

Spawning was assessed in Study II only. Viable embryos were collected from all treatments except for E2. Spawn quality and quantity was recorded only for selected days. The spawning observed in treatment tanks was not statistically compared to control tanks because spawning had not been established prior to collection, spawning collections were performed for only a limited duration, spawning recovery was not implemented , spawns represented different ages at collection, and

spawning count was estimated. No embryos were present on collection days in any of the E2 exposure tanks; however, some debris reminiscent of decayed embryos was found on the bottom of some E2 tanks on collection days. An absence of viable embryos could be due to one or more of the following reasons: embryos were not fertilized, embryos were abnormal, female oocyte development was disrupted, male sperm development was irregular, females did not drop eggs or perhaps social behavior was disrupted. Gonad modification and elevated vitellogenin have been associated with reduced fertilization in estradiol treated fish (Maack and Segner, 2004). An estimated number of viable embryos (not age-specific) collected per tank were averaged (Table 2). Table 2 illustrates that ATZ exposed zebrafish (up to 10 μM) produced viable embryos consistent with fathead minnow studies (Bringolf et al., 2004; Tillitt et al., 2010; USEPA, 2005). Further investigation into the potential for ATZ to disrupt spawning success and embryo viability could be explored to validate our findings.

Examining conflicts with previous reports

Zebrafish sex ratio was not affected by ATZ treatment in our study, which does not agree with the effects reported by Suzawa and Ingraham in 2008. Suzawa and Ingraham (2008) did not report the strain of fish utilized, the sample sizes for any treatment, stocking density, feeding regime, water quality, mortality for the chronic exposure study or other health evaluations such as incidence of mycobacterium or growth measurements. These conditions that could have affected sex ratios, sexual development, and growth were not discussed. Temperature and hypoxic conditions have been reported to affect fish sex ratios (Shang et al., 2006). Additionally no positive control was employed by Suzawa et al. (2008) to verify anticipated estrogenic effect (e.g., alteration in sex ratios). The positive control utilized in the present studies demonstrate that the zebrafish used were sensitive to chemically-mediated estrogenic effects. Because Suzawa and Ingraham (2008) did not report mortality data, it is not apparent whether mortality differentially affected the percentages of males and females observed at the end of the 6-month study. For the present studies we observed extremely low mortality over the entire 113-day study duration; therefore, it is not likely that mortality affected the results. It is also unclear in the Suzawa and Ingraham (2008) publication if there was sufficient sample size or replication, or if the sample population was randomized; additionally, nominal concentrations were not analytically verified. The analysis presented here of sex percentages indicates that the effects of ATZ on zebrafish in these studies do not agree with the results reported by Suzawa and Ingraham (2008).

CONCULSIONS

The results of the studies reported here demonstrate that chronic ATZ exposure does not significantly affect sex percentages or gonadal development in zebrafish. Zebrafish weight and length reduction in Study II is thought to have been caused by high ATZ (10 µM) concentrations; although, this was not observed in Study I. Zebrafish reproduction occurred in all atrazine treatments. Estradiol treatment altered the percentages of female and male fish significantly compared to control. Zebrafish exposed to estradiol did not produce viable embryos. Results from both highly controlled and similar studies were comparable.

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TABLES LEGENDS

Table 1 **Measured atrazine water concentrations approximated nominal concentrations throughout the study period.** Average measured concentrations ± one standard deviation (Study I: ATZ immunoassay, limit of detection (LOD) 0.05 µg/L; Study II: LCMS-MS, limit of quantitation (LOQ) 0.05 µg/L of unexposed tanks (0 µM ATZ) and ATZ and E2 exposure solutions for different periods of the 113-day studies. Average water quality measurements ± standard deviations for the studies are presented. *ATZ detected on some samples days ≥ 0.05 µg/L; ** ATZ detected >1.0 µg/L (see Results).

Table 2 **Viable embryos were produced in all atrazine exposure groups.** Average embryos spawned across eight replicate tanks. Each tank was collected and number of viable embryos estimated using a microscope. Because of the number of tanks to collect spawn, the age, quantity and quality of offspring were estimated.

FIGURE LEGENDS

Figure 1 **Chronic exposure to high levels of ATZ reduced zebrafish growth in Study II.** Study Weights and Lengths (n=8). Error bars represent SEM across eight tank replicates (n=8) (* $p < 0.05$; ** $p < 0.01$). Weight and length axes are different for Study I and Study II. Note: Fish that were <0.05 g were excluded from growth measurement graphs (two Study I and one fish Study II). **A)** Study I: Weight of female fish exposed to 10 µM ATZ and the weight male fish exposed to 1 and 10 µM ATZ was significantly less than the weight of unexposed females or males, respectively (0 µM ATZ) ($p= <0.05$). Female and male mean body weights for all other groups were not significantly different than zebrafish that were exposed to fish water only. **B)** Study I: Mean lengths of male and female fish for all treatments were not significantly different from unexposed mean length of the respective sex. **C)** Study II: Weight of male fish exposed to E2 was greater than the weight of males exposed to 0 µM ATZ ($p<0.01$). Exposure to 10 µM atrazine resulted in a significant decrease in female and male body weight ($p< 0.05$, <0.01). **D)** Study II: Length of average female (E2) was significantly less when compared to control ($p<0.05$). Average male zebrafish length was greater in E2 tanks when compared to 0µM ATZ ($p<0.05$). Exposure to 10 µM atrazine resulted in a significant decrease in female and male body length ($p<0.01$, <0.01).

Figure 2 **ATZ exposure did not affect zebrafish sexual development. A)** Percentage of mature male and female zebrafish across eight replicate tanks ± SEM. **B)** Percentage of abnormally developed males, testicular oocytes and undefined gonad (terms defined in Histopathology Section) following chronic exposure to untreated water, 1 nM E2, or 0.1, 1.0 or 10 µM ATZ ± SEM. Statistics described in methods section (*p < 0.05, ** p < 0.01). Data tables under graphs are actual counts of fish from all tanks, not percentages. Zebrafish were described with exclusively one gonadal developmental abnormality in the table; however, some testicular oocyte and undefined gonad fish were also identified as immature.

Figure 3 **Examination of ATZ exposed zebrafish gonadal tissues suggests the frequency of ectopic and testicular oocyte(s) is low while, E2 gonadal tissue changes were more dramatic than tissue changes in other treatments. A,B)** Mature gonadal tissue, male and female respectively (10 µM and 0.1 µM). **C,D)** Testicular oocytes defined as ova-testes or testes-ova found in two male fish exposed to E2. A cluster of primary oocytes are seen in Fig. 3d next to a small section of male gonadal tissue. **E,F)** Undefined gonadal tissue (0.001 µM E2) **G)** Primary oocyte trapped between sclera and outer connective tissue of eye from male zebrafish(1 µM ATZ). **H)** Primary oocyte found in mature testes from male zebrafish (0.1 µM).Symbols: oocyte primordia = arrow, sp = spermatids, sc = spermatocytes. Hematoxylin and eosin. Bar = 25 µm (A-C, E, F, H), 50 µm (D), 100 µm (G).

Supplementary Figure 1 **Novel material and design used for chronic toxicity testing. A)** Top view and bottom view of stainless steel 6 L tank inserts employed for Study II. Nested tank inserts permitted transfer of fish with minimal stress and researcher labor. The inserts also aided in maintaining the water quality by omitting residual exposure solution transfer during water changes. Nesting tank fit into stainless steel 6 L holding tanks (not pictured). **B)** Picture of a control (0 µM ATZ) fish tank mid-study. All tanks were equipped with air hose and bubbler. **C)** Picture of tank set up (only eleven of forty tanks pictured).

Table 1

Study Period	Measured Treatment Concentrations					Water Quality				
	0 µM ATZ	1 nM E2	0.1 µM ATZ	1.0 µM ATZ	10 µM ATZ	Dissolved Oxygen (mg/L)	Temp. (°C)	pH	Ammonia (mg/L)	Nitrate (mg/L)
Study I										
0-30	< LOD*	0.89 ± 0.16	0.089 ± 0.020	0.92 ± 0.22	9.3 ± 1.5	7.5 ± 0.22	27 ± 0.98	7.6 ± 0.11	0.23 ± 0.075	0.068 ± 0.12
31-60	< LOD	0.84 ± 0.47	0.081 ± 0.12	0.80 ± 0.15	8.6 ± 1.6	6.9 ± 0.39	27 ± 1.2	7.3 ± 0.12	0.063 ± 0.11	0.53 ± 0.38
61-90	< LOD**	0.58 ± 0.21	0.098 ± 0.025	0.92 ± 0.26	8.9 ± 1.5	6.8 ± 0.57	28 ± 1.1	7.2 ± 0.15	0.075 ± 0.12	1.0 ± 0.63
91-113	< LOD*; **	0.48 ± 0.038	0.085 ± 0.0089	0.88 ± 0.10	8.6 ± 1.2	7.0 ± 0.53	27 ± 0.42	7.2 ± 0.19	0.71 ± 0.49	0.54 ± 0.68
0-113	< LOD	0.72 ± 0.32	0.088 ± 0.019	0.88 ± 0.20	8.9 ± 1.4	7.0 ± 0.51	27 ± 1.1	7.3 ± 0.22	0.24 ± 0.33	0.50 ± 0.57
Study II										
0-30	< LOQ	0.36 ± 0.17	0.093 ± 0.0070	0.88 ± 0.17	9.2 ± 0.53	7.1 ± 0.28	27 ± 0.45	7.5 ± 0.24	0.79 ± 0.52	1.0 ± 0.63
31-60	< LOQ	0.28 ± 0.091	0.099 ± 0.0048	0.97 ± 0.049	9.7 ± 1.4	7.4 ± 0.28	27 ± 0.52	7.7 ± 0.07	1.05 ± 0.31	0.28 ± 0.55
61-90	< LOQ	0.37 ± 0.11	0.097 ± 0.011	0.92 ± 0.053	9.6 ± 0.61	7.4 ± 0.38	27 ± 0.89	7.5 ± 0.12	0.43 ± 0.35	1.3 ± 0.55
91-113	< LOQ	0.22 ± 0.13	0.094 ± 0.0025	0.92 ± 0.029	9.2 ± 0.31	7.2 ± 0.17	26 ± 0.37	7.4 ± 0.12	0.13 ± 0.35	0.78 ± 0.21
0-113	< LOQ	0.32 ± 0.13	0.096 ± 0.0074	0.92 ± 0.10	9.4 ± 0.84	7.3 ± 0.32	27 ± 0.70	7.6 ± 0.19	0.63 ± 0.49	0.85 ± 0.69

Table 2

Spawning Log : Average number of viable embryos produced

	0 µM ATZ	1 nM E2	0.1 µM ATZ	1 µM ATZ	10 µM ATZ
02/28/10	>50	0	>50	>50	>50
03/03/10	>50	0	>50	>50	>50
03/06/10	>50	0	>50	>50	>50
03/09/10	>50	0	>50	>50	>50
03/12/10	>50	0	>50	>50	0 < x < 50
03/15/10	>50	0	>50	>50	>50
03/18/10	0 < x < 50	0	0 < x < 50	0 < x < 50	0 < x < 50

Number of viable embryos
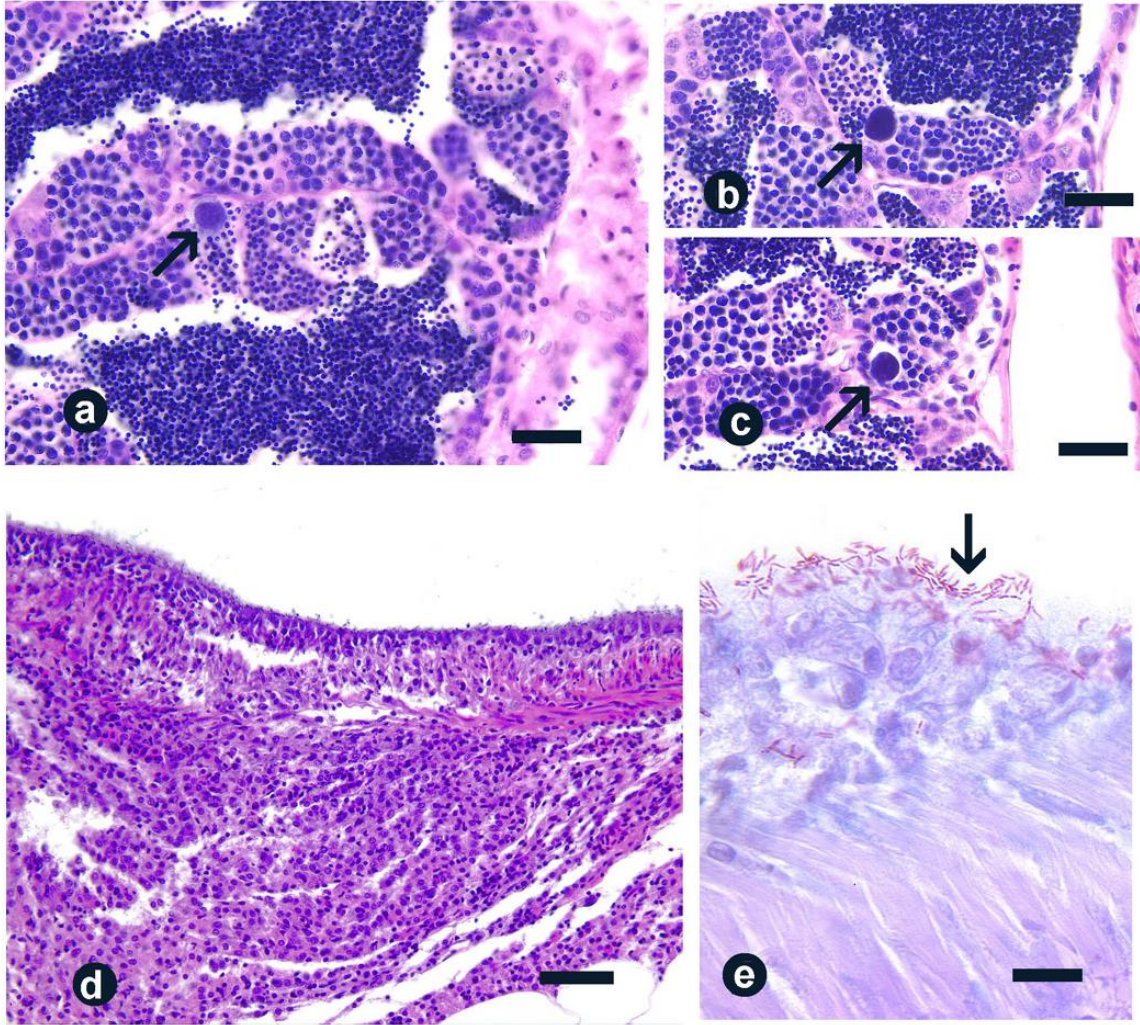
☐ 0 embryos

■ 0 < x < 50 embryos

▨ > 50 embryos

Fig. 1

Fig. 2

Fig. 3

Fig. 4