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Effect of parental exposure to trenbolone and the brominated flame retardant BDE-47 on fertility in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

We exposed sexually maturing male rainbow trout (*Oncorhynchus mykiss*) to BDE-47 (a polybrominated diphenyl ether) and female rainbow trout to trenbolone (an anabolic steroid). Male trout were orally exposed for 17 days to 55 µg/Kg/day BDE-47 and female trout continuously exposed for 60-77 days to a measured trenbolone water concentration of 35 ng/L. After the exposure, eggs and semen were collected and in vitro fertilization trials performed using a sperm:egg ratio of 300,000:1. In the BDE-47 study, eggs from control females were fertilized with semen from exposed males, while in the trenbolone study, eggs from exposed females were fertilized with semen from control males. All treatments were evaluated at two-three early developmental time-points representing first cleavage (0.5 day), embryonic keel (9 days), and eyed stages (19 days), respectively. The results indicated that BDE-47 exposure did not alter

fertility as embryonic survival was similar between control and exposed groups. Trenbolone exposure also did not alter embryo survival. However, in the embryos fertilized with eggs from trenbolone exposed females, a noticeable delay in developmental progress was observed. On day 19 when eye development is normally complete, the majority of the embryos either lacked eyes or displayed under-developed eyes, in contrast to control embryos. This finding suggests steroidal androgen exposure in sexually maturing female rainbow trout can impact developmental timing of F1 offspring.

Keywords: Trenbolone; Flame retardants; BDE-47; Rainbow trout; Reproduction

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In previous studies, we demonstrated that the synthetic estrogen ethynylestradiol (EE2) reduces the fertility of sexually maturing male rainbow trout (*Oncorhynchus mykiss*) by decreasing progeny survival (Schultz et al., 2003; Brown et al., 2007). At EE2 exposure rates comparable to environmental levels (e.g. < 15 ng/L), embryo mortality between developmental days 9 (embryonic keel formation) and 19 (eyed stage) approached 50%. This suggests some form of genomic instability rather than an effect on sperm morphology and/or motility is responsible (Brown et al., 2007). This raises important ecotoxicological questions in regards to whether this effect is limited to estrogens or can other classes of endocrine disruptors known to impact testicular development produce similar effects. Alternatively, can exposure to steroidal androgens impact female rainbow trout in a similar manner? In this study, we pursued these questions by exposing sexually maturing male rainbow trout to 2,2,4,4-tetrabromodiphenyl ether (BDE-47; commercially used as a flame retardant) and female rainbow trout to the anabolic steroid trenbolone. We chose to expose male trout to BDE-47 based on the findings from a previous study, using pair breeding fathead minnows (*Pimephales promelas*), which documented a reduction in spawning due to decreased production of mature spermatozoa in males (Muirhead et al., 2006). Trenbolone is a widely used growth promoter in livestock production that is known to cause masculinization of female fish (Ankley et al., 2003).

Sexually mature, male isogenic rainbow trout of the OSU x Arlee strain (Young et al., 1996) (22 months in age; body weight: 733 ± 166 g (mean \pm SD); $n=5$), raised at MSL holding facilities were orally exposed for 17 days to BDE-47 at a dose rate of 55 μ g/Kg/day. The BDE-47 was incorporated into the pelleted trout feed and assayed by GC-ECD using methods described in Muirhead et al. (2006) to verify the concentration and stored at -20°C until day of feeding. Each morning of the exposure, a known mass of BDE-47 feed was provided to individual trout. Complete consumption of the dose within 5 minutes was verified visually each

day. Control fish body weight: 727 ± 93 g; $n=4$; identical in age and genetics) were fed uncontaminated trout feed.

For the trenbolone exposures, sexually maturing female rainbow trout, 2⁺ years in age were obtained from a local hatchery (Nisqually farms, Lacey WA, USA) and exposed to a nominal waterborne concentration of 50 ng/ L. The trenbolone was continuously added to the exposure tanks dissolved in a methanol carrier (<0.004 % methanol in tanks), control fish were only exposed to the methanol carrier. Actual trenbolone exposure levels were determined by GC-MS using the method described by Marchand et al. (2000). The mean value of four separate determinations made throughout the exposure was 35 ng/L. After 60 d of exposure, all fish were checked for ovulation twice weekly until five control and treated fish had ovulated. (BW = 1450 ± 220 g exposed; 1375 ± 301 g control fish). Thus, the duration of exposure lasted between 60 - 77 days. At completion of the exposures, sperm or eggs were obtained by manual expression of the gametes from anesthetized fish and used in controlled fertility trials. For semen from the BDE-47 exposed males, the number of sperm/ml semen was quantified and fertilization trials performed using eggs from a single unexposed female donor at a sperm:egg ratio of 300,000:1.

Eggs collected from trenbolone exposed trout were immediately used in fertilization trials at a similar sperm:egg ratio using sperm collected from unexposed isogenic male trout. All treatments were evaluated at two or three developmental time-points: 0.5 days post fertilization (dpf), 9 dpf, and 19 dpf, representing first cleavage, embryonic keel, and eyed stages, respectively. An additional measure of eye pigmentation in the day 19 embryos from the trenbolone exposure was performed to assess the overall state of embryonic development. This was done subjectively by categorizing the level of pigmentation as: 0-no eye development, 1-eye development with no retinal pigmentation, 2-initial retinal pigmentation, 3-moderate retinal pigmentation, 4-fully eyed. Statistical analysis of the categorical data was done with SAS vs. 8.1

using a chi-square test of independence and a general linear model that specifically compared within treatment effects on pigmentation.

The results of the BDE-47 fertilization trials are shown in Fig. 1. Fertilization rates are consistently high and no apparent difference was observed between control and the BDE-47 exposure when embryo survival was assessed at 1st cleavage or at the eye stage of development. This result occurred despite the presence of BDE-47 in all semen samples (436 ± 109 ng/ml) from exposed trout used in the fertilization trials. Trenbolone exposure to female trout did not affect subsequent embryo survival, which was consistently above 80% in all treatment groups assessed at the three different developmental stages (Fig. 2A). During evaluation of embryo survival from the trenbolone experiment, it became apparent that the rate of embryonic development was delayed compared to control embryos. We chose to quantify this effect by subjectively categorizing the state of eye development at 19 days post-fertilization. These results are presented in Fig. 2B. The majority of embryos in the trenbolone exposed treatment group can be characterized as either having no eye pigmentation or a complete lack of eye development. This finding is in contrast to control embryos, the majority of which possessed fully developed embryonic eyes. Thus, exposure to an androgen in sexually maturing female trout does appear capable of impacting normal embryonic development. Our results combined with past studies of EE2 in male trout would indicate that exposure to steroids during sexual maturation impacts genomic stability and subsequent viability of the F1 offspring. This raises serious concerns about the consequences of municipal sewage release (primary source for xenoestrogens) and run-off from cattle feed-lots (primary source for anabolic androgens) on fish populations.

Acknowledgement

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Figure captions

Fig. 1. Embryonic survival assessed at 0.5 days of development (1st cleavage) and at day 19 (eyed stage) post-fertilization for offspring from BDE-47 exposed male rainbow trout. A minimum of 50 embryos were evaluated at each developmental stage. The bars (white= control; filled= trenbolone exposed) represent a mean \pm SD ($n=5$ exposed or control fish).

Fig. 2. (A) Embryonic survival assessed at 0.5 days (1st cleavage), 9 days (keel formation) and 19 days (eyed stage) post-fertilization for offspring from trenbolone exposed female rainbow trout. A minimum of 50 embryos were evaluated at each developmental stage. The bars (white= control; filled= trenbolone exposed) represent a mean \pm SD ($n=6$ exposed fish). (B) Eye development on day 19. See text for explanation of categories. *= $P<0.06$.

Figure 1

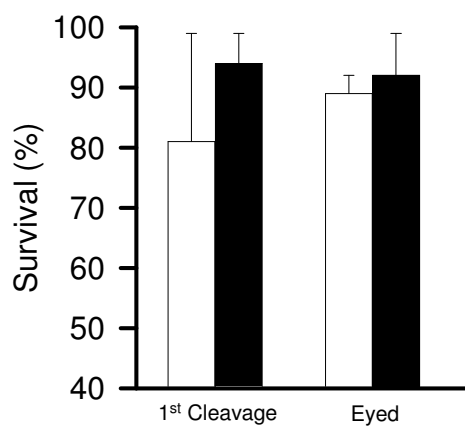


Figure 2

