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Lack of a heritable reproductive defect in the offspring of male rainbow trout exposed to the environmental estrogen 17 α -ethynylestradiol

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Abstract

Endocrine disruptors, including environmental estrogens, have been shown to induce heritable effects through both genetic and epigenetic mechanisms in mammals. Despite this information and the wealth of knowledge regarding the significant reproductive impacts endocrine disruptors impose on fishes, no studies have reported whether the observed effects are heritable. Without this information it is difficult to establish the long-term consequences for exposed populations. To determine potential consequences of long-term effects we must consider the possibility that induced reproductive defects in fishes may be heritable. Using rainbow trout (*Oncorhynchus mykiss*) as a model this study aims to determine whether a specific reproductive defect observed in 17 α -ethynylestradiol exposed male parents, diminished progeny survival, is heritable in the unexposed surviving F1 males. Semen was collected from anesthetized males of the F1 generation upon sexual maturation at two time-points, one year old precocious males and two years old males. *In vitro* fertilization was used to produce an F2 generation. F2 embryos were then analyzed for survival at 19 days post-fertilization (eye pigmentation) and the different treatment groups statistically compared to the controls. Analysis indicated that F2 offspring survival from F1 males propagated from both exposed and unexposed parents survive normally and no heritable effect was observed in males from the F1 generation for this specific reproductive defect. These results provide scope for the recovery of fish populations exposed to environmental estrogens should the contaminant be removed.

Keywords

Rainbow trout; Environmental estrogens; Reproduction; Heritability; Progeny survival; 17 α -ethynylestradiol

1. Introduction

Environmental release of estrogenic substances are a concern due to their known ability to significantly affect fishes through induced developmental abnormalities of the gonads and

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brain, which ultimately results in impaired reproductive success (Jobling et al., 1998; Janz, 2000; Jobling et al., 2002; Kuhl et al., 2005; Campbell et al., 2006; Kuhl and Brouwer, 2006; Filby et al., 2007; Cheshenko et al., 2008). The body of scientific evidence amassed evaluating environmental estrogen exposure in fishes indicates a strong likelihood for long lasting reproductive effects, some of which could ultimately be felt at the population level (Jobling et al., 1998; van den Belt et al., 2003; Nash et al., 2004; Kidd et al., 2007; Schafers et al., 2007). Despite the overwhelming support for this conclusion, no information is currently available on the heritability of observed reproductive effects.

The heritability of environmental estrogen induced reproductive defects could be accomplished through two different mechanisms: (1) a direct genetic mechanism such as an induced genetic mutation or (2) an epigenetic mechanism such as an altered methylation pattern. Direct genetic mechanisms of DNA damage have been observed using cell culture methods as well as *in vivo* exposures to estrogens and/or estrogen acting compounds (Notch et al., 2007; Kayani and Parry, 2008). These studies indicate that estrogens have genotoxic abilities that affect DNA repair mechanisms and in cell culture situations, induce the formation of aneuploid cells during mitosis. Epigenetic alterations are heritable changes that effect gene function without modifying the DNA sequence (Richards, 2006; Bird, 2007). Such changes can be induced through altered DNA methylation, chromatin structure remodeling by histone acetylation and/or methylation, and small RNA mediated regulatory processes on gene function (Berger, 2007). Studies of endocrine disruptors in mammals, including the estrogenic compound methoxychlor, indicate that induced reproductive defects can be inherited by future generations epigenetically (Anway et al., 2005; Crews et al., 2007). While these findings provide possible mechanisms for heritable effects due to environmental estrogen exposure there is no information on whether these defects can occur in fishes. The heritability of reproductive defects, either through genetic or epigenetic mechanisms, must be considered as it could have important real-world consequences for the survival of wild fish populations.

Rainbow trout (*Oncorhynchus mykiss*) exposed to the environmental estrogen 17 α -ethynylestradiol (EE2) immediately prior to full sexual maturation exhibit a significant and consistent reduction in progeny survival occurring between 9 and 19 days post-fertilization (Schultz et al., 2003; Brown et al., 2007). For these studies sexually maturing males undergoing active spermatogenesis were exposed to environmentally relevant concentrations of EE2 prior to the manual collection of semen and subsequent *in vitro* fertilization using eggs from unexposed females. By using *in vitro* fertilization the direct effect of the male germ cell on progeny development was determined. Given the fact that the exposures occurred during spermatogenesis there is scope for sperm DNA alterations, either genetic or epigenetic that could be heritable and may lead to a similar defect in surviving progeny. This study therefore sought to exploit the rainbow trout as an experimental model to determine whether the established defect observed as a result of male parental exposure to EE2 results in a heritable effect on the F1 generation that maintains decreased progeny survival in the F2 generation.

2. Materials and methods

2.1. Experimental animals and sample collection

Single pair mated families of rainbow trout were produced from crosses where only the male parents were exposed to different concentrations of EE2 beginning at 6700 degree days (0, 0.8, 8.3, and 65 ng EE2/L). The description of the experimental set-up and the reproductive defect documented in the EE2 exposed male parents is described in detail in Brown et al. (2007). The intent of this experiment was to test the F1 generation for the same reproductive defect documented in their male parents (Fig. 1). All surviving embryos in each of the 39 families (9:control, 10:0.8 ng EE2/L, 10:8.3 ng EE2/L, 10:65 ng EE2/L) from the Brown et al. (2007) study were individually marked with a unique passive inducible transponder (PIT) tag injected

into the coelomic cavity. The fish were held in tanks within a re-circulating system using temperature controlled, bio-filtered and UV treated well water at the University of Idaho Aquaculture Research Institute Cold Water Laboratory. On a monthly schedule average fish weights on a per tank basis were recorded. Semen was collected following previously established protocols (Brown et al., 2007) at two independent sampling times, (1) from some sexually precocious males in November 2006 at 1 year of age [1 year old; 0 (control) and 65 ng EE2/L groups], and (2) in November 2007 at 2 years of age [2 year old; all groups]. Precocious sexual maturation is typical within rainbow trout populations with a small proportion (<10%) of males producing semen at 1 year of age, while the majority typically attain sexual maturity for the first time at 2 years of age. Sexual maturity was assessed by manually expressing semen from anesthetized fish. Sexually mature males were identified to family and treatment group using recorded PIT tag numbers with no more than 3 individuals per family selected to represent any one treatment group. Sixteen precocious males were evaluated in 2007 (6 control males from 4 families; 10 males from 6 of the 65 ng EE2/L families) and 69 males in 2007 (11 control males from 6 families; 18 males from 7 of the 0.8 ng EE2/L families; 20 males from 8 of the 8.3 ng EE2/L families; 20 males from 8 of the 65 ng EE2/L families). All precocious males evaluated in 2006 were excluded from the 2007 sampling. Individual semen samples were collected and used for *in vitro* fertilization and developmental assay as described in Brown et al. (2007) with the exception that embryonic development was only evaluated at 19 dpf. This time-point was selected as it is the first and only time-point where embryonic lethality is observed. Fish used in this experiment were treated and maintained according to the guidelines established by the University of Idaho Institutional Animal Care and Use Committee.

2.2. Statistical analysis

Embryonic survival and sperm concentration data was statistically analyzed using a completely randomized design. The percentage of individuals reaching retinal pigmentation was analyzed after performing an arcsine transformation to normalize values. The linear model for all analyses was $y_{ij} = \mu + \alpha_i + e_{ij}$, where α_i represents the effect of paternal exposure to EE2 and e_{ij} the random error. ANOVA was performed to determine whether significant differences were present between treatment groups using PROC GLM in SAS/STAT (SAS Institute, Cary, NC, USA). When indicated by ANOVA, Fisher's protected LSD was performed to determine which treatments differed significantly from one another.

3. Results

3.1. One year old fish

Sixteen males of the F1 generation matured as one year olds from two of the treatment groups (0 and 65 ng EE2/L) and were evaluated for the reproductive defect documented in their male parents exposed to EE2 (Fig. 2A). No significant survival differences ($p = 0.7081$) were observed in the F2 offspring at the retinal pigmentation stage, 19 days post-fertilization, between the two groups. Mean sperm concentrations for these one year old, precociously spawning males were 6.72 billion/mL and 6.18 billion/mL for the control and exposed males with no significant differences observed ($p = 0.6636$).

3.2. Two years old fish

A total of 69 males of the F1 generation at two years of age from the four treatment groups were evaluated for the reproductive defect documented in their male parents exposed to EE2. There was no evidence that survival was decreased in the F2 progeny derived from F1 offspring whose male parents had been exposed to any of the EE2 treatments, compared to the control treatment (Fig. 2B). In one treatment group, F1 males whose fathers were exposed to 65 ng EE2/L, the mean percent survival was significantly higher than the other three treatment groups

(Fig. 2B). Mean sperm concentrations ranged were 4.20, 3.30, 4.23 and 3.78 billion/mL for the control, 0.8, 8.3 and 65 ng EE2/L exposed parent families, respectively with no significant differences observed ($p = 0.3312$).

3.3. Growth estimates

Growth estimates obtained monthly showed no differences between tanks until the October 2007 measurement (Fig. 3). At this time tank D6 had a larger average size, compared to all the other tanks, due to a reduction in fish density that occurred during September 2007. The system-wide average weight for fish during the October collection was 164 g, with individuals in the D6 system averaging 55 g higher at 219 g.

4. Discussion

Despite the wealth of knowledge from fishes regarding reproductive defects caused by environmental estrogen exposure, there is no information on whether these defects are heritable to subsequent generations. An understanding of heritable effects induced by environmental estrogens is critical for assessing population level consequences of exposure to EE2 and similar compounds. This is especially important given that long-term, continuous exposure of EE2 has been shown to cause significant deleterious population level effects in fish (Kidd et al., 2007). To grasp the environmental significance of previous findings demonstrating reduced progeny survival of male rainbow trout exposed to EE2, this study was performed to determine whether the observed reproductive defect was heritable. These results provide evidence that environmental estrogen exposure of sexually maturing males, in the form of EE2, does not cause this heritable defect in their F1 male progeny. No reductions in F2 offspring survival were observed when the different treatment groups were compared to the controls.

Although our results indicated that no heritable effect was present, an unexpected result was the observation of significantly higher progeny survival in the 2 year old treatment group derived from 65 ng EE2/L treated parents compared to the others (Fig. 2B). This difference is not believed to be a result of the parental EE2 treatment, but instead resulted from differential F1 growth that unavoidably occurred in several tanks during the course of this experiment. In salmonid fishes differences in growth rate and size at critical developmental time-points have been shown to affect the timing of sexual maturation and the quality of gametes (Dziewulska and Domagala, 2006; Larsen et al., 2006; Simonovic and Nikolic, 2007). Although every effort was made to maintain similar F1 tank densities of fish, which impacts fish size, density reductions in one tank occurred. This reduced density allowed a tank (D6) containing individuals from the 65 ng EE2/L exposure group to grow to a slightly larger size prior to semen collection (Fig. 3). This larger size appears to have resulted in higher semen quality that affected the number of F2 offspring from these F1 parents. This explanation is further supported by the fact that no difference in F2 progeny survival was observed in F1 males sampled from this group as one year olds (Fig. 2A) before the growth discrepancy occurred.

Most studies demonstrating effects of environmental estrogens in fishes use exposures encompassing the early life history, a period when male and female phenotypes of the gonads and brain are differentiating (i.e., sexual differentiation). Exposures at this time are known to cause significant detrimental impacts on physiology, anatomy and, most importantly, reproductive success (Jobling et al., 1998, 2002; Scholz and Gutzeit, 2000; van den Belt et al., 2003; Balch et al., 2004; Nash et al., 2004; Mills and Chichester, 2005; Kuhl et al., 2005; Campbell et al., 2006; Kuhl and Brouwer, 2006; Kidd et al., 2007; Maunder et al., 2007; Woodling et al., 2006). Although less frequently studied, exposure during later intervals of the life history also exhibit detrimental reproductive effects (van den Belt et al., 2002; Schultz et al., 2003; Brown et al., 2007). In male rainbow trout, exposures during late spermatogenesis lead to significantly reduced embryonic survival (Schultz et al., 2003; Brown et al., 2007). This

observed decrease is likely due specifically to an EE2 effect(s) on meiosis as only the male gamete was exposed to the environmental estrogen. While the specific mechanism of action (e.g., genetic, epigenetic, or other) for this observed defect is unknown, the possibility that it was being passed to future generations was considered an important question. Although the specific reproductive defect observed previously in rainbow trout, due to EE2 exposure, is not heritable, other unevaluated genetic or epigenetic effects may exist. Mammal study precedents exist for this possibility with endocrine disruptor exposures having been shown to produce both genetic (Hunt et al., 2003; Susiarjo et al., 2007) and epigenetic effects (Anway et al., 2005).

5. Conclusion

We have demonstrated that the specific reproductive defect, diminished offspring survival, observed in male rainbow trout parents exposed to various levels of EE2 is not observed in their F1 progeny once they reach sexual maturity. The lack of a heritable F1 effect in this study provides important evidence that the reproductive consequence suffered by EE2-exposed male parents does not preclude a population from recovery. The F1 embryos that survived and were tested in this study carried no defect forward that prevented the successful survival of their offspring. This finding is important because it provides support for the contention that even under contaminated conditions individuals can be present within a population which will reproduce normally. Furthermore, these apparently normal individuals could provide a base from which population recovery would occur, provided the contamination is removed.

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Fig. 1. Mean survival of F1 progeny propagated from males exposed to various concentrations of EE2 (Brown et al., 2007). Sexually mature surviving males from these F1 treatment groups were used to create F2 progeny.

Fig. 2.

Mean survival of F2 progeny propagated from the one year old and two years old F1 generation at 19 days post-fertilization. The F1 generation individuals were derived from families in which the male parent had been exposed to 0 (control) 0.8 ng EE2/L, 8.3 ng EE2/L or 65 ng EE2/L prior to spawning. Error bars represent ± 1 standard deviation. The trial performed at one year of age (A) included 6 and 10 individuals for the control and 65 ng EE2/L treatments, respectively. All groups were evaluated at two years of age (B) with 11, 18, 20 and 20 individuals from each of the control, 0.8 ng/L EE2, 8.3 ng/L EE2 and 65 ng/L EE2 treatments, respectively. Treatment groups with different letters in part B are significantly different at $p < 0.05$.

Fig. 3. Mean fish weights, by tank over time, for the F1 rainbow trout used to produce the F2 progeny. Fish used in the experiment from Tank D6 include 1-control, 1-8.3 ng/L and 10-65 ng/L. Additional experimental individuals used from other tanks were: controls from: C2 (1), C3 (3), C6 (2), D2 (2) and D3 (2); 0.8 ng/L from: C2 (3), C3 (2), C4 (2), C5 (4), D3 (2), D4 (3), D5 (2); 8.3 ng/L from: C1 (1), C4 (4), C5 (7), C6 (2), D5 (5); 65 ng/L from C5 (2), C6 (3), D4 (2), D5 (3).