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# Transcripts involved in steroid biosynthesis and steroid receptor signaling are expressed early in development in the fathead minnow (*Pimephales promelas*)

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ABSTRACT

Sex differentiation in organisms is correlated to sex steroid production and receptor signaling pathways involving androgens and estrogens. Timing of expression is critical, and characterization of sensitive windows is needed to determine how environmental stressors may perturb sex differentiation. The objectives of this study were to determine whether genes related to steroid biosynthesis, steroid receptor signaling, and those related to sex differentiation were expressed in pre-differentiated fathead minnow (FHM) embryos, an ecotoxicological model. Transcripts were measured over two weeks (1 day post fertilization (dpf) to 14 days), prior to sex differentiation. The first three time points investigated (1, 3, and 5 dpf) corresponded to the neurula stage, dorsal swim bladder pigmentation, and pre-hatch. The fourth time point (6 dpf) was collected immediately post-hatch and the fifth time point investigated was after 8 days of larval growth (14 dpf). The majority of transcripts investigated, for example estrogen, androgen, and thyroid receptors as well as steroid biosynthesis transcripts, were expressed within the first 72 hours of development; exceptions were *star* (steroidogenic acute regulatory protein) and *cyp19a*, which did not have detectable expression until 5 dpf (pre-hatch). Transcripts that increased in relative mRNA abundance over the first two weeks of development included *ar*, *dax1*, *hsd11b2*, *hsd17b*, *cyp19a* and *thra*. This study demonstrates that there is early expression of transcripts related to steroid biosynthesis, steroid receptor signaling, and sex differentiation in pre-hatch FHM embryos. Additional studies are required to determine their relative roles in male and female differentiation during these early developmental periods.


## 1. Introduction

There has been increased applications for embryos of small bodied fish in aquatic toxicology research. Small fish models such as zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*), and fathead minnow (FHM) (*Pimephales promelas*) have been used because they exhibit rapid development and are amendable to chemical screening (Leet et al., 2014; Villeneuve et al., 2014). Fish embryos have been used as models for characterizing toxicant mode of action (Scholz et al., 2008) and for annotating adverse outcome pathways of chemical exposure (Volz et al., 2011). As such, it is important to generate fundamental data on the temporal expression of transcripts over fish development, especially for transcripts that play a role in key physiological processes such as reproduction, central nervous system development, and cardiac function, among others.

A developmental gene profile is an experiment that examines expression patterns over the course of a defined time period. There are many key stages in the developmental life cycle of fish that can be used as landmarks, such as the completion of embryonic development (roughly 72–96 h post fertilization in many small bodied fish); post-embryonic development as yolk-sac larvae or fry larvae (yolk-sac is still attached and food supply is still endogenous), and the transition to free swimming larvae that marks the change from endogenous to exogenous food acquisition (Liu and Chang, 2002). During the final stages of this transition from embryo to free swimming larvae in zebrafish and other closely related species, there are morphological changes that occur such as the development of the gut and intestinal tract (segmentation beginning at 10 h post fertilization), development of the anterior–dorsal section of the mouth (48 h post fertilization), and inflation of the swim bladder (72 h post fertilization) as well as draining of the yolk sac; this is also the time when the fish first begin to move on their own, beginning to swim and flex their jaw muscles, fins, and move their eyes (Kimmel et al., 1995). Phenotypic sex determination,

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a system that determines the development of sexual characteristics, has also been shown to be an event that occurs early in all vertebrates, including the FHM and zebrafish. In zebrafish, ovarian development is initiated at 10 days post fertilization (dpf) and continues until 20 dpf. Testis development is estimated to begin ~21 to 30 dpf if the fish is going to be a male, and ovarian apoptosis begins (Hofsten and Olsson, 2005). The early onset of sexual differentiation, defined as the process in development where differences between males and females from an undifferentiated zygote are apparent, is a quality that makes small bodied fish ideal for embryonic toxicology testing, especially for chemicals that are either known or suspected endocrine disrupting chemicals.

Developmental processes are regulated by sex steroids that include androgens, estrogens, and thyroid hormones in addition to many others. There is evidence that steroids are present in early stage embryos. In rainbow trout, 17β-estradiol (E2) and cortisol levels increase during the final 2–4 days of embryogenesis and the androgen 11-ketotestosterone (11-KT) is produced 2 days after hatch (Yeoh et al., 1996). Moreover, maternal transfer of sex steroids occurs in rainbow trout, with initial concentrations being high, subsequently declining and then increasing again during embryonic development (Feist and Schreck, 1996). This suggests that steroid-mediated signaling pathways are required during the early stages of development. As steroids mediate teleost development, it is important to describe the developmental expression patterns of the enzymes involved in their biosynthesis, as well as their receptors.

The objectives of this study were to determine whether genes involved in steroid biosynthesis, steroid receptor signaling, and sex differentiation in the FHM were detectable early in development, commencing from a 1 day old embryo to a 2 week old fry larvae at five different time points (1, 3, 5, 6, and 14 dpf.). The first three time points corresponded to the neurula stage, the start of pectoral fin blood flow and dorsal swim bladder pigmentation, and pre-hatch (based on work in FHMs by Devlin et al. (1996); Fig. 1). The fourth time point (6 dpf) was immediately post-hatch and the fifth time point was collected after 8 days of larval growth (14 dpf). We measured transcripts related to steroid biosynthesis (*star*, *p450ssc*, *hsd11b2*) and receptor signaling (i.e. estrogen, androgen, and thyroid receptors) because these hormones are known to regulate development.

## 2. Materials and methods

### 2.1. Experimental design

Adult FHMs were taken from a breeding stock at the University of New Brunswick (Saint John) and placed into eight aquariums containing breeding tiles in a ratio of one male to two females per tank. Each tank was aerated using oxygen stones, which maintained a mean oxygen content of 86.9% oxygen per liter of water and had a mean water pH of 7.1. Dissolved oxygen percentage (% DO) was measured using a YSI Pro20 Dissolved Oxygen Meter (YSI Inc. Yellow Springs, OH). Dissolved $O_2$ readings were taken daily. Fish remained on a light/dark schedule of 16 h light to 8 h dark. The experimental room was maintained at approximately 20 °C while the water was maintained at approximately 25.5 °C. These conditions were maintained for 2 days until breeding was completed (six of the eight aquariums had produced eggs, yielding a total of approximately 360 fertilized embryos). Breeding occurred over 2 days to obtain a sufficient amount of fertilized eggs, however the collection of embryos was staggered to ensure that each pool was at the specific time point to be examined. All embryos were pooled and transplanted from the original breeding tiles into petri dishes. The pooled embryos were divided evenly across eight petri dishes, with each petri dish containing 35 embryos. Thus, eight biological replicates per time point were collected as each of the petri dishes were considered a biological replicate. We point out that not all replicates were used in the gene expression analysis as some RNA was not

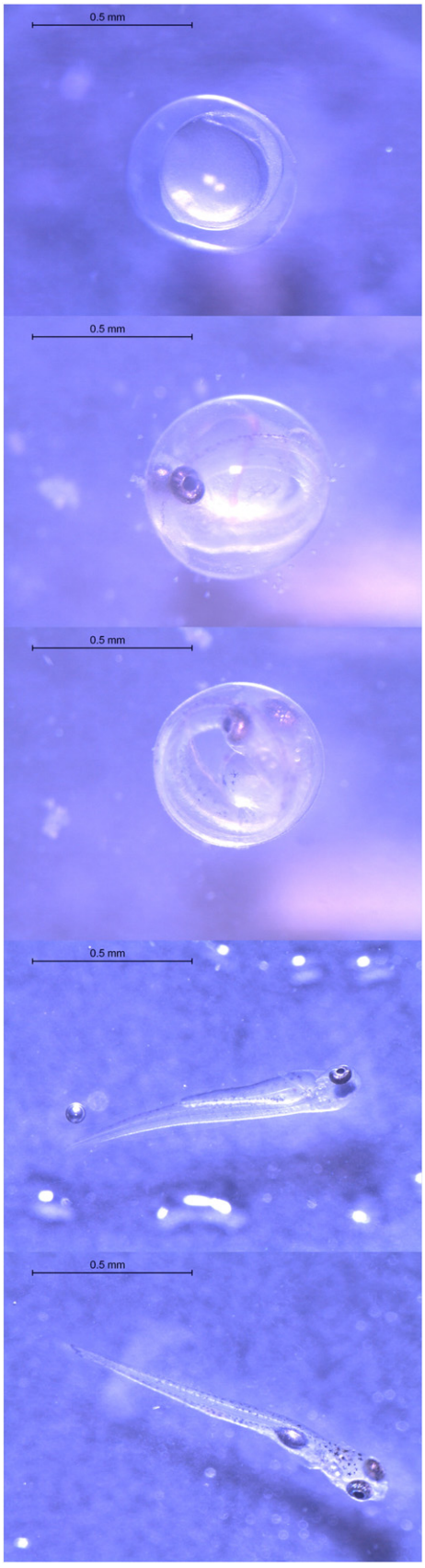


**Fig. 1.** Representative photographs of the developmental stages examined in this study for *Pimephales promelas* (micrographs from top to bottom are 1 dpf, 3 dpf, 5 dpf, 6 dpf, and 14 dpf).