Aromatase Inhibition in Fishes with Group-synchronous Oocyte Development: The Importance of Reproductive Traits

Jon Doering[1,2], Daniel Villeneuve[1], Brett Blackwell[1], Jenna Cavallin[3], Alexander Cole[4], Kendra Dean[4], Kellie Fay[5], David Feifarek[1], Kathleen Jensen[1], Michael Kahl[1], Ashley Kittelson[4], Carlie LaLone[1], Shane Poole[1], Eric Randolph[4], Charlene Tilton[4], Gerald Ankley[1]

[1] Mid-Continent Ecology Division, United States Environmental Protection Agency (U.S. EPA), Duluth, MN; [2] National Research Council, U.S. EPA, Duluth, MN; [3] Badger Technical Services, U.S. EPA, Duluth, MN; [4] ORISE Research Participation Program, U.S. EPA, Duluth, MN; [5] CSRA Inc, Science and Engineering, Duluth, MN

www.epa.gov

Introduction

- Aromatase (CYP19) is a steroidogenic enzyme involved in the conversion of androgens to estrogens and inhibition of CYP19 activity by chemicals can result in reduced concentrations of circulating estrogens, causing decreased synthesis of vitellogenin, and leading to impaired egg production.
- Reproductive failure resulting from exposure to chemicals that inhibit CYP19 activity has been extensively studied in several laboratory model fishes, including the fathead minnow (*Pimephales promelas*), and has led to the development of a quantitative adverse outcome pathway.
- These model species all have ovaries that undergo asynchronous oocyte development meaning that the mature ovary contains oocytes at all stages of maturation and recruitment of oocytes occurs continuously enabling frequent spawning events over an extended spawning season.
- Most fishes have ovaries that undergo group-synchronous oocyte development meaning that the ovary contains two distinct populations of oocytes, a synchronous population of larger oocytes (the clutch) and a heterogenous population of smaller oocytes which form the next clutch.
- Fishes with group-synchronous oocyte development typically ovulate the entire clutch in a single, annual spawning event proceeded by a dynamic seasonal cycle of reproductive parameters, including 4 key stages: (1) resting, (2) vitellogenesis, (3) ovarian recrudescence, and then (4) spawning.
- This dynamic seasonal cycle of reproductive parameters presents a significant challenge when studying reproduction in species with this reproductive trait in laboratory toxicity tests and no pragmatic laboratory model fish species has yet been developed, and consequently adverse effects of aromatase inhibition on reproductive performance in fishes with group-synchronous oocyte development is almost unknown.

Box 1. Study objective:
The primary objective of the present study was to investigate the use of mosquitofish (*Gambusia affinis*) as a suitable laboratory model fish species for group-synchronous oocyte development and investigate reproductive effects of aromatase inhibition in this species.

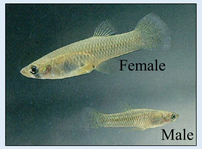


Figure 1. Mosquitofish (*Gambusia affinis*)

Materials and Methods

Box 2. Advantages of the mosquitofish model:
Mosquitofish could represent a suitable laboratory model for group-synchronous oocyte development with many advantages over some other group-synchronous species of fish (e.g. salmonids, perch), because:

1) The reproductive physiology of mosquitofish has been characterized in detail with demonstrated group-synchronous oocyte development.
2) Mosquitofish produce multiple, synchronous clutches per year with a complete reproductive cycle occurring at intervals of only 1 month as opposed to intervals of 12 months as common in fish.
3) Increasing temperature is the only environmental cue required to initiate the reproductive cycle (i.e. vitellogenesis).
4) Mosquitofish have a small adult size (maximum of 6 cm in length).
5) Mosquitofish are sexually dimorphic.
6) Mosquitofish are readily available and easily cultured in captivity.
7) Transcript sequence information is already available for key reproductive genes.

- Adult mosquitofish were acquired from a commercial supplier in early spring while being maintained at ambient temperatures of ≤ 12 C.
- 6 female mosquitofish were placed together in 20 L aquaria at 12 C under flow-through conditions. 13 replicate aquaria were used for each of 3 treatment groups representing 0, 2, or 30 ug/L of fadrozole. Fadrozole (FAD) is a model aromatase inhibiting chemical.

To initiate the reproductive cycle, water temperatures in the replicate aquaria were slowly increased from 12 C to 25 C over 48 hours.

- Replicate aquaria were sampled from each treatment group at 4 different time points representing exposure day -1 (at 12 C), 4 (at 25 C), 12 (at 25 C), and 19 (at 25 C) (illustrated in Figure 2B). These time points represent resting, early vitellogenesis, late vitellogenesis, and early ovarian recrudescence, respectively (Figure 2).
- Brain, liver, gonad, and plasma were sampled from fish at each time point and a broad suite of chemical, apical, biochemical, and molecular endpoints were investigated.
- Only CYP19 expression in gonad, 17b-estradiol (E2) concentration in plasma, vitellogenin (vtg) expression in liver, and gonadosomatic index (GSI) are shown here. These end points represent the primary reproductive parameters that could be effected by aromatase inhibition during group-synchronous oocyte development (Figure 2).

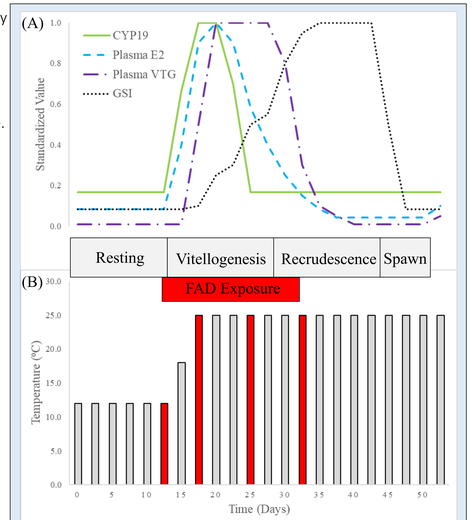
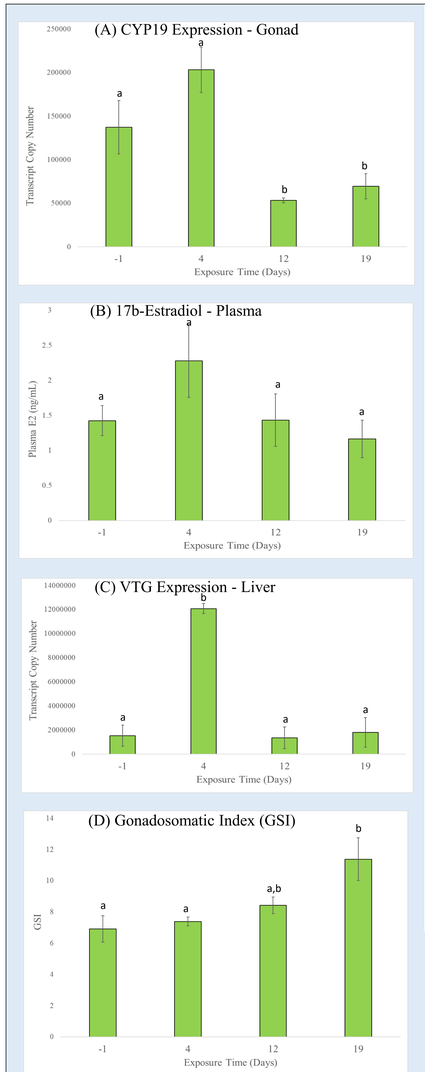


Figure 2. Cartoon showing normal temporal changes in CYP19 activity, plasma E2, plasma vtg, and GSI over the reproductive cycle in mosquitofish (A) relative to water temperature (B). The 4 sampling time points used in this experiment are highlighted in red (B) and the FAD exposure period is indicated by a red box (B).

Results and Discussion



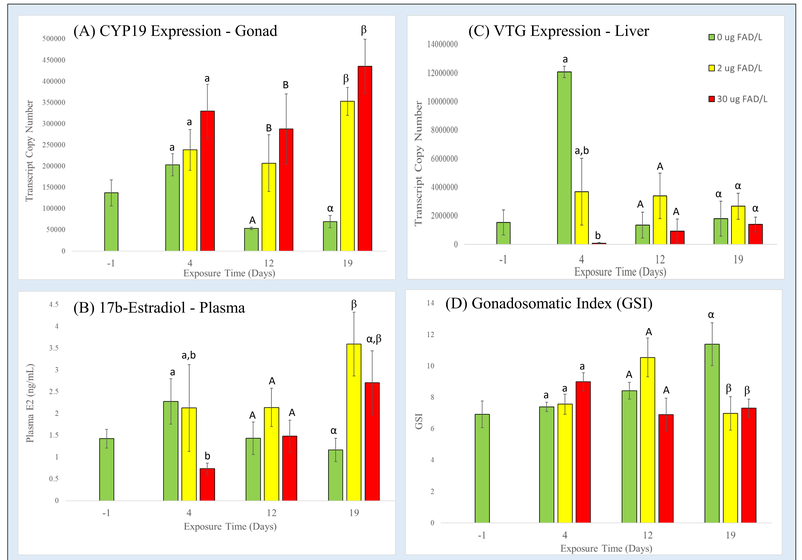


Figure 4. Responses to fadrozole exposure (0, 2, or 30 ug/L) of CYP19 transcript in gonad (A), E2 in plasma (B), vtg transcript in liver (C), and GSI (D) in mosquitofish at the 4 sampling time points outlined in Figure 2B. Statistical difference ($p \leq 0.05$) between treatments at each sampling time point is indicated by different letters.

- Control mosquitofish proceeded through a group-synchronous reproductive cycle following an increase in water temperature (12 C to 25 C) as demonstrated through a temporal increase then decrease in gonadal CYP19 expression, plasma E2, and hepatic vtg expression, followed by a gradual increase in GSI (Figure 3).
- Exposure of mosquitofish to 2 ug/L or 30 ug/L of fadrozole beginning during the resting stage and proceeding through the reproductive cycle caused unique temporal changes in parameters distinct from those of controls (Figure 4).
- Gonadal CYP19 expression remained elevated in exposed mosquitofish indicating that vitellogenesis did not reach completion (Figure 4A). Plasma E2 slowly increased in exposed mosquitofish by Day 19 (Figure 4B), possibly as a result of a compensatory increase in expression of CYP19 (Figure 4A). No clear increase in hepatic vtg expression (Figure 4C) or GSI (Figure 4D) was observed in exposed mosquitofish supporting the observation of incomplete vitellogenesis.
- Complete reproductive failure in mosquitofish exposed to 2 ug/L or 30 ug/L of fadrozole suggests that mosquitofish are at least as sensitive to aromatase inhibition by fadrozole as previously studied asynchronous fishes, such as fathead minnow.

Figure 3. Changes in CYP19 transcript in gonad (A), E2 in plasma (B), vtg transcript in liver (C), and GSI (D) in control mosquitofish over the 4 sampling time points outlined in Figure 2B. Statistical difference ($p \leq 0.05$) is indicated by different letters.

Conclusion

- This study demonstrates that aromatase inhibiting chemicals can cause reproductive dysfunction in fishes with group-synchronous oocyte development, but that temporal compensatory responses are distinct from those of fish with asynchronous oocyte development and therefore further research is warranted.

U.S. Environmental Protection Agency
Office of Research and Development