Stress and sex: A purely male phenomenon?
Examining the effects of air-emersion stress on sex ratio in zebrafish (*Danio rerio*)

Andrea Sim, Alexander Hare & Kathleen Gilmour
Department of Biology, University of Ottawa

Introduction

- Domesticated zebrafish (*Danio rerio)* have lost the chromosomal sex determination system that is present in wild zebrafish. Instead, multiple loci determine sex (i.e. a polygenic sex determination system), and therefore environmental factors may impact sex differentiation in domesticated zebrafish[4].
- For example, exposure to hypoxia very early in development (hours after fertilization) and the consequent increase in hypoxia-inducible factor-1 (HIF-1) induces masculinization in zebrafish[5], probably by decreasing aromatase gene expression which leads to an increase in testosterone levels[1,2].
- Similarly, high stocking density during early development induces a male-biased sex ratio[3,4], probably owing to chronic stress and accompanying elevation of cortisol, which is the primary glucocorticoid stress hormone in teleost fishes[2].
- In adult fish, elevation of cortisol mobilizes energy reserves to help the fish respond successfully to a stressor[2]. Accordingly, long-term cortisol elevation is associated with reduced growth and reproduction[2]. However the long-term effects of exposure to stressors and the consequent elevation of cortisol during early life are largely unknown.
- Thus, the present study tested the hypothesis that early life exposure to a stressor has long-term impacts on the physiology of developing zebrafish owing to the elevation of cortisol levels. Specifically, it was predicted that exposure to an air emersion stressor during early development would reduce growth and survival, reduce energetic investment into the reproductive organs, and induce masculinization in zebrafish.

Materials and Methods

- The experimental design (Fig.1) involved exposing zebrafish larvae to air at 4, 7, 15, or 35 days post fertilization (dpf) twice per day for two days[3].
- Whole-body cortisol levels were measured using a commercial EIA
- The forklength of zebrafish at 7, 15, and 35 dpf was measured from photographs using Image J.

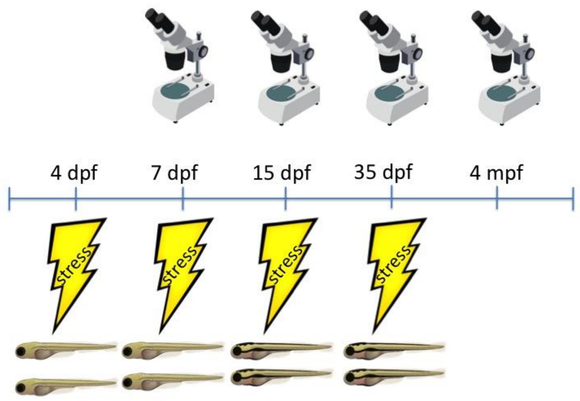

Figure 1. A schematic depicting the stress treatment (lightning bolt) and sampling points (microscope). The stressor consisted of air emersion[5], twice a day for 2 days. Cortisol levels, length and survival were assessed at all sampling points, while sex ratio and gonadosomatic index (GSI; gonad mass:body mass) were evaluated only at sexual maturity (4 months post fertilization; mpf).

Results

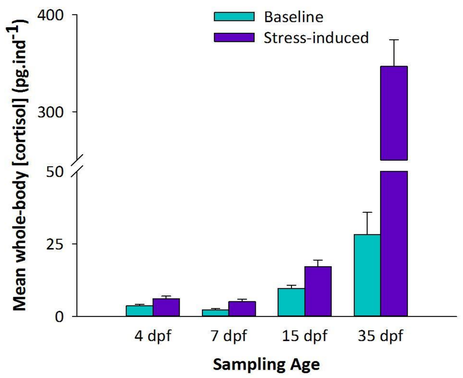

Figure 2. To confirm that exposure to the air-emersion stressor caused a cortisol response, baseline and stress-induced cortisol levels were measured at each treatment time. Exposure to air resulted in a significant elevation of whole-body cortisol concentrations at all treatment time-points, with older fish exhibiting higher cortisol levels. Additional tests revealed comparable elevation of cortisol across the individual exposures to air (data not shown). Values are means + SEM (n ≥ 5, where n = 1 represents a pool of 1 and 20 individuals depending on age). An asterisk indicates a significant difference in whole-body cortisol levels between baseline and stress-induced values (two way ANOVA, $F_{1, 57} = 7.0728$, $p = 0.0101$).

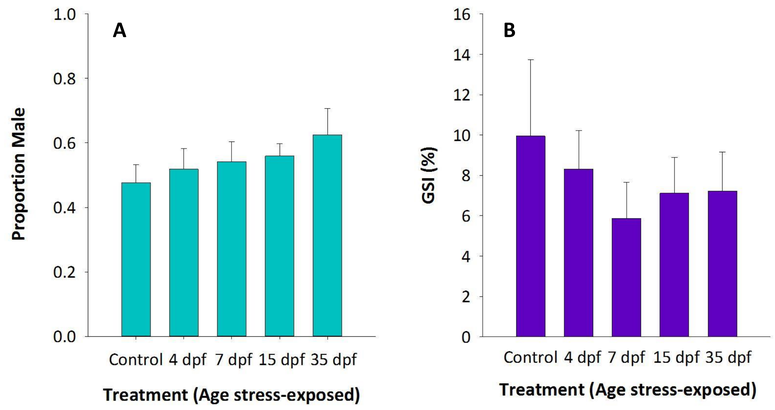

Figure 5. Early life exposure to an air-emersion stressor had no significant impact on sex ratio (A) or GSI (B) at sexual maturity. Values are means + SEM (n ≥ 4 for sex ratio, where n = 1 represents an independent tank of fish; n ≥ 93 for GSI, where n = 1 represents an individual fish). No significant differences were evident between control and treatment groups for the proportion of males within a treatment group (Chi-squared test, df = 4, $p = 0.2555$) or GSI (ANOVA, $F_{4,385} = 0.2577$, $p = 0.9059$).

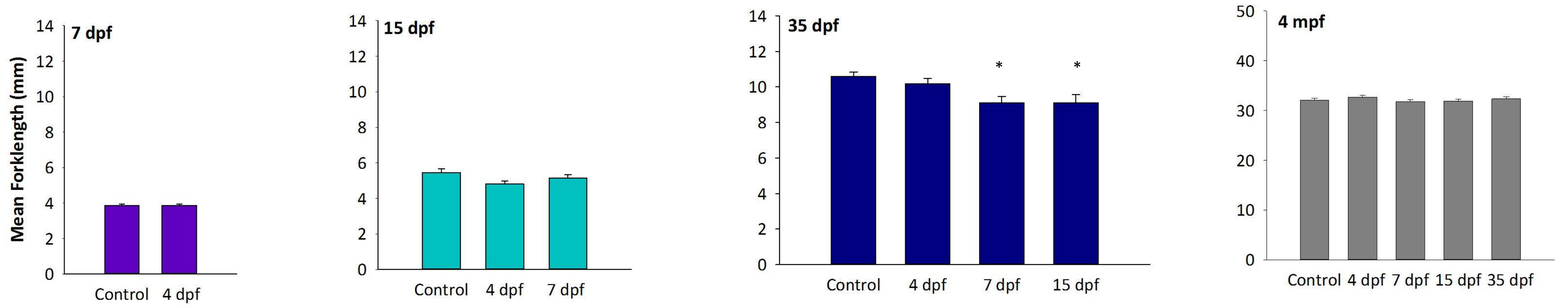

Figure 3. Exposure to the air-emersion stressor resulted in significant reductions in fork length at 35 dpf, but these differences were lost by the time of sexual maturity. Values are means + SEM (n ≥ 3, where each n represents the average of one tank for fish sampled from 7-35 dpf, and individual fish at 4 mpf). An asterisk indicates a treatment group that was significantly shorter than its corresponding control group (7 dpf, student's *t*-test, t=0.199, p = 0.845; 15 dpf, ANOVA, $F_{2,19} = 3.004$, $p = 0.076$; 35 dpf, ANOVA, $F_{3,47} = 5.014$, $p = 0.004$; 4 mpf, ANOVA, $F_{4,174.46} = 1.979$, $p = 0.1164$).

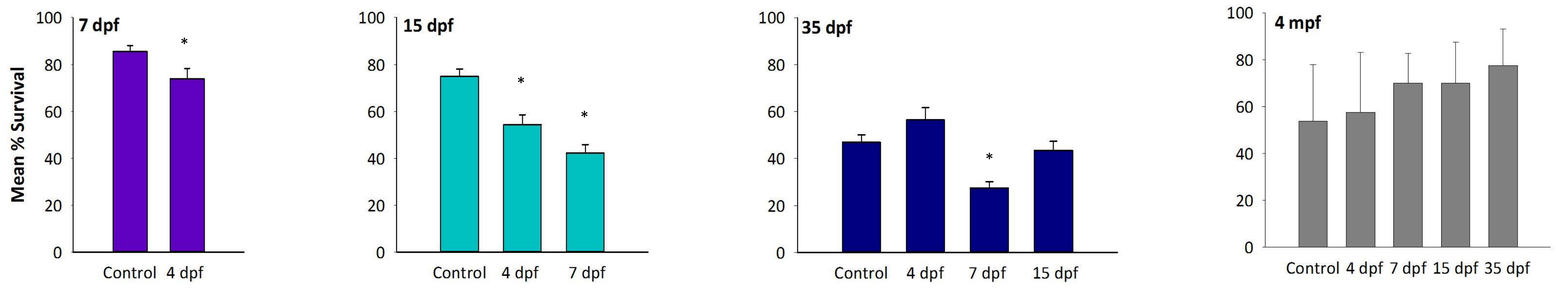

Figure 4. Exposure to the air-emersion stressor resulted in significant reductions in survival at 7, 15, and 35 dpf, but these differences were lost by the time of sexual maturity (4 mpf, ANOVA, $F_{4,17.096} = 0.16024$, $p = 0.9556$). Values are means + SEM (n ≥ 11, where each n represents the average of one tank). An asterisk indicates a treatment group that exhibited a significant decrease in survival relative to its corresponding control group (7 dpf, student's *t*-test, t= 2.479, p = 0.0197; 15 dpf, ANOVA, $F_{2, 33} = 20.604$, $p < 0.001$; 35 dpf, ANOVA, $F_{3,47} = 11.818$, $p < 0.001$).

Discussion

- Zebrafish were largely resistant to, or outgrew by sexual maturity, the effects of early life exposure to an air-emersion stressor.
- The air-emersion stressor was successful in eliciting a cortisol response at all treatment times, and transient effects of this repeated, acute stressor in terms of reduced length and survival were detected. These effects suggest that the mobilization of energy reserves that is expected to accompany a rise in cortisol levels diverts energy away from growth during early development.
- Although high stocking density[4] was associated with impacts on sex differentiation, no effect of repeated exposure to air was detected in the present study on adult fish. Thus, acute elevation of cortisol, even in a repeated fashion, does not appear to be sufficient to influence sex determination.

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