The Effect of Housing Density, Enrichment and Pathogens on Stress Levels in Zebrafish

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Background

- Despite the widespread popularity and applicability of the zebrafish as a biomedical research model, data on optimal husbandry parameters remains to be defined.
- Consequently, zebrafish continue to be infected with microorganisms acutely and subclinically that can confound research results.

Objectives

- Develop an evidence-based husbandry protocol to minimize stress in zebrafish.
- Parameters examined
  1. Stocking density
     - Low:
       - 1 female: 1 male and 1 female: 2 males
     - High:
       - 9 females: 9 males and 6 females: 12 males
  2. Enrichment
     - Silk plants vs bare bottom
  3. Pathogens
- Measurements of stress
  1. Serum cortisol concentrations
     - By enzyme linked immunoabsorbant assay (ELISA)
  2. Gene expression of hepatic stress receptor *nr3c1*
     - By quantitative PCR (qPCR)

Methods

Control Group

1F:1M	1F:2M
9F:9M	6F:12M

Enriched Group

1F:1M	1F:2M
9F:9M	6F:12M

Fig 1. Fish were housed in three tanks for each of the four stocking densities/sex ratios in bare bottom tanks (control) or tanks containing a silk plant (enriched) for a 1 week duration. A single female from each tank was then euthanized in 1µl/1ml of clove oil/dH_20 for blood collection and liver isolation. F=female; M=male.

Blood Collection

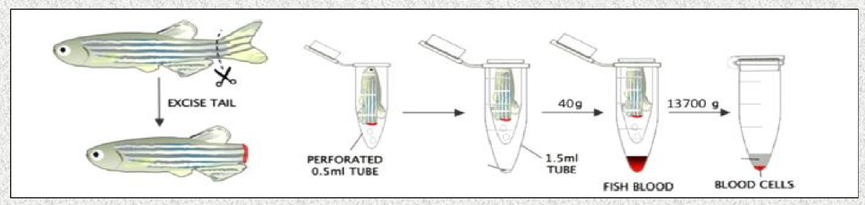

Fig 2. Fish were centrifuged twice at 40 x g for 5 minutes at room temperature, then 13,800 x g at 4 °C for 15 minutes. Procedure and figure were adopted from Babaei *et al* (2013)[1].

ELISA

[Cortisol]

Liver Dissection

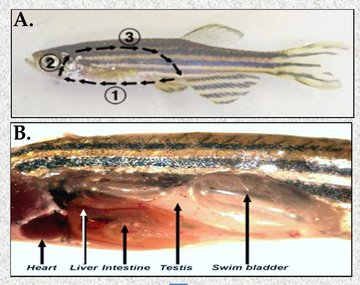

Fig 3. Livers were collected after centrifugation under dissection microscopes, and stored in RNAlater at -80°C for future analysis. **A.** Numbers correspond to order of incisions made. **B.** Location of organs are indicated following incision. Liver is indicated by the white arrow. Procedure and figure were adopted from Gupta and Mullins (2010)[2].

qPCR

nr3c1 upregulation

Results

Effect of Housing Density

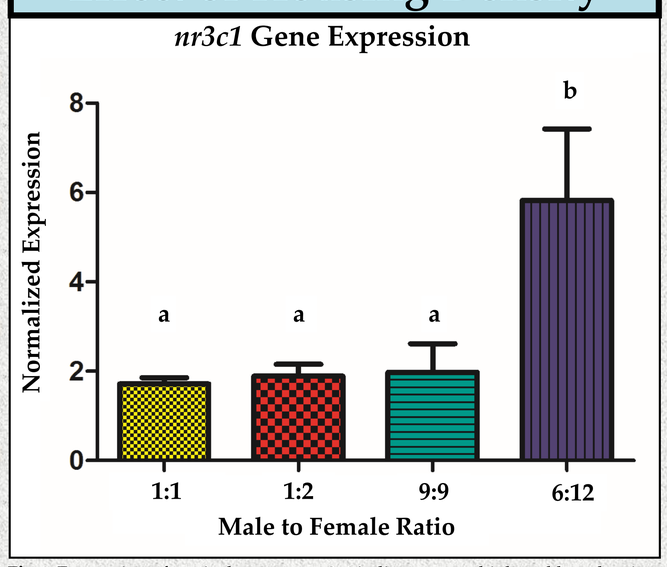

Fig 4. Expression of cortisol receptor *nr3c1* in liver across high and low density sex ratio housing conditions. Three tanks at each housing density were maintained and a single female from each tank was analyzed (n=3 for each density). 2 Way ANOVA of qPCR data showed an overall increase in expression of *nr3c1* in fish housed at the 6:12 **(b)** density compared to other groups **(a)** (6:12 vs 1:1 $p<0.038$, 6:12 vs 1:2 $p<0.020$, 6:12 vs 9:9 $p<0.009$). *Nr3c1* gene expression was normalized to the housekeeping gene, beta-actin (*actb1*) and analyzed as ΔΔCq with BioRad CFX Software Manager. Bars represent mean±standard error.

Effect of Enrichment

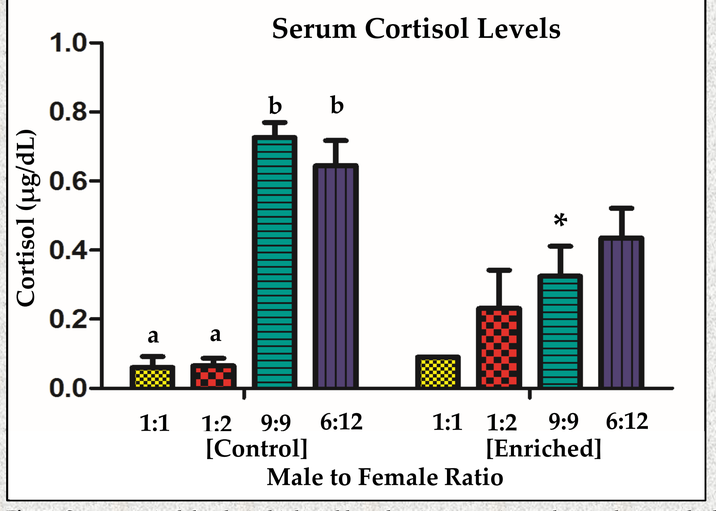

Fig. 5. Serum cortisol levels in high and low density sex ratios and control or enriched tanks. Six tanks at each housing density were maintained, three with plants (enriched) and three without plants (control). A single female from each tank was analyzed. 2 way ANOVA analysis of ELISA data showed significantly lower cortisol levels present in fish at low densities **(a)** compared to high densities **(b)** in control tanks ($p<0.001$). In tanks with plants, no statistically significant differences in cortisol levels were noted regardless of density. The asterisk indicates a statistically significant difference in cortisol levels at the 9:9 housing density between the control tanks and the enriched tanks. ($p<0.001$). Bars represent mean±standard error.

Effect of Pathogens

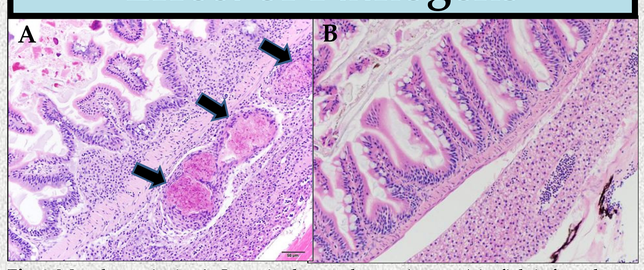

Fig 6. Mycobacteriosis. **A**. Intestinal granulomas (arrows) in fish infected with *Mycobacterium haemophilum* (n=2). **B.** Normal intestine in unaffected SPF fish (n=2). Samples were prepared by freezing in liquid nitrogen at -80°C (H&E; 20x).

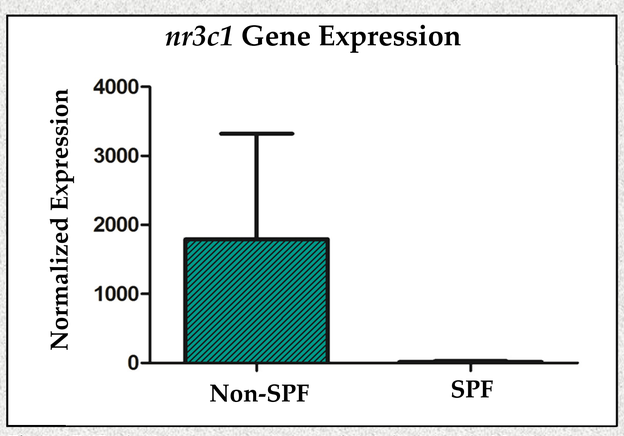

Fig 7. qPCR of *nr3c1* gene expression in fish infected with *Mycobacterium* vs SPF fish. SPF fish (n=6) showed a trend of decreased expression levels of the hepatic stress gene and cortisol receptor *nr3c1* compared to fish infected with *Mycobacterium haemophilum* (n=6) as determined by Mann-Whitney Rank Sum Test ($p=0.082$). Bars represent mean ± standard error.

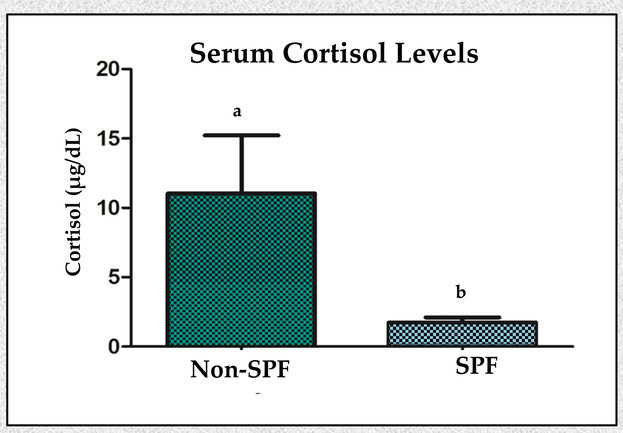

Fig 8. Serum cortisol in fish infected with *Mycobacterium* vs SPF fish. **(a)** SPF fish (n=6) had significantly lower levels of cortisol in their serum compared to fish infected with **(b)** *Mycobacterium haemophilum* (n=6) as determined by Mann-Whitney Rank Sum Test ($p = 0.019$). Bars represent mean ± standard error.

Conclusions

- Fish housed at densities above 5 fish/L appear to be more stressed, notably at unequal sex ratios. However, environmental enrichment with silk plants appears to have a protective effect against stress for all housing densities and ratios examined.
- Non-SPF fish with a detectable pathogen load have higher serum cortisol levels than SPF fish. Because a majority of facilities do not use SPF zebrafish, the presence of pathogens could create confounding effects on research data.

References

1. Babaei et al. (2013). Novel Blood Collection Method Allows Plasma Proteome Analysis from Single Zebrafish. *Journal of Proteome Research*.12:1580-1590.
2. Gupta, T., Mullins, M.C. (2010). Dissection of Organs from the Adult Zebrafish. *Journal of Visualized Experiments*.37: e1717, 1-5.

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