Development of Cardiovascular Metrics as Sublethal Endpoints for the Fish Embryo Toxicity Test

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Why:

- Current United States (US) and European Union (EU) regulations require that municipal and industrial effluents be tested for toxicity.[1–3] In the US we use the larval growth and survival (LGS) test, which utilizes larval fathead minnows as a model organism.
- The fathead minnow fish embryo toxicity (FET) test has been proposed as a more humane alternative to the LGS.[4–9] The use of fish embryos represents an improvement in animal welfare as younger organisms are considered to experience less pain and distress than more developed organisms.[9,10]
- The current FET test protocol does not include endpoints that allow for the prediction of sublethal adverse effects,[11] making it unlikely that the US will adopt the FET test as a replacement for current testing methods.
- Several cardiovascular related abnormalities have been noted in FET tests[4,12–14]. These abnormalities may be useful in identifying sub-lethal effects on exposed organisms, and their inclusion as endpoints may eventually allow for the estimation of chronic toxicity.

The objective of this study was to increase the predictive power of the FET by:

1) **developing methods to quantify sublethal endpoints related to cardiovascular development (*i.e.*, heart rate and pericardial area)**
2) **assessing the responsiveness of a battery of sublethal endpoints related to cardiovascular development and function to known reference toxicants**

How:

- Fathead minnow embryos (three replicates/treatment with twenty fish/replicate) were exposed to four reference toxicants in well plates for five days
  - Reference toxicants were: 3,4-dichloroanaline (3,4-DCA), sodium chloride, cadmium, and triclosan.
- Sublethal metrics associated with cardiovascular function and development were assessed throughout the exposure period (Figure 1).
- Relative gene expression for targeted genes (Table 1) was measured via real time qPCR using the standard curve method and normalized using the geometric mean of *l8* and *ef1*.
- **Cardiovascular function and development endpoints**
  - *Gene Expression* – measured via qPCR, see Table 1 for a list of genes and their functions.
  - *Pericardial Area* – measured as mm^2 via photograph at 120 hpf using DanioScope software.
  - *Heart Rate* – measured as beats per minute via video at 76 hpf or 96 hpf using DanioScope software.

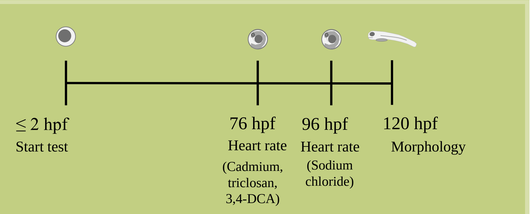

Figure 1. General timeline for the experiment, indicating when each endpoint was measured.

Gene Expression Analysis

Table 1. List of genes investigated in this study, with abbreviations and a brief description of function.

Gene		Function
atrial light chain-2	*myl7*	functions to modulate cardiac development and contractility
vascular endothelial growth factor a	*vegfa*	signal protein that stimulates vasculogenesis and angiogenesis
GATA-binding factor 1	*gata-1*	key mediator of the development of specific types of blood cells
bone morphogenetic protein 10	*bmp10*	cardiac growth and chamber maturation

- Significant differences in the expression of *vegfa* were detected between 3,4-DCA treatment groups (Fig. 2).
- No significant differences were seen between any of the sodium chloride treatment groups (Fig. 3)
- Neither *gata-1* or *bmp10* quantified in any of the samples.

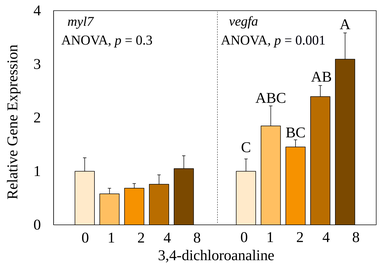

Figure 2. Average relative gene expression of genes related to cardiovascular function and development in 3,4-dichloroaniline exposed fish. Error bars represent one standard error; n = 5. Different letters above bars indicate significant differences between doses for each gene.

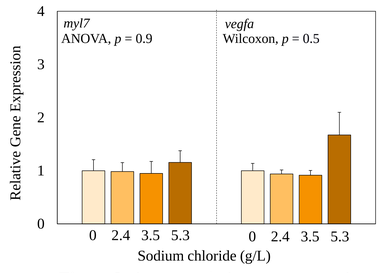

Figure 3. Average relative gene expression of genes related to cardiovascular function and development in sodium chloride exposed fish. Error bars represent one standard error; n= 5.

Pericardial Area

- Significant differences were found in pericardial area between 3,4-DCA (Fig. 4), sodium chloride (Fig. 5), and cadmium (Fig. 6) treatment groups at 120 hpf.
- For all three chemicals, the lowest observed effect concentration was lower than the calculated median lethal concentration for that chemical.
- No significant differences were noted in triclosan exposed embryos (Fig. 7). This is likely due to a lack of power resulting from small sample size.

Control Embryo

Embryo exposed to 8 mg/L 3,4-DCA

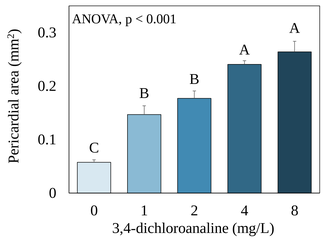

Figure 4. Average pericardial area for embryos exposed to 3,4-DCA. Error bars represent standard error; n = 3. Different letters above bars indicate significant differences between doses.

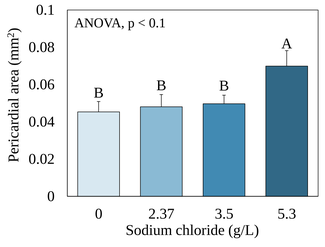

Figure 5. Average pericardial area for embryos exposed sodium chloride. Error bars represent standard error; n = 3. Different letters above bars indicate significant differences between doses.

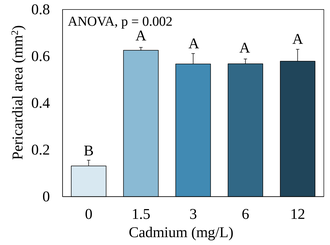

Figure 6. Average pericardial area for embryos exposed to cadmium. Error bars represent standard error; n = 3. Different letters above bars indicate significant differences between doses.

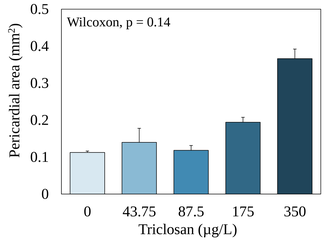

Figure 7. Average pericardial area for embryos exposed to triclosan. Error bars represent standard error; n = 2.

Heart Rate

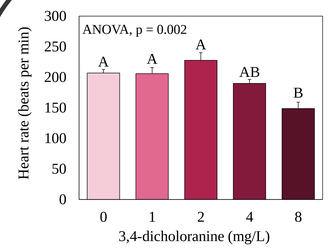

Figure 8. Average heart rate for embryos exposed to 3,4-DCA. Error bars represent standard error; n = 3. Different letters above bars indicate significant differences between doses.

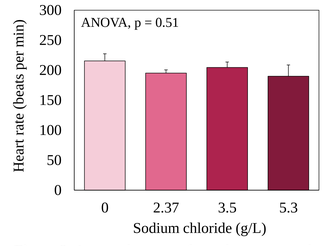

Figure 9. Average heart rate for embryos exposed to sodium chloride. Error bars represent standard error; n = 3.

QR code links to video of control and DCA exposed embryo, illustrating the **increased pericardial area** as well as the **reduction in heart rate**.

- Significant differences were detected in heart rate between 3,4-DCA treatment groups at 76 hpf (Fig. 8).
- There was a significant difference in the cadmium treatment groups, but post-hoc testing was unable to identify which groups were different (Fig. 10).

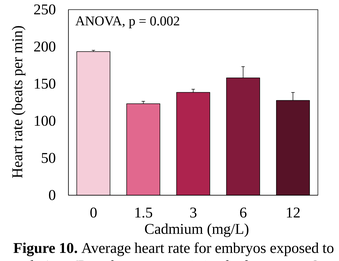

Figure 10. Average heart rate for embryos exposed to cadmium. Error bars represent standard error; n = 3. Post-hoc analysis was unable to identify significant differences.

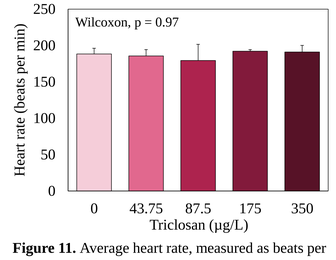

Figure 11. Average heart rate, measured as beats per minute, at 76 hpf for embryos exposed to triclosan. Error bars represent standard error; n = 2.

Conclusions and Future Directions

- **Gene expression showed the least promise. Significant alterations were only seen in one of the four genes targeted, and then only in response to one of the chemicals.**
- **Pericardial area shows the most promise for improving the sensitivity of the FET test. It was significantly increased in three of the four chemicals investigated, and in three out of the four chemicals it demonstrated a dose dependent trend.**

  Further research is needed to establish what the long-term adverse impacts of edema are on the health of fish.
- **Heart rate was less sensitive than pericardial area, but more sensitive than gene expression. Significant alterations were seen in two of the four chemicals.**

Acknowledgements

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References

1. *Federal Water Pollution Control Act Amendments of 1972* (1972). **2.** *Toxic Substances Control Act. 15* (1976) **3.** EU. *Off. J. Eur. Union* 396, 1–849 (2006). **4.** Jeffries, M. K. S. *et al. Environ. Toxicol. Chem.* 34, 1369–1381 (2015). **5.** Jeffries, M. K. S. *et al. Environ. Toxicol. Chem.* 33, 2584–2594 (2014). **6.** Embry, M. R. *et al. Aquat. Toxicol.* 97, 79–87 (2010). **7.** Belanger, S. E., Rawlings, J. M. & Carr, G. J. *Environ. Toxicol. Chem.* 32, 1768–1783 (2013). **8.** Lammer, E. *et al. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 149, 196–209 (2009). **9.** Strähle, U. *et al Reprod. Toxicol.* 33, 128–132 (2012). **10.** EFSA *EFSA J.* 3, 292 (2005). **11.** OECD. *Test No. 236: Fish Embryo Acute Toxicity (FET) Test*. (OECD Publishing, 2013). **12.** Chen, J. *Zebrafish* 10, 389–400 (2013). **13.** Hicken, C. E. *et al. Proc. Natl. Acad. Sci.* 108, 7086–7090 (2011). **14.** Antkiewicz, D. S. *Toxicol. Sci.* 84, 368–377 (2005).