Fish Early Life Stage: Developing AOPs to Support Targeted Reduction and Replacement

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Fish early life-stage (FELS) test

- Introduced >30 years ago as an alternative to FFLC – OECD 210 or OCSPP 850.1400
- Primary guideline test for estimating chronic toxicity
- Frequently used to support ERAs and chemical management programs around the world
- Europe: involves testing of protected life stages

**FRESHWATER**

- Fathead minnow (*Pimephales promelas*)
- Rainbow trout (*Oncorhynchus mykiss*)

**SALTWATER**

- Sheepshead minnow (*Cyprinodon variegatus*)
Need for an alternative testing strategy

• FELS test design is labor and resource intensive
  – Study duration is one to three months
  – Requires at least 360 fish, but usually >700 fish
  – Typical CRO cost per test is 50-125K USD

• FELS test endpoints provide little MOA information
  – Narrow focus on gross morphologic endpoints (i.e., survival, percent hatch, body length, etc.)
  – Chronic NOEC and/or EC_{10} thresholds not helpful for categorizing chemicals by MOA
Step 1: Build a conceptual model of biological events required for normal early fish development.

Step 2: Identify key events that, when impacted, will lead to adverse effects on FELS growth and survival.

Step 3: Outline hypothesized AOPs for these key events based on existing data within the literature.

Step 4: Develop targeted HTS/HCS assays that capture sub-organismal endpoints along these AOPs.

Step 5: Screen reference chemicals to test AOPs and define concentration-response extrapolations.

Qualitative AOPs

Quantitative AOPs
**Step 1 – Build a conceptual model of biological events required for normal early fish development.**

### Zebrafish embryogenesis

<table>
<thead>
<tr>
<th>Stage</th>
<th>Process/Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zygote 0 hpf</td>
<td>miRNA-mediated maternal transcript degradation</td>
</tr>
<tr>
<td>Cleavage 0.75 hpf</td>
<td>Genome remethylation by DNA methyltransferases</td>
</tr>
<tr>
<td>Blastula 2.25 hpf</td>
<td>Zygotic genome activation by transcription factors</td>
</tr>
<tr>
<td>Gastrula 5.25 hpf</td>
<td>Cell cycle (mitotic) regulation by Cdk/cyclins</td>
</tr>
<tr>
<td>Segmentation 10 hpf</td>
<td>Cell migration by F-actin polymerization and yolk cell microtubule towing</td>
</tr>
<tr>
<td>Pharyngula 24 hpf</td>
<td>Intracellular calcium signaling via ER release</td>
</tr>
<tr>
<td>Hatching 48 hpf</td>
<td></td>
</tr>
</tbody>
</table>

Step 2 – Identify key events that, when impacted, will lead to adverse effects on FELS growth and survival.

Criteria for identification of a ‘key event’ (e.g., cardiac looping)

**Criterion #1:** Observable and measurable

**Criterion #2:** Essential to survival and/or growth

Source: Bakkers (2011)

Source: Fish et al. (2011)
Step 3 – Outline hypothesized AOPs for these key events based on existing data within the literature.

Developing the weight of evidence supporting KERs

FELS AOP for AHR activation

Source: Antkiewicz et al. (2006)
Source: Antkiewicz et al. (2005)
Source: Antkiewicz et al. (2005)
Source: Henry et al. (1997)
Step 4: Develop targeted HTS/HCS assays that capture sub-organismal endpoints along these AOPs.
Step 5: Screen reference chemicals to test AOPs and define concentration-response extrapolations.
• The AOP built around cardiac looping was a case where well supported AOP could be derived from the extant literature.

• Not as simple to build AOPs around other key events.

• Swimbladder inflation as a case study
Does SB Inflation meet criteria of a KE?

Characteristics of Key Events (as defined for IPCS Mode of Action Framework*)

1. Measurable/observable
2. Plays an essential role in a causal chain from an MIE to AO (if KE is prevented, AO will not occur).

KE: Swimbladder inflation
- Key event is readily assessed via observation.
- Buoyancy control is vital to larval fish survival
  - Energy sparing
  - Diel migration
  - Predator avoidance

Can we develop linkages to the AO?

Developing the weight of evidence supporting KERs
Ecological Consequences of Swim Bladder Noninflation for Larval Yellow Perch


Organ

Reduced growth rate
(increased metabolic demand to maintain position in water column)
Increased O2 consumption

Organ System

Increased mortality under stress conditions

Individual

Population

Reduced y.o.y. survival

Impaired swim bladder inflation

• Toxicity would not necessarily manifest under laboratory conditions.

Woolley and Qin, 2010, Rev. Aquacult. 2: 181-190
Can we develop linkages to MIE(s)?

- MIE(s) unknown

- Should we invest the resources to identify MIE and support earlier KERs?
Could we directly observe the AO or KE in OFET?

FET = Fish Embryo Acute Toxicity Test (OECD TG 236)
OFET = Optimized FET

- Adverse outcome directly observable in FET?
  - NO
  - Key event(s) directly observable in FET?
    - NO
    - Need to extend to earlier KEs
    - Screen with HTS in vitro assay
    - High priority for additional AOP development

- Toxicity would not necessarily manifest under laboratory conditions.
  - Little to no competition for food
  - Lack of predators
  - Possibly as growth, but well after 96 hpf

- Unlikely
- Current FET Guideline: ends 96 hpf (D. rerio)
- Swimbladder inflation:
  - 1-3 dph (1-2 d later; D. rerio)
  - Generally near time active feeding begins

Hypothesis: Impaired thyroid hormone signaling can lead to impaired swimbladder formation in fish. (.....as well as other morphological outcomes likely to limit growth)

Liu and Chan, 2002. Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. Differentiation 70, 36-45

Putative AOP Development: Hypothesized MIE(s)
Potential MIEs linked to hypothyroidism: K. Paul presentation
- opportunity to leverage screening assay data to predict SB-mediated FELS toxicity
Thyroid peroxidase inhibition (TPO)
Inhibition of sodium iodide symporter (NIS)
Peripheral deiodinase inhibition (DI)

**Chemical**

- 2-mercapto benzothiazole (MBT)

**MIE**

- Thyroid peroxidase inhibition

**Cell**

- Reduced T3, T4 synthesis

**Blood**

- Reduced serum T3, T4

**Organ**

- Impaired swim bladder inflation

**Adverse outcome**

- Reduced young of year survival
Putative AOP - Testing

- 4 hpf through 8, 16 d
- Daily static renewal
- Verified concentrations
- 1 embryo per well
- Up to 8d not fed
- After 8d fed, transferred to cups

Timing of swim bladder inflation

<table>
<thead>
<tr>
<th>species</th>
<th>posterior</th>
<th>anterior</th>
</tr>
</thead>
<tbody>
<tr>
<td>zebrafish</td>
<td>4.5-5 dpf</td>
<td>20-21 dpf</td>
</tr>
<tr>
<td>fathead minnow</td>
<td>5.5-6 dpf</td>
<td>13-14 dpf</td>
</tr>
</tbody>
</table>
Putative AOP - Testing

- 8d – no significant effects
- 16 d - 1 mg MBT/L inhibited anterior, but not posterior sb inflation
- Anterior sb is an auditory organ in fish
  - Relevance to FELS growth and survival unclear
Do the results reject our hypothesized AOP?

Not necessarily......but the story is more complicated

Hypotheses –
• Insensitive to TPO inhibition until maternally-derived thyroid hormone is depleted

• Potentially sensitive to peripheral deiodinase inhibition while maternally-derived thyroid hormone is available. [inhibit T4 to T3 conversion]
Putative AOP - Testing

- 4 hpf through 5 d
- Daily static renewal
- Verified concentrations
- 2 embryos per well
- Not fed

5 ppm  
10 ppm  
20 ppm
• Observed lack of pigmentation in all surviving embryos at MBT concentration $\geq 5$ ppm starting at 48 hpf.

• Anti-sense knock-down of deiodinase I and II in zebrafish causes similar effects (Walpita et al. 2010 Gen. Compar. Endocrinol. 166:134-141.)

• Experiments in progress to examine whether 16 d exposure to DI inhibitor will impair posterior sb inflation in fathead minnow.
Conclusions

• Collaborative efforts to develop AOPs related to FELS toxicity on-going internationally.

• Strategies employed for AOP development for the purpose of developing alternative methods can different from those for other purposes.

• Thyroid disruption-related AOPs related to FELS toxicity provides examples of:
  1. Life-stage dependence of AOP applicability
  2. Chemicals with mixed modes of action and how that can influence the AOPs activated in dose-dependent and temporal dimensions of exposure.
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