A new standard for endocrine disruptor testing in fish -
The integrated Fish Endocrine Disruptor Test (iFEDT)

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“Development of a study protocol for regulatory testing to identify endocrine disrupting substances in biotic systems”

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Current challenges in endocrine disruptor testing with aquatic species

• Test guidelines for assessment of endocrine adversity are complex and long (= expensive and using many animals)
• Only few tests cover all relevant life stages and include population-relevant, apical endpoints
• Distinction from general toxicity is not possible in all tests
• Major gaps and weaknesses exist regarding the different EATS* modalities → EAS in fish, T in amphibians → need to run multiple tests to cover all

→ DG ENV call for tender (2018)

* Estrogen, Androgen, Thyroid, Steriodogenesis
Design of existing OECD test guidelines with fish

TG 229 (Fish short-term reproduction assay)
- Established endpoints: Vitellogenin, sec. sex characteristics, fecundity, gonad histology
- Life stages: Adult (> 6 mpf), Egg (< 1 dpf)
- Duration: 21 days

TG 234 (Fish sexual development test)
- Established endpoints: Growth, hatch, survival, vitellogenin, sex ratio, gonad histology
- Life stages: Embryo (< 5 dpf), Larva (> 5 dpf), Juvenile (1 – 5 mpf)
- Duration: 63 days

mpf: months post fertilization
dpf: days post fertilization
Proposal for a **new/merged** test protocol

**TG 229 (FSTRA)**
- Established endpoints
  - VTG, sec. sex characteristics, fecundity, gonad histology

**TG 234 (FSDT)**
- Growth, hatch, survival, VTG, sex ratio, gonad histology

**Life stages**
- | Stage | Description |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>(&gt; 6 mpf)</td>
</tr>
<tr>
<td>Embryo</td>
<td>(&lt; 5 dpf)</td>
</tr>
<tr>
<td>Larva</td>
<td>(&gt; 5 dpf)</td>
</tr>
<tr>
<td>Juvenile</td>
<td>(1 – 5 mpf)</td>
</tr>
</tbody>
</table>

21 days + 63 days = 84 days

→ OECD TG 240 „MEOGRT“: **133 days**!

mpf: months post fertilization
dpf: days post fertilization
## Gaps in fish OECD test guidelines 229, 230 & 234

<table>
<thead>
<tr>
<th>Test guideline</th>
<th>OECD TG 229(^{(c)})</th>
<th>OECD TG 230</th>
<th>OECD TG 234</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test duration</strong></td>
<td>21 days</td>
<td>21 days</td>
<td>60 days post-hatch</td>
</tr>
<tr>
<td><strong>Life stages</strong></td>
<td>Sexually mature male and spawning female (F0)</td>
<td>Sexually mature male and spawning female (F0)</td>
<td>From newly fertilised egg until completion of sexual differentiation (F0)</td>
</tr>
<tr>
<td><strong>Species</strong></td>
<td>Fathead minnow, Japanese medaka, zebrafish</td>
<td>Fathead minnow, Japanese medaka, zebrafish</td>
<td>Japanese medaka, threespined stickleback, zebrafish, fathead minnow (partially validated)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter name</th>
<th>Indicative of: (^{(a)})</th>
<th>VTG in females</th>
<th>VTG in males</th>
<th>Spiggin</th>
</tr>
</thead>
<tbody>
<tr>
<td>VTG in females</td>
<td>E, A, S</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>VTG in males</td>
<td>E, A, S</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Spiggin</td>
<td>A</td>
<td>X</td>
<td>X</td>
<td>X (^{(d)})</td>
</tr>
<tr>
<td>Male SSC in females</td>
<td>A</td>
<td>X</td>
<td>X</td>
<td>X (^{(d)})</td>
</tr>
<tr>
<td>Male SSC in males</td>
<td>E, A, S</td>
<td>X</td>
<td>X</td>
<td>X (^{(d)})</td>
</tr>
<tr>
<td>Specific gonad histopathology (^{(b)})</td>
<td>E, A, S</td>
<td>X (optional)</td>
<td>X (optional)</td>
<td></td>
</tr>
<tr>
<td>Sex ratio (female biased)</td>
<td>E, A</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex ratio (male biased)</td>
<td>E, A, S</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Test guideline**

<table>
<thead>
<tr>
<th>OECD TG 229(^{(c)})</th>
<th>OECD TG 230</th>
<th>OECD TG 234</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcriptional activity of cyp19a1b</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>Behaviour</td>
<td>N</td>
<td>X</td>
</tr>
<tr>
<td>Length</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Morphological abnormalities</td>
<td>N</td>
<td>X</td>
</tr>
<tr>
<td>Gonadosomatic index</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Embryo time to hatch</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Reproduction (fecundity, fertility)</td>
<td>N</td>
<td>X</td>
</tr>
<tr>
<td>Survival</td>
<td>N</td>
<td>X</td>
</tr>
<tr>
<td>Larval survival and length</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Survival of embryos</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Time to maturity (time to first spawn)</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Hatching success</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Histopathology (liver, kidney)</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Body weight</td>
<td>N</td>
<td></td>
</tr>
</tbody>
</table>

**Orange:** *in vivo* mechanistic  
**Blue:** EATS-mediated  
**Purple:** sensitive to, but not diagnostic of EATS

ECHA/EFSA guidance document 2018
Importance of the thyroid hormone system in fish

Thyroid hormones regulate „everything“

Developmental processes:
• Fins
• Pigmentation
• Craniofacial structures
• Swimbladder
• Neurodevelopment, sensory organs (eyes, ears, lateral line, olfactory epithelium)

Physiological processes:
• Energy metabolism
• Growth
• Stress response
• Immune system

Very little is known about adverse outcomes of environmental exposure of fish to thyroid hormone axis disruptors
Potential thyroid-related endpoints in fish

- Eye development
- Swimbladder inflation
- Pigmentation
- Thyroid histopathology
- Fin development

+ Thyroid hormone levels and gene expression in target systems
Proposal for the design of a new test protocol

Advanced TG (iFEDT: integrated Fish Endocrine Disruptor Test)

- TG 229 (FSTRA)
  - Established endpoints: VTG, sec. sex characteristics, fecundity, gonad histology
- TG 234 (FSDT)
  - Growth, hatch, survival, VTG, sex ratio, gonad histology
  - (Potential) new endpoints: Hormone levels, thyroid follicle histology
  - Hormone levels, thyroid follicle morphology/histology, eye development, skin pigmentation, swimbladder inflation

Life stages:
- Adult (> 6 mpf)
- Embryo (< 5 dpf)
- Larva (> 5 dpf)
- Juvenile (1 – 5 mpf)

21 days + 63 days
Strategy of EU Tender project « iFEDT »:

Hypotheses:

1. It is possible to merge the existing test guidelines 229 and 234 without major changes to the protocols
2. New endpoints in fish can be established in order to assess thyroid-related effects without using amphibians

Approach:

1. Run iFEDT experiment with a model thyroid hormone axis disruptor: Propythiouracil (PTU)
2. Run iFEDT experiment with a model estrogen disruptor: Ethinylestradiol (EE2)
Results: PTU experiment
Adults (21 days of exposure according to TG 229)

Reproduction

- PTU exposure caused impaired reproduction in adult fish
- A reduced cumulative number of eggs per female was found at the highest PTU concentration → No effect on hatch and survival of offspring
- No effect on adult survival
- No effect on adult weight and length

Cumulative number of eggs/female/day in adult zebrafish (Danio rerio) after 21-day exposure to different PTU concentrations (0 to 78.1 mg/L).
Adults (21 days of exposure according to TG 229)

Vitellogenin (VTG) levels

- PTU exposure had **no effect** on vitellogenin levels of adult fish

### VTG levels of female and male adult zebrafish after 21-day exposure to different PTU concentrations (0 to 78.1 mg/L)

(N=4; n= 4-6 per replicate, n=14-22 per concentration)
Adults (21 days of exposure according to TG 229)

Histology – Thyroid

- PTU exposure caused a **significant increase** in thyroid follicle epithelium thickness in adult fish
- PTU exposure induced a slight increase in size and number of thyroid follicles
- No histopathological effects in gonads, livers and eyes

**Thyroid follicle size and epithelium thickness of PTU-exposed zebrafish (**:** p<0.001; n=3-13)**

*Thyroid follicles of control vs. PTU-exposed zebrafish with proliferated epithelium*
Results: Larvae
PTU exposure caused a **significant increase** in thyroid follicle epithelium thickness in larvae.

* Thyroid follicle size and epithelium thickness of PTU-exposed fish (*: p<0.05; n=5-9)
F1 generation (25 days of exposure according to TG 234) : Larvae

Histology – Retinal pigment epithelium (RPE) layer of the eyes

- PTU exposure caused a **significant increase** in RPE thickness in the eyes of larvae

![Graph showing RPE thickness with PTU concentrations](image)

*RPE thickness of PTU-exposed fish (****: p<0.0001; n=5-8)*
Results: Juveniles
F1 generation (60 days of exposure according to TG 234): Juveniles
Morphological changes

- PTU exposure led to **impaired growth** of offspring

*Length and weight of juvenile fish (**: p<0.001, n= 64-100)*
F1 generation (60 days of exposure according to TG 234): Juveniles

Morphological changes

- PTU exposure led to formation of a goiter (proliferated thyroid follicles) in juvenile zebrafish. The images show the development of goiter in the F1 generation of zebrafish exposed to 78.125 mg/L PTU for 60 days.
F1 generation (60 days of exposure according to TG 234): Juveniles

Histology – Thyroid

- Number of thyroid follicles per fish:
  - 0 mg/L: 5-15
  - 12.5 mg/L: 15-30
  - 31.25 mg/L: 15-30
  - 78.125 mg/L: **15 to >60**

- Increase of epithelium thickness

- Strong changes in colloid structure

*Thyroid follicle size and epithelium thickness of PTU-exposed fish (****: p<0.0001)

Control  
78.125 mg/L PTU
F1 generation (60 days of exposure according to TG 234): Juveniles

Histology – Eyes

- PTU exposure induced a **significant increase** of eye diameter/body length ratio in juveniles.
- The thickness and pigmentation of the retinal pigment epithelium (RPE) was slightly decreased.

*Retina of control vs. PTU-exposed zebrafish with altered cellular structure*

*Ratio eye diameter/body length and retinal pigment epithelium (RPE) thickness of juvenile zebrafish exposed to PTU (n= 11-57)*
## Summary PTU « iFEDT » experiment

<table>
<thead>
<tr>
<th></th>
<th>Adults</th>
<th>Embryos</th>
<th>Larvae</th>
<th>Juveniles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reproduction</strong></td>
<td>↓</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Survival rates</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Weight</strong></td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>↓</td>
</tr>
<tr>
<td><strong>Length</strong></td>
<td>-</td>
<td>↑ at 12.5 and 78.125mg/L</td>
<td>ND at 31.25mg/L</td>
<td>↓</td>
</tr>
<tr>
<td><strong>Eyes</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>- eyes/body ratio</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>- RPE</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td><strong>Thyroid follicles</strong></td>
<td>↑</td>
<td>ND</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>- number</td>
<td>↑</td>
<td>ND</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>- size</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>- epithelium thickness</td>
<td>↑</td>
<td>ND</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td><strong>VTG</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Thyroid hormone</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Sex ratio</strong></td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>↑ females</td>
</tr>
<tr>
<td><strong>Gonad maturity</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td><strong>Other comments</strong></td>
<td></td>
<td>No effect on RPE but effect on some other layers (INL and Photoreceptors)</td>
<td></td>
<td>Change in behavior Eye malformations Goiter formation Change in colloid structure</td>
</tr>
</tbody>
</table>

↑ Increase  
↓ Decrease  
ND = No data  
- = No effect
Conclusions and outlook

- Not yet fully analyzed: hormone levels, swimbladder, low concentrations
- Preliminary results provide good evidence that merging of 2 existing TGs is possible
- Preliminary results provide good evidence that T-modality can be assessed in fish
- Thyroid histopathology seems to be the most sensitive endpoint (until now)
- Differences between life stages need to be considered when analyzing the eyes
- Experiment with Ethinylestradiol is ongoing
- Project was extended to end of 2021 due to delays caused by Covid-19 pandemic
Acknowledgements

Research teams in Antwerp, Odense and Heidelberg!

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Thank you very much for your attention !!