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

Team Odense:
Henrik Holbech,
Jane Morthorst

Team Heidelberg:
Lisa Baumann*, Pauline Pannetier
Lisa Goelz, Teresa Fagundes,
Thomas Braunbeck

Team Antwerp:
Dries Knapen,
Lucia Vergauwen,
Evelyn Stinckens

***Presenting author:** lisa.baumann@uni-heidelberg.de

Funding by:

EU tender project No. 07.0203/2018/794670/ETU/ENV.B.2

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

Steering Group:

Mara Ceridono
Aude Kienzler
Sharon Munn
Laia Quiros
Maristella Rubbiani
Georg Streck
Jordane Wodli

Scientific Expert Group:

Gerald Ankley
Zhichao Dang
John Green
Tom Hutchinson
Marc Leonard
Peter Matthiessen
Helmut Segner
Charles Tyler

Scientific Advisors:

Maria Arena
Francesca Pellizzato

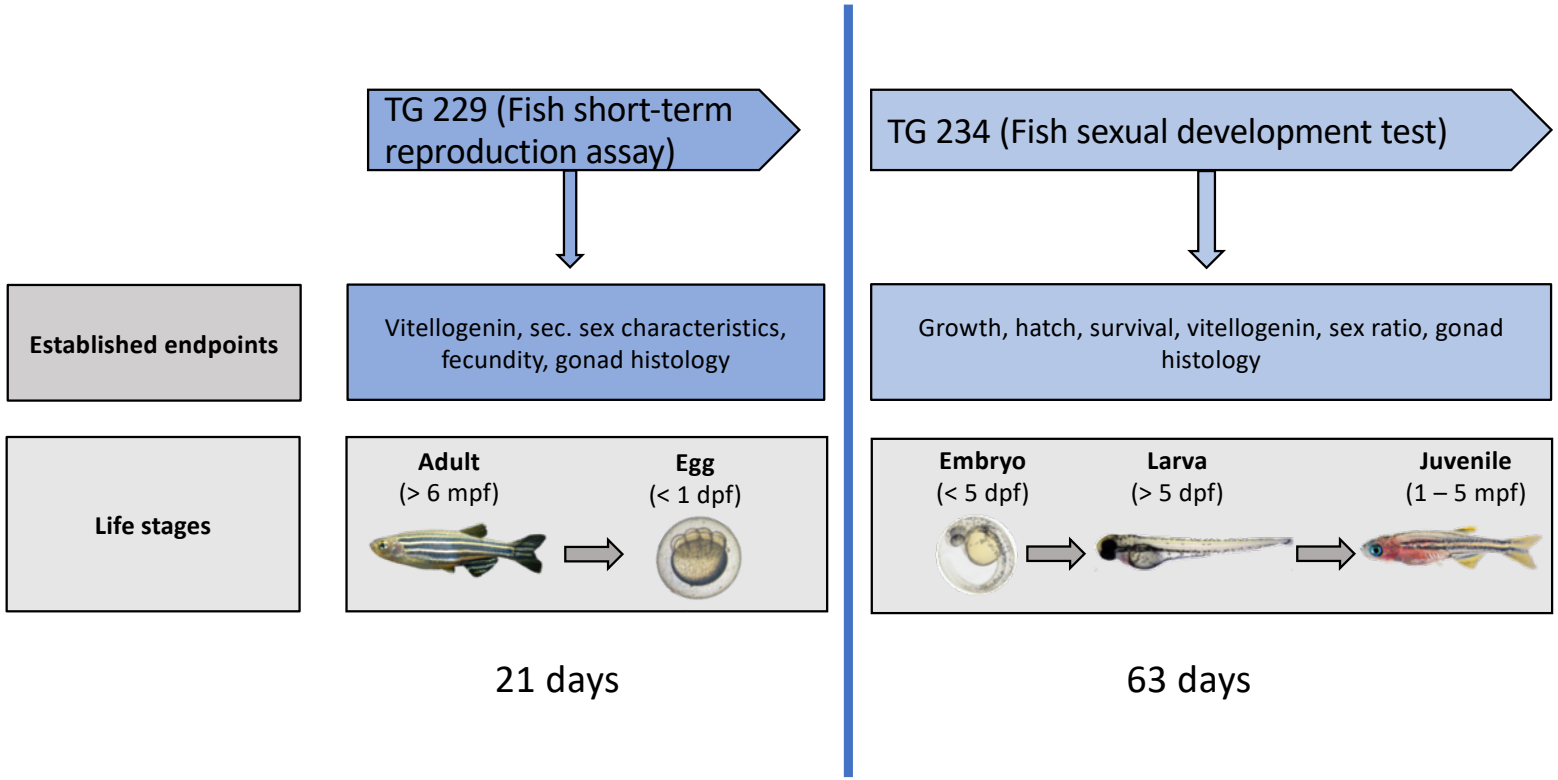
Current challenges in endocrine disruptor testing with aquatic species

- Test guidelines for assessment of endocrine adversity are complex and long (= expensive and using many animals)
- Only few test 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)**

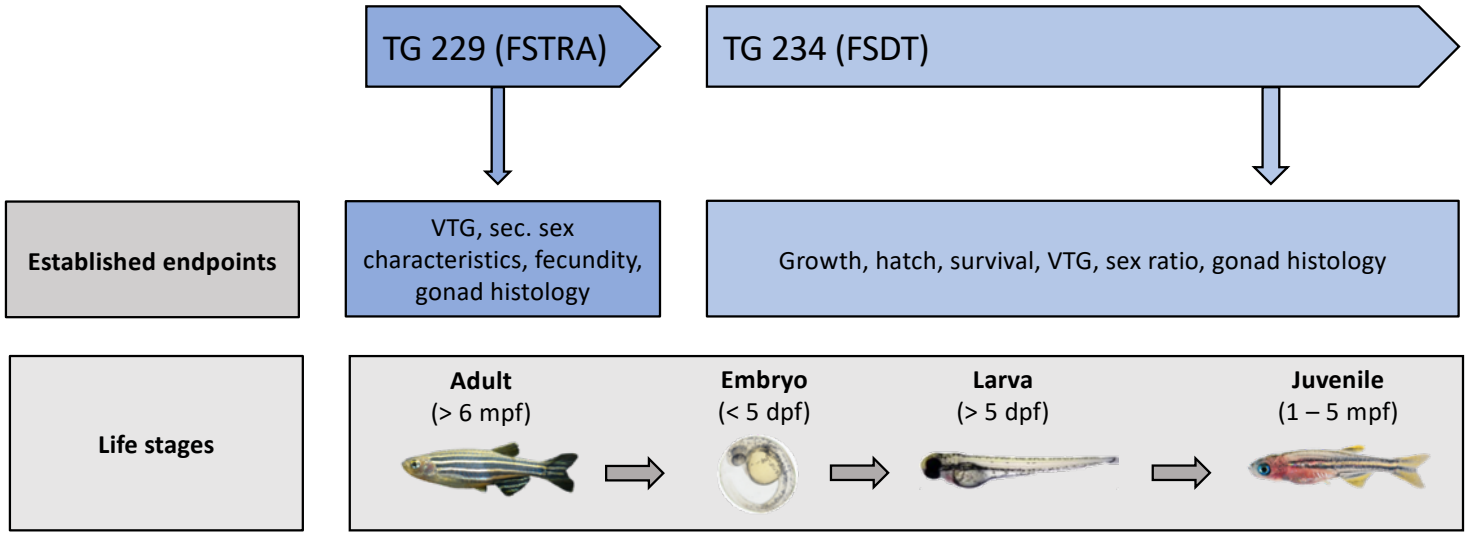
* **E**strogen, **A**ndrogen, **T**hyroid, **S**teriodogenesis

Design of existing OECD test guidelines with fish



mpf: months post fertilization
dpf: days post fertilization

Proposal for a new/merged test protocol



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

Test guideline		OECD TG 229[c]	OECD TG 230	OECD TG 234
Test duration		21 days	21 days	60 days post-hatch
Life stages		Sexually mature male and spawning female (F0)	Sexually mature male and spawning female (F0)	From newly fertilised egg until completion of sexual differentiation (F0)
Species		Fathead minnow, Japanese medaka, zebrafish	Fathead minnow, Japanese medaka, zebrafish	Japanese medaka, three-spined stickleback, zebrafish, fathead minnow (partially validated)
Parameter name	**Indicative of:**[a]			
VTG in females	E, A, S	X	X	X
VTG in males	E, A, S	X	X	X
Spiggin	A			
Male SSC in females	A	X	X	X[g]
Male SSC in males	E, A, S	X	X	X[g]
Specific gonad histopathology[b]	E, A, S	X (optional)		X (optional)
Sex ratio (female biased)	E, A			X
Sex ratio (male biased)	E, A, S			X

T?

Test guideline		OECD TG 229[c]	OECD TG 230	OECD TG 234
Transcriptional activity of cyp19a1b	E			
Behaviour	N	X	X	X
Length	N			X
Morphological abnormalities	N	X	X	X
Gonadosomatic index	N			
Embryo time to hatch	N			
Reproduction (fecundity, fertility)	N	X		
Survival	N	X	X	X
Larval survival and length	N			X
Survival of embryos	N			X
Time to maturity (time to first spawn)	N			
Hatching success	N			X
Histopathology (liver, kidney)	N			
Body weight	N			X

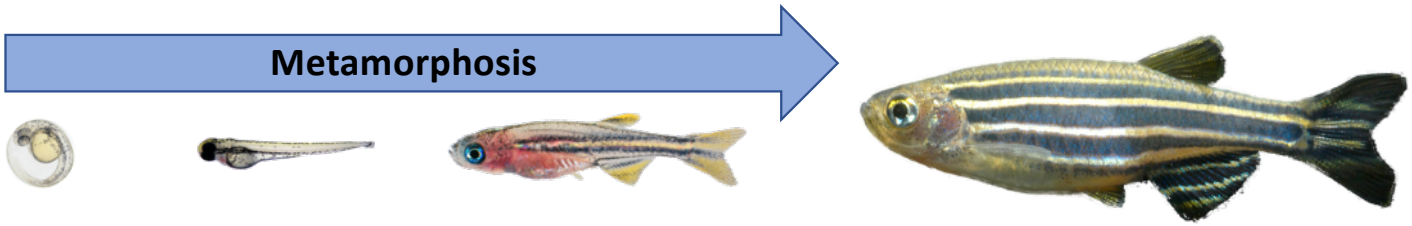
Orange*: in vivo* mechanistic

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:

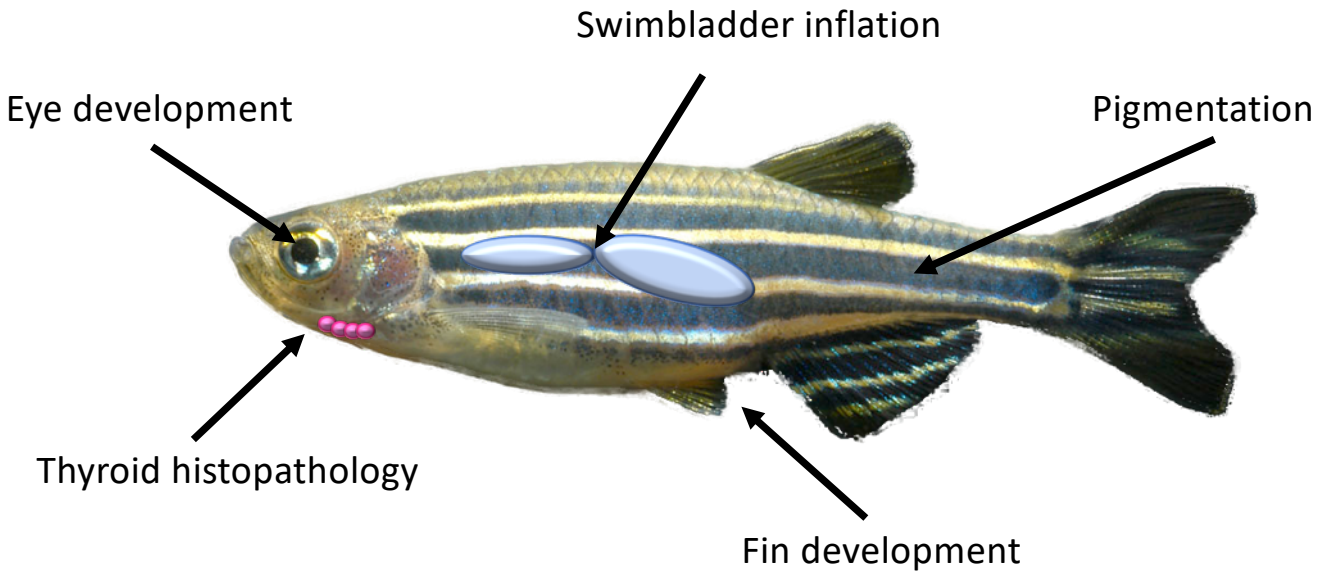
- Fins
- Pigmentation
- Craniofacial structures
- Swimbladder
- Neurodevelopment, sensory organs (eyes, ears, lateral line, olfactory epithelium)

Physiological processes:

- Energy metabolism
- Growth
- Stress response
- Immune system

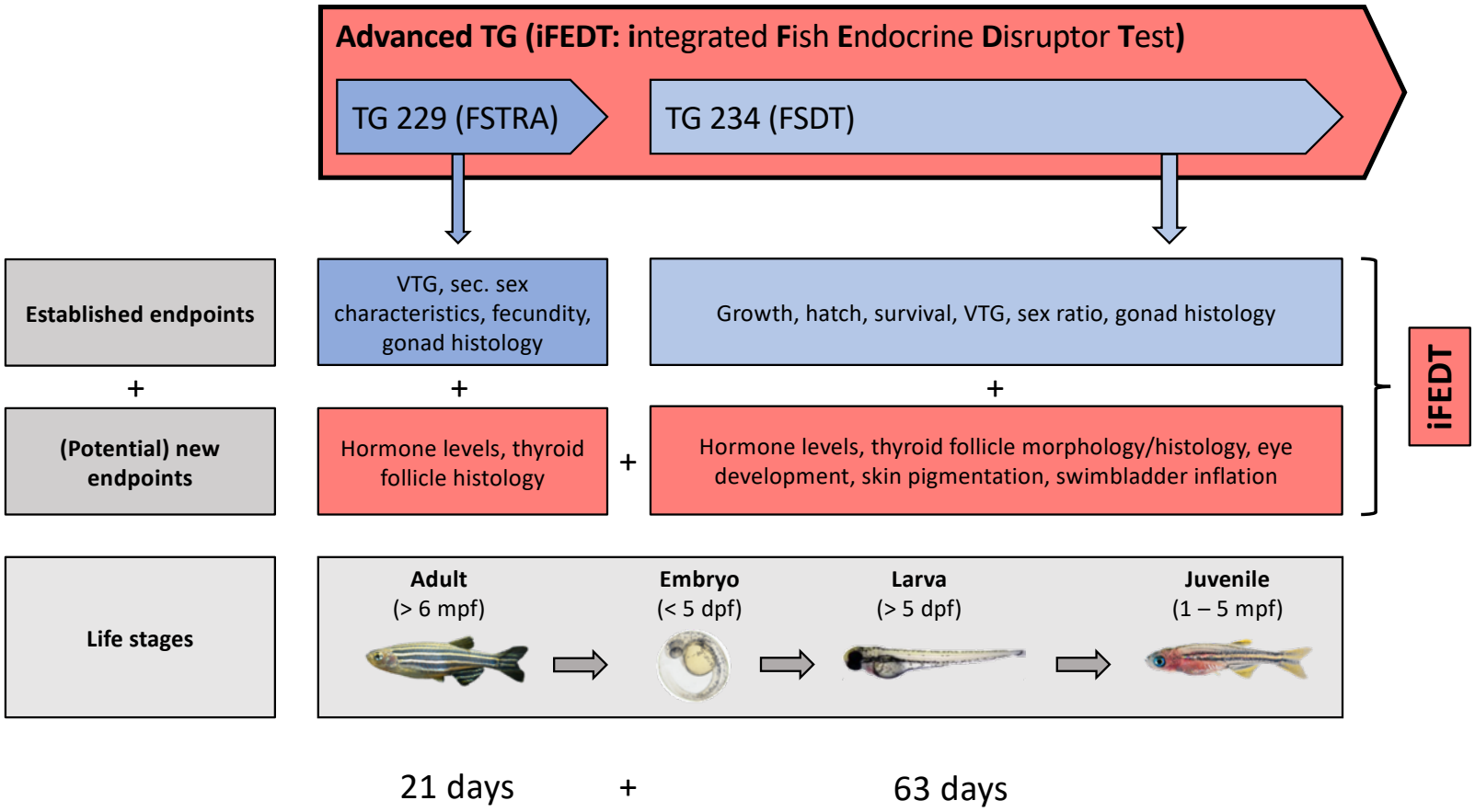
Very little is known about adverse outcomes of environmental exposure of fish to thyroid hormone axis disruptors

Potential thyroid-related endpoints in fish



+ Thyroid hormone levels and gene expression in target systems

Proposal for the design of a new test protocol



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

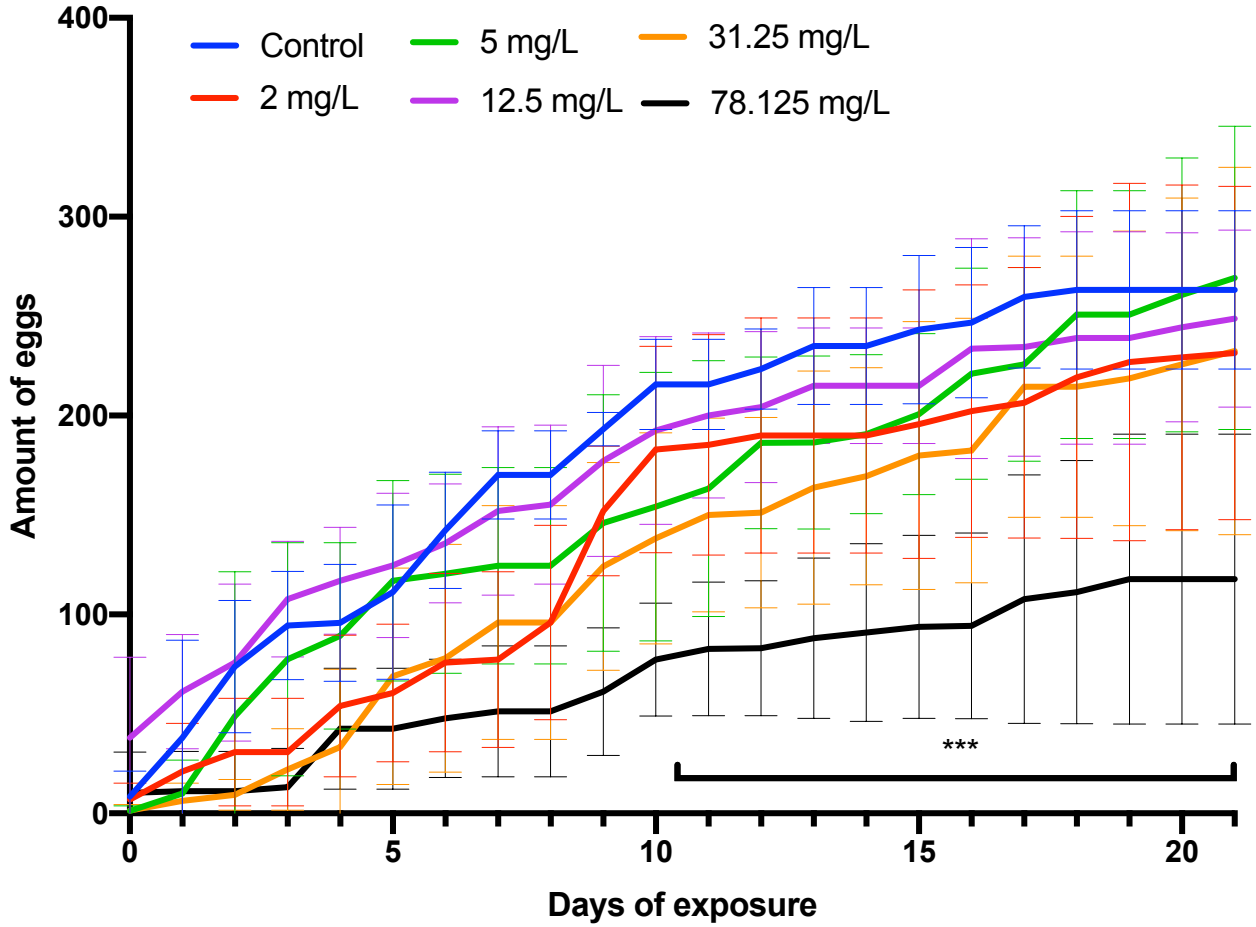
Approach:

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

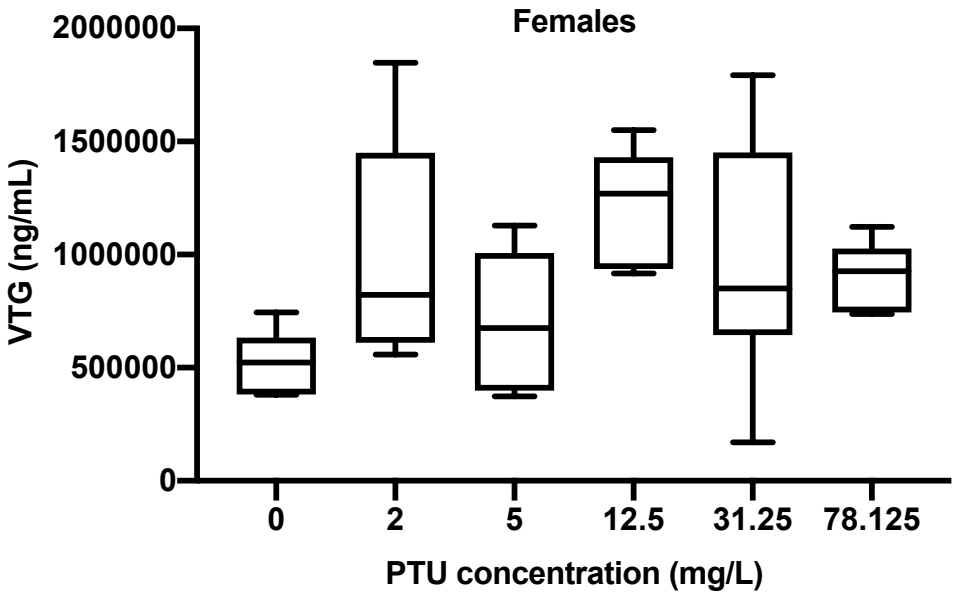


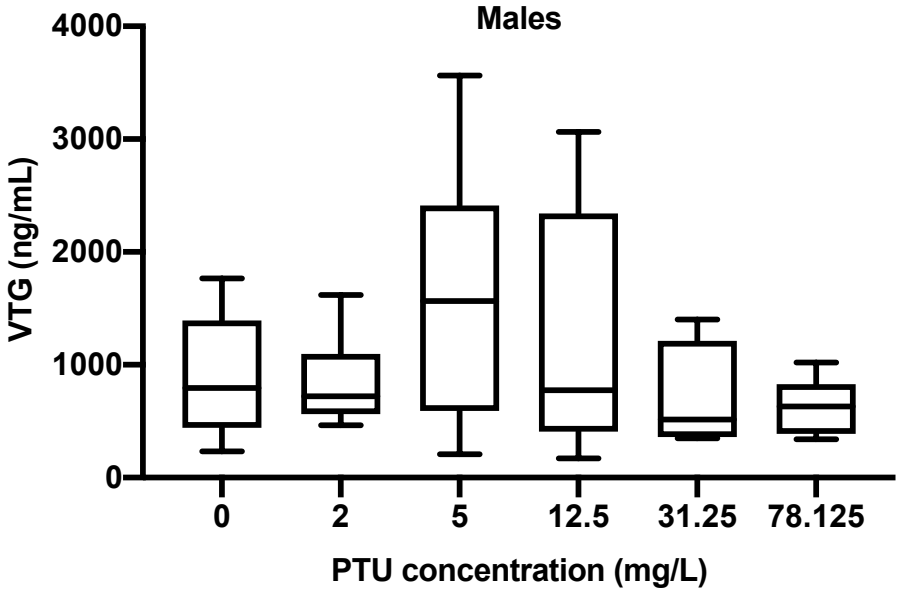
Cumulative number of eggs/female/day in adult zebrafish (*Danio rerio*) after 21-day exposure to different PTU concentrations (0 to 78.1 mg/L).

Adults (21 days of exposure according to TG 229)

Vitellogenin (VTG) levels

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





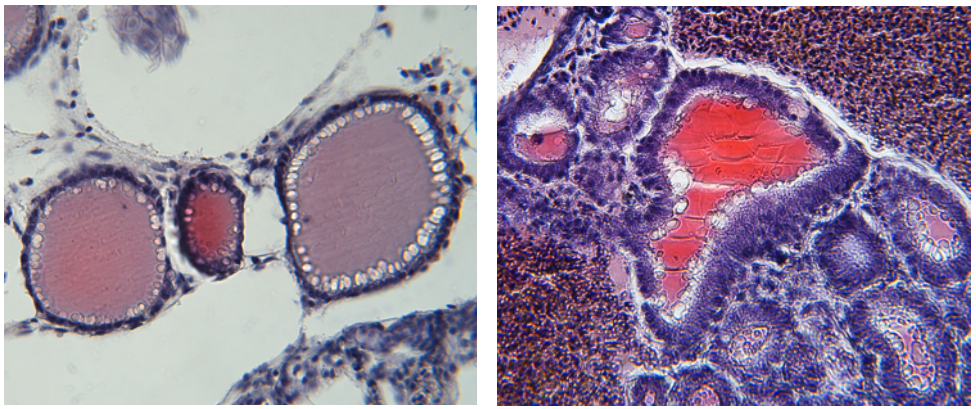
VTG levels of female and male adult zebrafish after 21-day exposure to different PTU concentrations (0 to 78.1 mg/L)
(N=4; n= 4-6 per replicate, n=14-22 per concentration)

Adults (21 days of exposure according to TG 229)

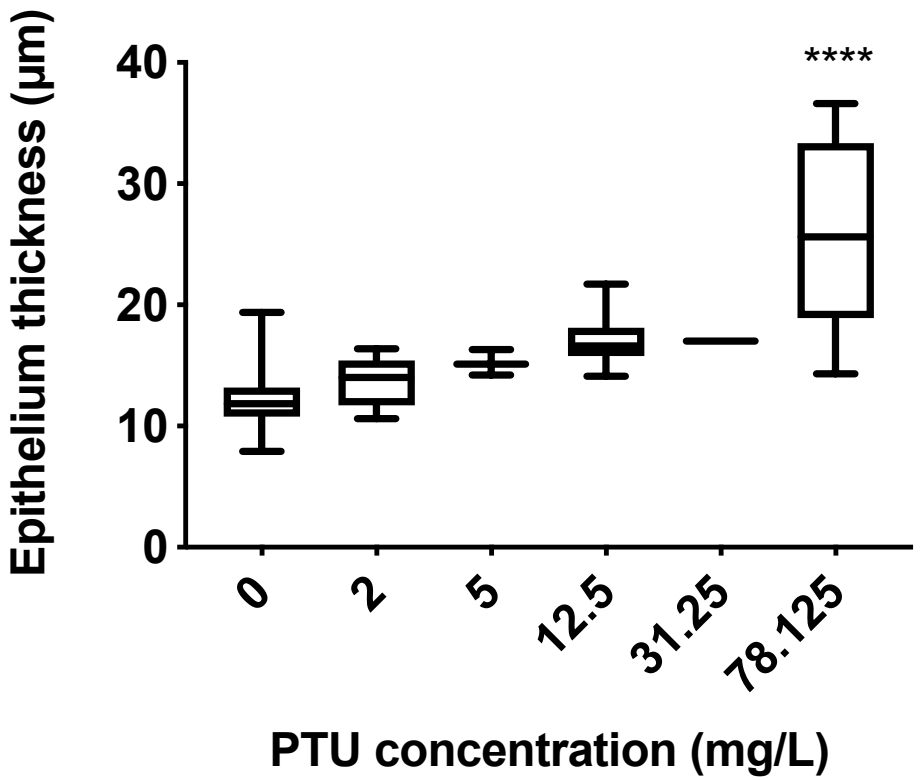
Histology – Thyroid

- 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

Control **78.125 mg/L PTU**



Thyroid follicles of control vs. PTU-exposed zebrafish with proliferated epithelium



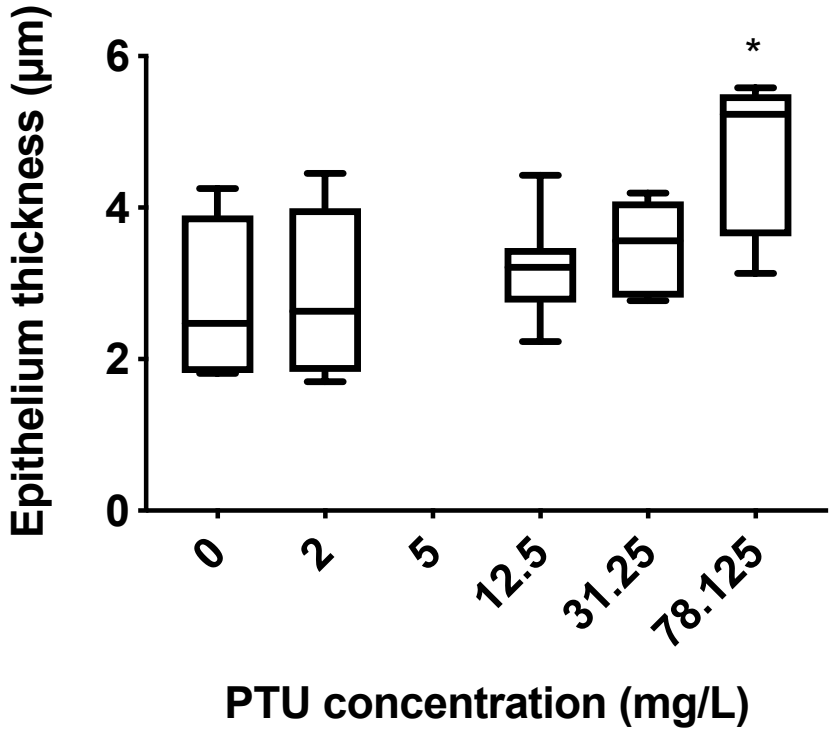
*Thyroid follicle size and epithelium thickness of PTU-exposed zebrafish (***: p<0.001; n=3-13)*

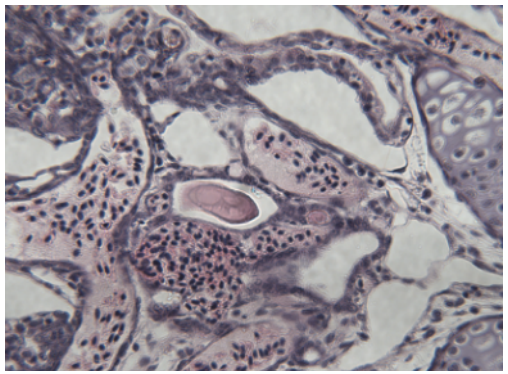
Results: Larvae

F1 generation (25 days of exposure according to TG 234) : Larvae

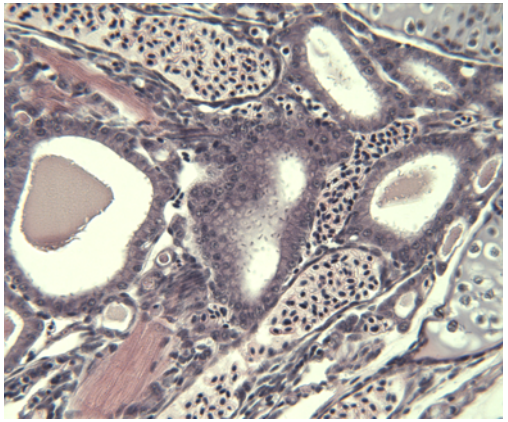
Histology – Thyroid

- PTU exposure caused a **significant increase** in thyroid follicle epithelium thickness in larvae





Control



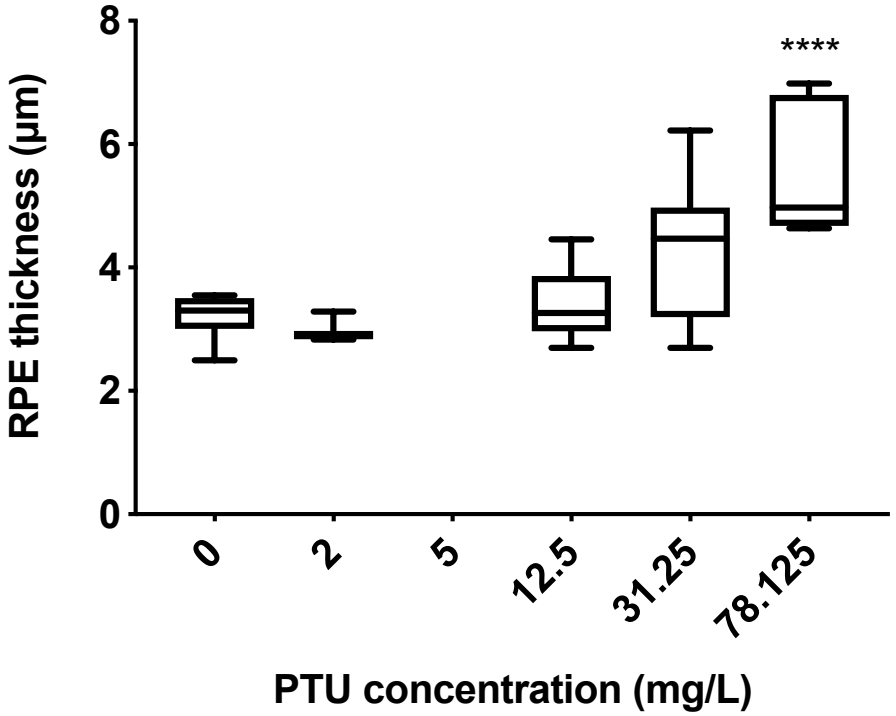
78.125 mg/L

Thyroid follicle size and epithelium thickness of PTU-exposed fish (: p<0.05; n=5-9)*

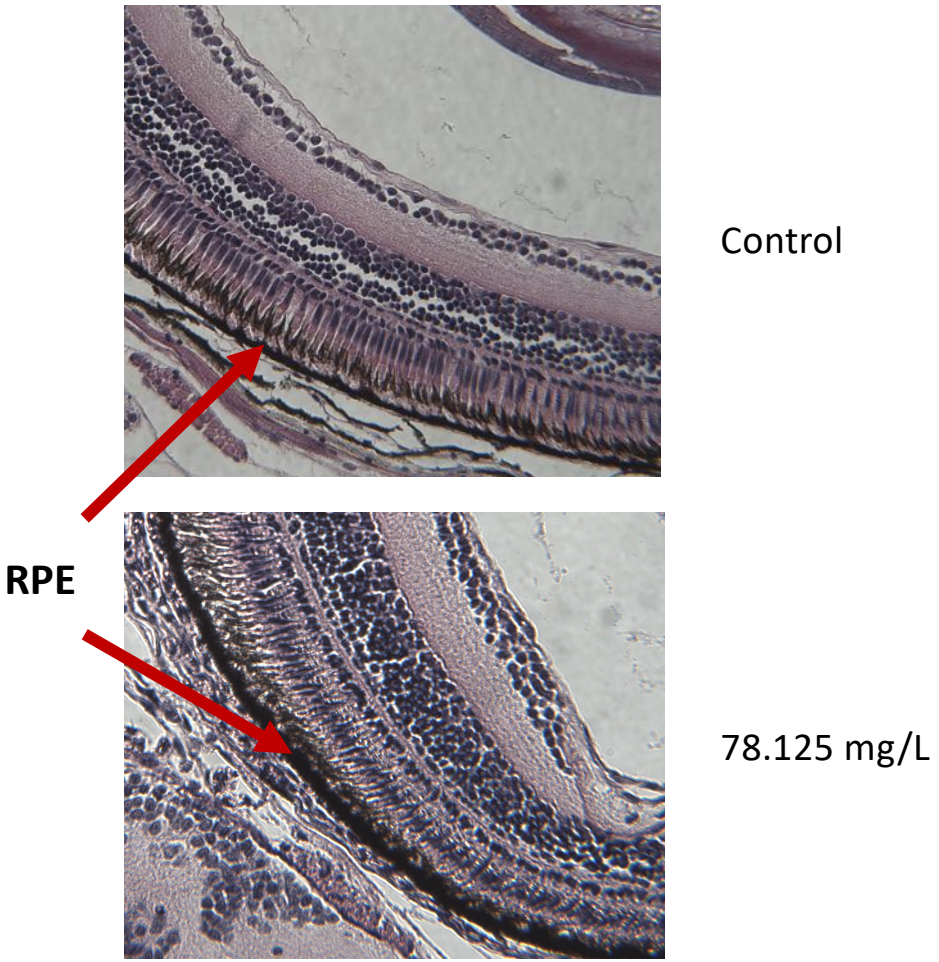
F1 generation (25 days of exposure according to TG 234) : Larvae

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

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



*RPE thickness of PTU-exposed fish (****: p<0.0001; n=5-8)*

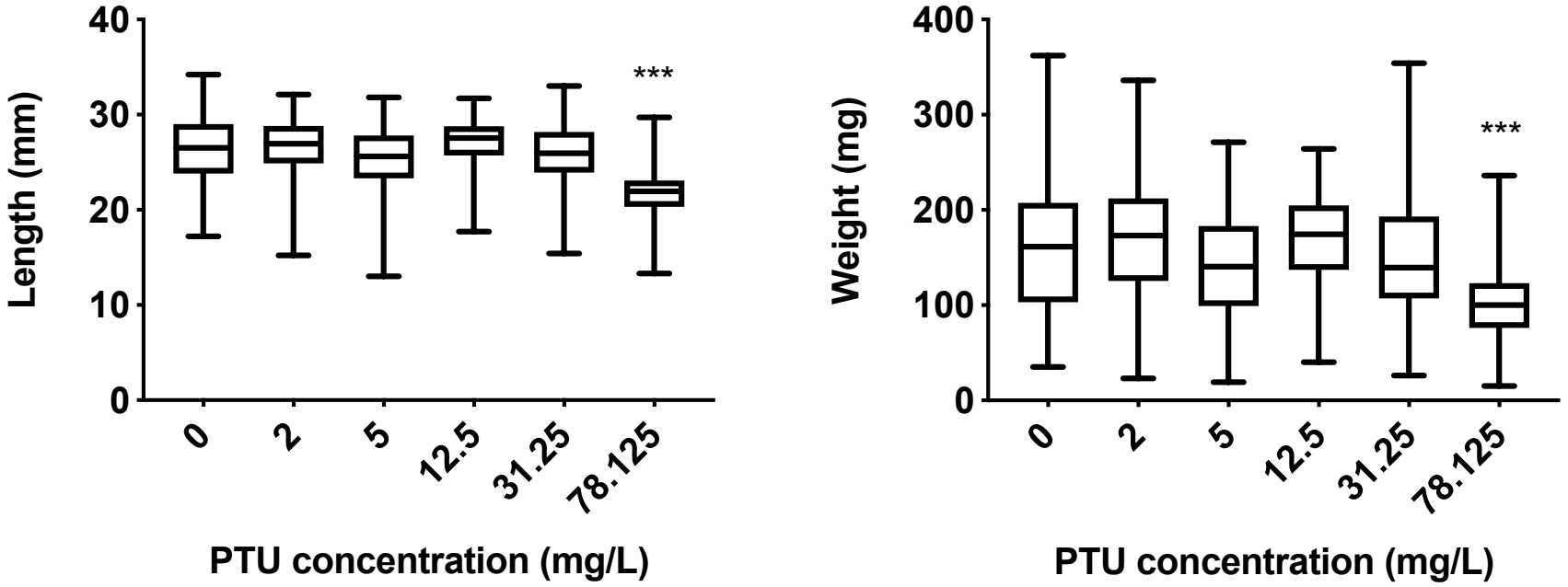


Results: Juveniles

F1 generation (60 days of exposure according to TG 234): Juveniles

Morphological changes

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

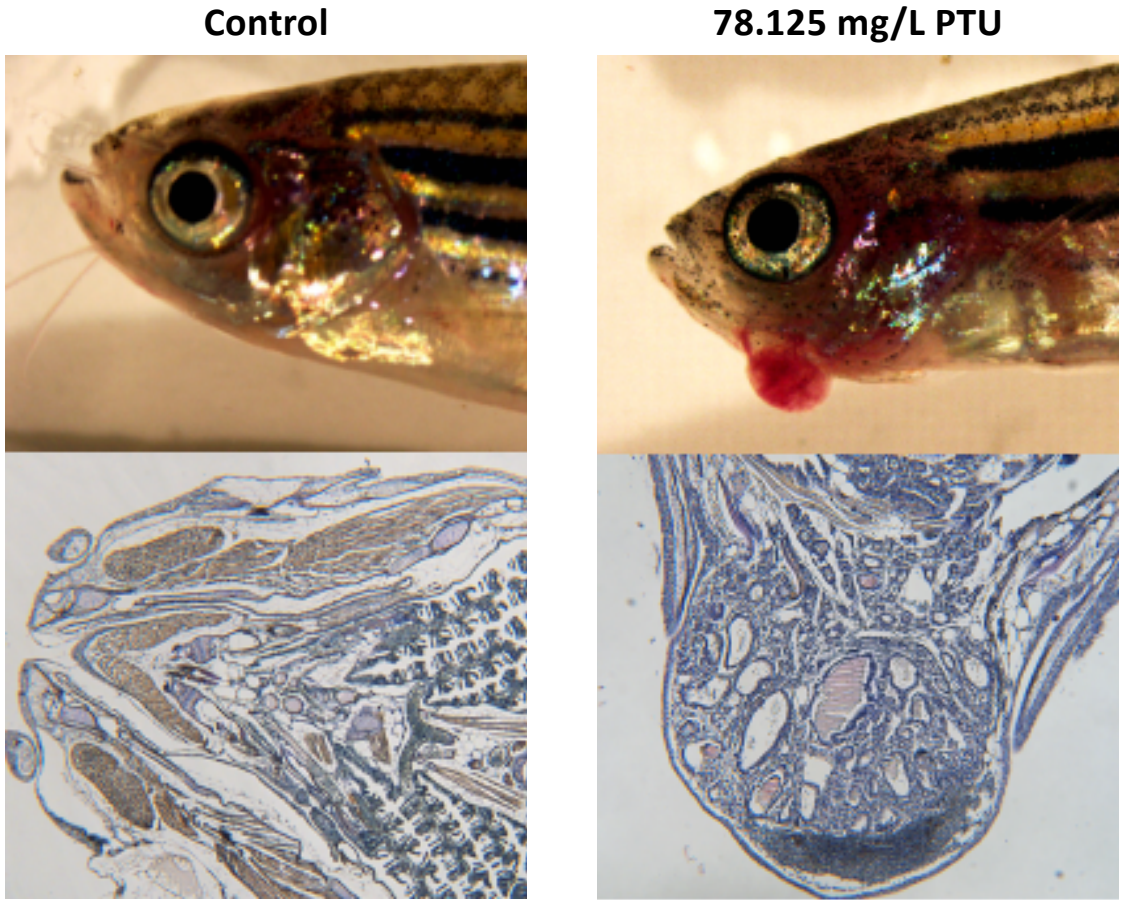


*Length and weight of juvenile fish (**: p<0.001, n= 64-100)*

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



Development of goiter in the F1 generation of zebrafish exposed to 78.125 mg/L PTU for 60 days

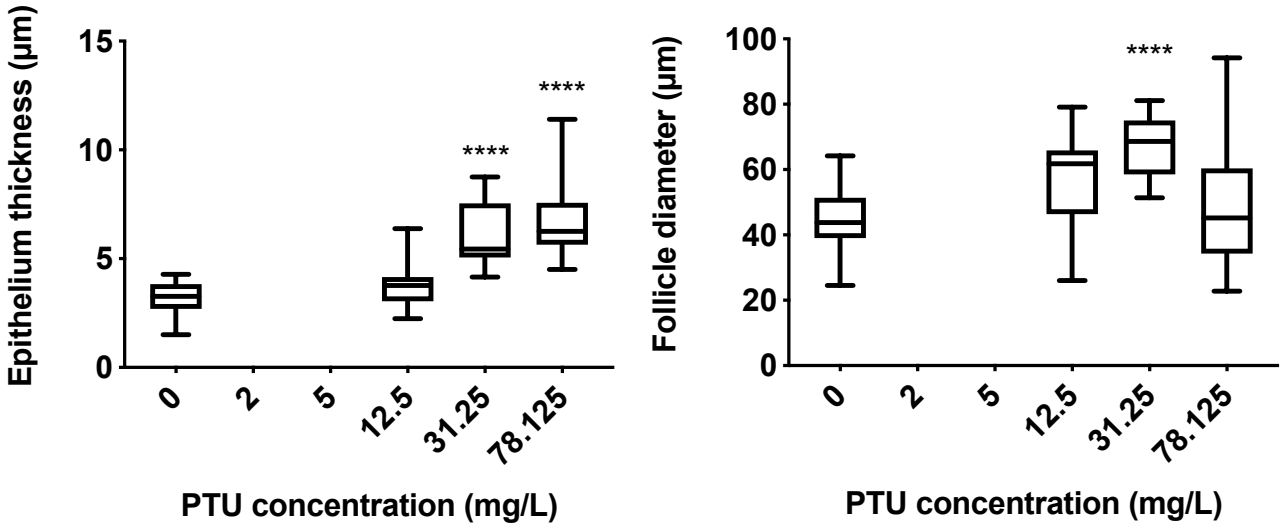
F1 generation (60 days of exposure according to TG 234): Juveniles

Histology – Thyroid

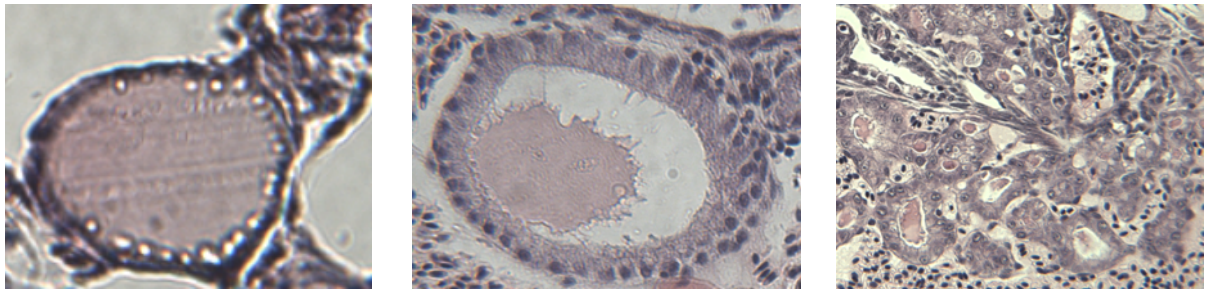
- Number of thyroid follicles per fish:
  - 0 mg/L: 5-15
  - 12.5 mg/L: 15-30
  - 31.25 mg/L: 15-30
  - 78.125 mg/L: **15 to >60**

- **Increase of epithelium thickness**

- Strong changes in colloid structure



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



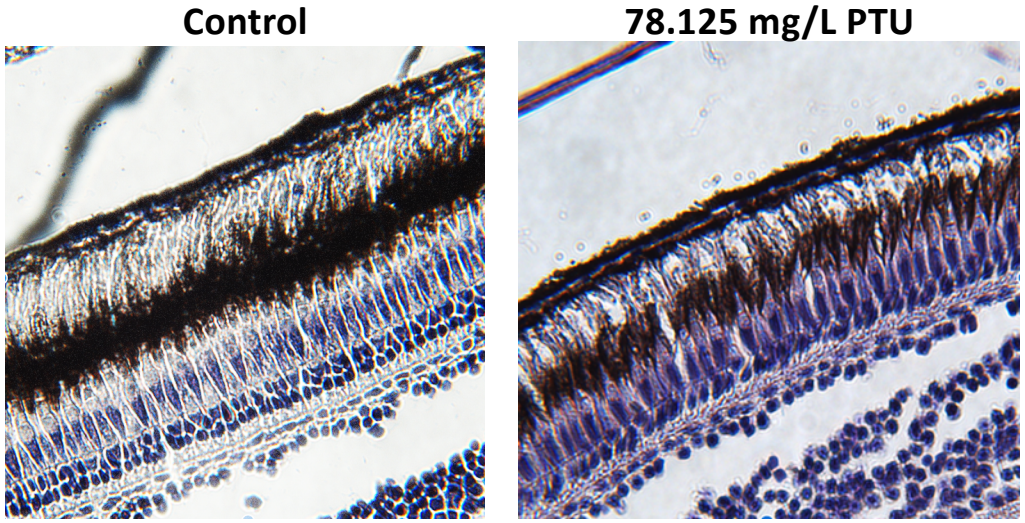
Control

78.125 mg/L PTU

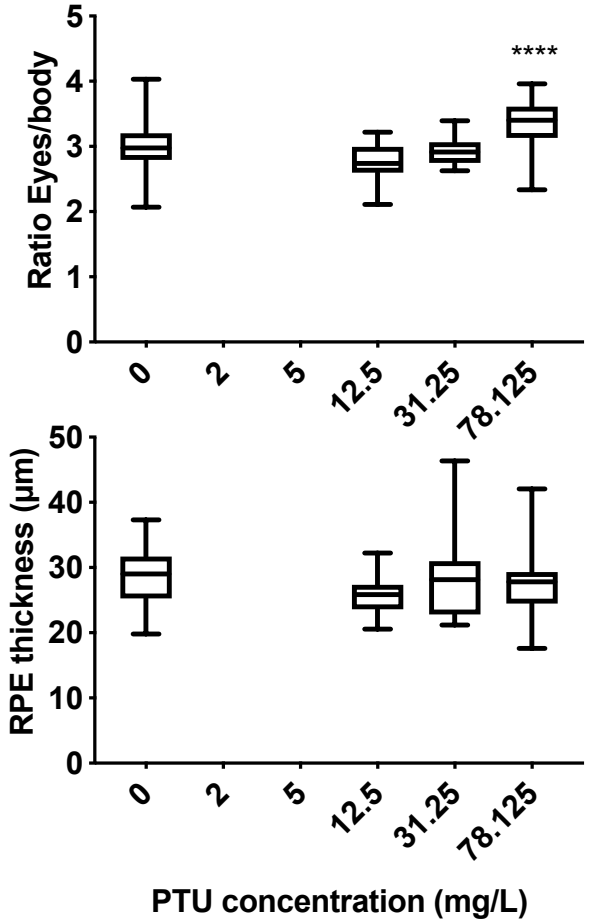
F1 generation (60 days of exposure according to TG 234): Juveniles

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

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

↗ 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!

Funding by:

EU tender project No. 07.0203/2018/794670/ETU/ENV.B.2

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

Steering Group:
Mara Ceridono
Aude Kienzler
Sharon Munn
Laia Quiros
Maristella Rubbiani
Georg Streck
Jordane Wodli

Scientific Expert Group:
Gerald Ankley
Zhichao Dang
John Green
Tom Hutchinson
Marc Leonard
Peter Matthiessen
Helmut Segner
Charles Tyler

Scientific advisors:
Maria Arena
Francesca Pellizzato

Thank you very much for your attention !!

European Commission | Horizon 2020 European Union funding for Research & Innovation