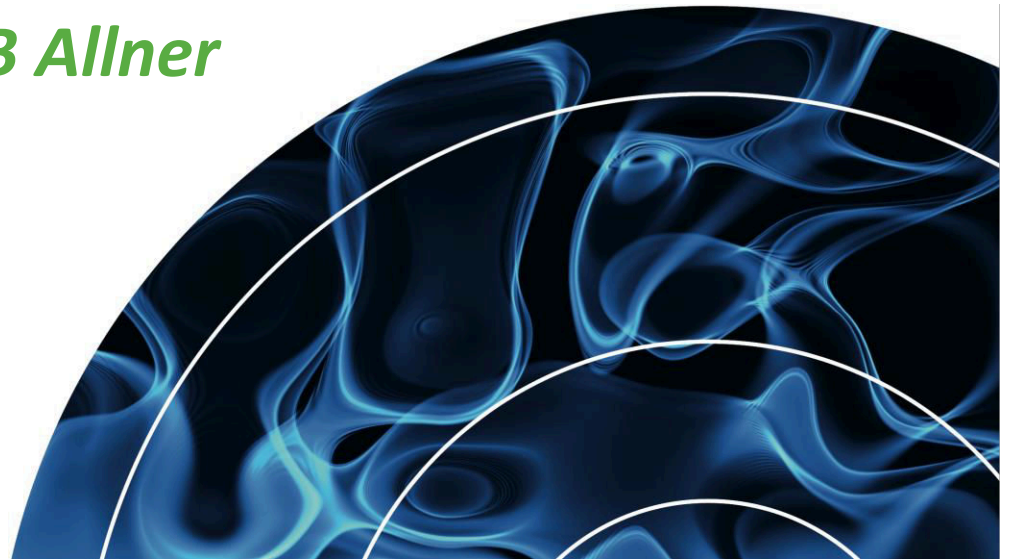


Three R's in endocrine disruptor assessment: Refining sampling, reducing numbers of tested animals and replacing in vivo testing

*CF Lerche, R Möller, T Schmidt, M Hennies,
P Stahlschmidt-Allner, B Allner*

Dr. Reinhard Möller
TECOmedical AG
Sales and Marketing



Endocrine disruptors (ED`s)

- Substances causing disturbances in the endocrine system of an organism
- Endocrine disruptors can be natural (phyto estrogens) or artificial (e.g. chemical and pharmaceutical products, cosmetics)
- ED`s are mainly distributed through the aquatic system
- Threat for the environment and humans

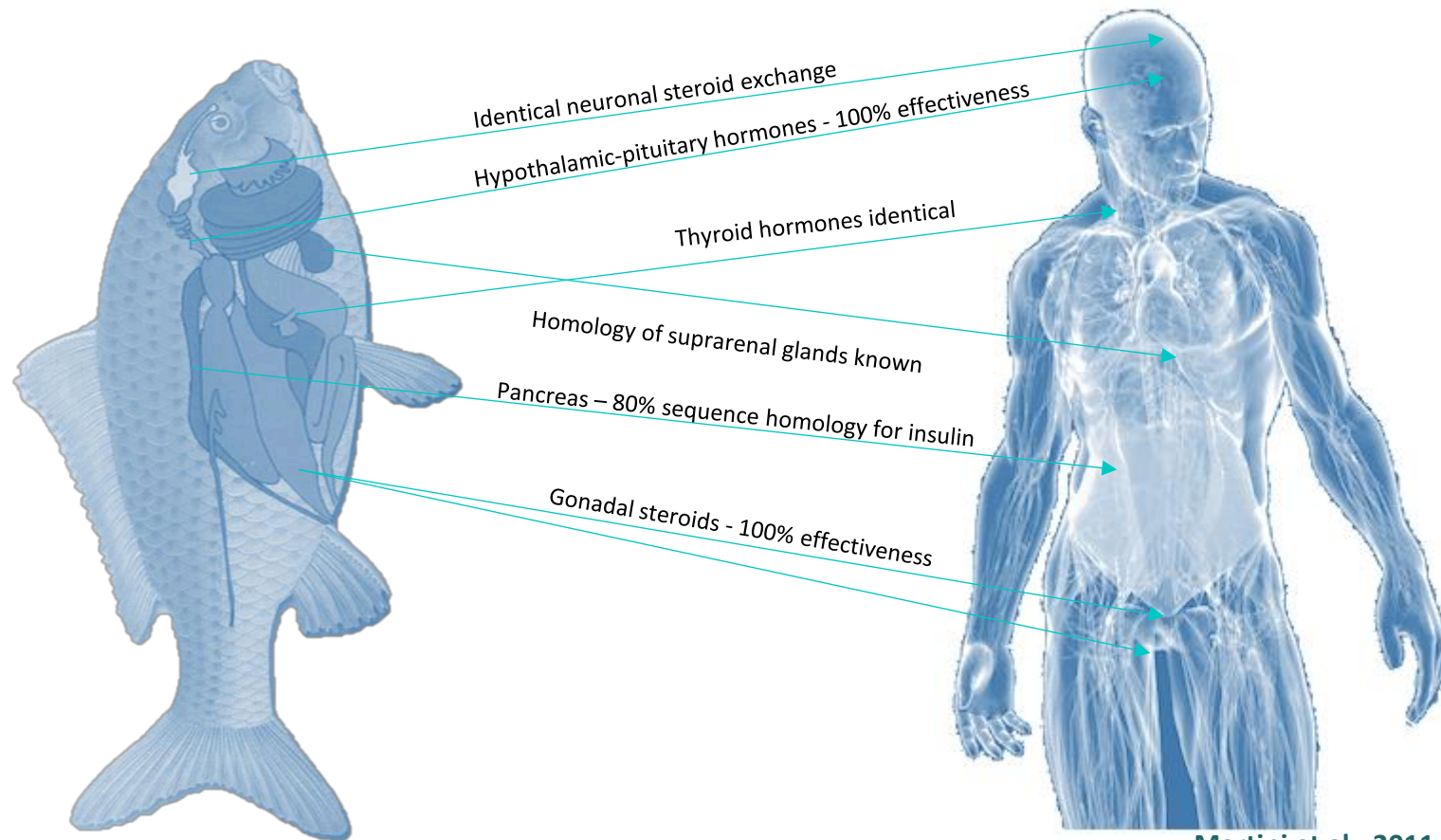
Testing for endocrine disruptors

- The public concern regarding the consequences of endocrine disruption triggered the implementation of additional animal testing
- Testing of chemicals, substances for endocrine disrupting activities according to OECD Guidelines (Lab testing)
- Ecotoxicological testing/monitoring (Field testing)
- Control of efficiency of sewage systems (Field testing)

Testing for endocrine disruptors with estrogenic activities

- Fish are important target organisms for testing
- Vitellogenin is a well accepted core endpoint of estrogenic activities in fish

The fish as a biomedical model organism

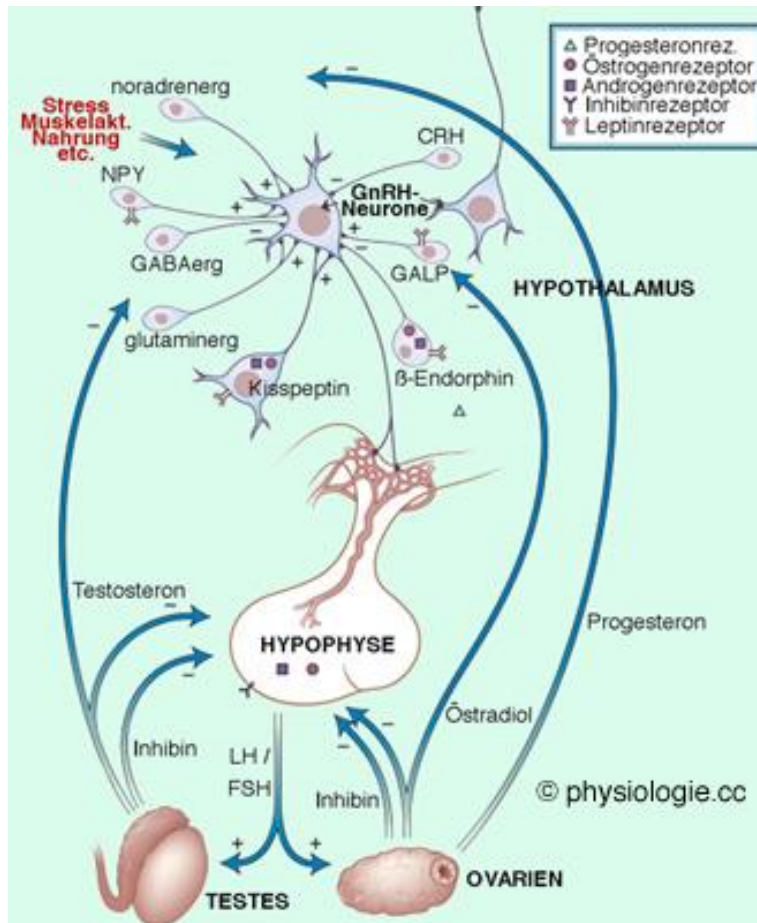


Martini et al., 2011. Human Anatomy
Pearson Ed. Lim..

ISBN 10: 0321688155

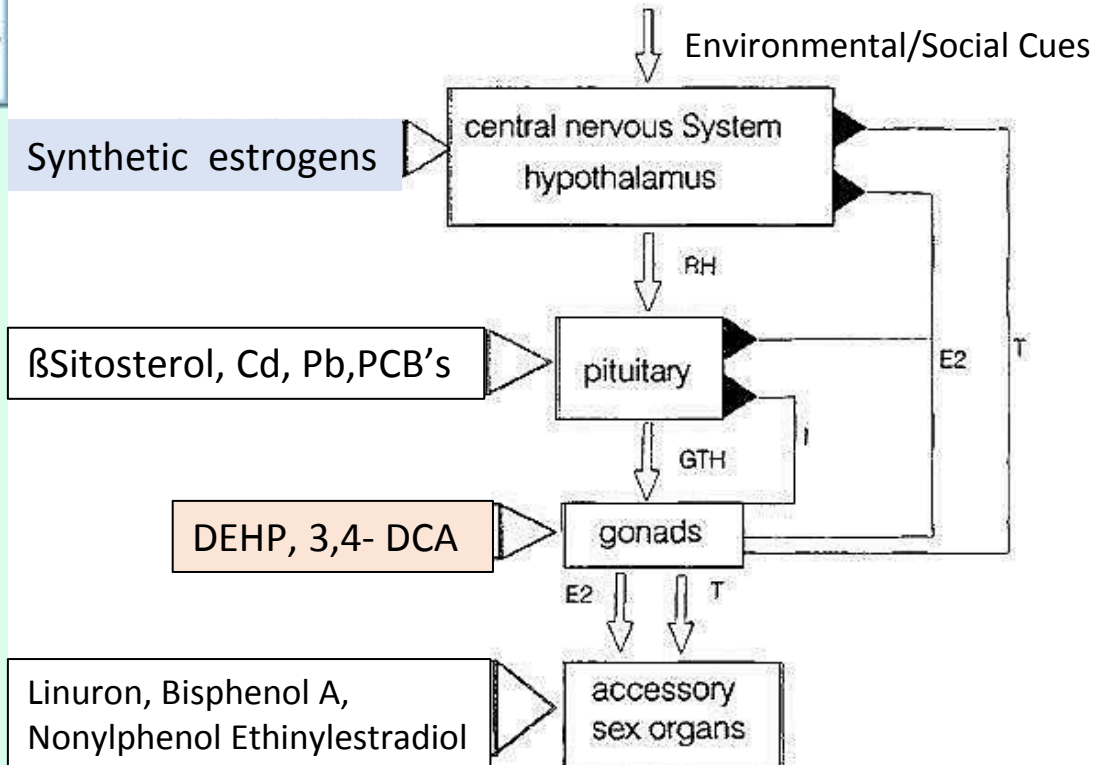
ISBN 13: 9780321688156

The hypothalamic–pituitary–gonadal axis



<https://quizlet.com/23864676/endocrine-12-hpa-axis-and-male-repro-flash-cards/>

www.tecomedical.com



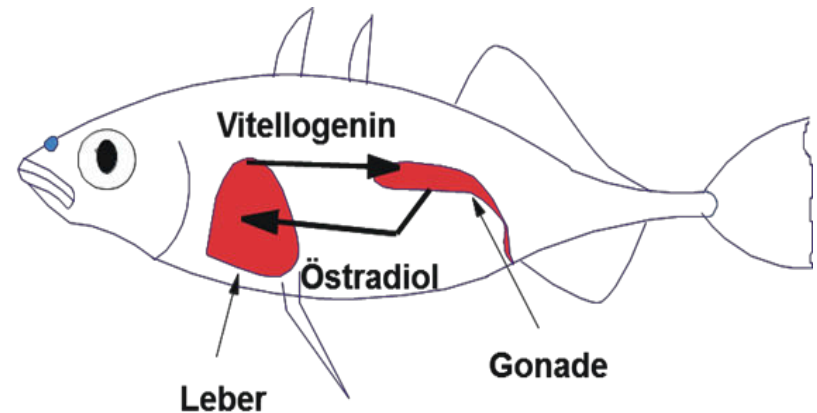
Stahlschmidt-Allner et al. 1997. Environ. Sci. & Pollut. Res. 4 (3): 155-162.

Dr. Reinhard Möller

Vitellogenin in fish

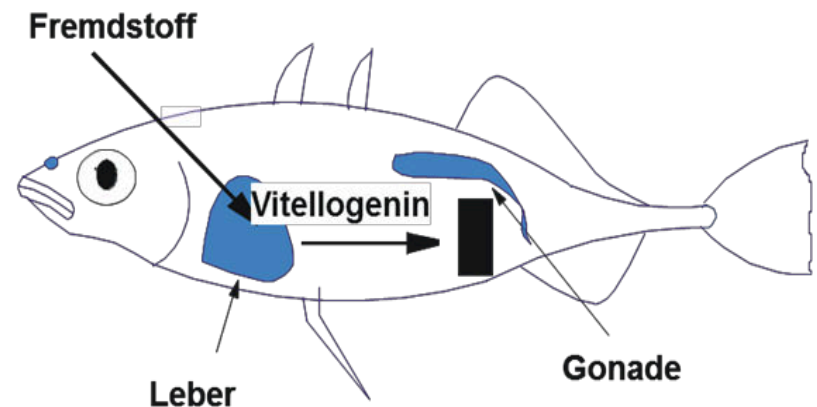
Considered as typical female protein

- a) produced under the influence of estrogens
- b) responsible for the nutrition of the embryos



Male and juvenile fish

- a) no or limited amount of estrogens and have therefore very low VTG level.
- b) non-physiologically increased VTG level is an indicator for exogenous estrogens





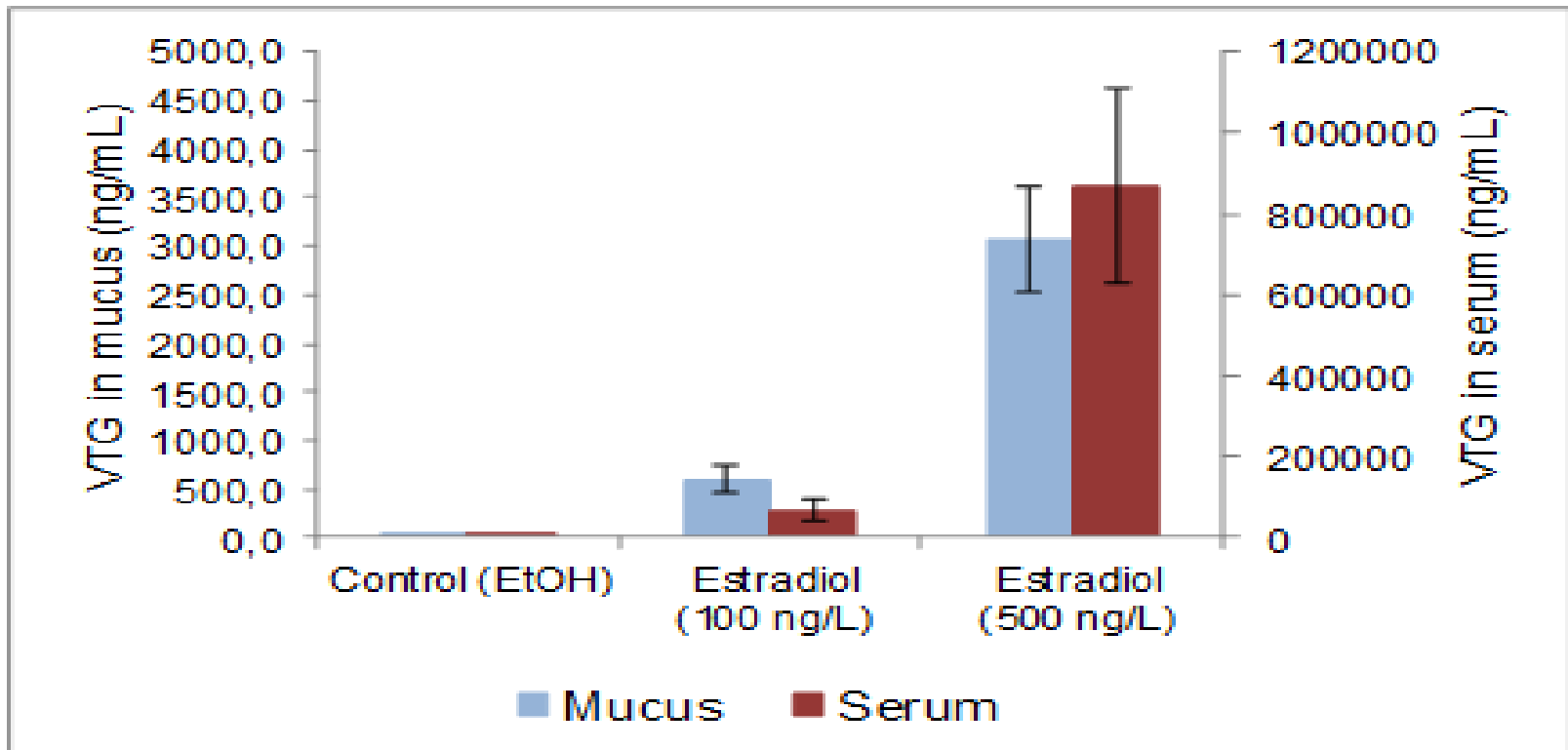
Vitellogenin testing in epidermal mucus

- **Recent studies show:**
- Both VTG and estrogen receptor genes are expressed in epidermal cells
- Immunoaffinity and mass fingerprint analysis show induction of identical VTG peptides in liver and epidermis
- Similar dose-response pattern in the epidermal mucus and blood
- VTG level in mucus: ng/ml; in blood/homogenate: $\mu\text{g/ml}$

TECO® Cyprinid Vitellogenin ELISA

Biological Evaluation (Zebrafish: Exposition 168 hours)

(A)



TECO Vitellogenin System

TECO Mucus Collection Set

Designed for effective, non-invasive, non-destructive sampling

Different sensitive ELISA Tests covering > 37 fish species

Ultra Sensitive ELISA Tests for Cyprinids and Salmonids

All ELISA tests are validated for the use of mucus samples

TECO®Mucus Collect Set (patent submitted)

Content of TECO® Mucus Collection Sets

Validated flocked swabs: 42

Eppendorf sample tubes: 42

Sample rack: 1

Package insert (Description of sampling) 1

Extraktion buffer: 1 bottle containig 25 ml



Mucus Collection Set: Validated Collection Set (GLP)

- ✓ Swabs: Flexible swabs with flocked tips (No risk of hurting the fish; high protein/mucus load capacity) and easy handling
- ✓ Sampling tubes: Swab tip will be stored in the sampling tube – the shape of bottom supports the extraction efficiency during vortex procedure
- ✓ Extraction buffer is a well designed buffer for fast and efficient extraction of mucus from the swab just before testing. Extraction guarantees maximum stability of Vitellogenin in extracted sample.
- ✓ Sample rack allows simple usage of mucus collection under field conditions
- ✓ Package inserts describes the mucus sampling procedure

TECO®Mucus Collect Set Sampling



TECO®Mucus Collect Set

2. Sample



TECO®Mucus Collect Set

Non-invasive, non-destructive procedure

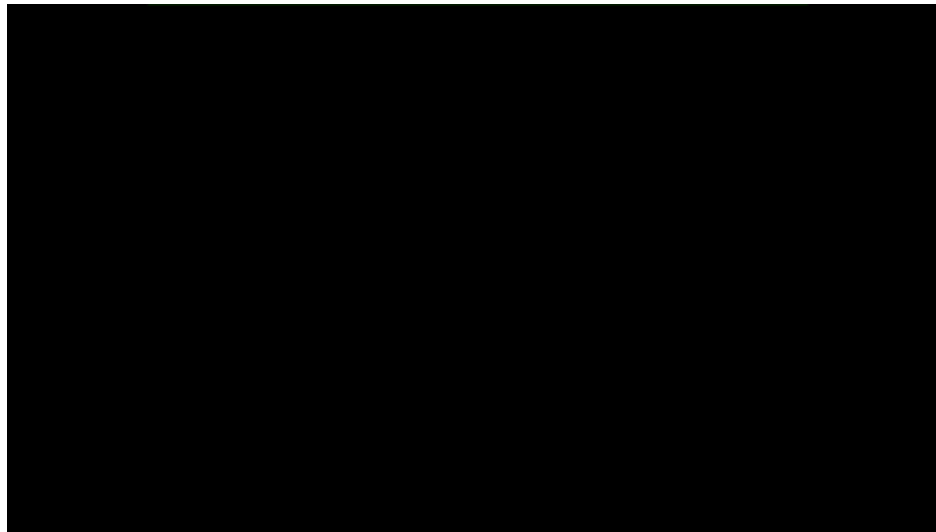


TECO®Mucus Collect Set

Transfer of swab tip into sample vial and store at -20°C

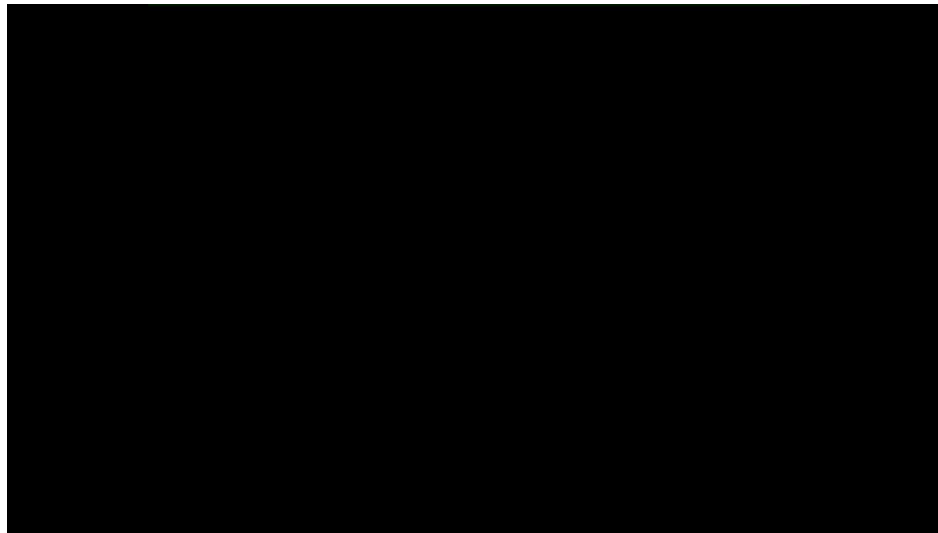


- Blood samples may be obtained by slicing the specimen dorsally at the caudal tip of the operculum so that the haemal arch is opened and a sample may then be drained with a pipette. Isolation of the plasma should follow immediately by centrifuging the sample for 3 minutes at 15.000 x g.

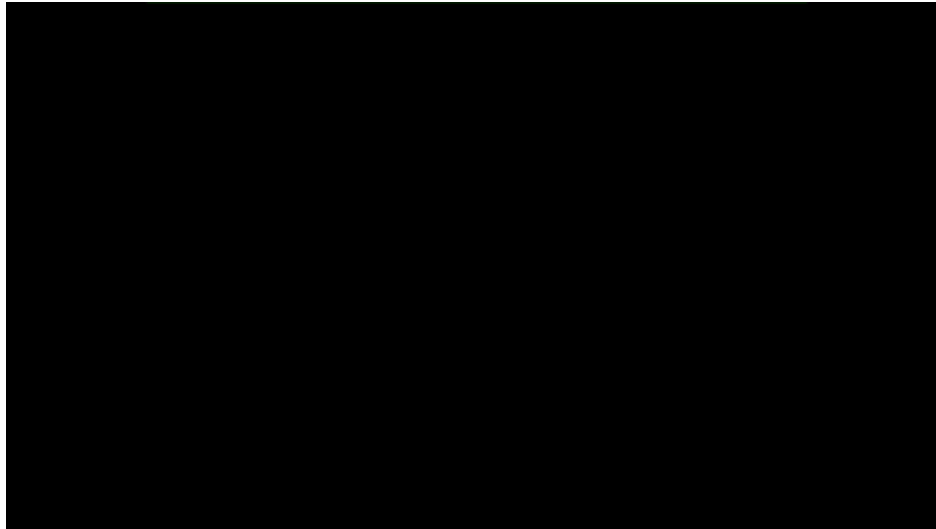


- Alternatively, the caudal peduncle may be partially severed with a scalpel blade and blood collected from the caudal vein/artery with a heparinized microhematocrit capillary tube. Blood may also be collected by cardiac puncture using a heparinized syringe.

- Head and tail homogenates are acquired by cutting the head behind the operculum and the tail after the dorsal fin. Both head and tail must be weighted before homogenizing the tissues in a homogenization buffer (Tris-HCl + protease inhibitor). Homogenates are to be centrifuged for 30 minutes at 50.000 x g.



- Smear samples are obtained simply by rubbing the heads of the swabs included in the TECO Mucus Collection Set on the skin of the specimen, turning the swab slightly during sampling (no need for anesthetics).



TECO Vitellogenin System

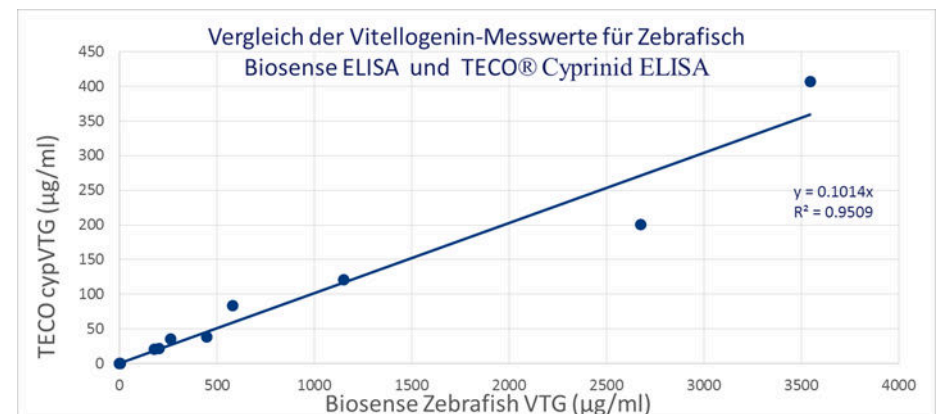
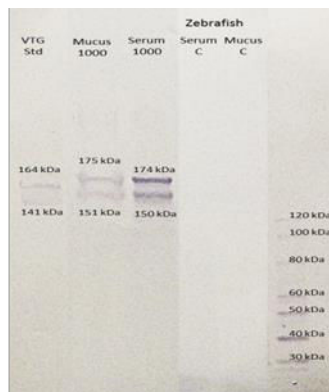
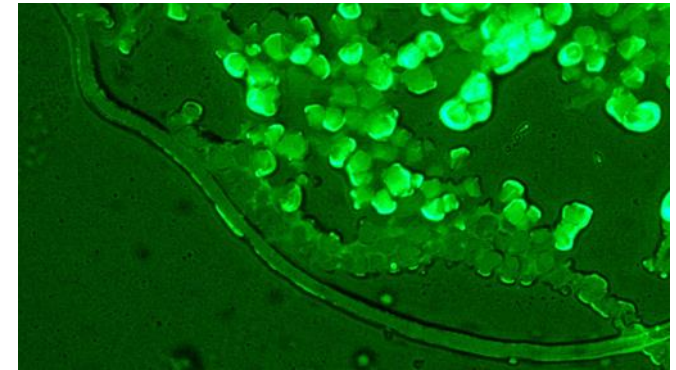
Epidermal Mucus

- Optimal procedure to obtain vitellogenin samples outdoors
 - a) Mucus Collection Set for fast and simple sampling
 - (all components required to collect the samples are included);
 - a) Mucus samples can be frozen immediately after collection on dry ice.



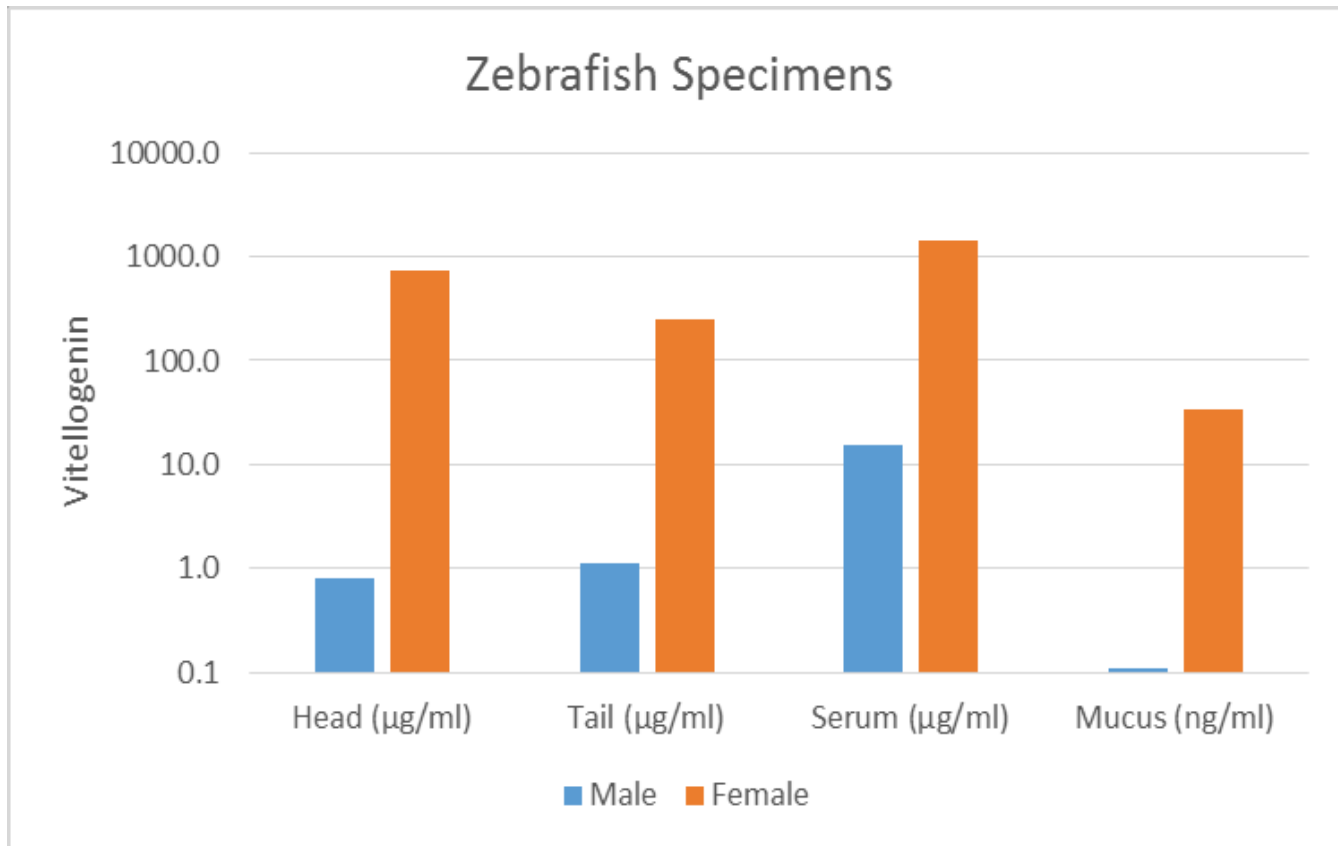
Validation of the specificity of the test system in blood, homogenate and epidermal mucus were confirmed using:

- ✓ Immunohistochemistry Staining
- ✓ SDS-PAGE and Westernblot Analysis
- ✓ Mass Spectrometry

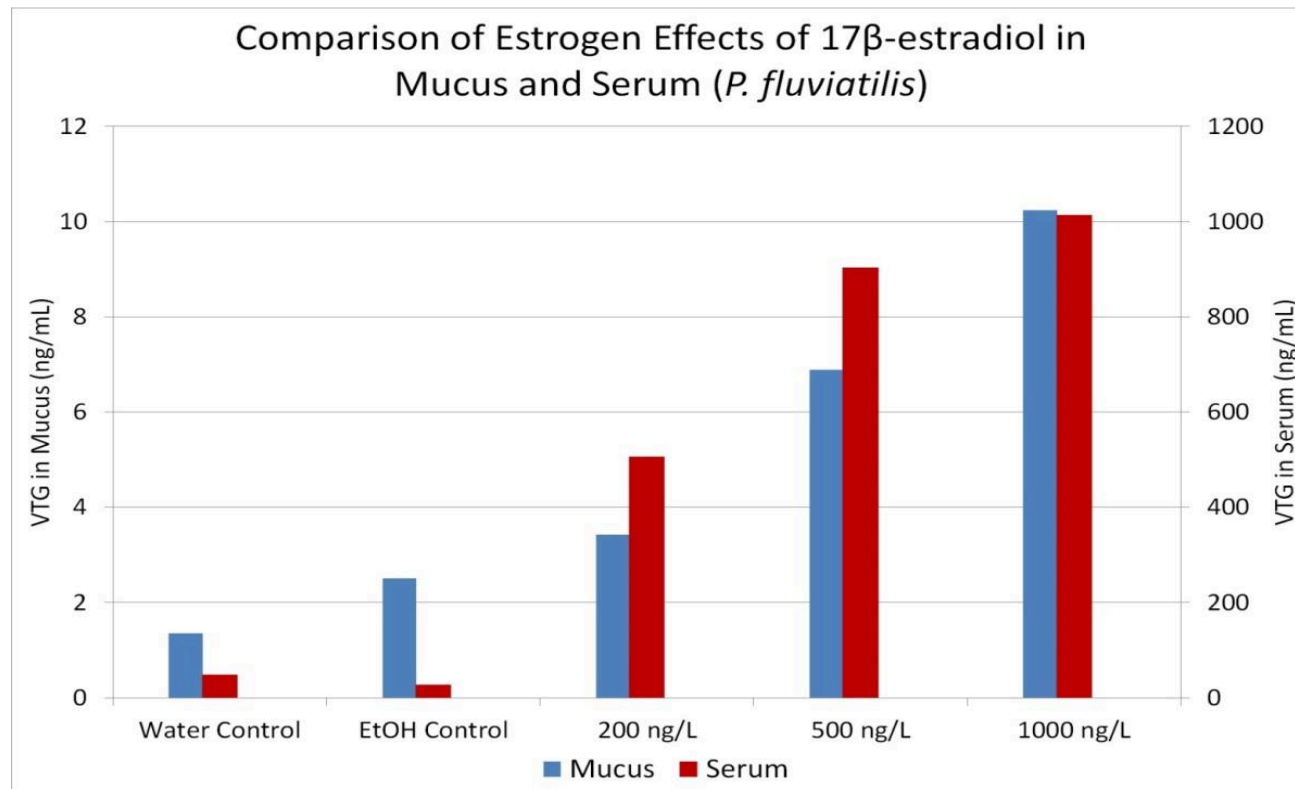


Vitellogenin in Blood, Homogenate and epidermale Mucus

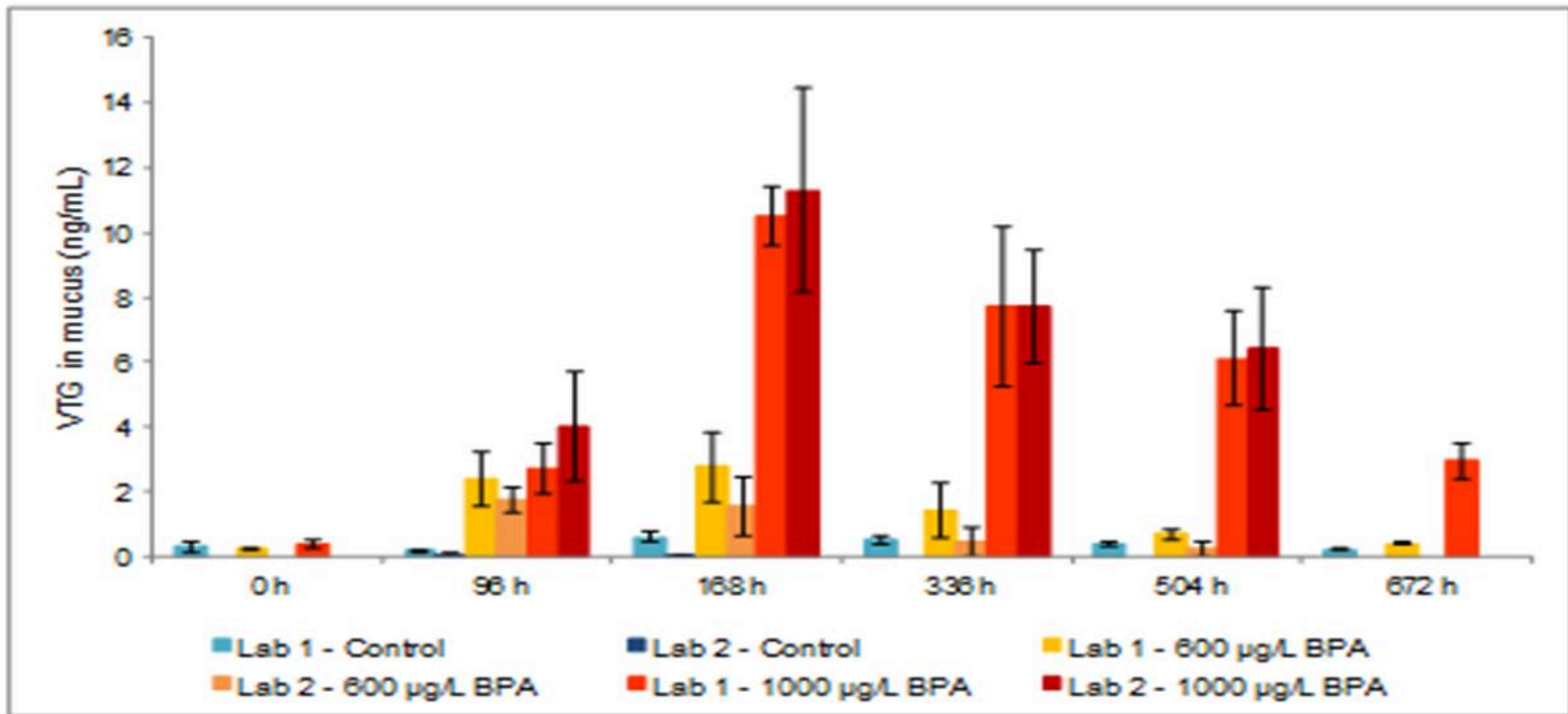
TECO® Cyprinid Vitellogenin ELISA



Exposition experiment with E2 in serum and mucus in European Perch at day 6



Comparison of results between two independent laboratories



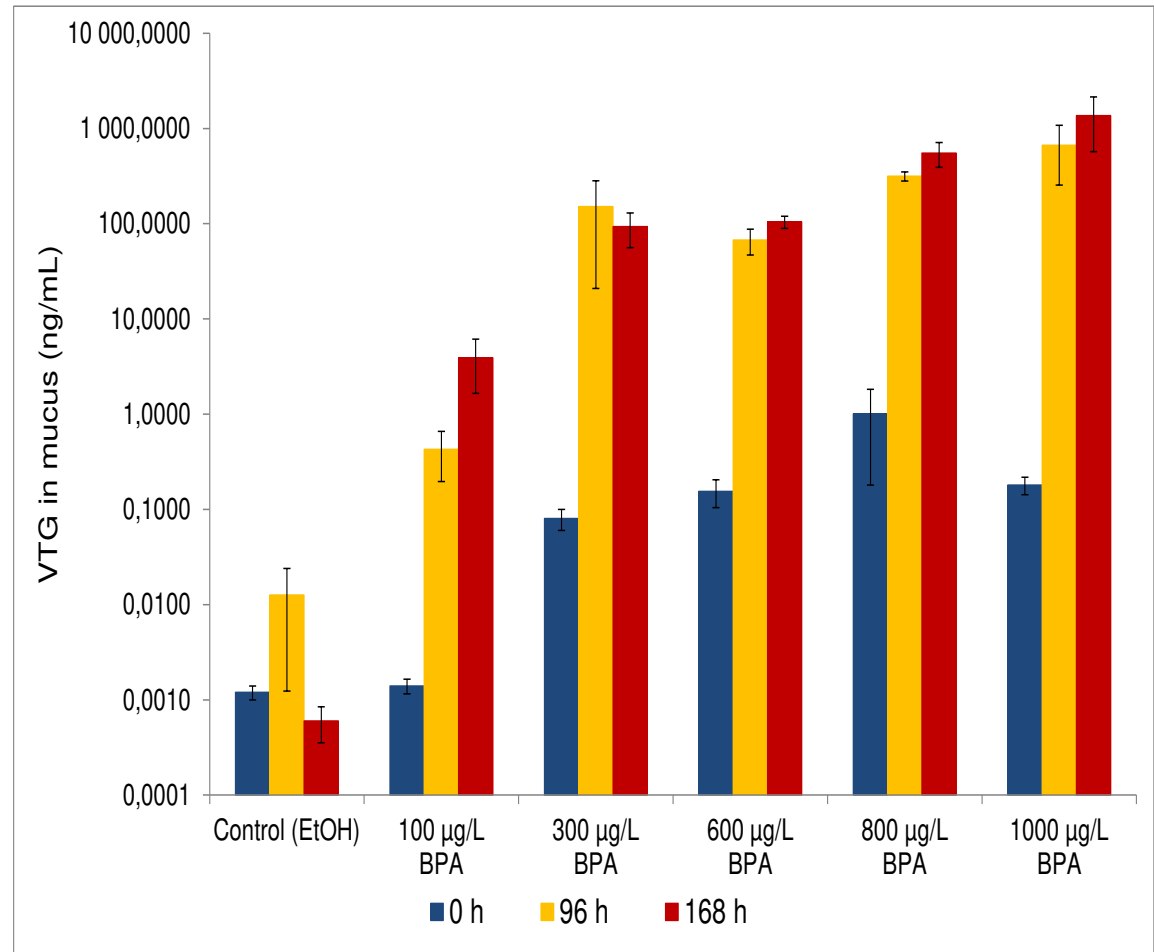
Measuring Vitellogenin in Mucus

- Monitoring due to multiple sampling over time:
- Vtg can be used to distinguish sex before exposure to ED
- Dynamik of VTG in response to Eds
- Shortening exposure experiments
- As mucus is a unique additional sample, it can be easily combined with other testing setups
- No effect on population in habitat
- Mucus and multi species testing allow testing of adequate fish species

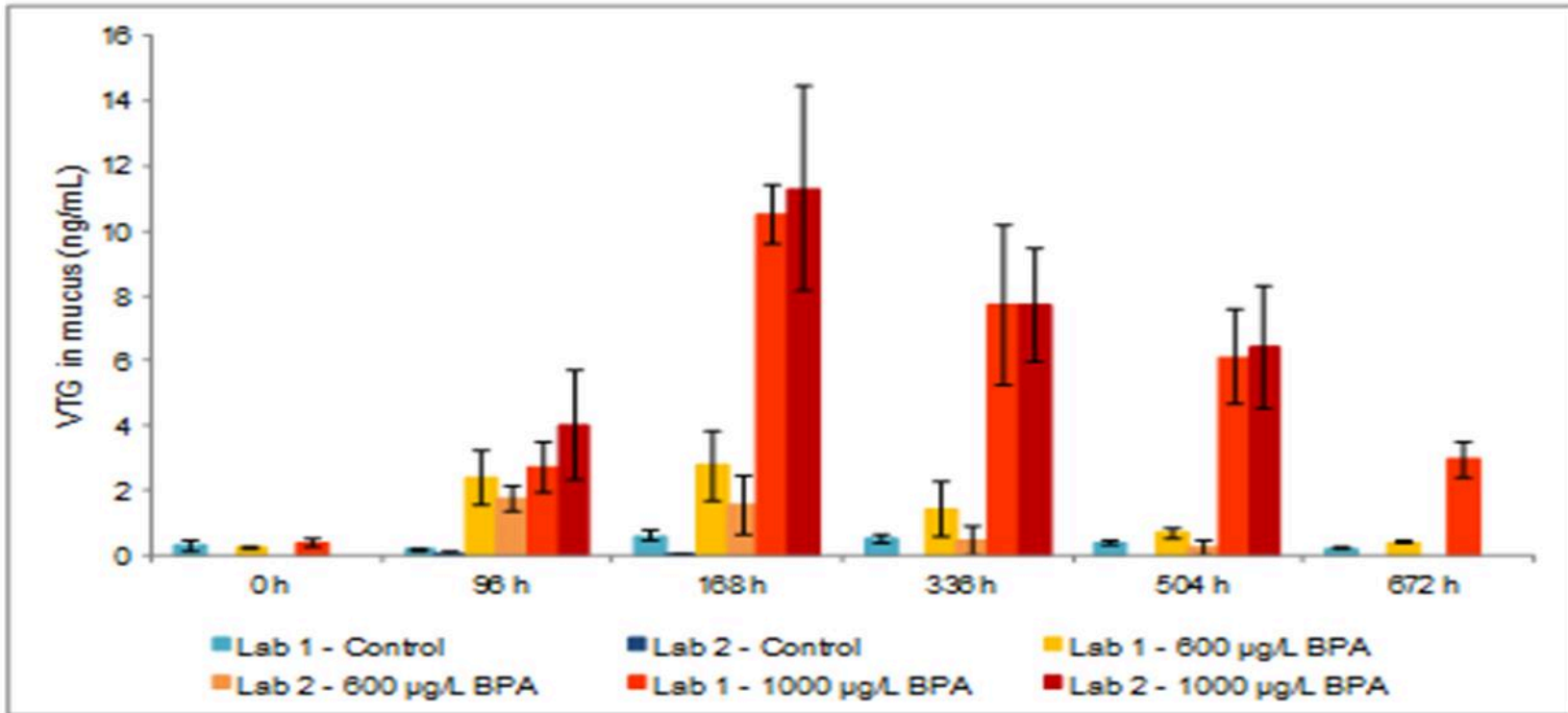
Refinement in chemical testing

Future Perspective:
Non destructive short
term screening possible

Shorter exposure
periods and non
destructive sampling
provide reliable results
on estrogenicity



Reduction in chemical testing



Additional endpoint in standard testing exemplified with OECD 215

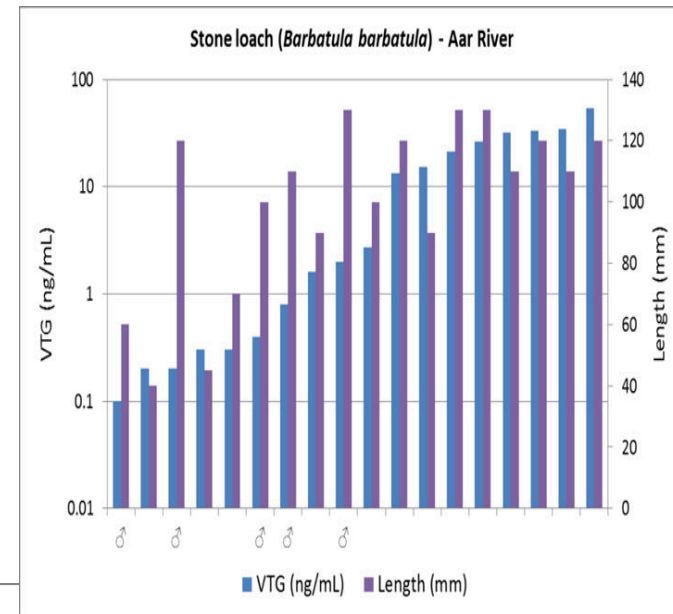
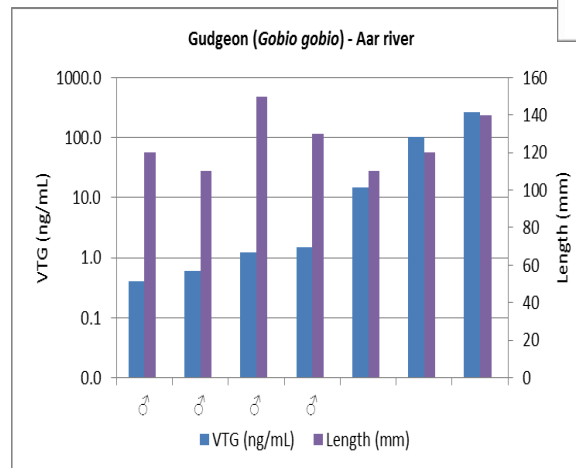
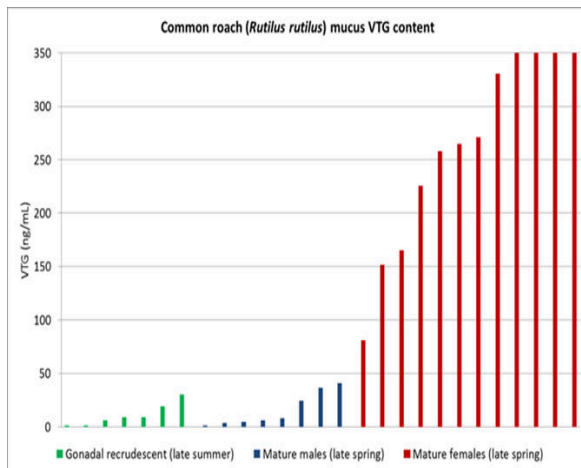
Bluegill (*Lepomis macrochirus*)

Environmental Monitoring

Destructive monitoring campaigns impact population under investigation

More information by monitoring seasonal changes

Not only paradoxical induction in males, also:
Suppression of ripening in females due to anti-estrogen effects



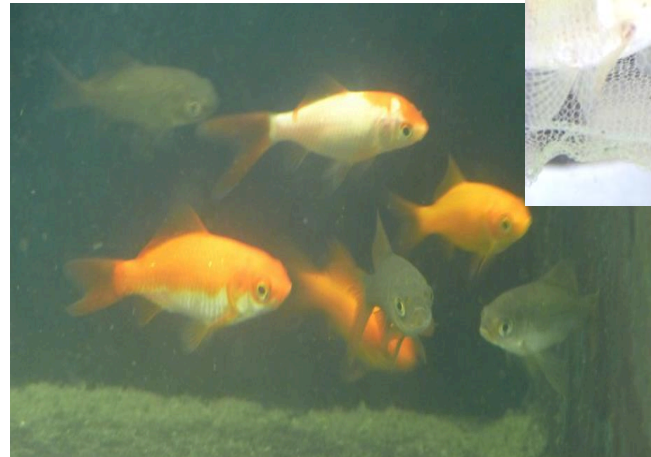
Sewage treatment plant management

Bypass experiments 4th cleaning level nonlethal kinetic

Caged experiments monitoring individual plants

Adequate species can be monitored as multi species assays are available

No exposure of trout downstream of salmonid regions



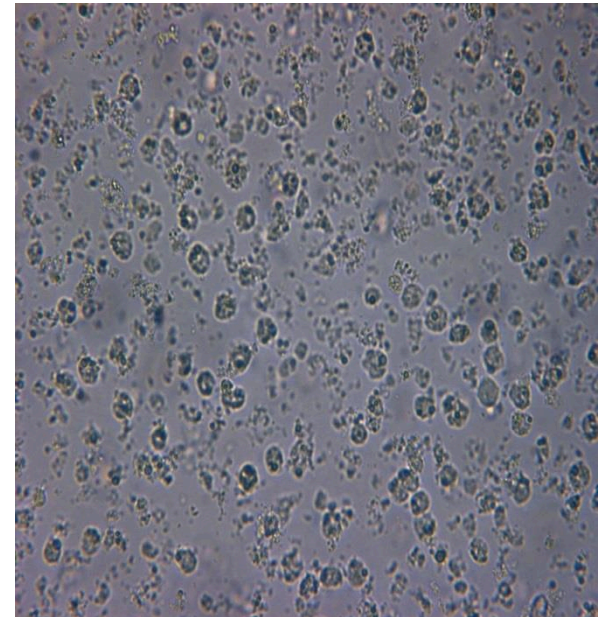
Replacement of animal testing by in vitro approaches

Translational approach using the same Endpoint/
Biomarker VTG in vitro and in vivo

State of the Art primary liver cells requires killing of
fish

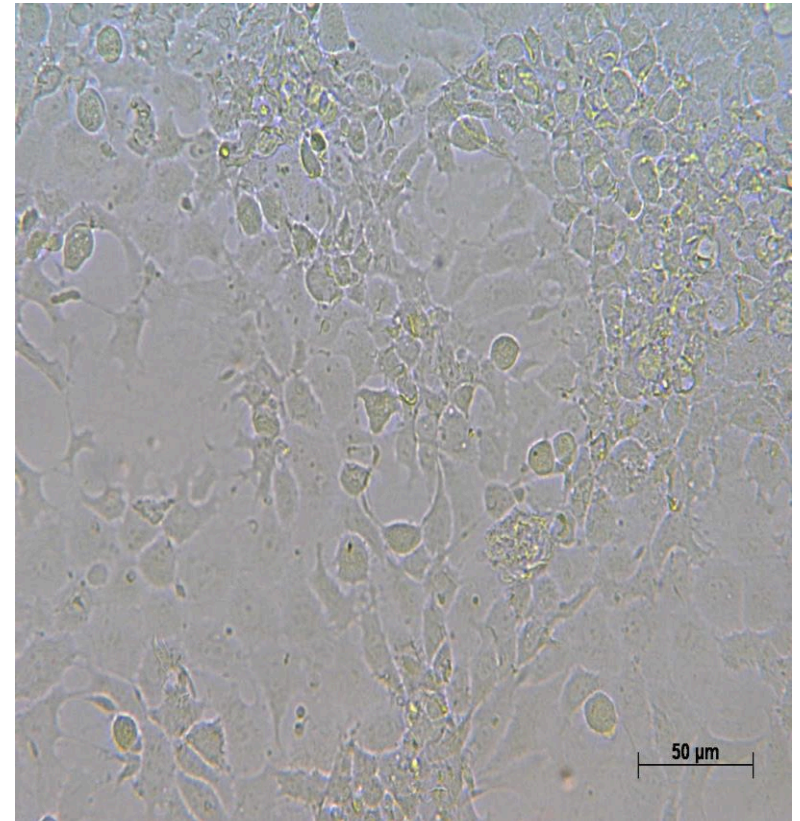
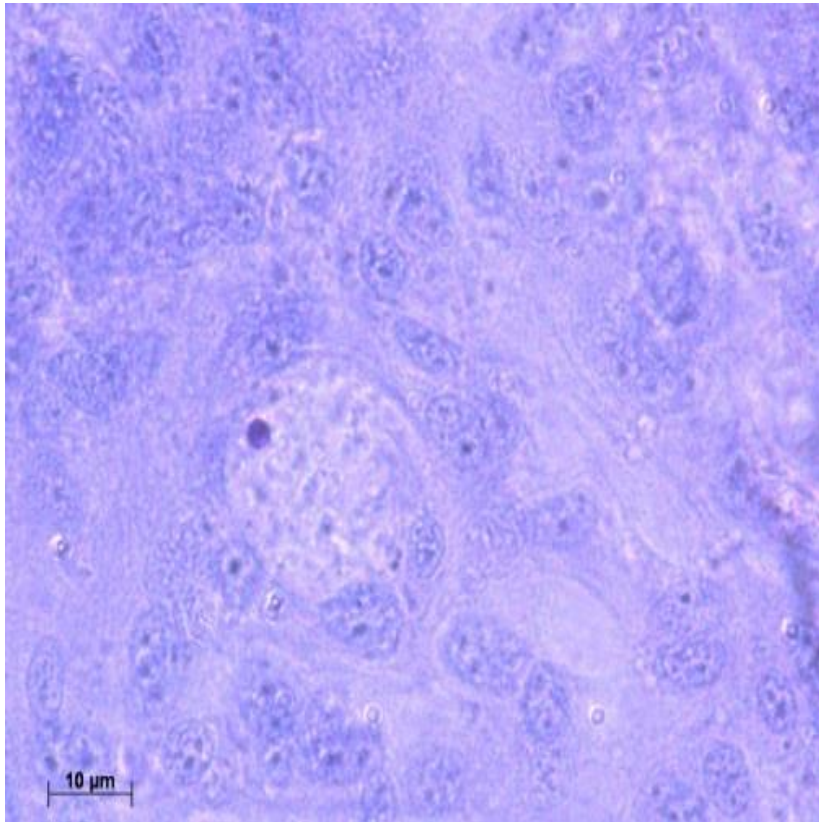
Method not suited for standardization

Cell viability and metabolic activity depends on the
condition of tissue removal

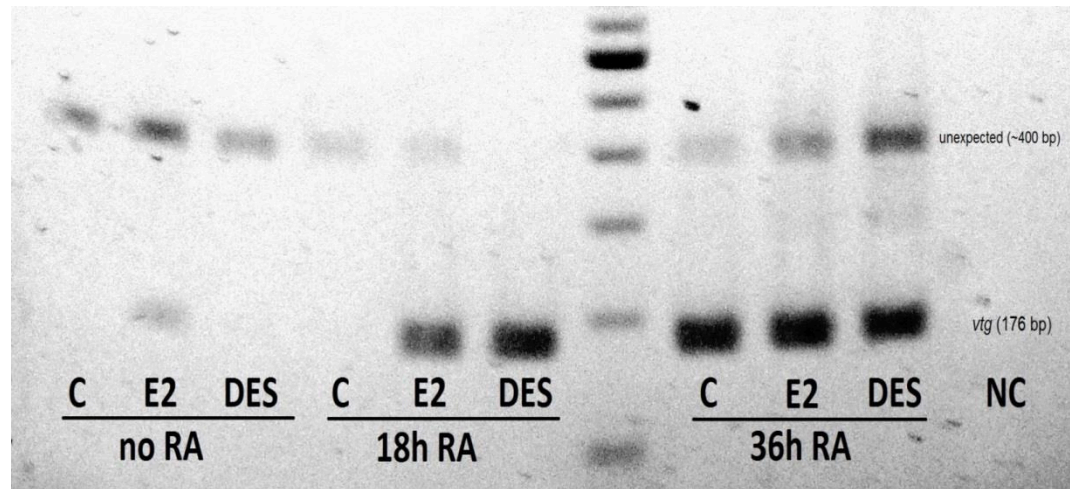


Primary liver cells
(*Cyprinus carpio*)

Stem cell line from the Koi carp brain (KCB)



Gene expression of estrogen receptors and vitellogenin



With no exposure to retinoic acid, only a band for E2, and with short exposure only for E2 and DES, while long exposure to RA leads to strong bands everywhere

Cyprinid VTG ELISA

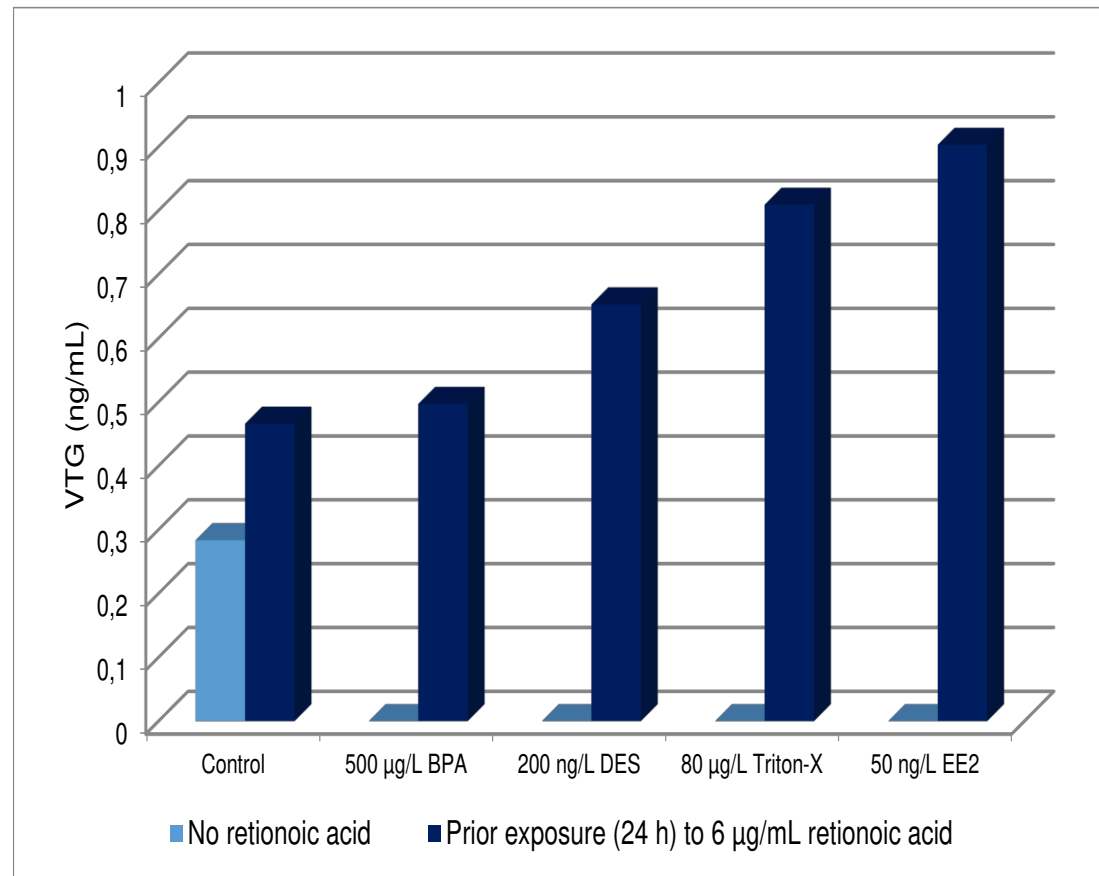
Preliminary results

BPA: bisphenol A

DES: diethylstilbestrol

Triton-X: "octylphenol"

EE2: ethinyl estradiol



Perspectives

- The stem cell line from the Koi carp brain (KCB) is a promising screening tool for lower tier testing
- The determination VTG in mucus allows a reliable monitoring of systemic effects
- The combination of in-vitro screening and monitoring systemic effects is the optimal way to realize responsible care.

Replacement, reduction, refinement

Refinement: minimized distress through non-invasive sampling

Reduction: less animals required due to successive sampling (kinetic monitoring); parallel investigation along with other endpoints

Replacement: potential *in vitro* assessment of EDC effects in the KCB cell line in lower tier experiments



<http://3rs.ccac.ca/en/research/reduction/>



Acknowledgements



TECOmedical Group



always your partner

EUSAAT

*European Society for
Alternatives to Animal Testing*