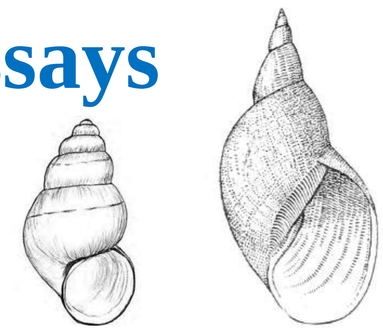
Histology Evaluation of Freshwater Snails *Potamopyrgus antipodarium* and *Lymnea stagnalis* used for Reproductive Assays



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Introduction

Water has become one of the world's most precious commodities. As more and more countries become industrialized contamination of our surface waters becomes a fundamental problem to human health and the availability of suitable resources to sustain human and aquatic life as we know it.

Invertebrates are important to the environment as herbivores, carnivores, decomposers of organic matter, and parts of the food chains. Toxicity testing on invertebrates aims at various aspects including product safety for the environment and humans, as well as economic aspects (e.g., honeybees, mussels in aquaculture, etc.).
The extent of possible effects of chemicals on mollusks came into public regard with the dramatic effects of tributyltin (TBT) compounds, which have broadly been used as antifouling agents for ships. The females of the Dog Whelk (*Nucella lapillus*) exposed to TBT further species developed male parts in addition to the female genital organs, a syndrome named 'imposex'. Almost 160 species are endangered. With a number of approximately 130'000 living species, the diverse molluscan phylum can be found in almost all biotopes world-wide. Consequently, a number of publications is available dealing with the effects of chemicals on wild living bivalves and gastropods.

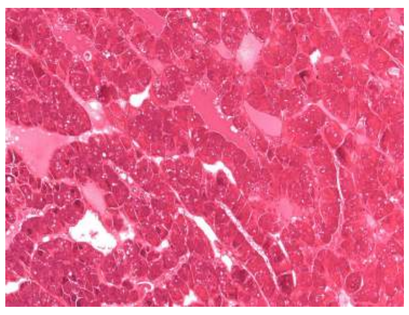
Development of Guidelines for Reproductive Toxicity Testing

Rittschof and McClellan-Green (2005) proposed strategies for the use of mollusks as multidisciplinary models in ecotoxicity studies mainly due the fact that some metabolic pathways show homologies to vertebrates. Since 1998, there is a guideline for the testing of chemicals in marine bivalves (ASTM E724-98).
The choice of the right model species is considerably affected by physiological conditions of the reproductive cycle. To test the endocrine activity of chemicals, Duft et al. (2007) proposed three prosobranch species, the New Zealand freshwater mud snail (*Potamopyrgus antipodarum*), the freshwater Ramshorn Snail (*Marisa cornuarietis*), and the marine Netted Dog Whelk (Nassarius reticulatus). Species proposed for a new OECD guideline on Full Life Cycle testing include *P. antipodarum* and *Limnea stagnalis* (Matthiesen, 2008). The review paper of the OECD (2010) recommended test procedures for optimisation and possible validation for partial life cycle (PLC) test P. antipodarum (freshwater, gastropod), full life cycle (FLC) with *L. stagnalis* (freshwater, gastropod) and with *Crassostrea gigas* (bivalve, marine). It was also recommended to develop protocols for FLC with *P. antipodarum* and PLC with L. stagnalis. The OECD (2016 a, b) published guideline for a reproduction test with *L. stagnalis* (Figures 1-3) and *P. antipodarum* (Figures 4-6). Histopathology was detailed in the guidelines but stated as '…other endpoint (e.g., histopathology)…' (OECD, 2016b) and was mentioned under point 3.3. and in the Annex for possible histopathology evaluation (OECD, 2016a).

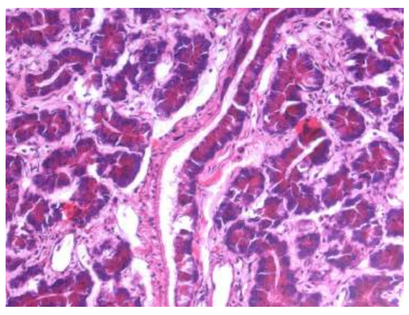
Histopathology on Model Species

The current guidelines target mainly endocrine disruptor effects. However, inflammatory, and degenerative processes or parasitic infestations can mask or mimic endocrine effects. Therefore, a histopathological examination should be performed. The cost and labor are not high. Nevertheless, the pathologist must be familiar with the morphology and physiology of the different species.

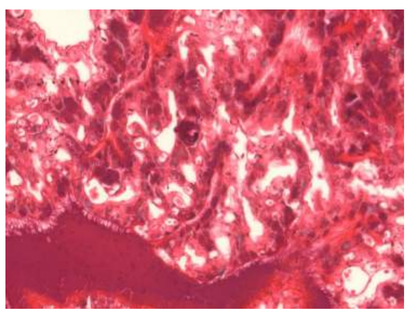
Figure Panel: Control tissues and induced lesions in test performed with *L.stagnalis*



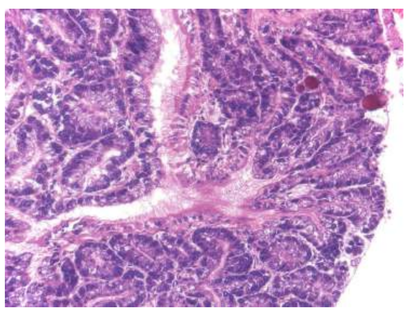
Albumen Gland. Normal. *L.stagnalis*



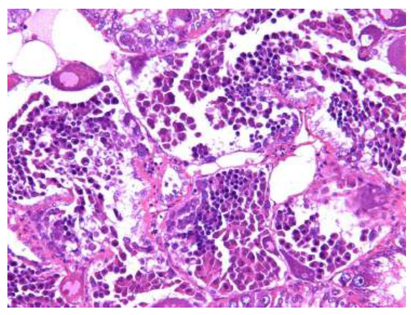
Albumen Gland. Immature/winter *L.stagnalis*



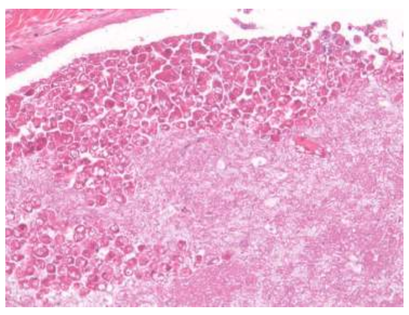
Albumen Gland. Degeneration. B-STE 100 ng/L. *L.stagnalis*



Albumen Gland. Atrophy. TBT 200 n/l *L.stagnalis*

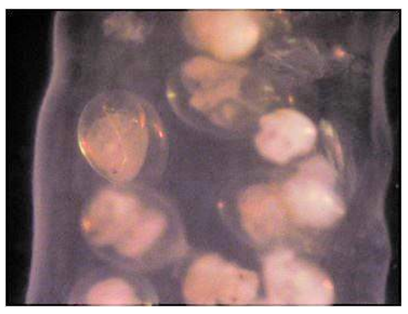


Hermaphrodite Gland. Decreased oogenesis, increased spermio genesis. TBT 200 n/l. *L.stagnalis*

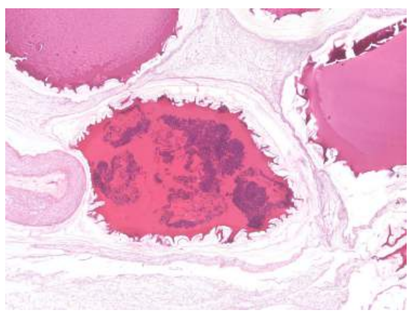


Sperm duct. Sperm granuloma. Cadmium 60-120 ng/L. *L.stagnalis*

Control clutch. *L.stagnalis*



Clutch with necrotic eggs. Cadmium 60-120 ng/L. *L.stagnalis*



Clutch with necrotic eggs. Cadmium 60-120 ng/L. *L.stagnalis*

Procedures

Fixation:

After death, transfer into Bouin's fixative for 24 hours, fixed in 70% ethanol, or rinsed in 70% ethanol and finally fixed in 4% neutral phosphate-buffered formaldehyde.

Bouin's fixative is of advantage due to a nearly complete dissolution of the shell.

Trimming;

L. stagnalis:

Three longitudinal sections to evaluate all organs on 1 glass slide (median-sagittal, one sagittal section approximately 2 mm to the left of the median axis to meet all glands of the reproductive system, and a third sagittal section approximately 2 mm adjacent to the lateral body wall to cut through the penial complex)

P. antipodarium:

Two longitudinal sections to evaluate all organs on 1 glass slide (each one sagittal section approximately 1 mm to the left and right of the median axis to meet all organs and glands of the reproductive system including brood sac)

Staining:

Hematoxylin and eosin is the best staining to evaluate all organs.

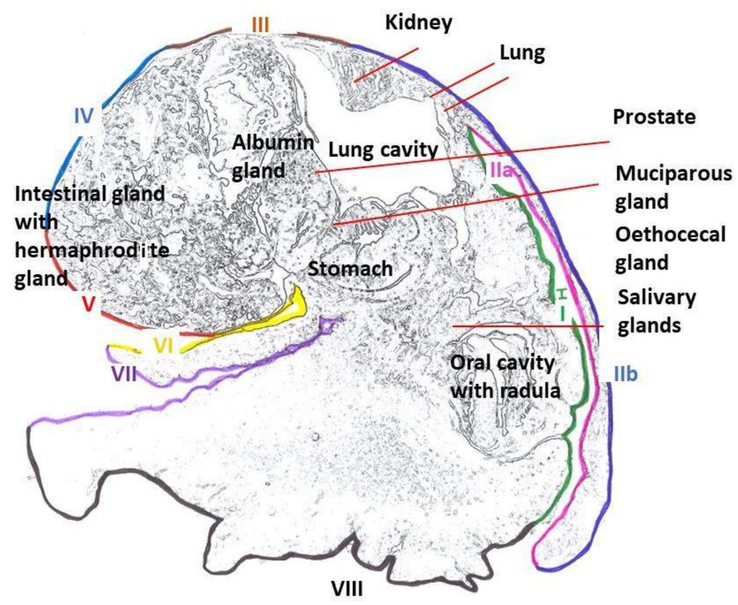


Figure 1. Overview on section with indication of different zones of surface epithelia (*L. stagmalis.*

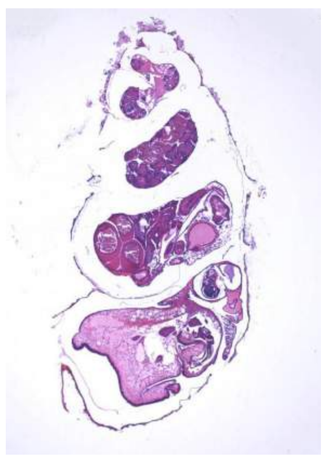


Figure 4. Overview *(P. antipodarium)*

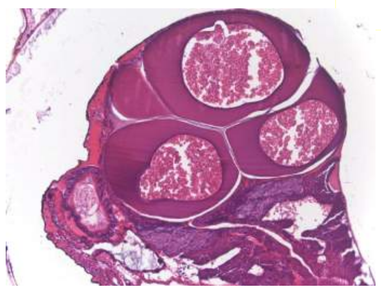


Figure 5. Ovary*(P. antipodarium)*

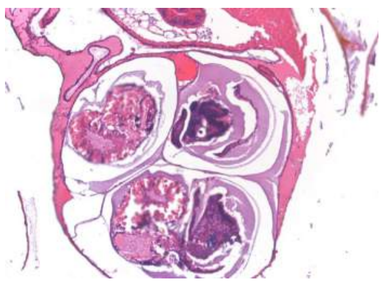


Figure 6. Brood sac *(P. antipodarium)*

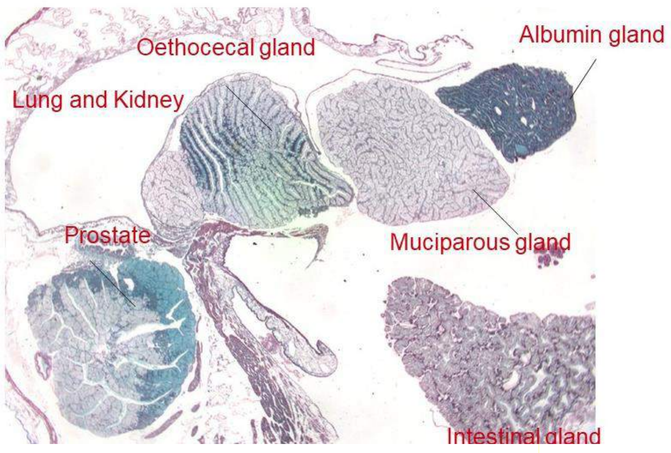


Figure 2. Overview on reproductive gland *(L. stagnalis)*

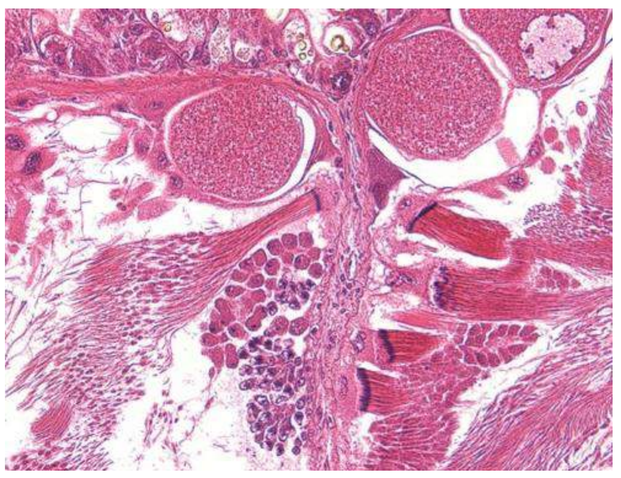


Figure 3. Hermaphrodite gland *(L. stagnalis)* with oocytes and sperm cells

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- Matthiessen P. An Assessment of endocrine disruption in mollusks and the potential for developing internationally standardized mollusk life cycle test guidelines. Integr Environ Assess Manag. 2008. 4: 274-284.
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- OECD. OECD Guideline for the Testing of Chemicals. Potamopyrgus antipodarum Reproduction Test. OECD 242. 29 Juy 2016a.
- OECD. OECD Guideline for the Testing of Chemicals. Lymnaea stagnalsi Reproduction Test. OECD 243. 29 Juy 2016b.
- OECD. Series on Testing and Assessment No. 121. Detailed review paper (DRP) on molluscs Life-cycle toxicity testing. ENV/JM/MONO(2010)9

Publication in Progress