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## Histological Study of Gonadogenesis in *Potamopyrgus antipodarum* and *Valvata piscinalis*

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### Abstract

This study focuses on gonad development in two freshwater gastropod prosobranch snail species (*Potamopyrgus antipodarum* and *Valvata piscinalis*).

These biological models have been shown to be sensitive to various reprotoxic substances and are known to be relevant organisms for studying endocrine-disrupting substances. Therefore we took up a histological study of their anatomy and the gametogenesis of the two species in order to key into biological elements potentially useful for reprotoxic stress studies.

Knowing that *P. antipodarum* is parthenogenetic, we studied all their gonadogenetic stages, taking caution to clearly describe the different oocyte developmental stages leading to the formation of the copulatory bursa, characterizing neighboring glands.

As for *V. piscinalis*, a hermaphrodite species, all the spermatogenesis development stages were described (spermatocytogenesis and spermiogenesis) as well as specification of nourishing cells and support cells, the objective being to define the species' reproduction mechanism.

This studied was carried out by observing the development of young *P. antipodarum* and *V. piscinalis*, exposing them to the same experimental conditions for 6 months. Weekly samples were taken for biometric imaging and conventional histology photonics in order to determine the age and to validate the appearance of early gonadogenesis. A fundamental, detailed study on tissue identification was conducted to accomplish this task.

**Keywords:** Early gametogenesis; Leydig cells; Sertoli cells; Germinal cells; Mitosis division; Meiotic division; Albumen gland; Ovogonium; Ovocyte; Spermatogenesis; Vitellogenesis

### Introduction

The present work involves an anatomical and histological study of two freshwater gastropod prosobranch snail species: 1) *Potamopyrgus antipodarum*, using only parthenogenetic females (Figure 1 and 2) *Valvata piscinalis* hermaphrodites (Figure 2), and paying particular

attention to their gonad development. They were chosen as models to study the effects of endocrine disruptors on production.



**Figure 1:** Adult *Potamopyrgus antipodarum*; Phylum: Mollusca; Class: Gastropoda; Family: Hydrobiidae; Genus: *Potamopyrgus*; photo: CEMAGREF Lyon, France.



**Figure 2:** Adult *Valvata piscinalis*; Phylum: Mollusca; Class: Gastropoda; Family: Valvatacea; Genus: *Valvata*; photo: CEMAGREF Lyon, France.

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The biological cycle of these two species depends heavily on environmental factors. The growth rate increases in spring and summer while in autumn it slows down and even halts in winter [1].

Like other gastropods, their diet varies, alternating between detritus and vegetation depending on the season. They principally feed on epiphytic algae in summer and on detritus when living among mudded foliage during cold periods. Their dispersion has been affected generally by humans, birds, fish, and even amphibians, insects, and currents [2].

These snails are relevant ecological indicators due to their wide distribution. These biological models are sensitive to different reprotoxic substances. *P. antipodarum* is known as a pertinent organism for studying endocrine-disrupting substances [3]. Taking advantage of this, we took up a histological study focusing on their anatomy and gonadogenesis to examine precise biological changes, such as late gonadogenesis and the decrease in embryo reproduction, during reprotoxicity studies.

### Classification and morphology

*P. antipodarum* is an invasive species from New Zealand, hence its common name “New Zealand mudsnail”, which over time has colonized Australia, Europe and the America.

As a general rule, this species is diploid in its native and sexual population though exclusively parthenogenetic and ovoviviparous in Europe.

They thrive mostly in fresh and brackish waters and are remarkably

resistant to harsh conditions, especially with regards to oxygen demands and thermal variations. They are also easily transportable from natural habitats to laboratory conditions (if the temperature is kept around 18°C).

As far as reproduction characteristics are concerned, egg-laying is continuous, although reproduction may be considered as seasonal, with optimal fertility of females in spring. *P. antipodarum* spends most of the winter in the juvenile stage and begins reproducing in June. Their life span is up to 1 year or more.

### General morphology

*P. antipodarum* has a soft body and a clearly distinct head. The visceral mass curls up in a helical shape and the bronchial cavity is in the front [4]. The mantel surrounds the visceral mass and forms a cavity containing feather-like gills. The tentacles are long and thin, with eyes situated on the tip ends.

Secreted by the mantel, the cone-shaped shell is thin although solid, with a yellowish-brown color, and has convex or rounded spirals [5].

The gonads are in the inferior half of the whorl, forming a spiral with a digestive gland and other individual organs [6]

*V. piscinalis*, the European stream valvata, is an autochthonous species found throughout France [2]. Present in all types of freshwaters, they mostly colonize muddy habitats having superior vegetation. They are abundant in sewer pipes as well in brooks and streams with slow water flow (Figure 2) [1].

This snail is a hermaphrodite with both distinct male and female organs. They have similar biological and ecological characteristics to other gastropods. Thus the characteristics discussed above for *P. antipodarum* can be applied to this species.

The shell is subdiscoiled with a circular opening and an almost centered corneous operculum. Yellowish in color, it has three and a half whorls.

Reproduction is seasonal and takes place between April and September.

### Materials and Methods

This experiment in controlled conditions was carried out in the laboratory using healthy individuals. It defined the size at which the two snail species (*P. antipodarum* and *V. piscinalis*) began sexual maturity and detected the different oocyte maturation stages.

Physicochemical experimental conditions for raising *P. antipodarum* and *V. piscinalis*

The study was conducted from February 6<sup>th</sup> to July 2<sup>nd</sup> 2008. Forty-five *P. antipodarum* and *V. piscinalis* juveniles, just 48 h old, were raised in the same experimental conditions at 16°C for 6 months (five organisms per beaker/L H<sub>2</sub>O for five beakers).

The conditions consisted of natural water with conductivity between 632 and 740 µs/cm for *P. antipodarum* and between 538 and 711 µs/cm for *V. piscinalis*. They were fed an artificial substance (TETRAMIN® Tetra GMBH, Melle, Germany; 0.3 mg/ind/day). The water quality remained acceptable throughout the experiment (7<pH<8, 7.4 mg/L<O<sub>2</sub>< 8.8 mg/L), NH<sub>4</sub> = 0, 16°C<T°<16.5°C).

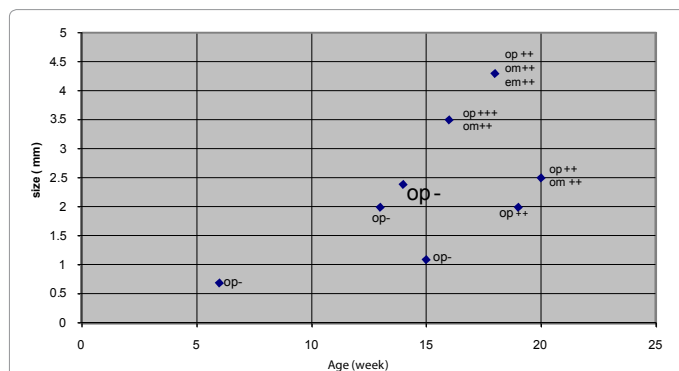


Figure 3: Development of gonad maturity in *Potamopyrgus antipodarum*; OP: Primary Oocyte; OM: Mature Oocyte; Em: Embryo.

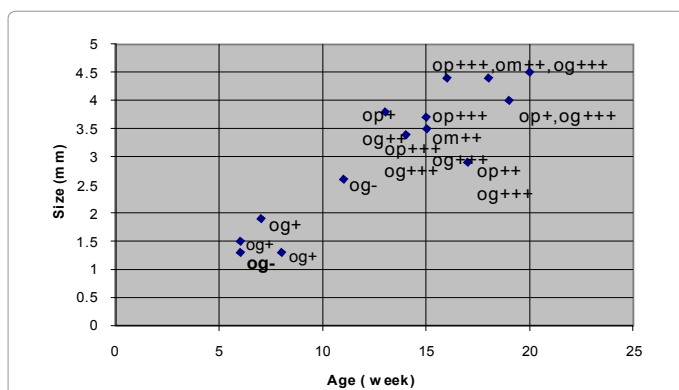


Figure 4: development of gonad maturity in *Valvata piscinalis*; OP: Primary Oocyte; OM: Mature Oocyte; Em: Embryo.

Six weeks into the experiment, the first organisms reaching 0.7 mm for *P. antipodarum* and 1.3 mm for *V. piscinalis* were sampled and conditioned for histological observations (Table 1).

### Histology

Two samples for each species were examined once a week. They were fixed to Bouin liquid for 24 h for juveniles and 48 h for adults. This step is essential in order to stabilize the tissue and cellular structures [7]. Bouin liquid was chosen because it does not disturb the morphology of the organisms, and it contains glacial acetic acid for dissolving the shell.

The material was rinsed with increasing alcohol concentrations for

complete dehydration. The “lightening” step follows, which involves a solvent bath (Butanol) to replace intracellular water with paraffin. Then 4- $\mu$ m cuts were made by the Leica® microtome, and then stained with hematoxylin-eosin stain. The hemalum stain dyes the acidophil structures (such as the nuclei) and the eosin dyes the basophile structures (such as the cytoplasm).

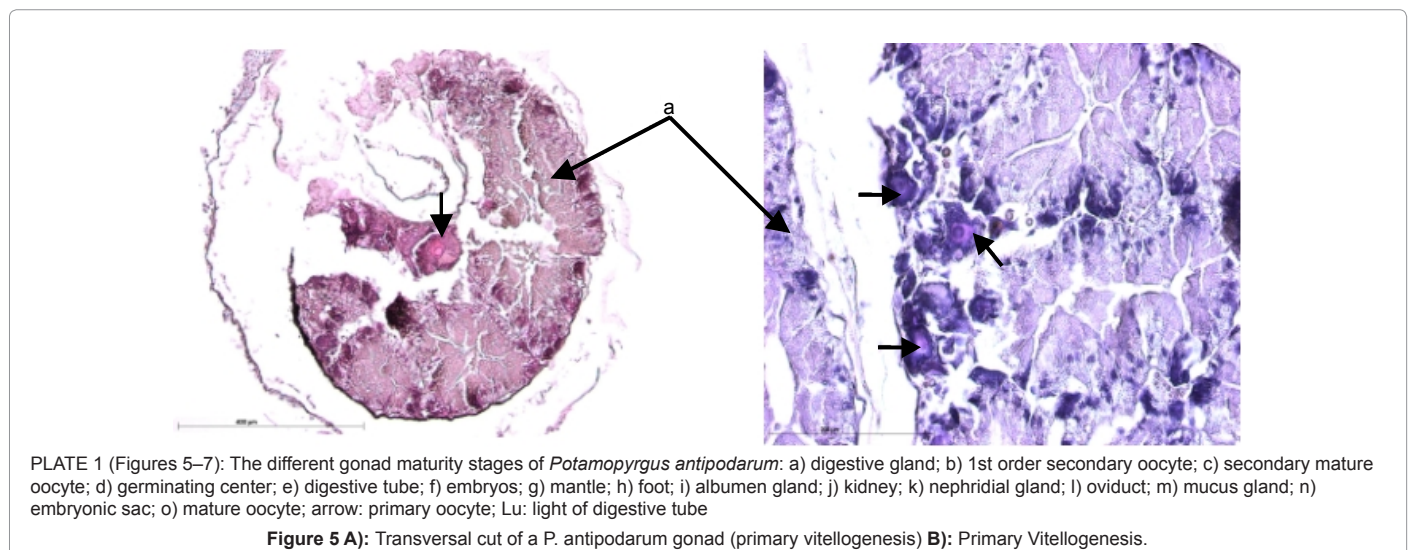
The Leica® microscope was used for observations.

### Results

The plates (1, 2, and 3) present a selection of dissections illustrating

Individual	Sampling date	pH	O <sub>2</sub> mg/l	T° °C	Cond $\mu$ s/cm <sup>2</sup>	Age (week)	Size (mm)	Gonad development			
								OP	OM	OG	EM
P1	19/03/2008	7.97	8.8	16.2	540	6	0.7	-	-		-
P1	06/05/2008	7.12	8.3	16.4	615	13	2	-	-		-
P1	14/05/2008	7.69	7.86	16	620	14	2.4	+	+		-
P1	28/05/2008	7.9	7.74	16.2	632	16	3.5	+++	++		-
P1	12/06/2008	8.04	8.12	16.2	608	18	4.3	+++	+++		+++
P5	22/05/2008	8.13	7.92	16.2	613	15	1.1	-	-		-
P5	19/06/2008	7.92	7.44	16	620	19	2	++	-		-
P5	26/06/2008	7.94	7.97	16.3	620	20	2.5	++	++		-
V3	19/03/2008	7.98	8.6	16.4	538	6	1.3	-	-		-
V3	27/03/2008	8.05	8.4	15.9	593	7	1.9	-	-	-	-
V3	24/04/2008	8.1	7.64	15.7	663	11	2.6	-	-		-
V3	29/04/2008	8.03	8.33	15.7	623	14	3.4	+++	-	+++	-
V3	15/05/2008	8.13	7.92	15.7	617	15	3.7	+++	+	+++	-
V3	22/05/2008	8.02	7.82	15.9	624	20	4.5	+++	-	+++	-
V5	19/03/2008	7.98	8.6	16.4	538	6	1.5	-	-	+	-
V5	03/04/2008	8.05	8.4	15.9	593	7	1.9	-	-	+	-
V5	06/05/2008	7.97	8.4	15.8	597	8	1.3	-	-	+	-
V5	22/05/2008	7.86	7.95	15.8	616	13	3.8	+	-	+++	-
V5	28/05/2008	8.13	7.92	15.7	617	15	3.5	+++	+	+++	-
V5	06/06/2008	7.78	8.39	15.8	630	16	4.4	+++	+++	+++	-
V5	12/06/2008	7.86	8.3	16.1	612	17	2.9	++	-	+++	-
V5	19/06/2008	7.92	8.21	16	605	18	4.4	+++	+++	+++	-
V5	12/06/2008	7.81	8.21	15.8	616	19	4	+++	++	+++	-

**Table 1:** The data presented consist of physicochemical results, size and age of organisms sampled for histological studies and for observations of the development of gonad maturity. The length of the shell (from the spire to the operculum) of each specimen was measured using a binocular magnifier with an integrated P: *Potamopyrgus antipodarum*, V: *Valvata piscinalis*, Cond: conductivity, OG: Oogonium, OP: primary oocyte, MO: secondary mature oocyte, EM: Embryon



the gonadogenesis development and the organization of the internal tissues of *P. antipodarum* and *V. piscinalis*.

### Potamopyrgus antipodarum

The dissections show that the gonad overlaps with the digestive gland taking up the basal half (Figures 5A, 6A,6B). Evolving near the foot, the albumen gland, whose role is to secrete albumen which envelops the oocytes, can be observed (Figures 6A,6B). Only female tissues were observed from the parthenogenetic specimens.

The histological study of gonad development for *P. antipodarum* showed early gonadogenesis when they reached 2 mm, at which point the primary oocytes increased in size (2.5 mm) (Figures 5A,5B) as well as the 1<sup>st</sup> order secondary oocytes (Figure 6A). At 3.5 mm, we observed a complete maturation of the gonad with a presence of all maturity stages (Figure 6B) and at 4.5 mm the embryos in the embryonic sac (Figure 7A). The maturation stages were still present at this size for other individuals (Figure 7B).

### The different oocyte maturity stages

**Oocytes in primary vitellogenesis:** During primary vitellogenesis, the cells were small and star-shaped. The central, nucleolated nucleus was hardly differentiated and the basophile cytoplasm was finely granulated (Figures 5A,5B).

**Oocytes in secondary vitellogenesis:** Two types of oocytes were differentiated: immature oocytes and mature oocytes (Figures 6A,6B).

**Immature Oocytes:** These were medium-sized cells with a

central, round, nucleolated, basophile nucleus and a cytoplasm that was very receptive to hematoxylin: oocytes in first-order, secondary vitellogenesis (Figure 6A).

**Mature Oocytes:** These are large cells with a basophile nucleus (germinating center) with a half-moon shape. Its movement from the center toward the periphery is an indicator of its maturity. At this stage, the cells were filled with lipid droplets and homogenous vitellus eosinophil globules with a half-moon diameter, which accumulate in the oocyte cytoplasm during the maturation phase: oocytes in secondary mature vitellogenesis (Figure 6B).

### Valvata piscinalis

The *V. piscinalis* gonad begins at the coiling extremity where the first male cells were observed. Maturity of the hermaphrodite glands occurs near the foot of the individual. Male cells can be observed in the center of the gland and female cells near the periphery (Figures 8A,8B,9). The hermaphrodite gland overlaps with the digestive organ. Continuing on the foot, the atrium and the ventricle can be observed just above the embryonic sac near where the mature female cells are channeled through the follicular cord (Figures 10C,11A,11B). The kidney is against the albumen gland, which itself touches the mucus gland (Figures 12A,12B,12C).

Histology of the *V. piscinalis* gonad makes it possible to detect early gonadogenesis at week 7 (1.9–2 mm), associated to the proliferation of male tissue. At the extremity of the coil, early acini formation can be observed in the middle of a thick eosinophilic (conjunctive) basal layer,

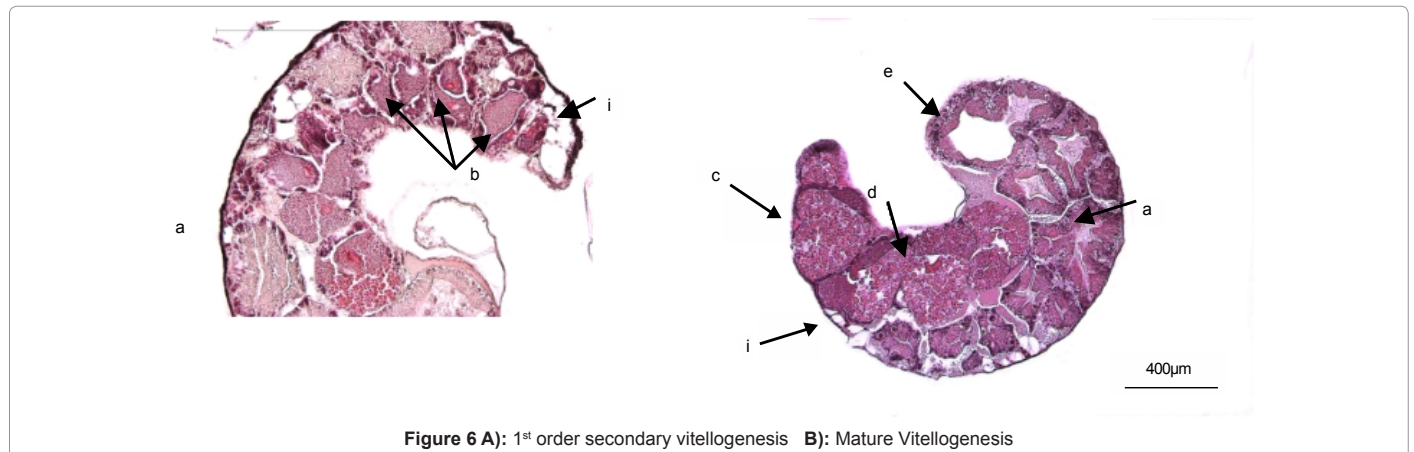


Figure 6 A): 1<sup>st</sup> order secondary vitellogenesis B): Mature Vitellogenesis

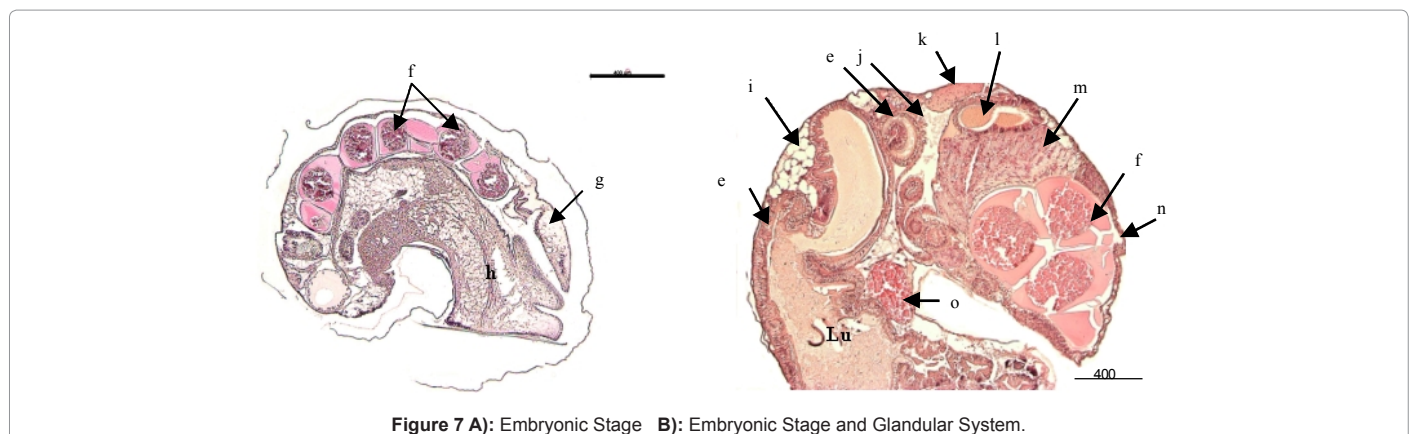


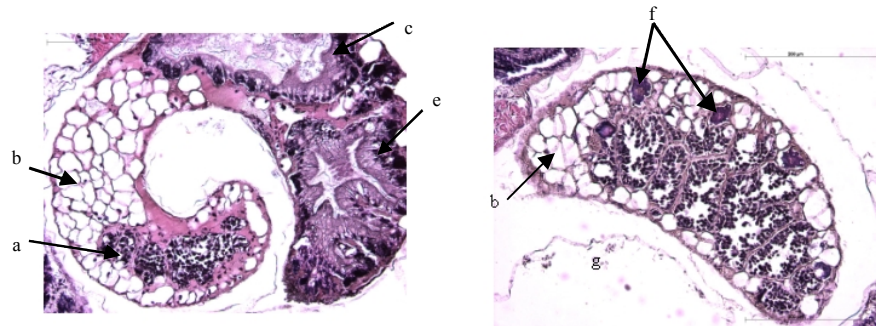
Figure 7 A): Embryonic Stage B): Embryonic Stage and Glandular System.

surrounded by primary oocytes, in turn surrounded by albumen gland cells (Figures 8A,8B). The empty follicular cord in the embryonic sac is visible (Figure 5B).

At week 11 (2.5 – 3 mm) the number of acini garnished with oogonia (Figure 8A) are visible near the inferior extremity of the gonad increases.

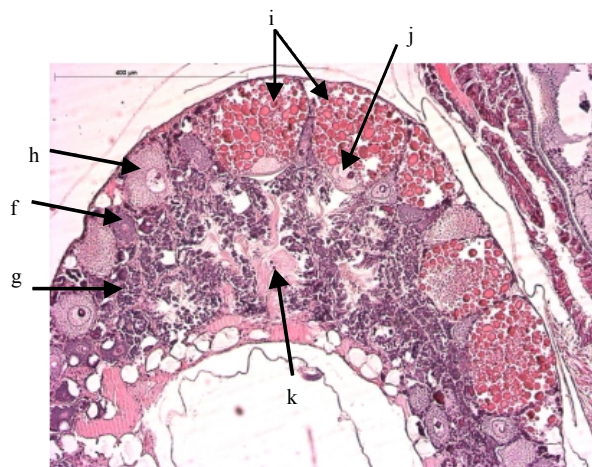
At week 16 (4 – 4.5 mm) complete gonad maturation can be observed, with perfect arborization of seminal receptacles, as well as the different oocyte maturity stages (Figure 9).

**Male tissue:** The histological cuts observed at high magnification (Figure 10A) revealed a tubular structure resembling seminiferous tubes separated by an amorphous tissue composed mostly of isolated

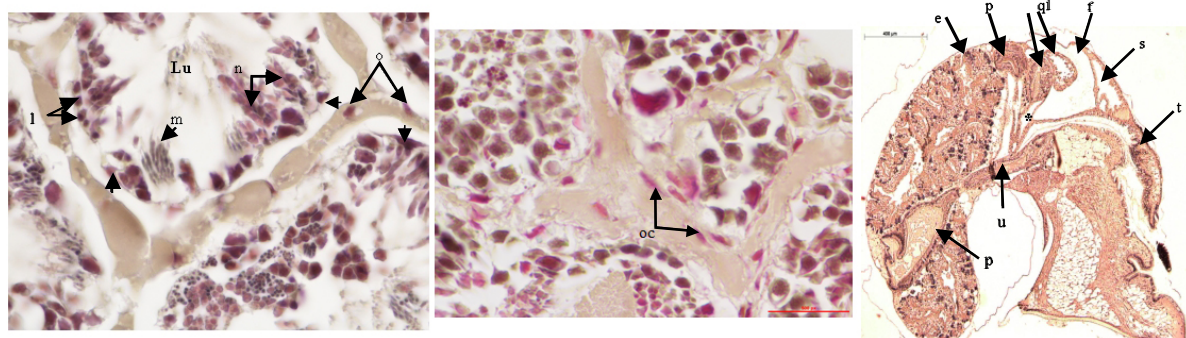


PLATES 2 and 3 (Figures 8–12): Different gonad maturity stages and internal organization of different organs observed in *Valvata piscinalis* a) acini; b) albumen gland; c) digestive tube; d) light of digestive tube; e) digestive gland; f) primary oocytes; g) acini covered with spermatogonia; h) 1st order secondary oocytes; i) secondary mature oocytes; j) germinating center; k) efferent canal covered with spermatozooids; l) germinating spermatogonia; m) spermatozooids; n) different maturity stages of spermatogonia, with light increasing along with maturity; o) cells resembling Leydig cells; oc: Leydig cells with Reinke crystalloids; p) intestine; q) ventricle; q1) atrium; r) embryonic sac; s) nephridial gland; t) mantle; u) columella muscle; v) follicular cord; w) oviduct; x) albumen gland; y) nerve cord; z) mucus gland; arrow: cells resembling Sertoli cells; Lu: light from the seminal receptacle; \*: kidney.

**Figure 8 A):** Transversal cut of a *V. piscinalis* gonad (early gonadogenesis sac) **B):** Acini Formation.



**Figure 9:** Gonad Maturity.



**Figure 10A):** Seminal Receptacle **B):** Leydig cells **C):** Glandular System.

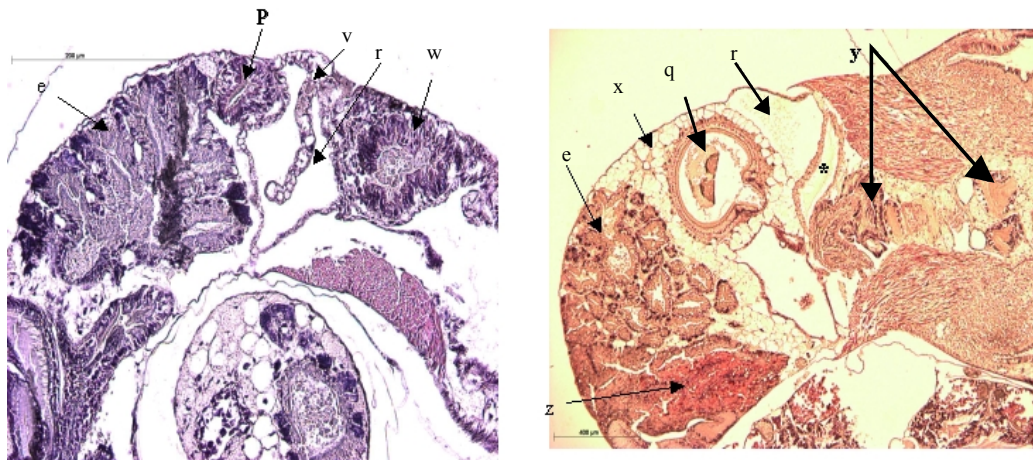


Plate 3. Organization of *Valvata piscinalis* internal tissues

Figure 11 A): Embryonic Sac B): Glandular System.

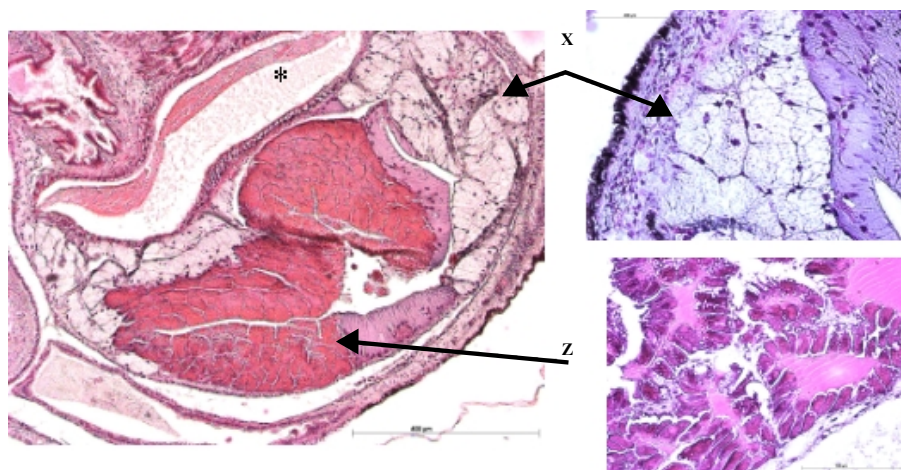


Figure 12 A): Glandular System B): Albumen Gland C): Mucus Gland (clockwise).

cells or regrouped ones surrounding blood vessels. These are known as Leydig cells recognizable by their stick-like, crystalloid inclusions known as Reinke crystalloids [8] (Figure 10B). The latter feature plays an important role in gonad mechanics.

Maturity of male gonads begins with spermatogenesis, a process by which the spermatids, as a result of meiotic division, go through the different maturation stages before being transformed into mature spermatozooids.

The transversal cut (Figure 10A) represents spermatogenesis forming where the arborization takes on a grape-like shape progressing in the light.

The seminiferous tubes are disposed in the center of the hermaphrodite gonad (Figure 8B) surrounded by female cells (oocyte). On the peripheral, they are surrounded by a stratified epithelium constituted of two cellular types.

**Germinal cells:** The type A spermatogonia in the basal compartment was undergoing several mitotic divisions and going on to form type B spermatogonia (future spermatozoid).

Type A spermatogonia may be characterized by their voluminous nucleus and condensed chromatin, whereas the type B spermatogonia have dispersed chromatin and dull colored cytoplasm. These latter were undergoing meiotic division leading to spermatozoid formation, which is recognized by their heads anchoring in the nourishing cells' cytoplasm with a flagellum extending towards the light of the seminiferous tube (Figure 10A).

At 14 weeks (size 3.4 mm), differentiated gametes were observed and three cellular types were taken into consideration: type A and type B spermatogonia, and then spermatids due to their anchoring in the cytoplasm of Sertoli cells (Figure 10A).

It's rare and often impossible to observe all the developmental stages on the same cut as they usually develop in clusters from the same cellular stages.

**Non germinal:** Cells alternating with type A germinal cells, Sertoli cells can be observed. These are voluminous cells with a pyramid shape and a cytoplasm blocking light due to their apical pole, which are recognized when anchoring with spermatids (Figure 10A). Their essential function is for nurturing young spermatozoids.

**Female tissue:** Once the male tissue was formed, the female tissue formed with the development of the first oocytes (Figure 8B) whose maturity stages are identical to those described for *P. antipodarum*. They have a follicular cord resting against the lower part of the gonad and pushed toward the oviduct throughout the maturation course in order to begin the fertilization chamber where their cells are produced. They start in the embryonic sac where embryo formation is completed (Figure 10C,11A,11B).

## Discussion

In our experimental conditions, primary oocytes from individuals measuring less than 2 mm were not observed, the minimum size at which we could count oocytes for individuals 19 and 20 weeks old. We can also assume that at 2 mm or more, oocytes in 13- and 14-week-old individuals were not observed (Figure 3).

The mature gonad is only visible in individuals with a minimal size of 2 mm in smaller individuals, in our laboratory conditions, primary oocytes were not observed. Nevertheless, this observation does not seem conclusive since certain individuals with this size limit and younger than 15 weeks did not reach the first observable oocyte maturity stage. Rapid growth of juveniles does not appear beneficial for oocyte maturation and reproduction.

These results observed in laboratory conditions should be confirmed on individuals in the field where trophic conditions may be more optimal for reproductive activities.

For *V. piscinalis*, sexual maturity seems to begin when individuals reach 1 mm and 6–8 weeks old, with ovogonia appearing. Primary oocytes were not observed among individuals smaller than 2.5 mm and 12 weeks old. All individuals exceeding these limits appeared to have advanced stages of oocyte maturation.

For the males, we observed an arborization of spermatogonia in the seminal receptacles of individuals between 2.6 mm and 3.8 mm in size and full oocyte maturation for those reaching 4 mm (Figure 4).

The lack of references is explained by the fact that our laboratory is the first to report a study of the histological anatomy of these two gastropod species.

## Conclusion

These results brought out the gonad maturity stages in the mud

snail species *P. antipodarum* and *V. piscinalis*. We were able to suggest a size limit around 2.5 mm at which point all individuals reach advanced gonadogenesis. Sizes below 1 mm are too small to detect gonad maturation activity (oogonia and oocytes absent).

With these results, we may now proceed with the second level of research which will involve reprotoxic stress studies.

## Acknowledgement

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