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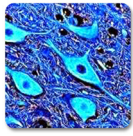
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Anatomical and histological characterization of the gametogenesis of *Radix balthica* (linnaeus, 1758) in comparison with *Lymnaea stagnalis* (linnaeus, 1758)

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Abstract

Freshwater gastropods are frequently used as model organisms to assess the effects of certain chemical substances. Among them *Radix balthica* and *Lymnaea stagnalis* are commonly used in the laboratory, mesocosm and field tests. In order to determine the effects of pollutants and more particularly endocrine disrupting substances on the reproduction of these organisms, histopathological analyses can be used. Because data are still scarce in the literature, knowledge development on anatomy of reproductive tissues and gametogenesis is a preliminary step before any studies on the impact of contaminants on the reproduction of these gastropods. The characterization of the anatomy and gametogenesis of *Radix balthica* and *Lymnaea stagnalis* was thus performed in this study. Important morphological differences exist between the two species. Despite this, the gonads of the two gastropods species have similar histological structure. In both species, spermatogonia are clustered; spermatozooids are anchored in the Sertoli cells and the male cells alternate with the female cells that rest on the epithelium of the hermaphroditic gland. This study is a prerequisite for any further histopathological studies on contaminated individuals.

Keywords: Anatomy, histology, gametogenesis, *Radix balthica*, *Lymnaea stagnalis*

Introduction

Gastropods are epibenthic organisms which are distributed in many types of aquatic ecosystems preferring lentic and brackish waters located near fields [8]. Considering their habitat preferences, they are directly exposed to pesticides and herbicides [20]. They become relevant ecological indicators and they are frequently used for water quality surveillance and monitoring [28] and in ecotoxicological bioassays [4,16].

As gastropods have a wide range of reproductive strategies (hermaphroditism, gonochorism, parthenogenesis) and feeding behavior, they are relevant models enabling to study the effects of endocrine disrupting chemicals [24]. Exposure of certain species of gastropods to TBT (tributyltin) at sub-lethal concentrations (ng/L), have lead to metabolic modifications [23,25,32]. Effects of BPA (Bisphenol A) on *Radix balthica* in lotic

mesocosm were studied by De Kermoisan [7]. The authors observed a significant increase in total abundance and a modification of population structure at 100 µg/L of BPA. An increase of population growth rate induced by BPA is suspected. The study of the effects of cadmium on *Lymnaea stagnalis* reproduction in a laboratory study has shown that the number of eggs per egg mass and stages of embryo development were particularly sensitive end points [15]. Furthermore, gastropods endocrine system is principally under the control of neuropeptides [19] and their hormonal system resembles vertebrate hormonal systems [26]. So, endocrine disrupting effects revealed on these organisms could be eventually extrapolated to vertebrates.

Histology is one of the methods used to evaluate the effects of toxic substances on aquatic organisms [18,33]. Effects of endocrine disrupting chemicals on the reproduction of certain

species of freshwater gastropods have also been revealed by histopathological analyses of gonads [12]. In some species, imposex was found [1,13,24,29]. As histopathological analyses provides further evidence on the eventual impairment of reproduction by endocrine disrupting chemicals, it seems important to incorporate them in ecotoxicological studies. Surprisingly, anatomical and histological characterization of the gametogenesis of most freshwater gastropods species in control conditions are not reported, even they are highly useful to eventually assess the effects of chemical substances on the reproduction of the organisms. In this context, we chose to study two gastropod species *Lymnaea stagnalis* (Linnaeus, 1758) and *Radix balthica* (Linnaeus, 1758) in order to specify some elements of their biology.

The pond snail (*Lymnaea stagnalis*) was recently introduced in our laboratory and is an ecologically relevant model species which is known to be sensitive to pollutants especially to endocrine disrupting chemicals [5,6,10]. The histological structure of the reproductive organs of *Lymnaea stagnalis* was described by [2,3,27] and by [19] using the former name of the species, *Helix palustris*. In our study, the description has been reviewed and completed.

Radix baltica belongs to the same family as *Limnaea stagnalis* that is *Lymnaeidae* [30]. The anatomy and histology of the gonads of *Radix balthica* have never been characterized. As this species is closely related to *Lymnaea stagnalis*, a comparison of the anatomy and histology of the reproductive organs has been made. Furthermore, this species is used as a model species in our mesocosm studies. As mentioned above, effects at the population level were reported after a 6 month exposure to an endocrine disrupting chemical [7]. As a phenomenon of "superfeminisation" of the individuals is suspected, histopathological analyses of the gonads of the contaminated individuals is necessary. Prior to this analysis, the study of the gonads of non-contaminated organism, reared in control conditions is thus fundamental.

Material and methods

Five adults of each species were fixed with Bouin's fluid for 72 h. Size of the organisms were between 15 and 18 mm for *Radix balthica* and 28 to 30 mm for *Lymnaea stagnalis*. The Bouin fixation is essential in order to stabilize the tissue and cellular structures [28]. The liquid was composed of the following mixture: 75% of a saturated solution of picric acid (Fluka, Gillingham, United Kingdom), 20% formaldehyde (Aldrich, Saint Louis, United States-Missouri) and 5% acetic acid (Carlo Erba, Val de Reuil, France). This mixture enables to both dissolve the shells and fix the tissues properly. To obtain a complete dehydration of the material, the samples were then rinsed with increasing alcohol concentrations (70% to 100%). Next, the "lightening" step was performed in using a solvent bath allowing the replacement of intracellular water with paraffin. Then 5- μ m sections were obtained using a microtome (Leica RM 2245, Wetzlar, Germany). They were then stained with

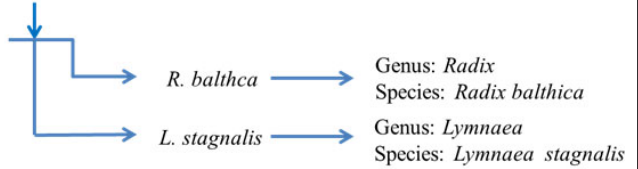
Groat hematoxylin (mixture v/v hemalum)(Sigma-Aldrich Aldrich, Saint Louis, Missouri USA) under acidic conditions and hematoxylin Sigma-Aldrich under alcoholic conditions, those dyes having a tinctorial affinity for nuclear acids. Eosin Diagnostic (RAL, Bordeaux Montesquieu, France) (1% in an aqueous medium) was used in order to stain cytoplasm. The Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) was used for the observations.

Results

Anatomy and general description of the gametogenesis

Phylogenetic classification of the two species is shortly presented in **Table 1**, showing that these two species belong to the same family and that their differences reside only in the species and the genus.

Table 1. Taxonomic classification of *Radix balthica* and *Lymnaea stagnalis* based on Glöer, 2003.

Kingdom: Animalia	
Phylum: Mollusca	
Class: Gastropoda	
Subclass: Orthogastropoda	
Order: Pulmonata	
Order: Pulmonata	
Family: Lymnaeidae	
	
	Genus: <i>Radix</i> Species: <i>Radix balthica</i>
	Genus: <i>Lymnaea</i> Species: <i>Lymnaea stagnalis</i>

The shell of *Lymnaea stagnalis* is massive, elongated and pointy with 6 to 8 spiral turns (H: 54, l:27) (**Figure 3A**). The shell of *Radix balthica* is subdiscoiled, belly shaped and appears like a droplet (**Figure 2A**). The apex is round and not as pointy as for *Lymnaea stagnalis*. It has three and a half spiral turns (H: 20, l:14) [14]. The mantle of *Radix balthica* has both pale and dark spots and has a dark line on the apical pole [11,30]. The foot is light green and covered by small shiny spots (**Figure 2A**) whereas the mantle and foot of *Lymnaea stagnalis* is clear and uniform (**Figure 3A**).

Gametogenesis of *Radix balthica* and *Lymnaea stagnalis* is summarized in **Figure 1**. **Figures 2** and **3** illustrate the general anatomy of an individual and show a selection of histological sections of the gonads for *Radix balthica* and *Lymnaea stagnalis* respectively.

For both species, female and male germ cells are produced in a hermaphroditic gland called ovo-testis. This gland is relatively massive and is located in the apical whorl inside an accessory lobe which is wrapped around the hepatopancreas (**Figure 1**).

In this area, in both species, the beginning of the gametogenesis showed only female germinal cells, surrounding

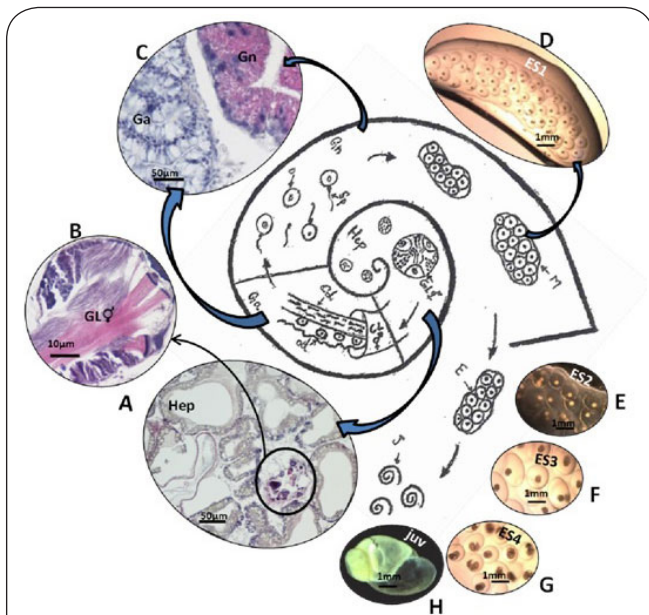


Figure 1. Simplified figure of the gametogenesis of *Radix balthica* and *Lymnaea stagnalis*.

A. Hep: Hepatopancreas; B. Gl: Hermaphroditic gland; C. Ga: Albumen gland, Gn: Nidamental gland; D. ES1: Stage 1 embryo; E. ES2: Stage 2 embryo; F. ES3: Stage 3 embryo; G. ES4: Stage 4 embryo; H. Juv: Juvenile, CL: Hermaphroditic duct, Cd: Spermiduct or vas deferens, Od: Oviduct; O: Oocyte, Sp: Spermatozoon, M: Egg mass. Scale bars, D, E, F, G, H=1mm; A, C=50µm; B=10µm.

the hepatopancreatic epithelium which is wrapped in the secretion of the albumen gland (Figure 1A). The hermaphroditic gland matures from the apex to the foot. Histological sections enable to show side by side the two types of gametes. Spermatozoa and oocytes are both discharged by respectively the spermiduct and the oviduct in a junction where the secretion of the albumen gland is also deposited (Figures 1A-1C). Surrounded by the albumen secretions, the gametes continue their journey in the gland of nidation where fertilization occurs; and so oocytes are fertilized and grouped in masses which are then discharged through the genital pore located on the head near the mouth (Figure 1D). Embryogenesis is pursued *ex-vivo* and juveniles are released between 14 to 15 days depending on the environmental conditions (Figures 1E-1H).

Histological characterization of the gametogenesis of *Radix balthica* and *Lymnaea stagnalis*

For two species, oocyte differentiation is essential allowing these cells to achieve the vitellogenesis stage that favors the production of exogenous vitellogenin. The latter is a precursor of vitellus and induces oogenesis favoring fertilization and embryogenesis.

Female cells are contained in a follicular cord located on the basal lamina of the gonad. As they mature, they are pushed little by little through the oviduct (Figure 2D). They end up

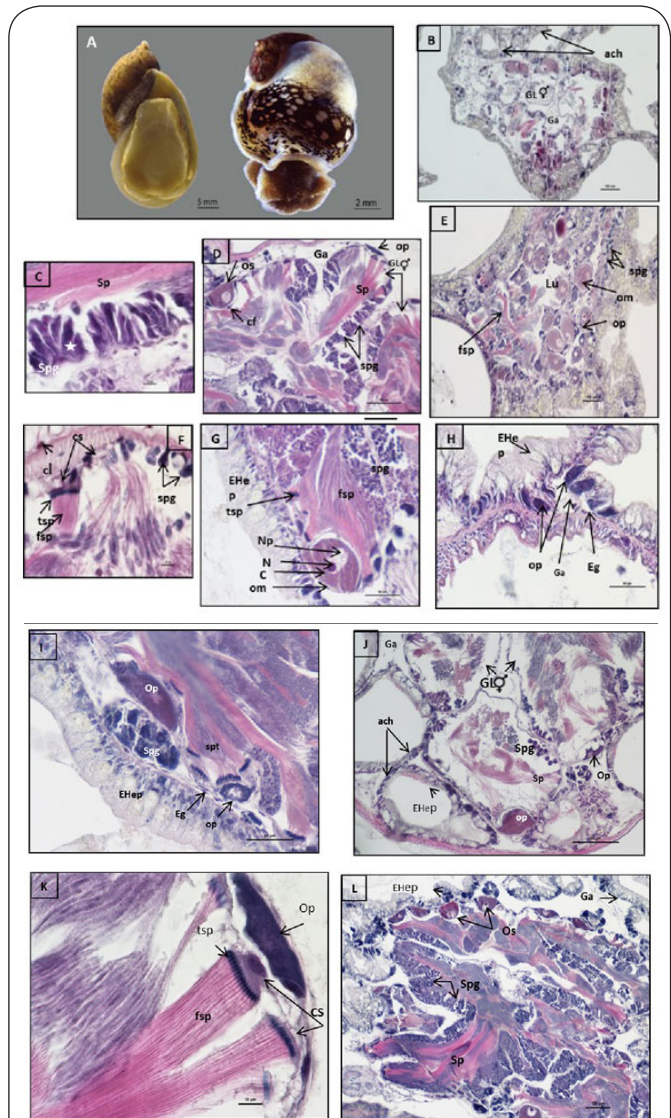


Figure 2. (A-H) Gonad histology of *Radix balthica*. A. Ventral view and general morphology of *Radix balthica* (Linnaeus, 1758). B-L: Transversal sections of *Radix balthica*; embedding paraffin; cutting width 4 µm; staining hematoxylin-eosin. B. GL: Hermaphroditic gland, ach: Hepatopancreatic acini, Ga: Albumen gland; C. Sp: Spermatozoon, Spg: Spermatogonium star, Clustered spermatogonia. D. cf: Follicular cells, Os: Secondary oocyte, Ga: Albumen gland, Sp: Spermatozoon, spg: Clustered spermatogonia; E. fsp: Spermatozoon flagella, spg: Spermatogonium, op: Primary oocyte, om: Mature oocyte, Lu: Gland lumen; F. cl: Leydig cells, cs: Sertoli cells; G. Om: Mature oocyte, C: Cytoplasm, N: Cell nucleus, Np: Nucleoplasm, tsp: Head of the spermatozoon, EHep: Hepatopancreatic epithelium; H. Eg: Germinal epithelium; Scale bars, B, E=100µm; D, G, H=50µm; C, F=10µm. (I-L) Gonad histology of *Radix balthica*. I. op: Primary oocyte, Eg: Germinal epithelium, EHep: Hepatopancreatic epithelium, spg: Clustered spermatogonia, op: Primary oocyte, spt: spermatozoon; J. ach: Hepatopancreatic acini; K. tsp: Head of the spermatozoon, cs: Sertoli cells; L. EHep: Hepatopancreatic epithelium, Sp: Spermatozoon, Spg: Clustered spermatogonia, Os: Secondary oocyte; Scale bars, L=100µm; I, J=50µm; K=10µm.

in a gland of nidation where they are fertilized. Three types of **Primary oocytes** cells were observed.

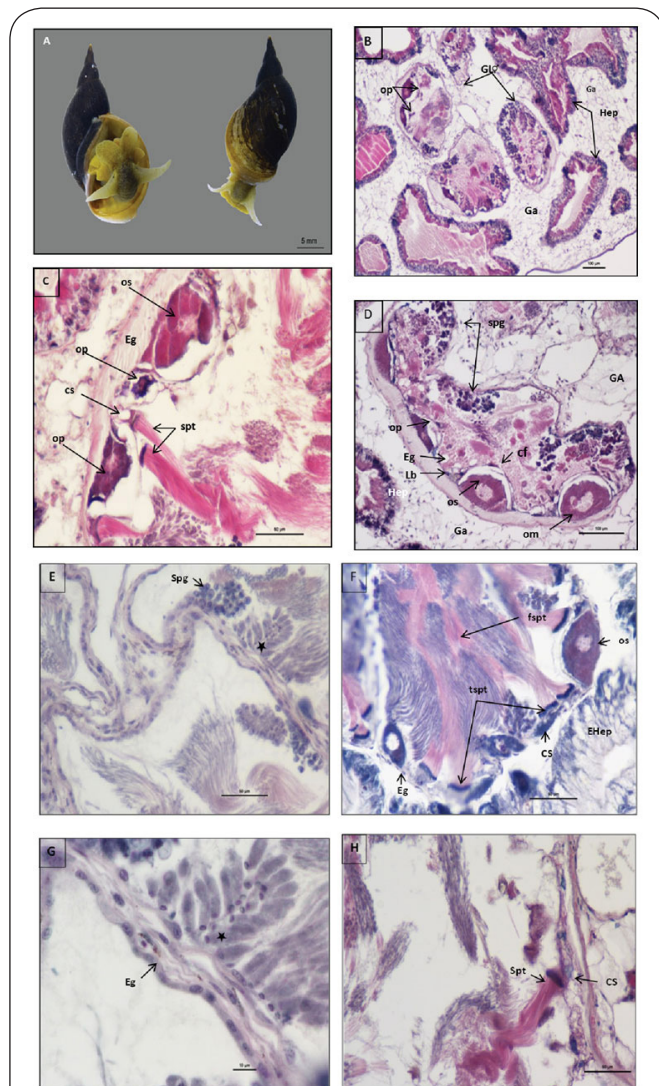


Figure 3. (A–D) Gonad histology of *Lymnaea stagnalis*.
 A. Ventral view and general morphology of *Lymnaea stagnalis* (Linnaeus, 1758). B–H: Transversal sections of *Lymnaea stagnalis*; embedding, paraffin; cutting width 4 µm; staining hematoxylin-eosin; B. op: Primary oocyte, GL: Hermaphroditic gland, Hep: Hepatopancreatic acini, Ga: Albumen gland; C. op: Primary oocyte, c: Sertoli cells, Eg: Germ epithelium, os: Secondary oocyte, Spt: Spermatozoon; D. om: Mature oocyte, os: Secondary oocyte, Lb: Basal lamina, Eg: Germinal epithelium, op: Primary oocyte, Spg: Spermatogonium, cf: Follicular cells, GA: Albumen gland; Scale bars, B, D=100µm; C=50µm=10µm. (E–H) Gonad histology of *Lymnaea stagnalis*. E. Star: Clustered spermatogonia interwoven between the Sertoli cells; F. Eg: Germinal epithelium, tsp: Head of the spermatozoon, fsp: Spermatozoon flagella, op: Primary oocyte, EHep: Hepatopancreatic epithelium; G. Eg: Germinal epithelium; H. Spt: Spermatozoon, cs: Sertoli cells; Scale bars, E, F, H=50µm; G=10µm.

The cells are small and star-shaped. The central nucleus is slightly differentiated and the cytoplasm is basophilic and is subtly granular (**Figures 2D, 2H, 2I, 2K** and **Figures 3C, 3D**).

Secondary oocytes

Two types of oocytes can be distinguished: oocytes at the beginning of maturation and mature oocytes. Oocytes that have started maturation are called secondary oocytes. These cells of medium size, with a central basophilic nucleus, show a large cytoplasm (**Figures 2D, 2E, 2L** and **Figures 3C, 3D, 3F**).

Mature oocytes

They are large cells with a round central nucleus, pigmented, basophilic which is trapped in a lipid cavity that could be equal to 2 to 3 times the surface of the nucleus (**Figures 2G** and **3D**). At this stage, the cytoplasm of the cells is filled with lipid droplets and homogenous eosinophilic globules of vitellus.

Male germ line cells of the two species were observed in the seminiferous tubes which are separated by connective tissue in which interstitial Leydig cells can be found (**Figure 2F**). Oogonia are discharged onto the germ epithelium which is anchored on the Sertoli cells. The latter are bulky and form a pyramid that lies towards the lumen of the seminiferous tubes allowing the anchorage of spermatogonia thus favoring their nutrition and explaining their clustered form (**Figures 2F, 2K** and **Figures 3C, 3H**). The male germ cells alternate with female germ cells (**Figures 2C, 2D, 2F, 2I** and **Figures 3C, 3E–3G**).

In the acini, a synchronized maturation of the male cells with the different stages of oogenesis of the female cells was observed (**Figures 2D, 2G** and **Figures 3D, 3F**). Indeed, female gametes rest on the acini epithelium cells and are surrounded with male gametes (**Figures 2D, 2I, 2L** and **Figures 3B, 3F**). These gametes are in pre-meiotic phase and then multiply by mitosis after their differentiation and constantly stay in contact with the membranes of the acini cells.

Spermatogonia

They are large sized cells located at the border of the seminiferous tubes and anchored between the Sertoli cells. They are organized in clusters. These cells undergo different stages of maturation leading to the formation of spermatozooids that are released in the lumen of the seminiferous tubes (**Figures 2C–2F** and **Figures 3E, 3G**).

Spermatozooids

The spermatozooids are very long cells composed of two distinct parts visible under a stereomicroscope: the head and the flagella. The head is oviform, slightly flattened, anchored between the Sertoli cells whereas the eosinophilic opposite part is tapered towards the lumen looking like ordered parallel silk filaments. This part contains the flagella which has the same structure for both species (**Figure 2F, 2I, 2K** and **Figures 3C, 3F, 3H**).

Discussion

Our results enabled to gain more knowledge on the structure and functioning of the gonad of *Radix balthica* compared to *Lymnaea stagnalis*. The latter has been frequently used in many studies dealing with toxic effects of pollutants [6,13,18]. All the authors acknowledged that this species belongs to one of the most sensitive groups of aquatic organisms.

Even if no difference in the histology of the gonads has been observed, important morphological differences exist between the two species. They concern the shell structure, the presence of alternating pale and dark spots on the mantle, the green color of the foot, the bright spots on the foot for *Radix balthica* compared to *Lymnaea stagnalis*. *Lymnaea stagnalis* has a long shell (H=29-54 mm) which is acuminate with 6 to 8 spiral towers, the last being bulky. *Radix balthica* has a globular shell with 4 to 5 spiral turns that increases rapidly, the last being very large.

This study allowed the description of the anatomy and internal structure of the gonad of *Radix balthica* in the absence of contamination. The next step is a histopathological analysis of organisms exposed to selected chemicals in order to eventually identify their effects on the reproduction of this species.

Radix balthica seems a possible candidate as a new model species allowing the identification of effects of certain chemical substance on the reproduction of gastropod mollusks, as the number of studies involving this species is growing, for example, Schniebs [30] studied the genetic variability of the cyt-b gene following a method used by Tamura [35]. They found a difference in length of the two fragments of the gene of 2.24% between the two species. These authors also mentioned a difference in the length and position of the bursa copulatrix. It is short and near the pericardia for *Lymnaea stagnalis* and long and near the provaginal duct for *Radix balthica* [30,31].

Important morphological differences exist between the two species. Despite this, the gonads of the two gastropods species have similar histological structure. In both species, spermatogonia are clustered, spermatozooids are anchored between the Sertoli cells and the male cells alternate with the female cells that rest on the epithelium of the hermaphroditic gland as already observed in the gastropod [34]. This study shows that the gonads of the two gastropods species, *Radix balthica* and *Lymnaea stagnalis* have similar histological structure. Indeed, in both species, the female cells rest on the epithelium of the hermaphroditic gland and alternate with the clustered structure of spermatogonia and the spermatozooids that are anchored between the Sertoli cells.

This study is a prerequisite for any further histopathological studies on contaminated individuals.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	KTA	SJ	JG	PB	JME	JMP	OG
Research concept and design	✓	--	--	--	--	--	--
Collection and/or assembly of data	✓	✓	--	--	--	--	--
Data analysis and interpretation	✓	--	--	--	--	--	--
Writing the article	✓	✓	--	--	--	--	--
Critical revision of the article	--	--	✓	✓	✓	--	--
Final approval of article	--	--	--	--	--	✓	✓
Statistical analysis	--	--	--	--	--	--	--

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