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ORIGINAL PAPER

Gonadal histology and gametogenesis of the Antarctic limpet *Nacella concinna* (Patellogastropoda, Nacellidae) collected at Potter Cove, 25 de Mayo (King George) Island, during austral summer

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Abstract

The limpet *Nacella concinna* is one of the most widely distributed gastropods along the Antarctic Peninsula. This species has been a useful tool in ecological and physiological studies for understanding Antarctic trophic interactions. Although the reproduction of limpets has been thoroughly studied, very little is known about gametogenesis in the genus *Nacella*. The purpose of this study is to describe the gonadal morphology and gametogenesis in *N. concinna* observed by light microscopy and to determine its chromosome complement. All the limpets were sexually mature at the time of sampling. Oocytes were separated by trabeculae with abundant glycogen reserves. There was a predominance of late vitellogenic oocytes. Many previtellogenic oocytes were pyriform in shape. Oogonia were clustered near the ovary wall or the trabeculae. Only two meiotic stages were found: metaphase I and anaphase I, both of which showed a regular arrangement of chromosomes. The oviduct contained mature oocytes surrounded by sperm. Testicular tubules were filled with spermatozoa. The elongated head of the spermatozoon resembles that of other Nacellidae members. One of the bivalents is heteropycnotic. The haploid complement is $n=4$ and the sex determination system is XO/XX. Although *N. concinna* has been commonly characterized as a broadcast-spawner, its unique spawning behavior, the presence of oocytes at anaphase I and spermatozoa within the oviduct suggests internal fertilization.

Keywords *Nacella concinna* · Gametogenesis · Internal fertilization · Meiosis

Introduction

The limpet *Nacella concinna* (Strebel 1908) (Patellogastropoda, Nacellidae) is one of the most conspicuous invertebrates along the coasts of the Antarctic Peninsula and adjacent islands (Cadée 1999; Amsler et al. 2015). It is frequently distributed in dense patches in the intertidal and subtidal zones up to 15 m in depth (Suda et al. 2015).

Different reproductive aspects, such as gonadal cycle, time of spawning, and spawning behavior, have been extensively studied in a number of limpet species. These gastropods often exhibit external fertilization (e.g., Southward and Dodd 1956; Picken 1980; Picken and Allan 1983; Niu and Fuji 1989; Brêthes et al. 1994; Stanwell-Smith and Clarke 1998; Morriconi 1999; Hodgson and Eckelbarger 2000; Rocha-Barreira 2002; McCarthy et al. 2008; Prusina et al. 2014). Information on the gonadal histology and gametogenesis of some limpet species is also available (Hodgson and Bernard 1988, 1989; Hodgson et al. 1996, 2007, 2012; Hodgson and Eckelbarger 2000; Hodgson 2009). Morriconi (1999) provided a description of the gonadal histology of *N. deaurata*, while Neuberger-Cywiak et al. (2009) presented a brief histological report of the gonads and the digestive gland of *N. concinna*. Moreover, Suda et al. (2015) published an exhaustive review on *N. concinna* that included phylogeny, thermal and osmotic tolerance, oxidative stress, anthropogenic influence, feeding, excretion, growth, population structure, and predators. In regard to reproduction,

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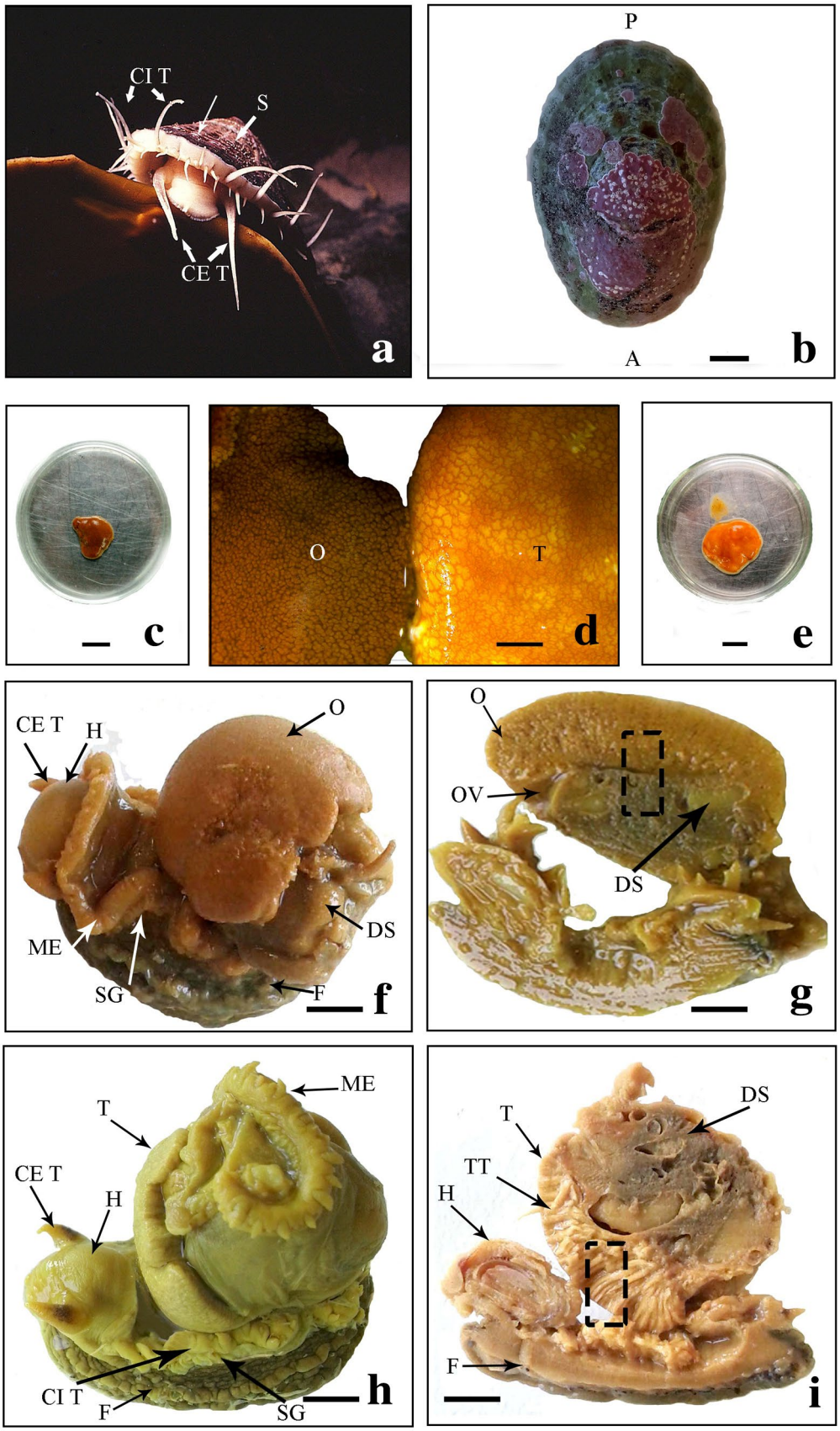

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◂**Fig. 1** *Nacella concinna*. General views. **a** Scuba diving photography of a live individual in the subtidal zone. Courtesy of Dr. G. Mercury. **b** Dorsal view of dissected shell. **c**–**e** Fresh gonads on a dissecting dish. **c** General view of an ovary. **d** Magnification of an ovary (left) and a testis (right). Oocytes and testicular tubules can be observed. **e** General view of a testis. **f** Laterodorsal view of a fixed female after shell removal. **g** General view of a sagittal section of a female. A histological section from the area enclosed by the dashed black rectangle is shown in Fig. 2d. **h** Laterodorsal view of a fixed male after shell removal. The position of the mantle edge was modified due to fixation. **i** Sagittal section of a male. A histological section from the area enclosed in the dashed black rectangle is shown in Fig. 3b. *A* anterior side, *CE T* cephalic tentacles, *CI T* circumpallial tentacles, *DS* digestive system, *F* foot, *H* head, *ME* mantle edge, *O* ovary, *OV* oviduct, *P* posterior side, *S* shell, *SG* secondary gills, *T* testis, *TT* testicular tubules. Scale bars: **b**, **c** and **e** = 1 cm; **d** = 2 mm; **f**–**i** = 0.5 cm

these authors only mentioned the unusual behavior of mature adults, which consists in aggregations of individuals forming stacks before spawning (see Picken and Allan 1983). To our knowledge, the gonadal morphology and gametogenesis of *N. concinna* have not been yet investigated. As it was suggested by Suda et al. (2015), *N. concinna* is a suitable model to analyze the effects of climate change and anthropogenic disturbance (Najle et al. 2000; Ahn et al. 2002; Ansaldo et al. 2005, 2007).

There are few reports on chromosome number for Nacellidae (Harasewych and McArthur 2000; Valdovinos and Rüth 2005). In some Gastropod taxa, the sex-determining mechanism is XO/XX (Nishikawa 1962; Thiriot-Quiévreux 2003), while it is unknown for *Nacella*.

The objectives of this study on the Antarctic limpet *N. concinna* were to (1) describe the gonadal histology in male and female adults, (2) describe the spermatogenesis and oogenesis processes, (3) describe the gametes by light microscopy, (4) determine the chromosome number and meiotic behavior, and (5) characterize the fertilization type in order to contribute to the knowledge of its unique reproductive behavior.

Materials and methods

Specimens of the Antarctic limpet *N. concinna* of similar size were collected during the austral summer (from January to March 2010–2011) at Potter Cove (Peñón de Pesca), 25 de Mayo (King George) Island, South Shetland Islands, Antarctica. All the limpets were collected from the subtidal habitat by scuba diving at a depth of 6–8 m. In accordance with the Protocol on Environmental Protection to the Antarctic Treaty, a total of 20 limpets were cryo-anesthetized and their shells carefully removed. Shell length, width, and height were measured using a Vernier caliper. The gonads were dissected and sex was determined by their color. Different parts of the gonad were either fixed in 70% ethanol for meiosis studies or immersed in Bouin's aqueous solution, for at least 12 h, for histological analysis.

For cytogenetic analysis, small pieces were immersed in 70% acetic acid at 50 °C for 3 h. They were immediately treated with Feulgen stain for 90 min and then hydrolyzed with 5 N HCl at 20 °C for 25 min. Finally, they were squashed in 2% acetic hematoxylin (Nuñez 1968; Dopchiz and Poggio 1999) using ferric citrate as a mordant. Some preparations were stained with lactopropionic orcein. Photomicrographs for cytogenetic analysis were taken using a Carl Zeiss Primo Star microscope.

For routine histology and histochemical techniques some subsamples fixed in Bouin's solutio were dehydrated in a graded ethanol series, cleared in benzene, embedded in Paraplast® plus (Kendall, Tyco Healthcare), and cut into 7-µm-thick sections with a Leica RM2125 RTS microtome. Other subsamples were dehydrated in a graded ethanol series and embedded in glycol methacrylate according to manufacturer's instructions (Leica Historesin, Germany); 4 µm sections were cut with a Leica RM2155 microtome for detailed observations. Slides were stained with either hematoxylin–eosin or modified Masson's trichrome (Carazzi's hematoxylin, xylidine ponceau, phosphomolybdic acid, and aniline blue or light green). Periodic acid–Schiff (PAS) and Best's carmine were used for the detection of neutral glycoconjugates, particularly glycogen. Photomicrographs were taken with a Zeiss Axioskop 2 microscope. Feret diameters of gametogenic, Sertoli, follicular, and trabecular cells were measured using the ImageJ 1.51 k software (Rasband 2017). This software was also used to measure the length of the sperm head and the thickness of the oviduct and sperm duct wall. Results are expressed in µm as mean ± SEM. Finally, the percentages of previtellogenic and vitellogenic oocytes were estimated counting cells from 25 random fields per slide at 40×magnification.

Results

Temporary stacks of limpets were seen at the time of sampling. We found no sexual dimorphism (Fig. 1a) and sex could be determined by dissection. Differences in shell dimensions between females and males (Fig. 1b), which

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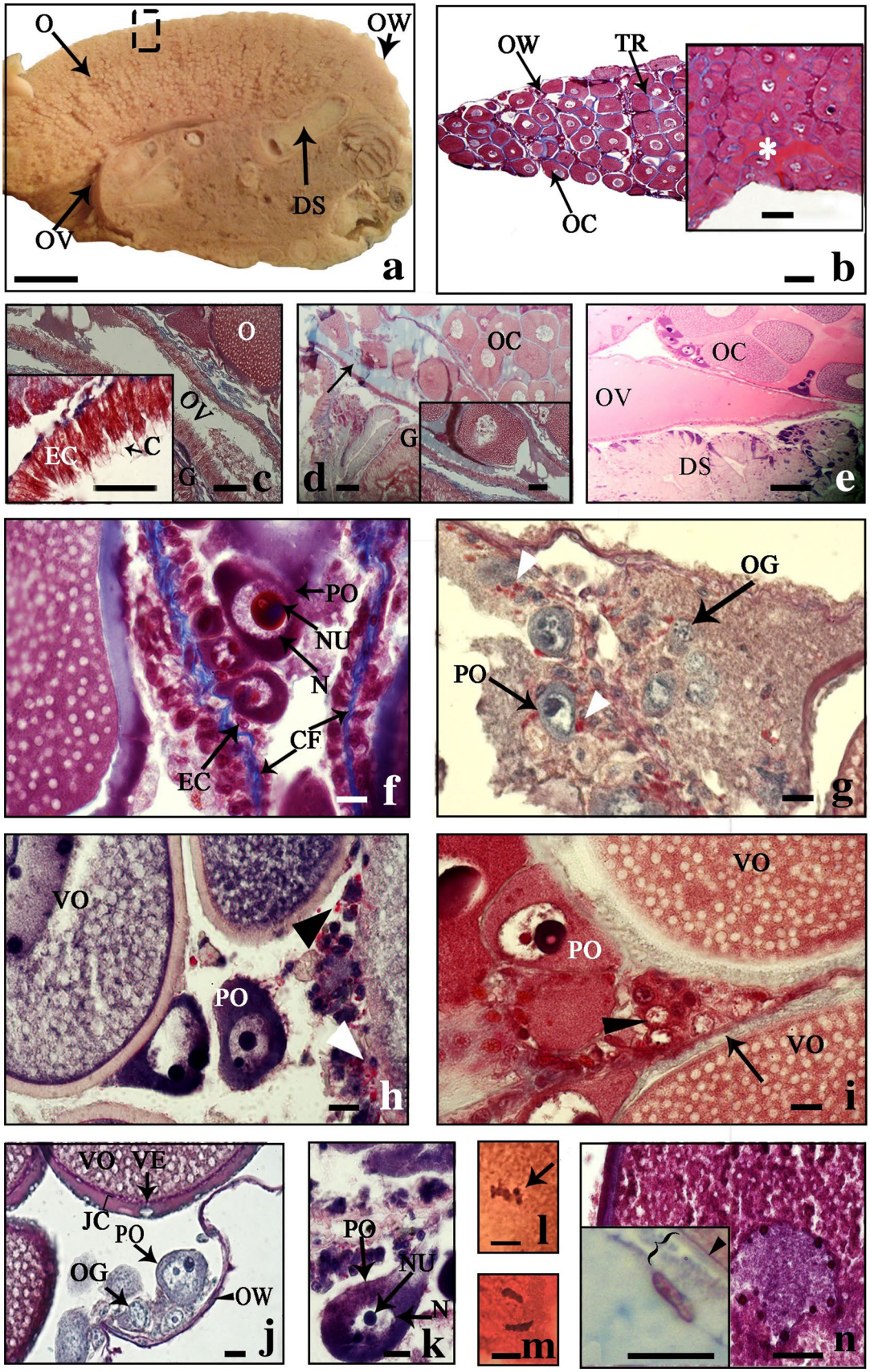

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◂**Fig. 2** *Nacella concinna*. Gametogenesis in females collected during austral summer. **a** General view of a sagittal section of the ovary and part of the digestive system. The histological section from the area enclosed in the dashed black rectangle is shown in Figure b. **b** General view of a longitudinal section of the ovary. Note the arrangement of trabeculae surrounding oocytes. Inset: Eosinophilic material between oocytes (*) on the ventral side of the ovary, close to the digestive system (the latter not shown). **c**–**e** Detail of the oviduct. **c** Distal part of the oviduct behind the limpet´s head. Inset: detailed section of the thickened epithelium composed of ciliated cells. **d** Mature oocytes in the middle section of the oviduct. The arrow indicates the oviduct. Inset: detail of the lumen containing a mature oocyte and acidophilic material stained light blue. **e** Proximal part of the oviduct. **f**–**h** Detailed sections of trabeculae using different stains. **f** Basophilic previtellogenic oocytes are attached to collagen fibers stained blue. Small epithelial (reserve) cells are also located on the trabeculae. **g**–**h** Arrowheads show glycogen particles stained magenta or red. **i** The arrowhead indicates nested oogonia surrounded by previtellogenic and vitellogenic oocytes covered with thick jelly coat (arrow). **j** Previtellogenic oocytes attached to the ovary wall. The vitelline envelope of a vitellogenic oocyte is stained magenta. **k** A pyriform previtellogenic oocyte. **l**–**m** Meiosis. **l** Metaphase I. The arrow shows sexual bivalent. **m** Anaphase I. **n** Detail of a late vitellogenic oocyte. Inset: squamous nucleus of a follicular cell. The arrowhead indicates the vitelline envelope and the bracket encompasses the jelly coat. *C* cilia, *CF* collagen fibers, *DS* digestive system, *EC* epithelial cell, *G* gut, *JC* jelly coat, *N* nucleus, *NU* nucleoli, *O* ovary, *OC* oocytes, *OG* oogonia, *OV* oviduct, *OW* ovarian wall, *PO* previtellogenic oocyte, *TR* trabeculae, *VE* vitelline envelope, *VO* vitellogenic oocyte. Scale bars: **a** = 0.5 cm; **b** and **e** = 200 μm; **c** and inset of **c**, **d**, **h,** and **n** = 40 μm; **d** = 100 μm; **f**–**g**, **i**–**k** = 10 μm; **l**, **m;** and inset of **n** = 5 μm. **b**–**d, f**, **i, n** and respective insets: aniline blue Masson's trichrome; **e**: light-green Masson's trichrome; **g** and **j**: PAS; **h** and **k**: Best's carmine; **l** and **m**: Feulgen–Hematoxylin

were measured separately, were not significantly different (Students *t* test, $p > 0.05$). The mean ± SEM values of length, width, and height were 40.1 ± 1.9, 29.4 ± 0.9, and 14.6 ± 1.2 mm, respectively.

All the limpets collected were sexually mature. Gonads are single and sex is easily determined by gonad color: brown for females (Fig. 1c, d) and light orange for males (Fig. 1d, e). Males have no copulatory organ. In both sexes, the gonad lies dorsal to the foot and surrounds the digestive system (Fig. 1f–i).

The ovary is lined by a thin wall (Fig. 2a, b, j) and consists of plate-like trabeculae extending ventrally toward the digestive system. These divide the organ into several compartments containing rows of oocytes (Fig. 2a, b). Late vitellogenic oocytes surrounded by an increased amount of eosinophilic material are placed on the ventral side, close to the digestive system (Fig. 2b inset).

The oviduct is located ventrally to the ovary and its distal end enters the pallial cavity (Fig. 2a). The wall of the distal oviduct is thick (17.86 ± 0.86 μm) and lined by vacuolated epithelial cells (Figs. 2c, 4e). The apical membrane of these cells bears cilia in the distal and middle part of the oviduct (Fig. 2c inset, d). The proximal oviduct has a reduced diameter, is lined by flattened cells (3.05 ± 0.12 μm), and its lumen is filled with acidophilic material (Figs. 2e, 4f).

The trabeculae are thin and consist of bundles of collagen fibers and small trabecular (epithelial) cells whose nuclei contain clumps of chromatin (Fig. 2f). The cytoplasm of these small epithelial cells contains abundant glycogen particles that stain magenta with PAS (Fig. 2g) or red with Best's carmine (Fig. 2h). In addition, there are a few muscular cells scattered among collagen fibers (not shown in the figures). Oogonia clustered in nests (Fig. 2i) and previtellogenic oocytes are attached to the ovary wall or to the trabeculae (Fig. 2f–j). Oogonia have a central nucleus occupying most of the cell; the chromatin is distributed in fine granules and forms small clumps beneath the nuclear membrane. The nucleus is surrounded by a fine ring of cytoplasm (Fig. 2g, i, j). The previtellogenic oocytes have a homogeneous, basophilic cytoplasm; the nucleus is centrally located and contains a large eccentric nucleolus and several smaller nucleoli. Previtellogenic oocytes are small and remain attached to the trabeculae by a peduncle; they are first pyriform and then become irregular in shape (Fig. 2f–k; Table 1). Early vitellogenic oocytes are less than 5% (data not shown) and a predominance of late vitellogenic oocytes (74–92%) is evident at low magnification (Fig. 2b). The latter cells are large, roughly circular, and have acidophilic cytoplasm. As vitellogenesis proceeds, lipid droplets (3.64 ± 0.08 μm in diameter) and yolk platelets (0.77 ± 0.03 μm in diameter) accumulate within the oocyte (Fig. 2h–j, n). The nucleus is almost central, with granular chromatin and several nucleoli and micronucleoli located at its periphery. A fibrous, radially striated jelly coat (6.32 ± 0.12 μm thick), appears between the vitelline envelope and the overlying follicle cells (Fig. 2n inset). The vitelline envelope is thin (0.78 ± 0.04 μm thick) and contains neutral glycoconjugates, as revealed by Best's carmine (Fig. 2h) and PAS (Fig. 2j). The haploid number was found to be 4 (Fig. 2l). Meiotic cells exhibit a very

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Table 1 *Nacella concinna.* Morphometric parameters of ovarian cells determined from the Feret diameter

	Cell	Nucleus	Nucleolus < 10 µm	Nucleolus ≥ 10 µm
Oogonia	10.81 ± 0.61 (8)	7.82 ± 0.25	–	–
Small previtellogenic oocytes < 80 µm	39.97 ± 2.31 (42)	20.81 ± 1.00	5.60 ± 0.25	13.49 ± 0.55
Irregular previtellogenic oocytes ≥ 80 µm	101.96 ± 5.92 (18)	39.31 ± 1.99	6.63 ± 0.32	13.75 ± 0.64
Late vitellogenic oocytes	189.10 ± 2.50 (59)	79.00 ± 1.80	4.80 ± 0.10	–
Follicle cells	–	4.67 ± 0.4 (14)	1.13 ± 0.1	–
Trabecular cells	–	4.82 ± 0.2 (37)	1.97 ± 0.2	–

Values are expressed in µm as mean ± SEM ($n = 8$ animals). The number between brackets indicates the number of cells measured per limpet

dense cytoplasm. Metaphase I and anaphase I were the only meiotic stages observed in all samples and showed a regular arrangement of chromosomes. One bivalent is heteropycnotic and the remaining three have a terminal chiasma (Fig. 2l). Sticky chromosomes are seen at anaphase I (Fig. 2m).

The testes consist of finger-like tubules (Fig. 1i) lined by a germinal epithelium and separated by thin trabeculae, which are composed of connective tissue and scarce trabecular (epithelial) cells (Fig. 3a–d). The Sertoli cells are located between the connective fibers and the germinal epithelium. Each Sertoli cell possesses an euchromatic oval nucleus with a small nucleolus (Fig. 3c, d). Spermatogonia contain the largest nuclei, which have conspicuous nucleoli (Fig. 3c, d). These cells divide mitotically (Fig. 3e, f). The nuclei of primary spermatocytes show chromatin clumps, as shown in Fig. 3d. These cells undergo meiosis (Fig. 3g, h) and turn into secondary spermatocytes, which can be recognized by their small nuclei and homogeneous chromatin (Fig. 3d, Table 2).

The studied males showed regular meiosis (Fig. 3g, h). Some cells were at prophase I. At leptotene, the nucleus becomes basophilic and the chromatin has a fibrillar pattern of condensation (not shown). At diakinesis (Fig. 3g), the haploid complement is $n = 4$, comprising three acrocentric bivalents with a terminal chiasma and one X chromosome. Sticky chromosomes are detected in spermatocytes at metaphase I and anaphase I (Fig. 3h).

The onset of spermiogenesis is evidenced by chromatin condensation with intensely basophilic staining. Spermatids are highly polarized cells (Fig. 3i–l). The spermatid differentiation can be divided into three stages: early, middle, and late. They are round at the early stage (Fig. 3k) and acquire a drop-like shape at the middle stage (Fig. 3i, j). At the late stage, spermatids become elongated and polarized, and develop an incipient tail with a lanceolate ending (Fig. 3k). Then, the tail elongates showing an abrupt ending and the head adopts an ovoid shape (Fig. 3l). Further differentiation leads to a mature spermatozoon possessing a long tail, a middle piece, and an elongated head with a large acrosome (Fig. 3m, Table 2). Bundles of mature spermatozoa occupy the lumen of each testicular tubule (Fig. 3b, d).

The sperm duct is filled with spermatozoa; it is translucent and hardly visible to the naked eye, and its wall is lined by flattened cells (3.33 ± 0.40 µm thick) (Fig. 4a, b). In regard to fertilization, only four limpets had spermatozoa,

Fig. 3 *Nacella concinna*. Gametogenesis in males collected during austral summer. **a** Sagittal section of a testis. Testicular tubules with spermatogenic cells are surrounded by trabeculae. **b** Histological section from the area enclosed in the dashed black rectangle in Fig. 1i. The tubule wall is composed of thin connective tissue and epithelial cells forming trabeculae. **c** Detailed section of three testicular tubules. The white arrowhead shows Sertoli cell nuclei. The black arrow shows connective tissue of the trabeculae. **d** Detail of different stages of spermatogenesis. The black arrowhead shows a Sertoli cell nucleus. **e–f** Mitosis in spermatogonia. **e** Metaphase. **f** Anaphase. **g–h** Meiosis. **G**. Diakinesis. The arrowhead points out an X chromosome. **h** Spermatocytes in meiosis I. The arrow shows cell at anaphase I. The arrowhead shows polar view of a cell in metaphase I. **i–m** Spermiogenesis. Arrowheads point out the incipient head and tail. **k** The arrow shows an early spermatid. **l** The head differentiates from the tail. **m** Mature sperm with an elongated head and tail. *A* acrosome, *CF* collagen fibers, *DS* digestive system, *EC* epithelial cell, *H* head, *MP* middle piece, *PS* primary spermatocyte, *SG* spermatogonia, *SPD* spermatids, *SPZ* spermatozoa, *SS* secondary spermatocyte, *T* tail, *TR* trabeculae, *TT* testicular tubule. Scale bars: **a–b** = 200 µm; **c–h** and **m** = 10 µm; **i–l** = 5 µm. **a–c**: aniline blue Masson's trichrome; **d**: light-green Masson's trichrome; **e–f** and **i–l**: Feulgen–Hematoxylin; **g–h**: Lactopropionic orcein; **m**: Hematoxylin

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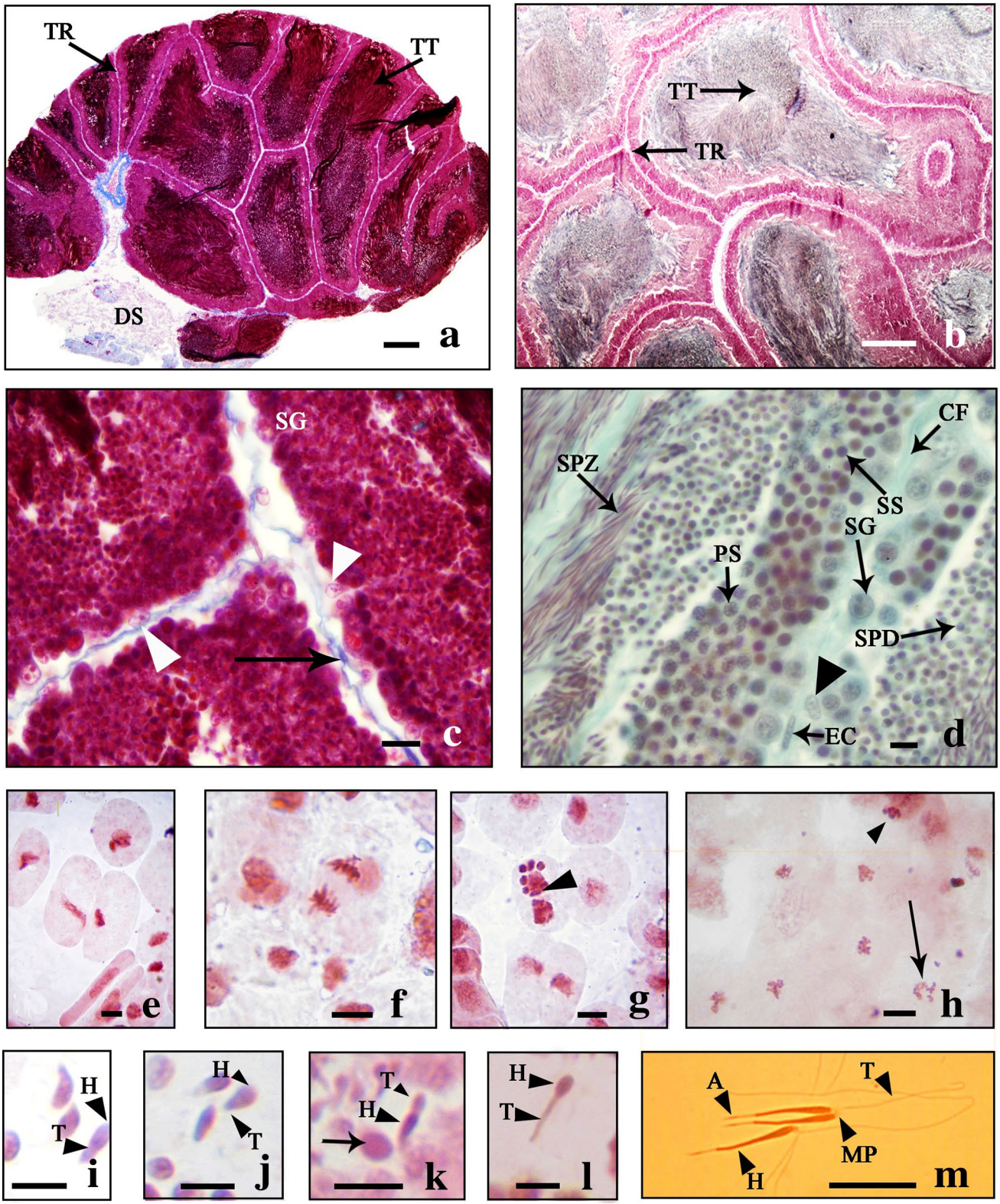

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Table 2 *Nacella concinna.* Morphometric parameters of testicular cells

	Nucleus	Nucleolus < 10 µm	Nucleolus ≥ 10 µm
Spermatogonia	5.07 ± 0.16 (22)	1.68 ± 0.07	–
Primary spermatocytes	3.90 ± 0.06 (24)	–	–
Secondary spermatocytes	3.04 ± 0.06 (31)	–	–
Early spermatids	1.90 ± 0.03 (55)	–	–
Middle spermatids	2.84 ± 0.17 (14)	–	
Spermatozoa head	12.70 ± 0.25 (24)	–	–
Sertoli cells	5.61 ± 0.15 (18)	1.22 ± 0.20	–
Trabecular cells	5.30 ± 0.38 (7)	–	–

The Feret diameter was determined for all spermatogenic cells except spermatozoa, for which head length was measured. Values are expressed in µm as mean ± SEM ($n = 12$ animals). Numbers between brackets indicate the number of cells measured per limpet

and these were found in the distal part of the oviduct (Fig. 4c–f). Spermatozoa and round spermatids are located very close to the oocytes (Fig. 4c, d).

Discussion

In the present study, we describe the gonadal histology and gametogenesis in males and females of the Antarctic limpet *N. concinna*. We also report the chromosome number of this species.

The ovaries and testes of the analyzed individuals of *N. concinna* collected during the austral summer are at the same ripe stage and developing stage, respectively, as those of the Subantarctic limpet *N. deaurata* studied by Morriconi (1999), who described its annual reproductive cycle based on a histological analysis. In addition, *N. concinna* females exhibit large ovaries which form extensions surrounding the digestive system, as also reported for *N. deaurata* by Morriconi (1999). Within the ovary, oocytes are placed in numerous compartments separated by unbranched trabeculae, as seen in other patellid limpets (Branch 1974; Hodgson and Eckelbarger 2000). These trabeculae are in close contact with small oocytes and their epithelial cells have glycogen reserves, as revealed by the histochemical staining. It has been suggested that trabeculae may serve as a conduit for the delivery of precursors to oocytes and that the hypertrophic trabecular epithelial cells may be the source of these nutrients (Hodgson and Eckelbarger 2000; Najmudeen 2008). On this basis, we propose that the epithelial cells (reserve cells) of *N. concinna* transfer nutrients to developing oocytes.

Synchronous oogenesis has been assumed to take place in six *Patella* spp. (Hodgson and Eckelbarger 2000; Najmudeen 2008) and in *N. concinna* (Picken and Allan 1983). Synchronous release of gametes may enhance fertilization success by increasing the probability of contact between them. We found a predominance of late vitellogenic oocytes and mature oocytes in the oviduct of *N. concinna* individuals, suggesting that they were ready for spawning. Moreover, the presence of nested oogonia and basophilic oocytes in the ovary would indicate that there was a second batch of previtellogenic oocytes for the next spawning.

Histological examination by the light microscope indicated that there were remarkable differences between previtellogenic and late vitellogenic oocytes; for example, the cytoplasm content in the former is basophilic, typical of cells specialized in protein synthesis, while in the latter it is acidophilic by accumulation of yolk platelets and lipid droplets. Both previtellogenic and vitellogenic oocytes contain a small amount of carbohydrates, as revealed by PAS and Best's carmine staining. Although the chemical nature of the yolk granules has been studied in a few gastropod species (Dreon et al. 2006), ultrastructural data support the concept that auto-synthetic yolk production is a primary process in Mollusca, serving as a nutrient source during embryogenesis

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(Kessel 1982; Benmeradi 1992; Hodgson and Eckelbarger 2000).

Neuberger-Cywiak et al. (2009) provided a first but brief description of the testis of *N. concinna*, but without characterizing the cells or the spermatogenesis process. Our observations indicate that the male gonad is organized similarly to that of *N. deaurata* (Morriconi 1999). The morphology of the spermatogenic cells in *N. concinna* resembles those of other gastropods (Hodgson and Bernard 1988; Rocha-Barreira 2002; Hodgson et al. 2012; Prusina et al. 2014; Chen et al. 2015). There is little information on the morphological types of spermatozoa in members of the family Nacellidae, with the nucleus being triangular in *Cellana radiata capensis* and elongated in *N. delesserti* (Hodgson and Bernard 1988, 1989), resembling that of *N. concinna*. Ultrastructural studies in patellids (Hodgson and Bernard 1988; Hodgson et al. 1996) suggest that there are at least five morphological types of spermatozoa based on head morphology (nucleus shape and complexity of the acrosome). This classification is useful for distinguishing between closely related species (Branch 1974; Hodgson and Bernard 1988; Reunov and Hodgson 1994; Hodgson et al. 1996; Ridgway et al. 1998; Collado and Brown 2006).

The spermatozoa of aquatic invertebrates are classified into two categories: introsperm and aquasperm, involved in internal and external fertilization, respectively. In turn, aquasperm is subdivided into ect-aquasperm, typical of species with true external fertilization, and ent-aquasperm, produced by species in which fertilization occurs in, for example, the mantle cavity (Hodgson et al. 2012). In *N. concinna*, we detected spermatozoa in the oviduct of 50% of the females, suggesting internal fertilization in contrast to Picken (1980), who reported that this species has external fertilization. The aggregation of individuals forming stacks involves a close contact between sexes and may facilitate the transfer of ribbon sperm (Stanwell-Smith and Clarke 1998; Powell et al. 2001; pers. obs.). Moreover, the spermatozoon of *C. radiata capensis*, *N. delesserti*, and *N. concinna* differs from the typical morphological pattern of ect-aquasperm (Hodgson and Bernard 1988, 1989; our results). Hodgson et al. (2012) challenged the traditional relationship between spermatozoa morphology and reproductive mode by postulating that spermatozoon structure is not linked to the site of fertilization, but to specializations of the egg or its envelope, e.g., spermatozoa with long nucleus are related with large yolky eggs. Previous studies have reported internal fertilization in few acmaeid limpets (Hodgson 2010). Future TEM studies providing a detailed comparison of spermatozoon and oocyte ultrastructure in *N. concinna* will help elucidate its fertilization mode.

The Phylum Mollusca shows a wide variation in chromosome number, ranging from $n=5$ to 72 (Nishikawa 1962; Vitturi et al. 1982; Thiriot-Quiévreux 2003). Chromosome numbers of $n=8$ and $n=9$ have been reported for Caenogastropoda (Thiriot-Quiévreux 2003) but no data are available for Patellogastropoda. We determined that *N. concinna* has a haploid complement of $n=4$ and an XX/XO sex determination system. Additionally, we found that the heteropycnotic bivalent corresponds to the XX pair. Our results are in agreement with those reported for several species belonging to Caenogastropoda (Buccinidae, Carinariidae, Cerithiidae, Columbellidae, Fasciolariidae, Littorinidae, Muricidae, Pomatiopsidae, Rissoidae, Turritellidae), Neritimorpha (Neritidae), and one species of Vetigastropoda (Trochidae) (Nishikawa 1962; Thiriot-Quiévreux 2003). A reduction in chromosome number is essential for oocyte maturation (Von Stetina and Orr-Weaver 2011). Mollusc oocytes are classified into classes I and II, according to oocyte development and fertilization. Class I oocytes (e.g., *Spisula* and *Barnea*, Bivalvia) are fertilized at the prophase I stage and meiosis is induced to proceed to completion. Class II oocytes (e.g., *Patella*, Gastropoda; *Mytilus* and *Ruditapes*, Bivalvia) are initially arrested in prophase I allowing oocyte differentiation; then, they resume meiosis and progress to metaphase I where they undergo a second arrest, to complete meiosis after fertilization (Colas and Dubé 1998; McNally and McNally 2005). Our study suggests that *N. concinna* oocytes may belong to Class II, as has also been reported for other limpets. Furthermore, the joint presence of anaphase I oocytes and sperm in the oviduct supports the hypothesis that this limpet reproduces by internal fertilization. This mode of fertilization may have evolved as a successful reproductive strategy in response to the harsh environmental conditions in Antarctica.

We agree with Hodgson (2010) in that information on ovarian morphology and oogenesis may be useful to resolve systematic and phylogenetic questions in gastropods, while spermatozoon head morphology may help to clarify the systematics of prosobranchs. Moreover, these reproductive features may be used as biomarkers of pollution (Ahn et al. 2002).

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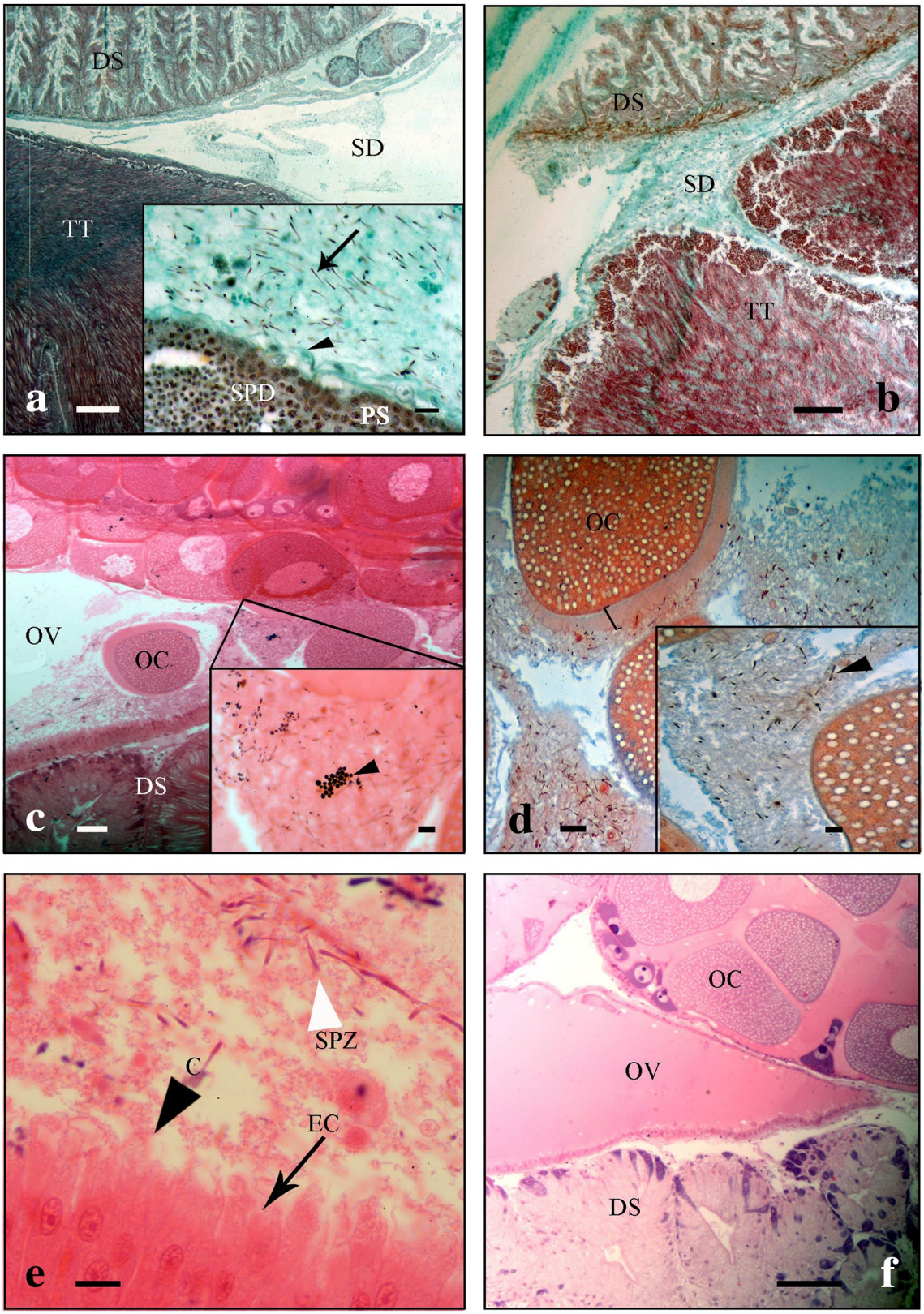

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◂**Fig. 4** *Nacella concinna*. Genital ducts. **a**–**b** Distal and proximal part of the sperm duct, respectively. Testicular tubules are located ventrally. Inset: the arrowhead shows the sperm duct with thin epithelial lining and the arrow shows the lumen filled with sperm. **c**–**d** Distal part of the oviduct. Mature oocytes are surrounded by spermatozoa. Inset of **c** The arrowhead shows atypical early spermatids. **d** The arrowhead indicates the sperm, which is close to the thick jelly coat (square bracket). **e** Magnification of the thick epithelium lining the distal part of the oviduct. The black arrowhead indicates the columnar ciliated epithelial cells and the white arrow points to sperm in the lumen of the oviduct. **f** Magnification of the thin epithelium lining the proximal part of the oviduct. Note the lumen filled with eosinophilic material. *C* cilia, *CT* connective tissue, *DS* digestive system, *OC* oocytes, *OV* oviduct, *PO* previtellogenic oocyte, *PS* primary spermatocytes, *SD* sperm duct, *SPD* spermatids, *SPZ* spermatozoa, *TT* testicular tubules, *VO* vitellogenic oocyte. Scale bars: **a** = 200 µm; **b** = 100 µm; **c** = 50 µm; **d** and **f** = 20 µm; **e** and inset of **a**–**d** = 10 µm; **a**–**b**: light-green Masson's trichrome; **c** and inset, **e**–**f**: Hematoxylin–eosin; **d** and inset: aniline blue Masson's trichrome

The fact that *N. concinna* is known to be preyed upon by some seabirds, echinoderms, and the fish *Notothenia coriiceps* (Barrera Oro and Casaux 1990, Favero et al. 1997; Suda et al. 2015) denotes its importance in the Antarctic marine food web. On this basis, research on the reproduction of this species contributes to conservation goals.

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