INTRAOVARIAN SPERM STORAGE IN *HELICOLENUS DACTYLOPTERUS DACTYLOPTERUS*: FERTILIZATION, CRYPT FORMATION AND MAINTENANCE OF STORED SPERM

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***ABSTRACT.** –* The females of the bluemouth rockfish, *Helicolenus dactylopterus dactylopterus* (DelaRoche, 1809), store sperm within their ovaries for periods of up to 10 months. Twenty six females with standard lengths between 152 and 257 mm and six males with standard lengths between 253 and 290 mm were caught for this study during the course of a year. They were analysed in order to observe the ultrastructure of the storage crypts with stored spermatozoa and to describe their evolution over the year. After internal fertilization and once sperm reaches the ovary, a crypt forms probably by an epithelial inclusion at the base of the lamellae of one or several spermatozoa groups that are floating freely in the interlamellar space of the ovarian lumen. Stored spermatozoa have a large cytoplasm bag surrounding their heads. This bag could serve as a nutritive reservoir during the long storage period. Many desmosomal and tight junctions between the crypt cells ensure that male sex cells are protected against the female immune system.

***KEY WORDS.** – Helicolenus dactylopterus*, sperm storage, ovarian crypts, ultrastructure.

INTRODUCTION

The bluemouth rockfish, *Helicolenus dactylopterus* (De la Roche, 1809), is a benthic species that usually inhabits the sea bottom at a depth of between 200 and 1,000 m (Whitehead et al., 1986). The distribution pattern of this species is very complex. Eschmeyer (1969) identified two Atlantic subspecies, *H. dactylopterus dactylopterus* and *H. dactylopterus lahillei*. The former is made up of four separate populations (in the Northeast Atlantic Ocean and Mediterranean Sea, the Gulf of Guinea, off South Africa and in the Northwest Atlantic Ocean) while the latter is found off the Argentine and Uruguayan coasts. It is a commercial species that is caught extensively and is the target of semi-industrial and artisanal fishing. It can often be found in markets. Besides the obvious interest for fisheries research, the unusual reproductive characteristics of *H. dactylopterus dactylopterus*, makes it a very interesting species from the point of reproductive biology.

Helicolenus dactylopterus dactylopterus has cystovarian type ovaries as described by Hoar (1969). They are saccular with a transverse section that is approximately circular. They have a muscular-connective stroma crossing them longitudinally and from which sprout some ovigerous lamellae suspended within the ovarian cavity by highly irrigated fibromuscular trunks. On the surface of these fibromuscular trunks, we found oocytes in different stages of development that increased in maturity as we moved further away from the central stroma. Previous work by our research group (Muñoz et al., 1999, 2000) discovered intraovarian sperm storage that can last up to 10 months, which is an extremely long period for a teleost. Optical and electron microscopy have corroborated the existence of rounded cryptal structures that store the sperm at the lamellar base.

Sperm storage in teleosts within the ovarian cavity has been studied in some species using light microscopy. The morphological characteristics related to sperm storage in the ovary have also been studied in several species using light and electron microscopy (Fraser & Renton, 1940; Mendoza, 1943; Ishii, 1957; Tateishi et al., 1958; Igarashi, 1961; Moser, 1967; Jalabert & Billard, 1969; Gardiner, 1978; Lisovenko, 1979; Shinomiya, 1985; Pusey & Steward, 1989; Takahashi et al., 1991; Grier & Parenti, 1994; Burns et al., 1997; Koya et al., 1997; Muñoz et al., 1999, 2000, 2002a; Meisner et al., 2000; Potter & Kramer, 2000).

In the majority of cases, there were no specialized differentiated storage structures and the spermatozoa float freely within the ovarian lumen. However, they always remain there for only relatively short storage periods [e.g., *Alcichthys alcicornis* (Koya et al., 1997)]. In other cases, such as *Cymatogaster aggregata* (Gardiner, 1978), spermatozoa insert their heads into the ovarian epithelium into a kind of sperm pocket for several months. This is typical in poeciliids

such as the female platy which are also capable of storing viable sperm for up to several months due to specific epithelial cells lining the oviduct (Potter & Kramer, 2000). However, other species that have slightly more specialized storage mechanisms have also been documented, such as *Sebastodes paucispinis* (Moser, 1967), where the male gametes can be surrounded by the ovigerous lamellae epithelium and in *Helicolenus dactylopterus*, in which relatively specialized structures allow spermatozoa to be stored within the ovary for a long period of time.

In this study, we describe the morphological changes that the sperm cells undergo from their formation in the testes to intraovarian fertilization, after having passed through different stages in the storage period. We also analyse the crypt formation process and look at the mechanisms that permit male sexual cells to be kept for a long period of time despite the absence of a specialized storage organ.

MATERIALS AND METHODS

The 26 female and 6 male *Helicolenus dactylopterus dactylopterus* used in this study were chosen from catches made over a 1 year period in Palamós (Costa Brava, the Northwest Mediterranean Sea). Ovary samples from specimens caught throughout the year were analysed in order to observe the annual evolution of the crypts. We also obtained 6 testicular samples to study the ultrastructure of the spermatozoa.

After capture, the samples were immediately fixed in a 10% formalin solution. The gonads were weighed and then stored in 70% ethyl alcohol. They were then imbedded in glycol methacrylate and cut into 5 mm sections. Toluidine blue was used as a general stain and the Pas-Schiff stain was used to detect mucopolysaccharides.

For the ultrastructural analysis, transverse sections were made of different parts of the male and female gonads, all of which were dissected in vivo. The samples (less than 1 mm^3) in volume were fixed in a mixture of glutaraldehyde (2.5%) and paraformaldehyde (2%) with a 0.1 M cacodylate buffer. After being fixed for 2 hours at 4ºC, they were washed with a 0.1 M cacodylate buffer. Post-fixation was conducted in 1% osmium and a cacodylate buffer at 4ºC for 1 hour. The samples were then washed several times, dehydrated with an alcohol series and finally embedded in Spurr's mixture. Sections of about 40 - 50 nm were made with a Reichert ultramicrotome, stained with uranyl acetate and lead citrate and examined under a Zeiss EM-910 transmission electron microscope.

Transverse sections of ovaries were used for proliferating cell nuclear antigen (PCNA) analysis. They were fixed in 10% formalin, embedded in paraffin and 5 μm thick sections were cut for the analysis. After de-paraffination, the sections were then washed with phosphate-buffered saline (PBS) (5 mM, pH 7.6). After inhibiting endogenous peroxidases, the sections were washed with PBS. They were incubated continuously with the primary antibody for the PCNA analysis, then with the immunoglobulin-linked biotin and washed again with PBS. After incubation with the avidin-biotin-peroxidase (ABP) complex, the sections were washed again with PBS and revealed with tris-buffered saline (TBS) which contained diaminobenzidine (DAB) and H_2O_2. They were then contrasted with methyl green, dehydrated and mounted. This technique is based on Pallarés (2002).

RESULTS

Each of the testes has a main spermatic duct which runs longitudinally along its latero-dorsal edge and which is connected to short efferent ducts. These efferent ducts spread out in all directions, forming a mass of seminiferous lobules which are small in diameter and which come to a dead end in the gonad periphery. The two spermatic ducts remain independent, passing very close to the urinary bladder and continuing to the urogenital papilla, where they join together to form a single duct. This urogenital papilla is large and conical in shape and can be projected towards the exterior to eject the semen (Muñoz et al, 1999).

Helicolenus dactylopterus spermatozoa have the three typical, clearly differentiated zones: head, middle-piece and tail or flagellum. The nucleus has homogenously condensed chromatin without specific accumulations. Within the testes, the spermatozoa retain some debris of the nuclear membrane that remains from the large reduction in size of the nucleus during spermiogenesis. The head morphology is long and has a lateral prolongation that covers the diplosome (Fig. 1). The middle-piece is moderately long, approximately 2 mm and is formed by four mitochondrial layers, each of which is integrated with four to 10 mitochondria. The spermatozoa flagella have a classical axonemic structure with two central
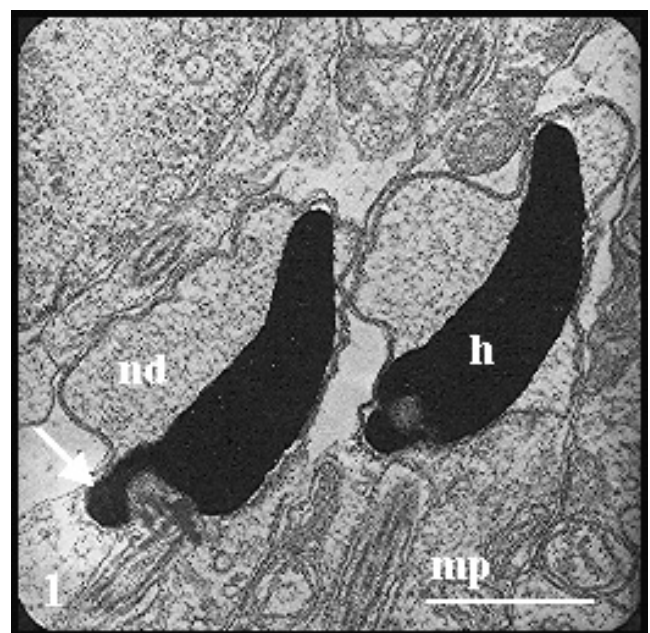

Fig. 1. Spermatozoa within the testes. The arrows show the lateral prolongation of the nucleus. Scale bar = 1 μm. h = sperm head; mp = middle-piece; nd = nucleus debris.

microtubules and nine peripheral pairs. The flagellar membrane usually forms one or two lateral extensions which appear as two small wings of varying lengths.

The morphology of the sperm cells detected within the ovary is notably modified as they have a large cytoplasm bag around their heads (Fig. 2). This bag is evident both in spermatozoa that are floating freely within the interlamellar space and the spermatozoa that are inside the storage structures. It should also be pointed out that the sperm samples, obtained by pressing directly on the testicles of captured specimens, also

had a large cytoplasmic bag surrounding the nucleus and there were numerous vacuoles in the middle piece. As the spawning period approached, the amount of material within the cytoplasmic bag decreased until it nearly disappeared in the months of February and March. In the same period, the quantity and size of the mitochondria also decreased considerably, to the point where they were hardly detectable in several spermatozoa (Fig. 3).

The crypt or specialized storage structure in *Helicolenus dactylopterus* is made up of a cryptal epithelium surrounding
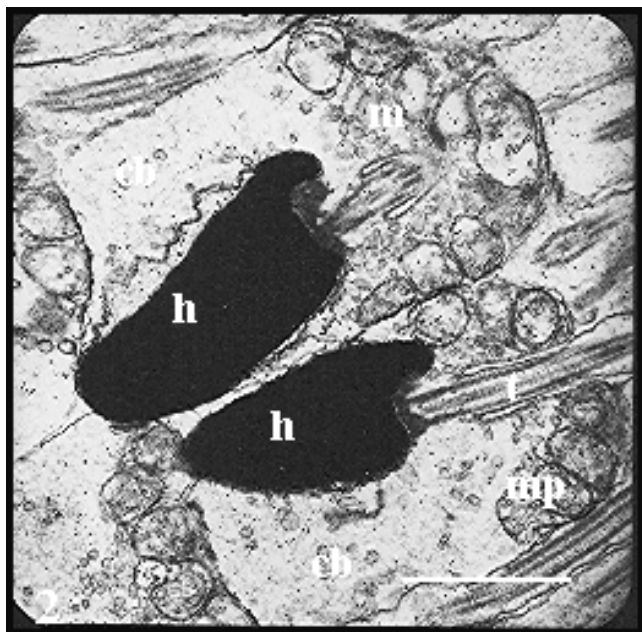

Fig. 2. General structure of the spermatozoa within the crypt. Scale bar = 1 μm. cb = cytoplasmic bag; h = sperm head; m = mitochondria; mp = middle-piece; t = spermatozoon tail.
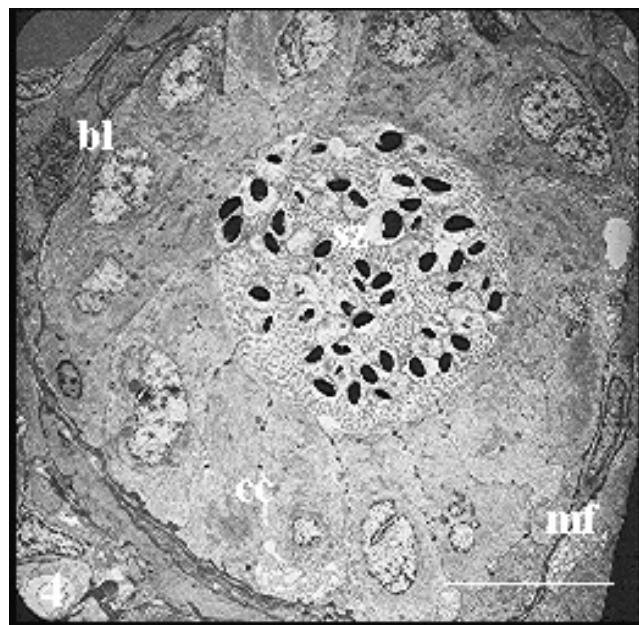

Fig. 4. General view of a crypt in which we can clearly see the cryptal sheath. Scale bar = 10 μm. bl = basal lamina; cc = cryptal cells; mf = muscular fibres; sz = spermatozoa.

Fig. 3. Reduced mitochondria in the middle piece of a spermatozoon at the end of the storage period. Scale bar = 1 μm. m = mitochondria; v = vacuoles.
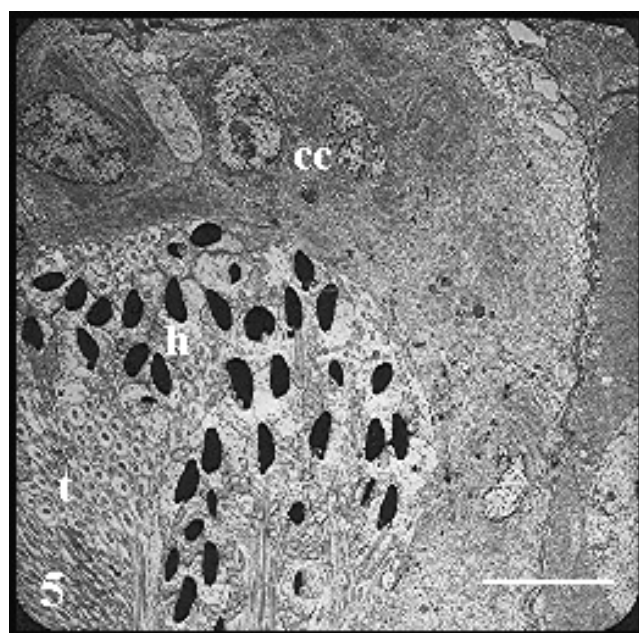

Fig. 5. The organization within the crypt shows some spermatozoa clusters of heads and some clusters of sperm tails. Scale bar = 5 μm. h = sperm heads; cc = cryptal cells; t = sperm tails.

that encloses the stored spermatozoa (Fig. 4) and is connected to the ovarian lumen by a duct. During the storage period (from May onwards), there are a large amounts of spermatozoa that are very compacted within the crypts and are arranged in different clusters. In each cluster, the spermatozoa are perfectly aligned, despite their moderately elongated form (Fig. 5).

The crypt epithelium is made up of a simple cubical epithelium resting on a basal lamina surrounded by a layer of smooth muscular fibres (Fig. 6). The cell nucleus of the epithelial cells has an oval, spherical shape during the spawning period and is deeply folded during the post-spawning period. This nucleus is usually conspicuous, being slightly electron-dense and surrounded by cytoplasm that is rich in perinuclear myofilaments. In the cytoplasm, there is a Golgi apparatus, a large number of secretory vesicles, lysosomes and residual bodies (Fig. 7). All of these indicate that the epithelium has secretory functions during the storage period. The number of lysosomes, secretory vesicles and vacuoles seem to increase as the spawning period approaches. At the same time, the number of blood vessels around the crypts also appear to increase.
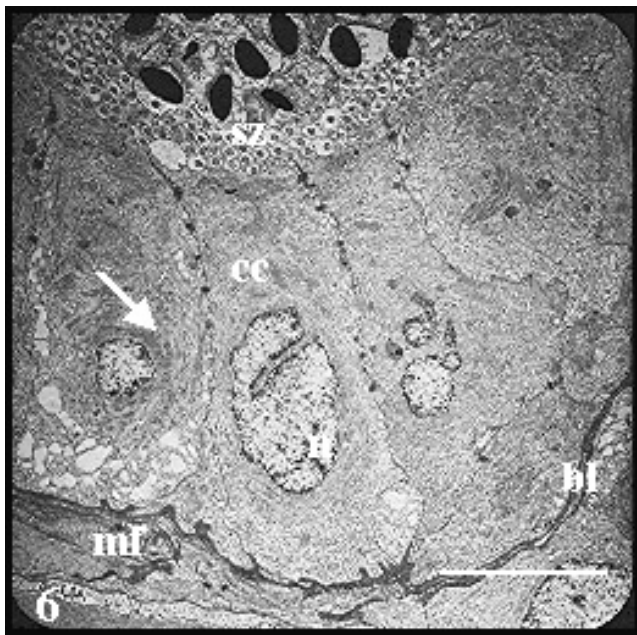

Fig. 6. Ultrastructure of the cryptal epithelium. Note the round-elliptical shapes of the nucleus' cryptal cells. The arrow shows the perinuclear myofilaments of the cryptal cells. Scale bar = 5 μm. bl = basal lamina; cc = cryptal cells; mf = muscular fibres; n = nucleus; sz = spermatozoa.

Fig. 8. Intercellular junctions between two cryptal cells. Scale bar = 1 μm. cc = cryptal cells; d =desmosome; i = interdigitation; sz = spermatozoa.
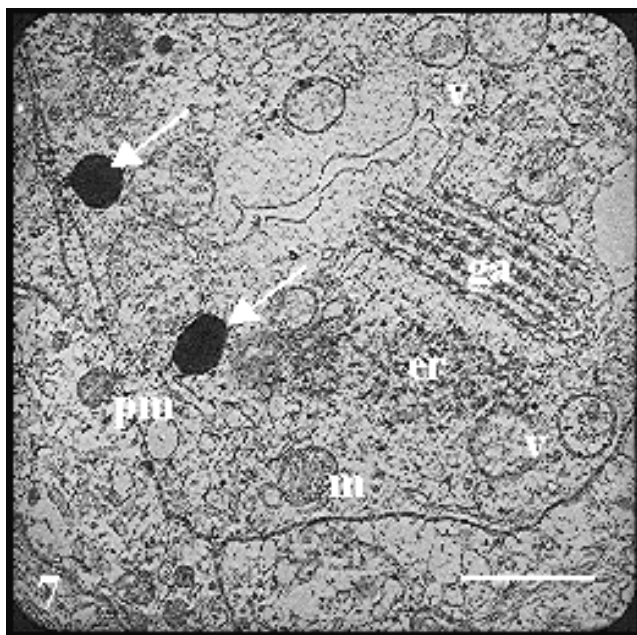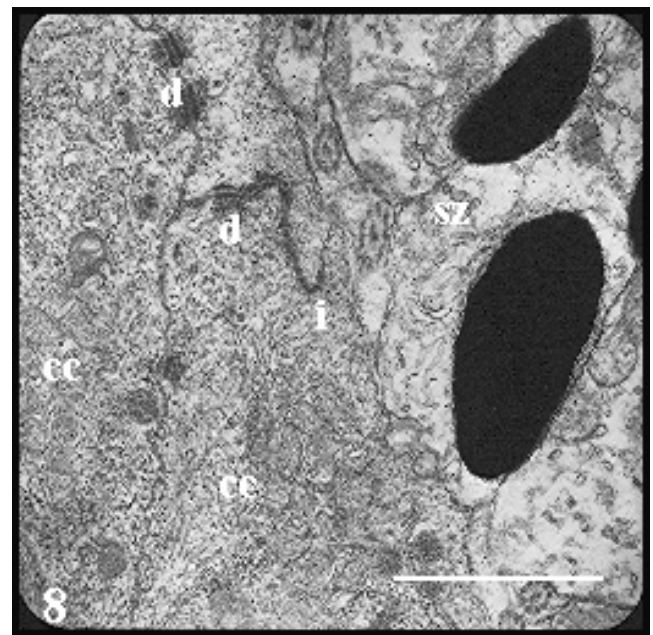

Fig. 7. Cytoplasmic constituents indicating the notable secretory activity of the cryptal cells. The arrows show lysosomes. Scale bar = 1 μm. er = endoplasmic reticulum; ga = Golgi apparatus; m = mitochondria; pm = plasma membrane; v = vesicles.

Fig. 9. Desmosome separation process between two cryptal cells in the post-spawning period. Scale bar = 1 μm. cc = cryptal cells; d = desmosomes; pm = plasma membrane.

The crypt epithelium may originate from a specialization of the lamellar epithelium as their cellular morphology is very similar. PCNA analysis revealed that the lamellar epithelium has an evident proliferation at the margins and some marginal cells undergo division.

The epithelial cells surrounding the crypts are joined together firmly by tight intercellular junctions and an enormous quantity of desmosomes (Fig. 8). During the post-spawning period, in March and April, we observed a decrease in the number of desmosomal and tight junctions. We also observed that some were broken (Fig. 9).
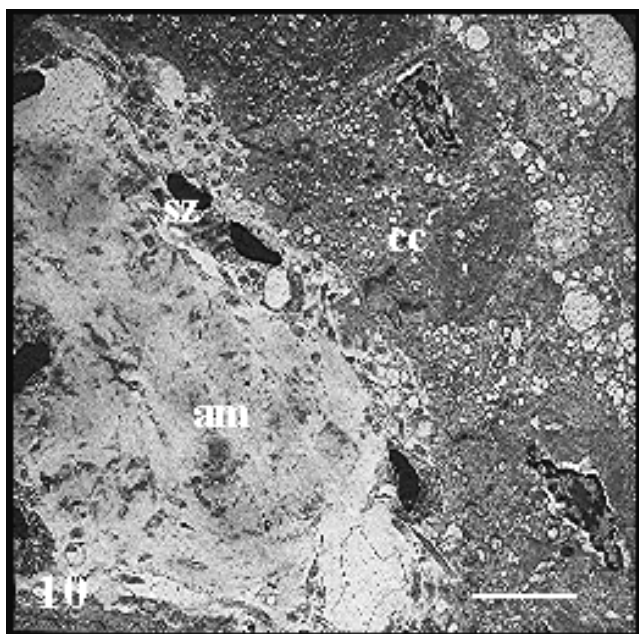

Fig. 10. Partially empty crypt at the final stage of the storage period. Scale bar = 5 μm. am = amorphous material; cc = cryptal cells; sz = some spermatozoa.

Furthermore, we found some partially empty crypts with very few spermatozoa and amorphous material (Fig. 10). These crypts also showed a lower level of vascularisation. Moreover, the residual spermatozoa inside the crypts were less compacted and did not follow any organized pattern. The existence of intraepithelial phagocytes near the crypts were especially evident at the end of the spawning period (Fig. 11) and was probably related to the phagocytosis of the residual spermatozoa or what had remained of them (Fig. 12).

DISCUSSION

The spermatozoa of *Helicolenus dactylopterus dactylopterus* can be classified as Type I anachrosomal aquasperm as defined by Jamieson & Leung (1991). In general, spherical-headed spermatozoa with a short middle-piece are thought to be evolutionarily primitive and related to external fertilization. Morphologically specialized sperm heads (often elongated) and a long middle-piece, are thought to be more advanced and related to internal fertilization, as in the case of the bluemouth rockfish. Elongation of the sperm nucleus may be related to sperm transport and storage in internally fertilizing species (Jamieson, 1987). The streamlined nucleus could aid the passage of spermatozoon through narrow areas and viscous fluids of the female reproductive tract (Gardiner, 1978) or help form sperm bundles (Atwood & Chia, 1974). Thus, the elongation of the sperm nucleus may facilitate the side-by-side alignment of spermatozoa moving through the testicular ducts (Burns et al., 1995). In fact, after the bluemouth rockfish mates, the sperm penetrates deep into the ovary, reaching the interlamellar space. Hence, elongation of the middle piece is necessary to accommodate the energy stores for sperm movement. This energy is supplied by the mitochondria which respire and consume the endogenous substrate of the middle-piece (Baccetti & Afzelius, 1976).
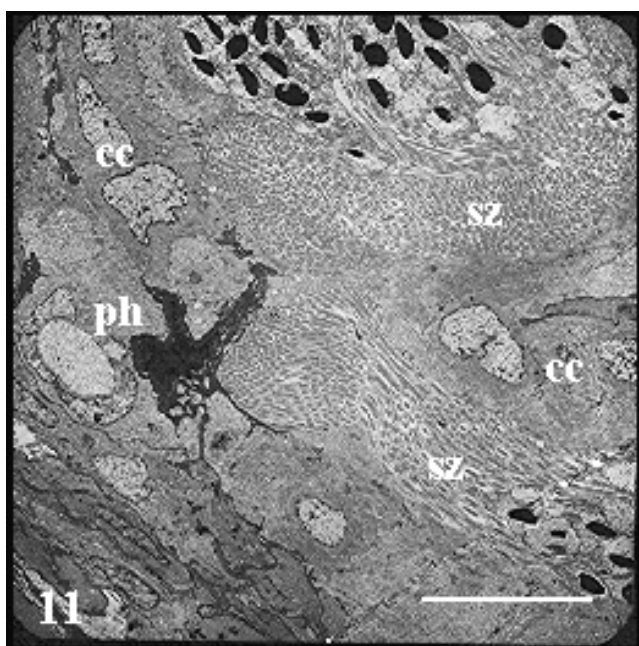

Fig. 11. Intraepithelial phagocyte next to a crypt. Scale bar = 10 μm. cc = cryptal cells; ph = phagocyte; sz = spermatozoa.

Fig. 12. Initial degeneration phase of one spermatozoon. Scale bar = 1 μm. dsz = degenerative spermatozoon.

Both the sperm cells extracted directly from the testicles and those stored within the ovaries, show a large amount of cytoplasm around their nucleus, which indicates that they enter the ovary in this state. The spermatozoa that float freely within the lumen also have this cytoplasm bag. Thus, we believe that this cytoplasmic bag is formed when the mature sperm cells pass through the seminal ducts before they are released into the female's body. The spermatozoa retain the remains of the cytoplasmic bag around their heads and middle-pieces. This seems to be a characteristic specific to this species as an extensive review of literature for this study found no mention of this phenomenon in any other species.

Once the sperm cells are completely mature, they are introduced through the genital papilla into the female genital tract. These sperm cells enter the ovaries mainly in small clusters and we believe that when these clusters make contact with the lamellar epithelium, the latter will incorporate into one or several groups of spermatozoa, which leads to the sperm storage crypt being formed. This process is repeated many times to form the large numbers of crypt structures that can be found in a transverse section of the ovaries. Hence, the crypt is probably formed due to an epithelial inclusion at the base of the lamellae of one or several spermatozoa groups that are floating freely in the interlamellar space of the ovarian lumen. This hypothesis is supported by the results obtained with the PCNA analysis which showed that the cells located at the margins of the lamellar epithelium had an evident cycle of cellular divisions. This hypothesis would explain the existence of various small groups of male sex cells found in the majority of crypts, each of them oriented in a different direction and also the extraordinary resemblance between epithelial lamellar cells and cryptal cells. Therefore, crypts are probably formed when several groups of spermatozoa make contact with the basal lamellar epithelium.

During the long sperm storage period, the male sex cells need to be maintained nutritionally and protected from the female's immune system until fertilization. We have mentioned previously that the spermatozoa inside the crypts as well as the ones floating freely within the lumen have large cytoplasmic bags around their heads which decrease in volume as the spawning period approaches. Once inside the female, this cytoplasm serves as a reservoir of nutrients consumed during the long storage period. The cytoplasm, along with the nutritive secretions produced by the cryptal epithelium could also feed the stored spermatozoa. During the entire storage period, cryptal cells show considerable secretory activity. Secretory vesicles remain near the plasma membrane of the cell until an extracellular signal causes their contents to be released (Alberts et al., 1996). In fact, mucopolysaccharide granules released by the cryptal epithelium into a cavity retaining spermatozoa have been documented in this species using light microscopy (Muñoz et al., 2002b). The mucopolysaccharide granules, as well as large amounts of rough endoplasmic reticulum, a well-developed Golgi apparatus, secretory vesicles and free ribosomes, are evident characteristics of protein synthesis.

It should also be highlighted that the large number of tight junctions and desmosomes located between the cryptal cells are an efficient barrier around the crypt that protects spermatozoa inside it from the female's immune system. This fact has been documented in other species characterized by intraovarian sperm storage such as *Alcichthys alcicornis* (Koya et al., 1997), *Cymatogaster aggregata* (Gardiner, 1978) and *Xiphophorus maculatus* (Potter & Kramer, 2000), as well as in oviparous teleosts such as *Fundulus heteroclitus* (Brummett et al., 1982) and *Syngnathus scovelli* (Begovac & Wallace, 1987).

The storage period lasts until the moment of fertilization when the spermatozoa cells are released within the ovarian lumen to fertilize the mature eggs. This is the reason for the significant decrease in the number and size of the storage structures in the post-spawning period. Nevertheless, by that time several new crypts are formed when the female can be re-inseminated.

After fertilization, which is known to be internal in the genus *Helicolenus* (Wourms & Lombardi, 1992), the eggs are retained within the female's genital tract, although the gestation period of this species is unknown. White et al. (1998) proposed a 20 - 30 days gestation period as yolk globule-stage oocytes were present in early December and embryos were observed in late January.

This reproductive strategy is called zygoparous oviparity and ensures a larger percentage of fertilized eggs than oviparous species.

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