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Histological Description of the Gonad, Reproductive Cycle, and Fertilization of *Pisidium amnicum* (Müller, 1774) (Bivalvia: Sphaeriidae)

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Abstract. A detailed study of the reproductive cycle of a Spanish population of *Pisidium amnicum* (Müller, 1774) based on monthly histological gonadal samples is presented. Results of the study of the gonadal cycle perfectly match previously reported data on the dynamics of this population, with mature gametes of both sexes present between July and October. Specimens surviving one reproductive cycle undergo a second gametogenesis resulting in a new brood. Nevertheless, most of them die before birth because of the adults' limited life span. We suggest that cross-fertilization in these freshwater bivalves occurs in summer in the gills or in the suprabranchial chamber instead of in the gonoduct as has been proposed by other authors. Illustrations of all stages of the gametogenic processes, both male and female, as well as of the first stages of embryonic development, are given. Differences between the reproductive strategies of *P. amnicum* and other sphaeriid species are also discussed.

INTRODUCTION

All species of the family Sphaeriidae studied are hermaphroditic and incubatory, retaining fertilized eggs in brood sacs developed in the inner gills. Following Mackie (1978), who reviewed the terms ovoviviparity and viviparity, these freshwater bivalves are ovoviviparous. The main literature about reproduction, with histological studies of species of the family Sphaeriidae, deals with the genera *Musculium* Link, 1807 (Okada, 1935a, b, c, 1936; Heard, 1977) and *Sphaerium* Scopoli, 1777. (Gilmore, 1917; Woods, 1931, 1932; Thomas, 1959; Heard, 1977). The only authors who studied the gonadal histology of species of the genus *Pisidium* Pfeiffer, 1821, were Heard (1965), who focused on the reproductive strategies of the North American species, and Meier-Brook (1970) who dealt with several European species, not including *Pisidium amnicum* (Müller, 1774).

The population dynamics of *P. amnicum,* the largest species of the genus, has been studied in Germany (Danneel & Hinz, 1976), England (Bass, 1979), Canada (Vincent et al., 1981), and Spain (Araujo et al., in press), but no data exists about its gonadal development or its reproductive cycle from a histological point of view. Recently, Araujo & Ramos (1997) described the gonadal morphology and evidence of intrafollicular fertilization in several specimens of this species, discussing the possibility of self-fertilization.

This paper describes histologically gametogenesis, the cellular types of germinal lineage, reproductive cycle, and fertilization process of a Spanish population of *P. amnicum* previously studied by Araujo et al., (in press). It shows that this isolated population, the southernmost of the species in Europe and in the world (except for the North African population cited by Kuiper in 1972), is very well adapted to local conditions as indicated by its breeding success compared with North European populations of the species (Araujo et al., in press).

MATERIALS AND METHODS

Specimens of *P. amnicum* were collected monthly between June 1990 and May 1991 in the Miño River in the NW of the Iberian Peninsula. A description of the sample site, sampling methods, and water physico-chemical characteristics are provided by Araujo et al., (in press). In the laboratory, each monthly sample was sorted into 1 mm size classes. Four specimens of 6–7 mm were used to determine which of four protocols was best for fixation: (a) immersion of specimens in hot water (6 seconds, 60°C) and fixation with 70% ethanol; (b) direct fixation in formalin saline solution (100 mL 40% formalin, 9 g sodium chloride and 900 mL distilled water); (c) immersion in hot water (6 seconds, 60°C) and fixation with formalin saline solution; and (d) relaxing specimens with menthol (24–72 hrs), and fixation with formalin saline solution. The second method produced the best results and was used in the study. Fixed specimens were removed from their shells, dehydrated in a graded ethanol series (30, 50, 70, 96, and 100%) and embedded in paraffin. Sections were made between 7–10 μm and stained with hematoxylin-eosin and Heidenhain's azan. Those specimens containing shelled larvae in the gills were submerged in a mixture of 70% ethanol and 1% acetic acid

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for 3 days in order to decalcify the embryonic shells, as proposed by Okada (1935a). We were normally successful when we decreased this time to 24 hr.

As the months in which embryos and/or larvae grow inside the maternal gill, and the month of juvenile release were already known (Araujo et al., in press), the fertilization and first stages of cleavage were mainly studied in specimens from August and September. Thus, we studied 13, 14, six, four, and two specimens from September, August, June, July, and October, respectively, and one from the rest. In order to analyze the gametogenetic stages, adult specimens of 7–8 mm were observed. Once we knew the months of greater gametogenic activity and the size at which the species reaches sexual maturity, specimens of all size classes from these months were observed.

RESULTS

P. amnicum is a simultaneous hermaphrodite, as both male and female gametes develop in the gonad of each sexually mature specimen at the same time. Male and female tissues are organized in follicles, the male fraction being much larger than the female and occupying the anterior part, while the small ovarian fraction is posterior (Figure 1A, B). Although there are no hermaphroditic follicles, both gonadal fractions overlap near the hermaphroditic ducts, which are exterior and lateral, running parallel to the cerebro-visceral connectives (Figure 1C). As has been demonstrated by Araujo & Ramos (1997), male and female gametes are commonly found together in this area.

Gametogenesis

The simultaneous presence of mature spermatozoa and ripe oocytes was restricted to the period from July to October. Specimens born in April–May undergo gametogenesis in summer. The larger gravid specimens found from June to September were the survivors of the previous year in which the gametogenetic activity restarts, although most of them die before the new cycle finishes. Fertilization and settlement of the zygotes occur in late summer (August and September) in both newborn spec-

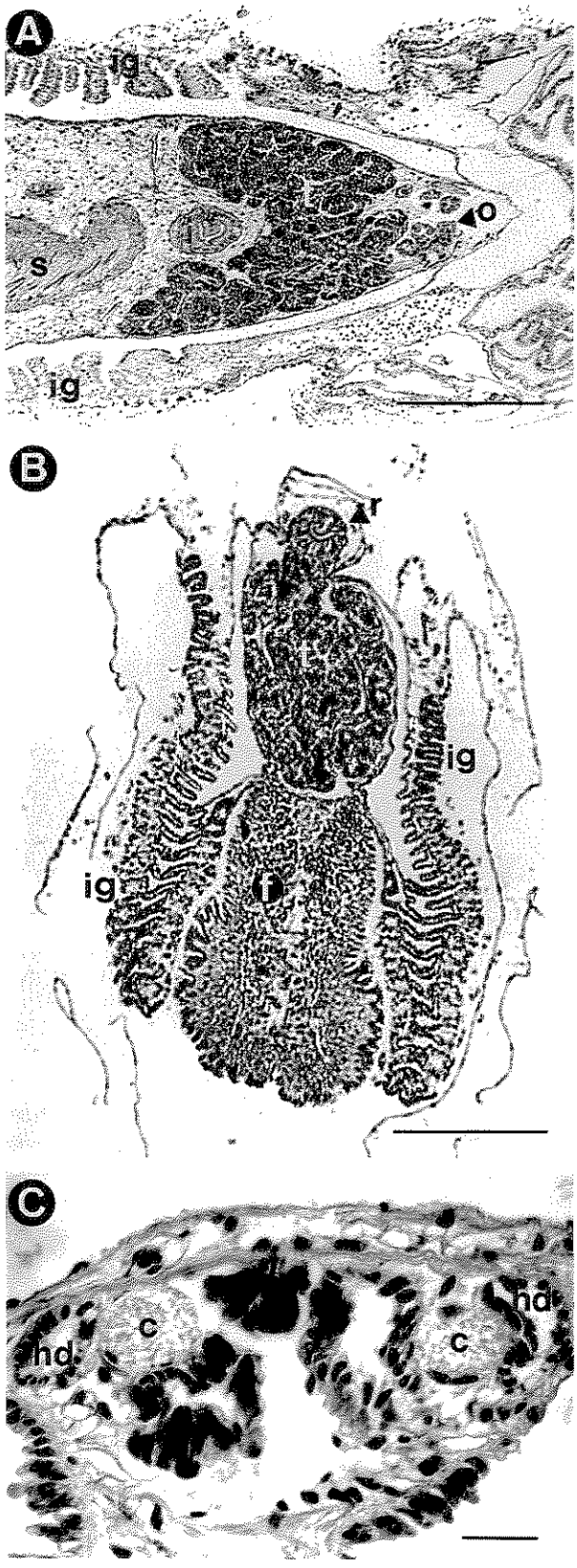

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Figure 1

Gonad of *P. amnicum*. **A.** Longitudinal section of an 8–9 mm specimen from August. The anterior part is at left. Scale bar = 1 mm. **B.** Transverse section through the gonad of a 4–5 mm specimen from August. Scale bar = 0.5 mm. **C.** Transverse section through the end of the gonad showing the hermaphroditic ducts and the connectives. Scale bar = 50 μm. c, connectives; f, foot; hd, hermaphrodite ducts; i, intestine; ig, inner gill; o, ovary; r, rectum; s, stomach; t, testis.

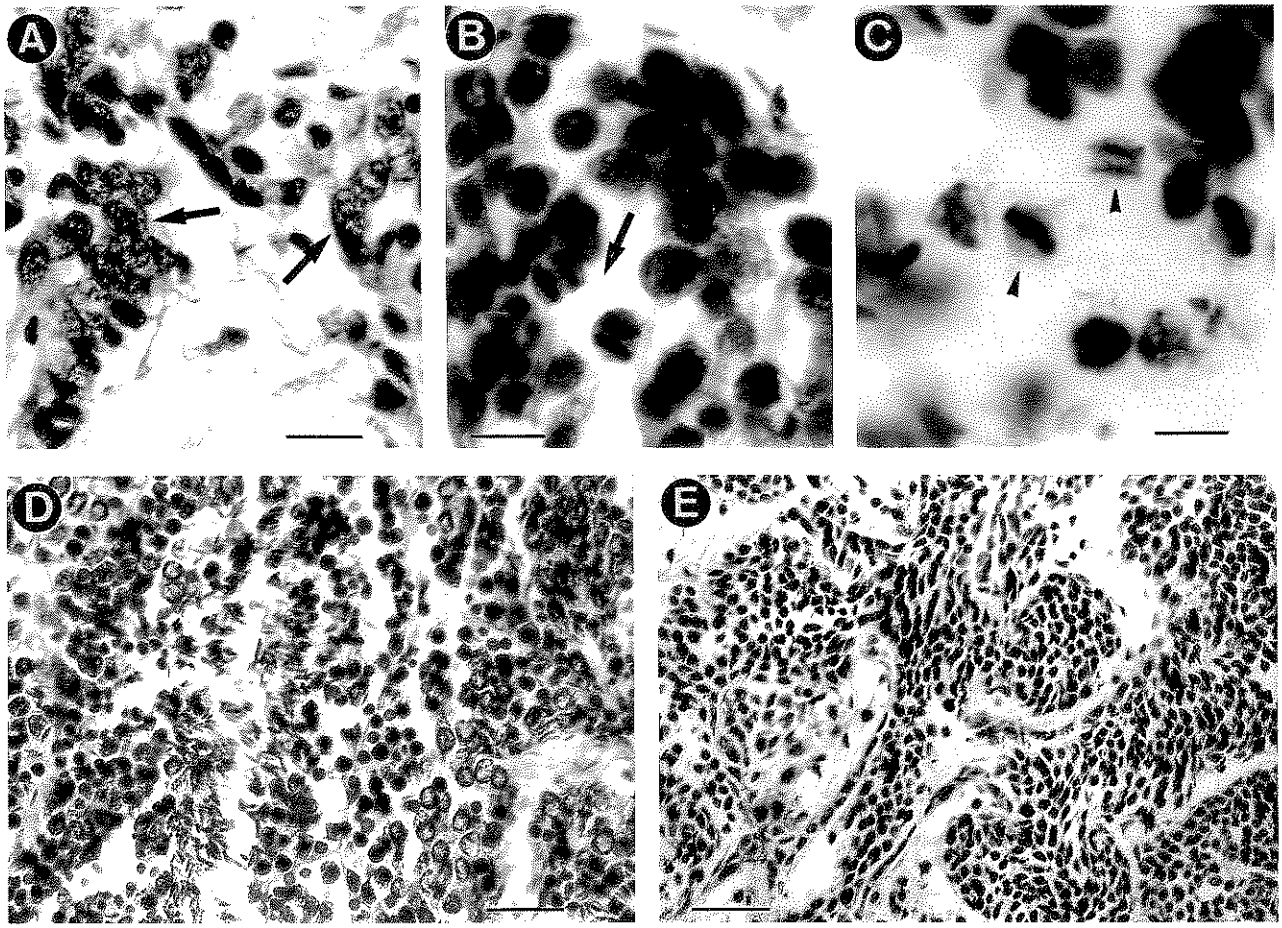

Figure 2

Spermatogenesis of *P. amnicum*. **A.** Spermatogonia (arrows). Scale bar = 30 μm. **B.** Spermatocytes I. The arrow shows the anaphase of first division. Scale bar = 20 μm. **C.** Spermatocytes II. The arrow heads show the metaphase and anaphase of second division. Scale bar = 12 μm. **D.** Section of testis showing different stages of spermatogenesis. Scale bar = 40 μm. **E.** Testis of a specimen from January. Scale bar = 75 μm.

imens and ones from the previous generation. Gravid animals collected during these months still presented many mature oocytes and spermatozoa.

Maturation of the male gametes occurs in specimens from May to October, the light in the middle of the follicles increasing at the same time as spermiogenesis occurs. In October, the follicles are empty, with many spermatogonia growing from their walls; this evacuation phase is followed by a proliferation phase from November to April, with full and compact follicles without light inside.

Spermatogonia were present in the testis from April to December. They are large cells (cell diameter = 10–12 μm) with very little cytoplasm, adhering to the follicle walls (Figure 2A). The nucleus is full of chromatin granules so it is difficult to ascertain the number of nucleoli. First order spermatocytes (Figure 2B) are cells with a diameter of 7–8 μm, scant cytoplasm, and the chromatin spreading in a large nucleus. When these cells are found in the middle of the follicle, the chromatin appears at the edge of the nucleus. Second order spermatocytes are smaller (4–6 μm), and we have also found them in metaphase and anaphase II (Figure 2C) prior to development of the spermatids (2–3 μm). The condensation of the genetic material at this last stage makes it easier to see the cytoplasm than at previous stages. The head of the mature spermatozoa is about 5–6 μm, and the tail is very difficult to see. In July all the male cellular lineage is easily observed in the same follicle (Figure 2D).

In specimens between 4 and 6 mm from August and September (those born in May of the same year), the spermiogenesis is in the latter stages. From July to September, spermatogenesis has restarted in the largest (> 7 mm) and old specimens. In October the testis is massive,

full of polyhedral cells (Figure 2E), with conjunctive tissue in the interfollicular space. The testis remains at this stage for the rest of the winter until late spring and summer when spermatogenesis restarts. The process in the female tissues is very similar. Previtellogenetic oocytes are present in August and September in specimens born in May of the same year. After spawning, the ovogenesis restarts in the oldest specimens. The oogonias adhere to the follicle walls. They are cells similar to the spermatogonias with a diameter of about 20 μm. They have a large nucleus that occupies most of the cell, having a refringent light nucleolus and scattered particles of chromatin, very visible both in azan and hematoxylin-eosin stained slides. A second nucleolus and accompanying cells attached to the oogonias can be observed (Figure 3A). The previtellogenic oocytes are still attached to the follicle wall; they are about 20 μm in diameter with a 15 μm nucleus, showing one or two nucleoli (Figure 3B). Previtellogenic oocytes still maintain accompanying cells (Figure 3C) and sometimes have an anphinucleolus in the nucleus. At the end of the previtellogenic stage, the heterogeneity of the nucleus increases and the accompanying cells disappear. At this state, spherical corpuscles resembling the nuclei of the oocytes can be seen in the female follicles of specimens up to 6–7 mm (Figure 3D). At the beginning of the vitellogenesis, the oocyte size increases and the nucleus still contains the nucleoli and the anphinucleolus (Figure 3E). The ripe oocytes, now free from the acinar wall, are about 40–60 μm and have a nucleus of 12 μm with one or more nucleoli, the larger ones sometimes having an anphinucleolus (Figure 3F).

In specimens from September, the above mentioned spherical corpuscles only appear in specimens over 4 mm.

No signs of reabsorption were observed within the ovarian tissues after ova release, but differentiation of the oogonia immediately follows this event.

During August and September, the largest amount of mature male and ripe female gametes appears, often occurring together within female follicles near the hermaphroditic duct, allowing the occurrence of self-fertilization.

P. amnicum becomes sexually mature at a shell length of about 3–5 mm, the male gonad probably maturing first (we detected one mature specimen of 3–4 mm) and the female later (4–5 mm). Testis maturation proceeds from the anterior to the posterior area, and from the center to the periphery.

Fertilization and Brooding

Only once did we observe a mature oocyte in the hermaphroditic duct, and it had not been fertilized.

No gravid specimens appeared among those from June and July that were studied histologically. In the specimens from August, three histological observations indicated recently fertilized oocytes (zygotes) in the inner gills. In one case, the zygote had a nuclear membrane with many nucleoli and the male pronucleus (Figure 4A). In the other two, the zygote had lost the nuclear membrane, the male pronucleus was located at the edge of the cell, and the female pronucleus was in meiotic metaphase (Figure 4B, C, D). Embryos at several stages of cleavage were also found. Figure 4E, F shows respectively, one embryo in a stage prior to blastula and one blastula, and Figure 4G shows the arrangement of the embryos within the parental gill.

During August and September when the first stages of the embryos were observed, several gravid specimens carried embryos in even more advanced stages, i.e., the ova had been fertilized and had begun the cleavage process once they fell between the gill filaments.

The germ cells from which the gonads develop were observed during all the cleavage of the embryo from the blastula (Figure 5A) to the prodissoconch larvae (Figure 5B) (the one shelled and still covered by the brood sac within the marsupium).

DISCUSSION

The simultaneous occurrence of mature male and female gametes in *P. amnicum* specimens over 5 mm collected from July to October, particularly in August and September, means that this species, like the other of the family Sphaeriidae studied by Meier-Brook (1970), is a simultaneous hermaphrodite (see Araujo & Ramos, 1997). This agrees with Meier-Brook's (1970) point that the simultaneous occurrence of mature gametes of both sexes in 3 mm specimens of *Musculium heterodon* (Pilsbry) suggests that the species is, for most of its life, a simultaneous hermaphrodite, although the male fraction probably matures before the female one. This agrees with Okada's (1935b) concept of protandric maturation. However, following Hoagland (1984), the term protandric should describe animals that can change their sex from male to female without reverting to male. Therefore, this term does not apply to the Sphaeriidae.

Regarding the reproductive habits of this species, histological analysis of the gonads of *P. amnicum* confirms the reproductive strategy (semelparous and univoltine) postulated by Araujo et al. (in press) on the basis of population dynamics. This strategy differs from the rest of the European species of the genus, which, according to Meier-Brook (1970), are iteroparous and multivoltine.

After spawning (gamete release), there is a growth period of the germinal cells in the gonads, which is very long and slow in *P. amnicum*, especially in the female. In *P. lilljeborgii* Clessin, 1886, this growth is quicker, allowing two reproductive periods in the same year. The lack of mature female gametes inmediately after spawning (as occurs in *P. lilljeborgii*) and the long gravidity period in *P. amnicum* (nearly 9 months) compared with

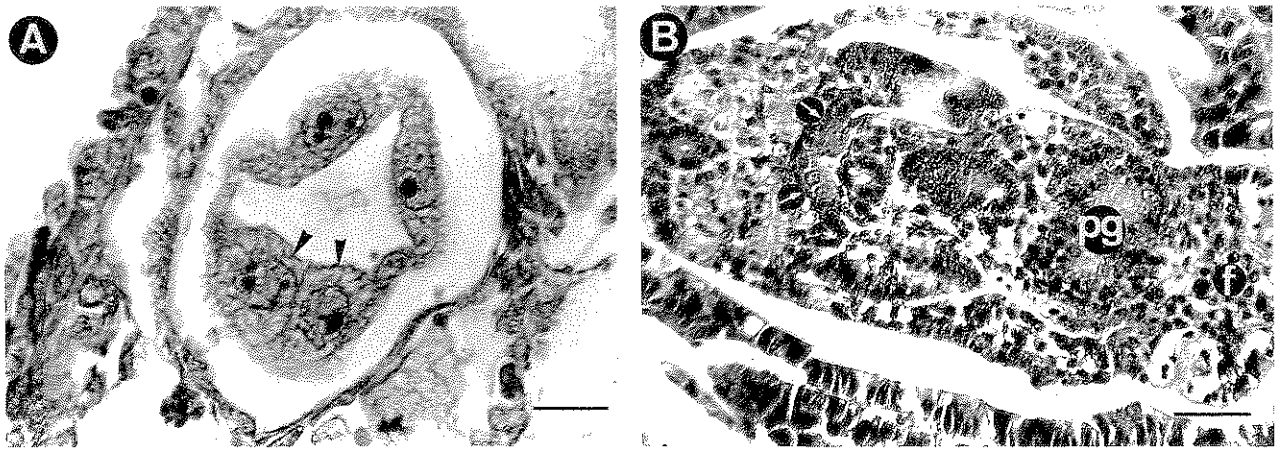

Figure 5

A. Blastula with germ cells (arrow heads). Scale bar = 30 μm. **B.** Prodissoconch larvae with germ cells (arrows). Scale bar = 75 μm. f, foot; pg, pedal ganglia.

cies only occurs in summer, with mature male gametes appearing only during a short period in mid summer (Heard, 1965). Other comparisons between the reproductive strategies of Spanish and other European populations of *P. amnicum* have already been discussed in Araujo et al. (in press). The similarity in the way the testis matures in *P. amnicum* and *Musculium heterodon* is also interesting, as in both species it occurs from the anterior to the posterior region. In the Japanese species, mature spermatozoa are present all year, although a smaller number is observed in winter (Okada, 1935a).

Lucas (1965) proposed that the number of nucleoli differentiates spermatogonia with two nucleoli from oogonia with only one. In the Spanish *P. amnicum* specimens, it was impossible to test this hypothesis because the large amount of chromatin granules in the nucleus of the spermatogonia makes it difficult to determine the number of nucleoli present. Lucas (1965) also cited the difficulty of observing the spermatocyte II due to the speed of the second meiotic division in mollusks. However, we observed this cellular stage in *P. amnicum,* and Okada (1935a) did so in *Musculium heterodon.* Regarding the morphology of the spermatozoa, there are conflicting data in the literature. For Monk (1928) the spermatozoa of *Sphaerium notatum* (Sterki, 1927) lacked a tail, probably due to the difficulty of observing these structures. For Okada (1935a) they were very easy to observe in *Musculium heterodon.* In *P. amnicum,* the tails of the spermatozoa are very difficult structures to identify.

The existence of primary oocytes in spring and autum in *M. heterodon* (Okada, 1935a) corresponds to the peculiar type of reproduction in this species (and all the Sphaeriinae) in which the embryos are present in the gills of the maternal specimens all year.

According to Okada (1935a), the size of the mature ovum ("primary oocyte") in *M. heterodon* is about 40 μm, with an eccentric nucleus of 15–20 μm. Woods (1932) illustrated a mature ovum of about 70 μm in *Sphaerium striatinum* (Lamarck), while the maximum size detected in Spanish *P. amnicum* is about 60 μm and corresponds to metaphase I oocytes. Okada (1935a) cited the presence of accessory plasmosomes, a common structure in the nucleus of growing oocytes in mollusks. He suggested that the increase in these structures is transitory due to the increase in nuclear contents; that explains its absence in the first and final stages of oocyte growth. For Stauffacher (1894, in Okada, 1935a), these accessory nucleoli arise from the budding of the main nucleolus. However, in *P. amnicum* these nucleoli are observed in growing and ripe oocytes, and, indeed, in the zygotes recently settled in the gills (Figure 4A), making it difficult to accept such an explanation. As regards the oocyte accompanying cells, our observations also agree with Okada (1935a) in the sense that their relation with the oocyte become less clear as the oocyte grows, supporting Woods' (1932) idea that one or several epithelial cells surrounding the growing oocyte are joined to it, totally or partly, aiding oocyte formation.

No references have been found in other species of *Pisidium* regarding the refringent corpuscles located in the mature female follicles of *P. amnicum.* According to Ituarte (1997), who studied the oosorption process in *Eupera platensis* Doello Jurado, 1921, a South American sphaeriid, the germinal vesicle of degenerative oocytes are not phagocytosed as occurs with the cell cytoplasm; they remain in the lumen of the follicle. Assuming that the corpuscules may be nuclei of degenerated oocytes, this phenomenon could explain their presence in the gonad of *P. amnicum* if other signs of oosorption are found, and their presence indicates a recent spawning process.

Our results suggest that cross-fertilization in *P. amnicum* takes place in the inner gills of the parents, where the meiosis of the ova starts. Nevertheless, Araujo & Ramos (1997), reported and illustrated one specimen in which most of the oocytes, with a diameter between 40–60 μm, had lost the nuclear membrane and appeared in meiotic metaphase inside the ovary. These authors also reported several cases suggesting that intrafollicular fertilization can occur in *P. amnicum*.

Although no gravid specimens appeared among those histologically studied from June and July in this study, some gravid ones were detected by dissections of survivors from a previous cycle (see Araujo et al., in press), suggesting that the fertilization process might begin before August.

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