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LATE GAMETOGENESIS IN *Leptodactylus labyrinthicus* (Amphibia, Anura, Leptodactylidae) AND SOME ECOLOGICAL CONSIDERATIONS

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ABSTRACT

Histological aspects of late gametogenesis in *Leptodactylus labyrinthicus* and of unfertilized oocytes collected from clutches in the field were studied by light microscopy. Specimens were collected during the reproductive period to determine why only 10% of the oocytes deposited in foam nests are fertilized. Sections of ovaries and oocytes were stained with hematoxylin and eosin, mercury bromophenol blue and toluidine blue. During the reproductive phase, the ovaries were completely developed and consisted of a sack-shaped, multilobular structure, with each lobe containing many oocytes in advanced developmental stages. Atretic oocytes were also seen in the ovaries during the reproductive phase. Oocyte development in the ovaries was considered synchronous, although few oocytes were seen in the early developmental stages. There were no differences in the morphology or staining of oocytes in the ovary and of unfertilized oocytes. Testicular development was synchronic with that of the ovary, with the testes also being fully developed during the reproductive period. Each seminiferous tubule had many cysts containing all of the phases of spermatogenesis, especially spermatids with different levels of nuclear condensation. Free spermatozoa were also observed in the lumen of the seminiferous tubule. The significant proportion of unfertilized oocytes present in many clutches may indicate that males produced an insufficient number of spermatozoa to fertilize all of the oocytes or that females deposited additional oocytes subsequent to spawning. These unfertilized oocytes are ingested by the larvae and may represent a reproductive strategy for increasing tadpole survival.

Key words: Anura, development, gametogenesis, *Leptodactylus labyrinthicus*, reproduction

INTRODUCTION

Gametogenesis in ectothermic vertebrates has been divided into many stages or phases according to the nuclear and cytoplasmatic changes. The number of stages described for each animal group varies considerably, depending mainly on the authors' criteria and species peculiarities [1,12]. However, in general, oogenesis in vertebrates may be divided into previtellogenic and vitellogenic, or primary and secondary growth stages [24]. Spermatogenesis may also be classified into pre-spermiogenic and spermiogenic stages [6,18].

As in most vertebrates, in female anurans meiosis in diplotene (prophase of the first meiotic division) is suspended until ovulation. At this stage, chromosomes in oocyte nuclei decondense and a large amount of RNA is translated from some specific chromatin regions [2,5,9,13,15]. This RNA is condensed into numerous, small, nucleoli-like structures known as micronucleoli, that are distributed close to the inner face of the nuclear envelope [7,8,12,14,25]. A large, vesicular nucleus with an irregular nuclear envelope and numerous peripheral micronucleoli is characteristic of young pre-vitellogenic oocytes in the primary growth stage [1,8,12,24]. The secondary growth stage involves preparation for reproduction, with the formation of cortical granules and vitellogenesis that culminates in ovulation [24].

In contrast to oogenesis, meiosis in male germ cells is completed before spermatogenesis is finished [2]. The pre-spermiogenic stage is characterized by mitotic division (proliferation) of the spermatogonia and meiosis, that will give rise to the spermatids [6]. The spermatids subsequently undergo a series of changes involving chromatin condensation, elimination of cytoplasm, and flagellum formation to produce the spermatozoa, a process referred to as spermiogenesis [6,18].

The frog *Leptodactylus labyrinthicus* is a member of the family Leptodactylidae, currently placed in the *Leptodactylus pentadactylus* group [19,20]. The species occurs throughout open formations of central

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and northeastern Brazil, coastal Venezuela, and in more mesic vegetation formations from southeastern Brazil to Misiones, Argentina [20]. The reproductive mode of this species consists of eggs embedded in white foam nests that are deposited in depressions at the edges of ponds; exotrophic tadpoles subsequently develop in the water [19,22].

The percentage of anuran eggs that is fertilized generally varies from 75% to 100% [3,4,23]. However, only 10% of the eggs deposited in foam nests of *L. labyrinthicus* are fertilized, with the unfertilized eggs being consumed by the tadpoles [22]. In this work, late gametogenesis was studied in the ovaries and testes of *L. labyrinthicus* during the reproductive period in order to understand why so many oocytes in the foam nests were not fertilized. The morphology of the unfertilized eggs collected from recently deposited clutches was also compared with that of oocytes in the ovaries.

MATERIAL AND METHODS

One adult male (SVL=142.2 mm) and one gravid female (SVL=127.0 mm) of *L. labyrinthicus* were collected in the municipality of Rio Claro (22°25' S, 47°33' W), in São Paulo State, southeastern Brazil. The frogs were collected during the reproductive period, in October 2002, in order to ensure that the gonads were completely developed. A recently deposited clutch, which was also collected in October 2002, in the municipality of Rio Claro, contained ca. 1,817 eggs, but only 11.4% were fertilized. The clutch and specimens were preserved and deposited in the Célio F. B. Haddad collection, housed in the Departamento de Zoologia, Universidade Estadual Paulista, Rio Claro (male, CFBH 6020; female CFBH 5551).

Light microscopy

Samples from the gonads of the adult male and female and from unfertilized eggs were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 24 h. After rinsing in the same buffer, the tissues were dehydrated in an increasing ethanol series (70 - 95%), (10 min for each concentration, and embedded in Leica Historesin: sections 5 µm thick were cut and stained with hematoxylin and eosin. The sections of ovary were also stained with toluidine blue and with mercury bromophenol blue (300 ml of 2% acetic acid, 0.15 g of bromophenol blue and 3 g of $HgCl_2$) for at least 2 h to assess variations in the pattern of chromatin condensation and in the protein content of the oocytes, respectively. The sections were subsequently rinsed in 0.5% acetic acid in butylic acid, for 5 min each. The sections were mounted in balsam and observed and photographed in a Zeiss photomicroscope.

The maturation of frog oocyte has been divided into six stages (I-VI) based on the oocyte size and nuclear-cytoplasmatic changes seen in *Xenopus laevis* [12]. This classification, which has been applied to other species, was used to determine the oocyte stages in *L. labyrinthicus*.

Atretic oocytes were not considered as an oocyte stage.

The standard oocyte diameter in each stage was calculated based on measurements of 11 oocytes from each stage. The results measurements were obtained from photomicrographs containing appropriate scale bars and presented an accepted standard deviation. Anova test was used for statistical analysis, with $p< 0.5$ considered significant.

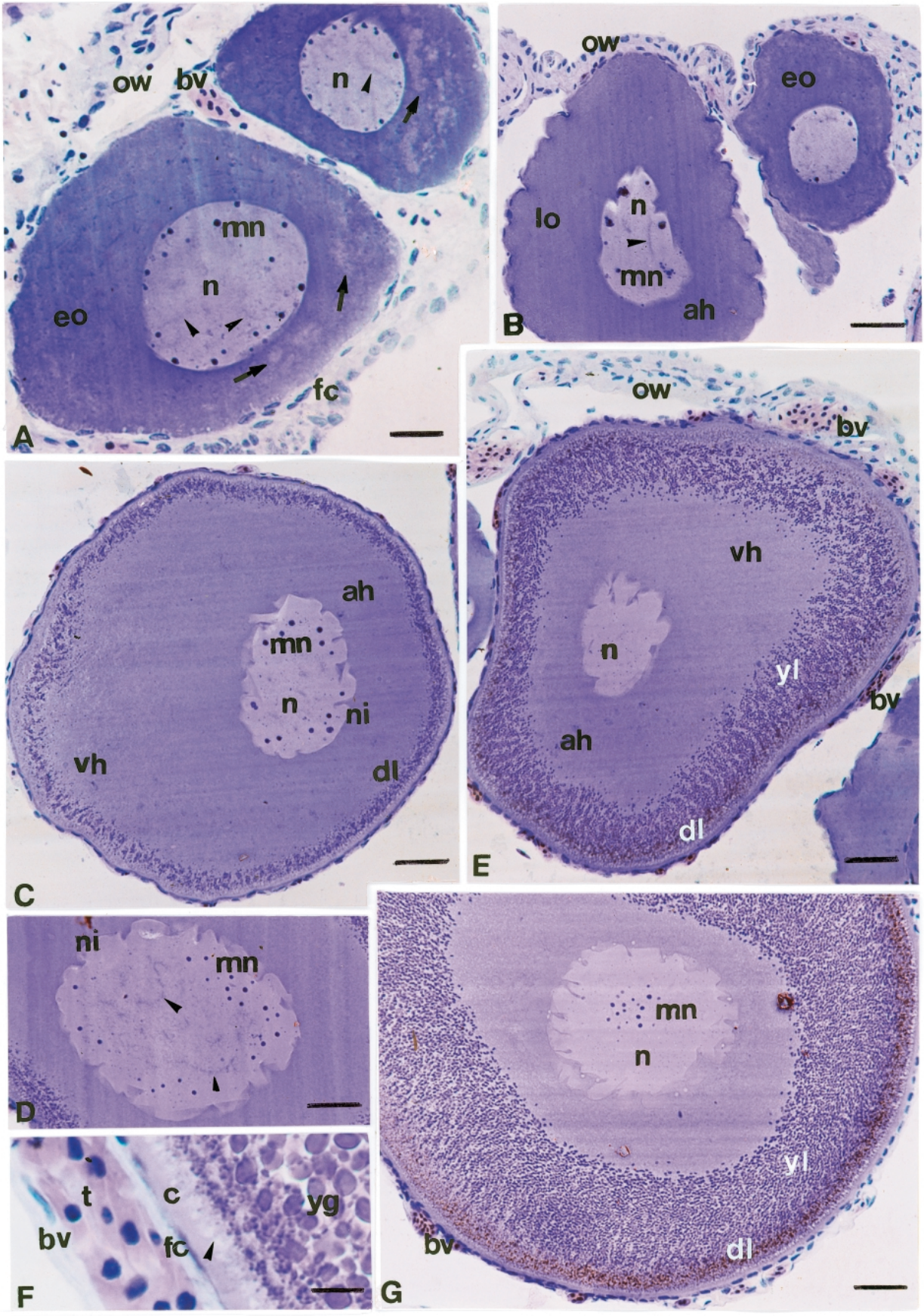
RESULTS

Oogenesis

The ovaries of *L. labyrinthicus* were paired, latero-dorsal, sack-shaped structures, each consisting of many lobes. Each lobe contained groups of oocytes in almost the same developmental stage.

Oogonia were not observed in the ovaries during the reproductive phase, although a few oocytes in early developmental stages occurred in one lobe along with many others in advanced stages. The first oocyte developmental stage (stage I) is a pre-vitellogenic stage. Stage I oocytes were globular cells, about 300 µm in diameter, that were always found attached or very close to the ovarian wall (Fig. 1A,B) and were the smallest cells in the ovary during the reproductive phase. Early stage I oocytes had a large, central, spherical nucleus with decondensed chromatin and many micronucleoli, located mainly on the inner face of the nuclear envelope (Fig. 1A).

Figure 1A. Early stage I oocytes (**eo**) attached to the ovary wall (**ow**). **Arrowheads** = lumpbrush chromosomes, **bv** = blood vessel, **fc** = follicular cells, **mn** = micronucleoli, **n** = nuclei. HE, Bar = 50 µm. **B**. Late stage I oocytes (**lo**). Note the irregular contour of the nucleus (**n**) and the more basophilic, homogeneous cytoplasm compared to the early oocyte (**eo**). In late stage I oocytes, the nucleus (**n**) is positioned in the animal hemisphere (**ah**). **Arrowhead** = lampbrush chromosome, **ow** = ovary wall. HE, Bar = 200 µm. **C**. Stage II oocyte showing deposition of a dense, granular layer (**dl**). Note the nucleus (**n**) located in the animal hemisphere (**ah**), the nuclear envelope infoldings (**ni**) and the micronucleoli (**mn**). **Vh** = vegetal hemisphere. HE, Bar = 200 µm. **D**. Stage II nucleus in which the nuclear infoldings (**ni**) can be seen. Note the many micronucleoli (**mn**) associated with the lampbrush chromosomes (**arrowheads**). HE, Bar = 200 µm. **E**. Deposition of the first yolk layers (**yl**) in a stage III oocyte. Note the dense, granular layer (**dl**) at the oocyte periphery, the nucleus (**n**) located in the animal hemisphere (**ah**), and the weak yolk and dense granule deposition in the vegetal hemisphere (**vh**). **bv** = blood vessel, **ow** = ovary wall. HE, Bar = 200 µm. **F**. Stage III oocyte, showing the theca (**t**) surrounding the very flat follicular cells (**fc**). **Arrowhead** = thin projection of the oocyte plasma membrane, **bv** = blood vessel, **c** = first chorion layer, **yg** = yolk granules. HE, Bar = 62 µm. **G**. Stage IV oocyte, showing the migration of micronucleoli (**mn**) to the center of the nucleus (**n**). Note that the dense, granular layer (**dl**) is darker in the animal hemisphere and that the yolk layer (**yl**) is thicker. **bv** = blood vessel. HE, Bar = 200 µm.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The cytoplasm of early stage I oocytes was basophilic because of the large amount of RNA present in this phase. This RNA was produced by the expanding lampbrush chromosomes that were discernible in the nuclei of oocytes in stages I and II (Fig. 1A,D), and agglutinated as micronucleoli. The material produced by the loops of the lampbrush chromosomes during transcription to form the micronucleoli was released from the nucleus through the nuclear pores and aggregated in the cytoplasm to form material called nuage. This material associates with mitochondria and membranes to form Balbiani bodies. The weakly stained, granular material seen in certain regions of the oocyte cytoplasm (Fig. 1A) probably represented nuage or Balbiani bodies.

Each oocyte in stage I was already enveloped by a layer of follicular cells (Fig. 1A). From early to late stage I, the oocyte almost tripled in size to reach a diameter of about 1000 µm. By this late stage, the nucleus was also much larger and the micronucleoli were located exclusively at the nuclear periphery. In late oocyte stage I, the cytoplasm was intensely basophilic and homogeneous, and no longer showed weakly stained regions (Fig. 1B). The nucleus became irregular in contour and moved to the future animal hemisphere (Fig. 1B). The region of attachment of the oocyte to the ovary wall bore no relationship to the orientation of the nucleus.

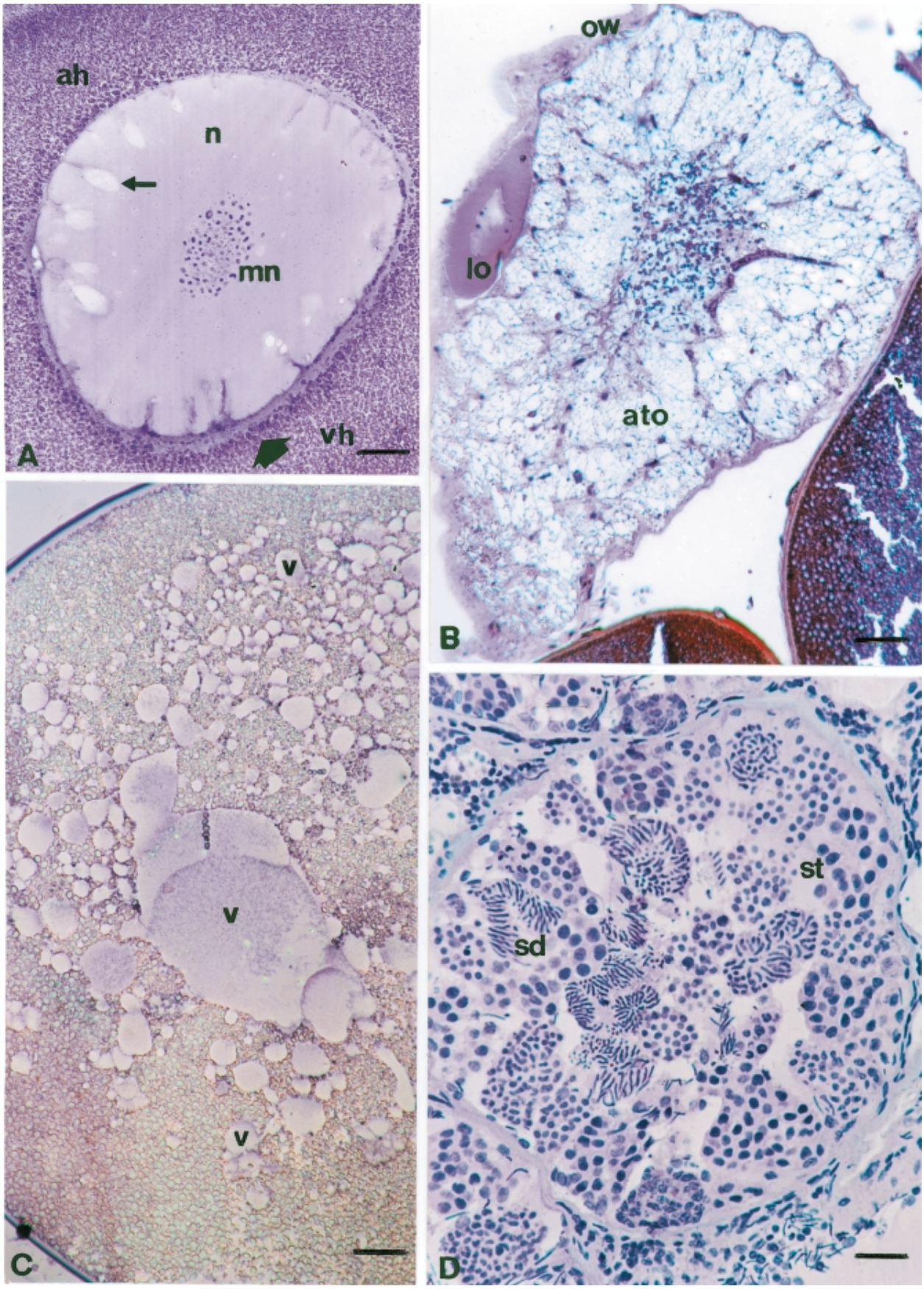
Stage II oocytes were about 1700 µm in diameter. The large nucleus (about 620 µm in diameter) had many infoldings of the nuclear envelope (Fig. 2C,D), but showed no specific localization or concentration. The micronucleoli that were previously very close to the inner face of the nuclear envelope withdrew from the envelope and formed a halo at the nuclear periphery (Fig. 1C,D). Lampbrush chromosomes were clearly visible in the nuclei and were associated with the micronucleoli (Fig. 1D). In this stage, the oocytes were detached from or close to the lateral wall of the ovary. The cytoplasm was not as basophilic as before and contained a layer of small granules at the periphery (Fig. 1C), that stained weakly with mercury bromophenol blue and was purple after staining with hematoxylin and toluidine blue (Fig. 1C,E,G). Deposition of the chorion was initiated by the follicular cells, which acquired microvilli. Blood vessels were more frequent around the oocytes in this stage.

Vitellogenesis started in stage III, with the deposition of yolk granules at the periphery at first, followed by their migration to the oocyte center and increased yolk deposition (Fig. 1E). These granules stained intensely with mercury bromophenol blue, toluidine blue, and eosin (Fig. 1E-G). Some layers of the chorion had already been deposited and the follicular cells were very flat, with microvilli that were indistinguishable from those of the oocyte plasma membrane (Fig. 1F). Blood vessels were more frequent in the oocyte theca (Fig. 1F). The nucleus was located in the animal pole, the micronucleoli started to migrate to the center of the nucleus, and lampbrush chromosomes were no longer seen (Fig. 1E). The strongly stained granules, initially deposited during stage II, persisted and were easily differentiated from the yolk granules. These stained-granules formed a layer beneath the oocyte plasma membrane (Fig. 1E). Oocytes in stage III were slightly larger than those in stage II (about 2000 µm in diameter).

Stages IV, V, and VI were not very discernible. The oocyte size increased considerably from stage III to stages IV-VI and reached about 5000 µm in diameter at stage V. This increase in size resulted entirely from expansion of the cytoplasm since the nuclear diameter (about 1300 µm) remained the same from stage II onwards. The main characteristic of stage IV oocytes in *L. labyrinthicus* was the migration of the micronucleoli to the center of the nucleus and the presence of a halo free of yolk granules around the nucleus (Fig. 1G). The animal hemisphere contained the nucleus, small yolk granules, and a very dark layer of granules (Fig. 1G).

During stage V, the cytoplasm was filled with yolk granules (Fig. 2A) and the infoldings of the nuclear envelope became deeper and acquired a racket-like aspect; these infoldings were more concentrated towards the animal pole (Fig. 2B). Some granular ma-

Figure 2 A. Detail of a nucleus (**n**) in a stage IV oocyte. Note the aggregation of micronucleoli (**mn**) and heterochromatin in the center of the nucleus. The nuclear infoldings have become deeper and produce some racket-like expansions (**arrow**) that are reduced in the vegetal hemisphere (**vh**). **ah** = animal hemisphere. Note the very thin space (arrowhead) formed around the nucleus (**n**) that is free of yolk granules and darker stained in the vegetal hemisphere (**vh**). HE, Bar = 200 µm. **B**. Detail of an atretic oocyte (**ato**), showing many vacuoles and remaining yolk granules dark stained in the center. A late stage I oocyte (**lo**) can be seen attached to this atretic oocyte and to the ovary wall (**ow**). HE, Bar = 500 µm. **C**. Detail of an unfertilized oocyte, showing many vacuoles (**v**). HE, Bar = 500 µm. **D**. Detail of a fully developed testicular tubule of *L. labyrinthicus* collected in the same period as the female gonads. Note the many cysts of spermatocytes (**st**) and spermatids (**sd**) with different degrees of chromatin condensation. HE, Bar = 150 µm.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terial was present within the vesicular portion of these infoldings. Strongly stained material was present around the nucleus and the halo free of yolk granules became thinner (Figs. 1G and 2A), and appeared to be displaced to the vegetal hemisphere of the nucleus (Fig. 2A). The micronucleoli showed some vacuolization and aggregates in the center of the nucleus, together with other thin granulations (Fig. 2A) that probably represented condensed chromosomes. The chorion was well developed, with the follicular cells being very flat or in an inactive state. The blood vessels around the oocytes were highly developed. In stage VI, the nucleus was usually no longer visible and the oocytes had a diameter of ~ 1000 μm. Mature oocytes (stages V-VI) predominated in the ovaries and no oogonia were observed.

Some oocytes may degenerate inside the ovaries to produce a condition known as atresia (possible stage VII). These atretic oocytes formed an irregular vacuolated mass with some yolk granules remaining inside (Fig. 2B). By analogy with other vertebrates, this structure may be referred to as a *corpus atreticus*.

No marked morphological differences were observed between developing oocytes in the ovaries and unfertilized oocytes from clutches collected in the field, except for the presence of some signs of degeneration (vacuolization of the cytoplasm) in unfertilized eggs (Fig. 2C).

Spermiogenesis

Histological analyses of late spermatogenesis in a male *L. labyrinthicus* during the reproductive phase showed that the testes were fully developed (Fig. 2). The testes where paired organs, each consisting of a yellowish kidney-shaped structure (1.0 x 0.4 cm) encapsulated by a thick layer of connective tissue. Each testis had many seminiferous tubules that contained various cysts or groups of synchronously developing germ cells in the wall (Fig. 2D). Free spermatozoa were present in the lumen of the seminiferous tubule following rupture of the cysts. A large number of seminiferous tubules contained more spermatocytes and early spermatids than free spermatozoa (Fig. 2D).

DISCUSSION

Although few immature oocytes were present in the ovaries, oocyte development in *L. labyrinthicus* was considered to be synchronous in the reproductive period, with a predominance of mature oocytes. The *L. labyrinthicus* oocyte stages were classified according to Dumond [12], although some of our results did not coincide completely with the original classification which was based on oocytes of the frog *Xenopus laevis*. The main differences were in stages IV to VI. In contrast to *L. labyrinthicus*, in *X. laevis* the region of attachment of the oocyte to the ovary wall has no relationship to the orientation of the nucleus, as also observed for other amphibians [12]. In addition, the infoldings of the nuclear envelope seen in early stage II oocytes were more frequent in the vegetal hemisphere in *X. laevis* [12] than in *L. labyrinthicus.*

According to Dumond [12], the disperse, weakly stained material seen in stage I oocyte cytoplasm in *X. laevis* is composed of lipids, nuage and Balbiani bodies, as shown by transmission electron microscopy. Balbiani bodies are involved in yolk formation or mitochondrial replication, although Clerót [7] and Eddy [14] have shown that the nuage may also contribute to the oocyte germ plasm. In *X. laevis*, the first layer of granules to appear at the periphery of stage II oocytes is composed of cortical granules, mitochondria, small yolk platelets, lipids, and mainly pre-melanosomes [12].

The structure and morphology of *L. labyrinthicus* testes were similar to those of other anurans in the same family, including *Pleurodema thaul* [11] and *Physalaemus cuvieri* [21]. Cysts or groups of synchronously developing germ cells were seen in the testes of *L. labyrinthicus*. Oliveira *et al*. [21] also observed these structures in the testes of *P. cuvieri* and suggested that since they occurred in other anurans, the arrangement of germ cells in cysts must be an important trait of anuran amphibians and of other anamniotes [17]. Although the number of early-developed spermatids in the seminiferous tubules was greater than that of free spermatozoa, full development of the male gonads apparently coincided with the spawning of reproductive females in *L. labyrinthicus*. A similar result was found by Díaz-Páez and Ortiz [11] for another leptodactylid, *P. thaul*, in Chile.

Following ovulation, oocytes complete the first meiotic division and are ready to be fertilized. However, in *L. labyrinthicus*, only about 10% of the eggs are fertilized, with the unfertilized eggs serving as a supplementary food source for the tadpoles, which can survive in the nest for almost 30 days before entering water to complete their metamorphosis [22]. Histological analysis of developing and unfertilized

oocytes revealed no marked morphological differences between these two groups, except for some signs of degeneration (extensive vacuolation of the cytoplasm) in the unfertilized eggs.

There are at least two possible explanations for the large proportion of unfertilized eggs in foam nests of *L. labyrinthicus*. The first explanation is based on the occurrence of a large number of seminiferous tubules containing more early-developed spermatids than free spermatozoa. This may indicate that the number of spermatozoa produced by males is insufficient to fertilize all of the eggs, and may represent a reproductive strategy in which there is excessive egg production. An assessment of the number of spermatozoa produced is needed to confirm this hypothesis**.** Histological analysis of the testes of another species, *Leptodactylus fuscus*, revealed the same developmental pattern as seen in *L. labyrinthicus*, with many lobes containing no mature spermatids or spermatozoa (unpublished results). However, in contrast to *L. labyrinthicus*, almost all *L. fuscus* eggs are fertilized (C.P.A. Prado, pers. obs.).

A second, more plausible explanation is based on the reproductive behavior of *Leptodactylus fallax*, another species in the *L. pentadactylus* group. This species has a totally terrestrial mode of reproduction, with the eggs in foam nests being deposited in burrows far from water. In this case, the tadpoles develop within the nest [10,22]. The females of *L. fallax* return to the nest after spawning to deposit additional oocytes to nourish the tadpoles [16]. We suggest that a similar behavior could also be present in *L. labyrinthicus*, which would explain the lack of fertilization of a large number of oocytes. Since *L. labyrinthicus* occurs mainly in regions with a seasonal rainfall [20], the additional eggs would serve as a very rich supplementary food source for the developing tadpoles in such habitats by functioning as trophic eggs and enhancing larval survivorship.

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