Received: 14 May 2018 | Revised: 5 December 2018 | Accepted: 26 December 2018

DOI: 10.1002/jmor.20950

WILEY JOURNAL OF morphology

RESEARCH ARTICLE

Ultrastructural and histochemical study of previtellogenic oogenesis in the desert lizard *Scincus mitranus* (Squamata, Sauropsida)

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Funding information
Deanship of Scientific Research, King Saud University, Grant/Award Number: RG-164

Abstract

The structure of the granulosa in reptilian sauropsids varies between groups. We investigated the follicle development in the desert lizard *Scincus mitranus*. In the germinal bed, oogonia, and primary oocytes were identified and found to be interspersed between the epithelial cells. Previtellogenesis was divided into three stages: early, transitional, and late previtellogenic stages. During the early previtellogenic stage (diplotene), the oocyte is invested by small epithelia cells that formed a complete single layer, which may be considered as a young follicle. The transitional previtellogenic stage was marked by proliferation and differentiation of the granulosa layer from a homogenous layer consisting of only small cells to a heterogeneous layer containing three cell types: small, intermediate, and large cells. The late previtellogenic stage was marked by high-synthetic activity of large cells and the initiation of cytoplasmic bridges between large granulosa cells and the oocyte. Small cells were the only type of granulosa cells that underwent division. Thus, these cells may be stem cells for the granulosa cell population and may develop into intermediate and subsequently large cells. The intermediate cells may be precursors of large cells, as suggested by their ultrastructure. The ultrastructure of the large granulosa was indicative of their high synthetic activity. Histochemical analysis indicated the presence of cholesterol and phospholipids in the cytoplasm of large cells, the zona pellucida, among the microvilli, in the bridges region, and in the cortical region of the oocyte cytoplasm. These materials may be transferred from large cells into the oocyte through cytoplasmic bridges and provide nutritive function to large cells rather than functioning in steroidogenesis or vitellogenesis.

KEYWORDS

follicle development, reptillian

1 | INTRODUCTION

In vertebrates, the growing oocyte is associated with auxiliary cells that perform essential functions during the initial phase of oogenesis. These cells are frequently involved in the formation of the vitelline membrane and have been implicated in the production of hormones associated with maturation and ovulation. However, and as an exception to this, the large cells of the granulosa of squamates, which have been suggested to be derived from germ cells, are equivalent to nurse cells in *Drosophila melanogaster* (Timmons, Mondragon, Meehan, & McCall, 2017). During early development, the vertebrate oocyte becomes surrounded by cells from the adjacent epithelium which constitutes the "granulosa layer" of the ovarian follicle. When an oocyte is surrounded by a layer of small granulosa cells, it is referred to as a primordial follicle (Tokarz, 1978).

The structure of the granulosa in reptilian sauropsids varies between groups: Testudines (turtles), Sphenodontida, Squamata (lizards and snakes), and crocodiles (Arrieta, Sandova, & Alvarez, 2017; Hernandez-Franyutti, Uribe-Aranzabal, & Guillette, 2005; Machado-Santos, Santana, Vargas, Abidu-Figueiredo, & de Brito-Gitirana, 2015;

Vieira, Perez, & Ramırez-Pinilla, 2010). In crocodiles, sphenodon, and turtles, the granulosa layer remains as a single layer of small cells throughout follicle development (Narbaitz, 1973; Hubert, 1985). In squamates, however, the granulosa layer first develops as a single layer of small cells, but later differentiates into three different types of cells: small, intermediate, and large pyriform cells (Boyd, 1940; Eimer, 1872; Filosa, Tadde, & Andrecuttei, 1979; Gegenbaur, 1861; Hubert, 1971a, 1971b, 1976; Klosterman, 1987; Laughran, Larsen, & Schroeder, 1981). However, there are exceptions, such as in lizards where the granulosa layer persists throughout development as a single layer (Goldberg & Bezy, 1974), with only two types of cells (small and large) in the granulosa layer rather than three cell types (Corso, Lissia Frau, & Pala, 1978; Ibrahim, 1977; Ibrahim & Wilson, 1989).

The ultrastructure of oogenesis of lizards has been described in few species, including *Zootoca vivipara* (Hubert, 1970b), *Lacerta sicula* (Filosa & Taddei, 1976), *Acanthodactylus scutellatus* (Bou-Resli, 1976), and *Elgaria coerulea* (Klosterman, 1983). Notably, relatively few species of squamates have been studied (Vieira et al., 2010), including the family Scincidae, the largest family of lizards. The presence of both oviparous and ovoviviparous species in this family indicates the presence of corresponding diversity of the granulosa and oocyte structures. This study conducted the first ultrastructural investigation on the folliculogenesis of the Arabian lizard species *Scincus mitranus*. There were two main objectives to this study: (1) to document the ultrastructural characteristics of follicle development in neglected groups of the family Scincidae and determine whether these characteristics have undergone changes in response to the desert environment, and (2) improve the understanding of cellular features of the histology and cytology of the developing follicle in lizards, particularly during the previtellogenic phase. This included the investigation of two types of lipids, cholesterol and phospholipids, which were examined using histochemical analysis. These lipids were chosen because of thier well-known contribution to the construction of the cell plasma membranes (PM), and therefore presumably to the oolemma of rapidly growing oocytes. Also, cholesterol is a precursor of steroid.

2 | MATERIALS AND METHODS

2.1 | Animal collection and housekeeping

This study (including capturing, handling, and killing of the animals) was approved by the Research Ethics Committee at King Saud University (Anderson, 1871). Thirty adult female specimens of the sand skink, *Scincus mitranus* (Anderson, 1871), were captured during the active sexual period (April to May) from the Thumamah region (25° 10′ N, 46° 50′ E), north–east of the city of Riyadh, Saudi Arabia. Animals were housed in separate cages and maintained for short periods in plexiglass boxes filled with 10 cm of clean sand. Only 19 female animals were used for this investigation whereas the rest of females were used for another study. They were sacrificed by ether anesthesia and dissected to remove the ovaries.

2.2 | Tissue preparation for light microscopy

For light microscopy, the ovaries isolated from seven adult females were fixed in 10% buffered formalin or Bouin's fluid and preserved in 70% alcohol. Histological sections were prepared with a section thickness of 5 μm and stained with hematoxylin and eosin.

2.3 | Tissue preparation for transmission electron microscopy

Ovarian tissues from different four females were cut into cubes (1 mm^3) and fixed in 3% buffered glutaraldehyde for 4 hr at 4 °C (0.1 mol L^{-1} sodium cacodylate buffer; pH: 7.2). Samples were then fixed in 1% osmium tetroxide for 2 hr. Dehydration of the tissues was performed using ascending grades of ethanol, and then the samples were cleared in propylene oxide before embedding in pure resin (SPI, Toronto, Canada; Reynolds, 1963).

Semithin sections were cut using a glass knife to locate the study area. Ultrathin sections (50–65 nm) were then cut using an ultramicrotome (Leica, UCT; Wetzlar, Germany) with a diamond knife (Diatome, Hatfield, PA); sections were placed on 300-mesh copper grids and stained with uranyl acetate (20 min) and lead citrate (5 min). Micrographs were taken using a transmission electron microscope (JEOL JEM-1011, Tokyo, Japan) operating at 80 kV using Tengra™ (TEM CCD camera and iTEM software, Olympus, Tokyo, Japan) at the Central laboratory, King Saud University. Electron micrographs were finalized using Adobe Photoshop CS 5.1.

2.4 | Detection and localization of cholesterol and phospholipids in the oocyte and the granulosa layer

2.4.1 | Cholesterol

The method used in this investigation for cholesterol detection is similar to that described by Scallen and Dietert (1969). It is based on the use of digitonin in the gluteraldehyde fixative, omission of propylene oxide during processing and the use of Epon 812 as the embedding media.

The use of digitonin in the primary fixative clearly enhances the morphological preservation of lamellated structures and membranes. Ovarian tissues from four animals were fixed for about 20 hr at room temperature, washed in 0.1 mol L^{-1} Na-cacodylate for 2 hr and then post fixed in 1% osmium tetroxide for 2 hr. After, the tissues were washed in 0.1 mol L^{-1} Na-cacodylate for an hour prior to dehydration in graded concentrations of ethanol. Then, they were infiltrated with a mixture of 70% ethanol and Epon 812 as follows:

- Two parts of 70% ethanol + one part of Epon 812 for 2 hr at room temperature.
- One part of 70% ethanol + one part of Epon 812 for 2 hr at room temperature.
- One part of 70% ethanol + two parts of Epon 812 for 2 hr at room temperature.
- Leave in fresh Epon 812 for 4 hr and then into another change of Epon 812 for overnight.

Finally, the tissues were embedded into a fresh Epon 812 and leaved to polymerize for 24–48 hr. Tissues are then cut and stained with the double staining procedure (uranyl acetate and lead citrate).

2.4.2 | Phospholipids

The identification and localization of phospholipids in the oocyte and the granulosa layer cells of *S. mitranus*, is based on that used previously by Bluemink (1972) with slight modification. This method is mainly based on the use of potassium ferricyanide and calcium chloride in the secondary fixative (osmium tetroxide) after primary fixation in Karnovsky's fixative.

After the primary fixation of ovarian tissues from four animals in Karnovsky's fixative for about 12 to 20 hr, tissues were washed in two changes of 0.1 mol L^{-1} Na cacodylate buffer for 2 hr. After washing in buffer they were postfixed for 3 hr in a modified fixative containing 1% osmium tetroxide, 0.05 mol L^{-1} potassium ferricyanide and 0.05 mol L^{-1} calcium chloride in 0.1 mol L^{-1} Na-cacodylate buffer at pH 7.2. Tissues are then washed in two changes of cold 0.1 mol^{-1} Na-cacodylate buffer for about an hour prior to dehydration. The dehydration and embedding procedure is similar to that described earlier in the method for cholesterol detection.

3 | RESULTS

3.1 | Light microscopy

Two germinal beds were found on the dorsal surface of each ovary. Three layers were distinguished in the germinal bed area: (1) outer ovarian epithelium (OOE), (2) inner ovarian epithelium, and (3) intermediate stromal layer (Figure 1a). In the germinal bed, oogonia, and primary oocytes were identified and found to be mixed with epithelial cells (Figure 1b). Oogonia were observed in the OOE, with most of these cells in interphase (Figure 1b). During a later stage, the oogonia increased slightly in size and became oocytes, but they remained in the OOE layer. At the diplotene stage, the oocyte is surrounded by small epithelia cells and moved from the germinal bed area toward the stroma layer (STR; Figure 1b). At this stage, the oocyte was surrounded by a complete, single layer of small cells and was considered as a young (primordial) follicle.

The follicles may be broadly divided into previtellogenic follicles (without yolk deposition) and vitellogenic follicles (with yolk deposition). Previtellogenesis is a stage of development that includes a variety of follicles, ranging from primordial follicles, which are confined to the germinal bed, to large follicles immediately prior to vitellogenesis. Thus, based on the size of the oocyte and structure of the associated granulosa, previtellogenic follicles can be divided into three stages: early, transitional, and late previtellogenic stages.

During the early previtellogenic stage, the oocyte was surrounded by the granulosa layer that appeared as a single layer consisting only of small cells (Figure 1b,c). The germinal vesicle (GV) in most cases was eccentric in position, had a round to oval shape, and occupied a large proportion of the oocyte (Figure 1d). A striking feature of this stage was the presence of nuclei in the oocyte cytoplasm (Figure 1c, d). The early previtellogenic follicles were surrounded by a thin layer of fibroblasts and collagen fibers, which formed the initial thecal layer (Figure 1d,e).

At the beginning of the transitional previtellogenic stage, follicles were found in the area adjacent to the germinal bed and moved toward the interovarian space. This stage was marked by the proliferation and differentiation of the granulosa layer from a homogenous layer consisting of only small cells to a heterogeneous layer containing three cell types: small, intermediate, and large cells (Figure 1e,f). Thus, the granulosa layer was thicker in some regions than in others because some parts of the granulosa layer were occupied by small, intermediate, and large cells, whereas other parts were occupied only by small cells (Figure 1g).

The onset of the late previtellogenic stage was marked by the initiation of cytoplasmic bridges between large granulosa cells and the oocyte (Figure 1h). The large cells clearly dominated the granulosa layer, which also contained small and intermediate cells (Figure 1i,j). Termination of the late previtellogenic stage was indicated by the appearance of yolk granules in the ooplasm and breakdown of cytoplasmic bridges followed by degeneration of large granulosa cells. The granulosa layer decreased in thickness and eventually reverted to a single layer of small cells, marking the start of the vitellogenic stage (Figure 1k).

3.1.1 | Transmission electron microscopy

3.2 | Germinal bed

The oogonia (20–40 μm in diameter) varied in appearance, depending whether they were in interphase, undergoing mitosis, or entering meiosis. Most oogonia in the germinal bed appeared to be in interphase and exhibited some distinct characteristics. They had a large and round nucleus containing relatively diffuse chromatin dispersed as tiny granules in the nucleoplasm, and occasionally in small scattered clumps (Figure 2a). The nucleoli were single or multiple, ring-shaped, and condensed or vacuolated. The oogonia ooplasm generally contained relatively few organelles which aggregated near the nucleus. Mitochondria were the most common organelles, and were most often elongated, and in few cases dumb-bell shaped, circular, or vacuolated. Many small vesicles were also observed to be scattered in the ooplasm.

With the onset of meiosis, the oogonia increased slightly in size and entered Prophase I of meiosis and thus became oocytes. Oocytes were oval or round in shape, with a large round nucleus (Figure 2b). During leptotene and zygotene, homologous chromosomes began to attach to each other to eventually form the synaptonemal complex (Figure 2c). During the diplotene stage, the nucleus was relatively large and had an irregular surface with diffuse chromatin, which was no longer organized in strands (Figure 2d). The nuclear envelope was studded with pores. The peri-nuclear space between the two nuclear membranes enlarged sometimes to contain large dense granules (Figure 2e). The cytoplasmic organelles remained aggregated toward the center and around the nucleus. Mitochondria, commonly showed an elongated shape, and were abundant in the central and cortical cytoplasm (Figure 2f). Golgi complexes were rarely observed, whereas many longitudinal elements of the smooth endoplasmic reticulum

(SER) and small lipid droplets appeared in the oocyte ooplasm (Figure 2d,f). Another prominent feature of this stage (diplotene) was the presence of various sizes of membrane-bound vesicles distributed randomly in the cytoplasm (Figure 2d,g). Some of these vesicles appeared to be empty, whereas others contained fibrous and electron dense materials (Figure 2g). Epithelial cells in the germinal bed region were connected by desmosomes, which were absent between the oogonia and granulosa epithelial cells (Figure 2h,i).

The main feature of the diplotene stage was the presence of nuclei in the ooplasm of the oocyte, some of which were surrounded by an intact nuclear envelope, while others were not (Figure 2j,k). Chromatin clumps were associated with the nuclear envelope and dispersed in the nucleoplasm. These nuclei were relatively large and appeared as dense clumps embedded in fibrous materials.

3.3 | Previtellogenic stage

3.3.1 | Early previtellogenic follicles

In early follicles (range from 40 up to 150 μm in diameter), PM of both the oocyte and granulosa cells were in close contact and the interface

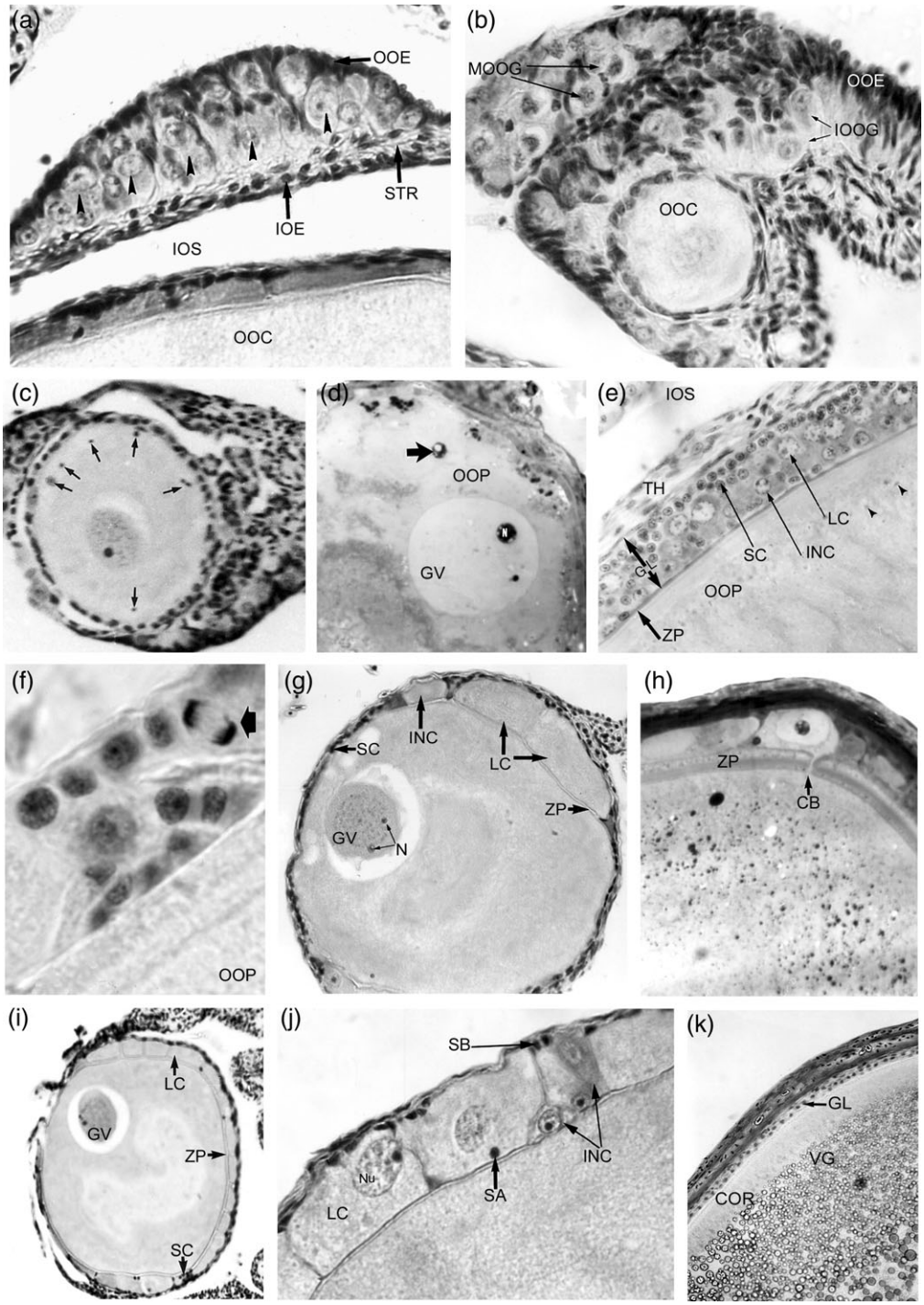

FIGURE 1. Legend on next page.

had a smooth outline (Figure 3a). With further growth of the oocyte, microvillus structures were developed in the zona pellucida region from both, the oocyte and the granulosa cells, which later increased in length and density and interdigitated with each other to increase their interaction surface (Figure 3c,f). In some areas, the granulosa cells were connected to the oocyte by desmosomes. At the end of this stage, many micropinocytotic vesicles appeared and budded off from the oolemma (Figure 3c). At this early previtellogenic stage, differences were not detected between granulosa cells, which were still small. They formed a single layer of oval to round epithelial cells (Figure 3a,c). They contained a relatively large nucleus and the cytoplasm contained numerous bundles of microfilaments (Figure 3d). These microfilaments were typically found in close association with desmosomes (Figure 3e). With respect to these bundles of microfilaments, elements of the rough endoplasmic reticulum were frequently found in the ooplasm (Figure 3f).

3.3.2 | Transitional previtellogenic follicles

Transitional previtellogenic follicles (range from 150 μm up to 1 mm in diameter) were mainly characterized by a granulosa layer that was irregular in thickness because it contained intermediate and developing large cells (Figure 4a). In the oocyte, the cytoplasmic organelles increased in number and size (Figure 4b), and aggregated in an area around the GV, were, distributed more evenly, both in the center and cortical region. The GV was found to be slightly eccentric in position. The nuclear envelope was studded with nuclear pores (Figure 4c). Membrane-bound vesicles, containing tubular, and granular materials, were occasionally found in contact with the inner side of the nuclear membrane (Figure 4c).

Additionally to the size and staining properties, the differences between the three types of cells in the granulosa layer were primarily in the relative abundance of organelles and their distribution. Small cells were dense and round to oval (Figure 4a). They contained a nucleus limited by an irregular nuclear membrane and were frequently observed to be undergoing mitosis (Figure 1e).

Intermediate cells stained more lightly than small cells but were slightly denser than large cells (Figure 4a). They were commonly found to be associated with the oolemma and were not undergoing mitosis. They showed a relatively large nucleus limited by a nuclear membrane with well-defined inner and outer membranes. The numerous microfilaments characteristic of the cytoplasm of small cells were not detected in intermediate cells.

Large granulosa cells showed a pale staining cytoplasm and a large round nucleus (Figure 4a,d). They had an oval shape and a nucleus that was eccentric in position, occupying approximately one-third of the large granulosa area. The nuclear membrane was well-defined with distinct inner and outer membranes with a clear perinuclear envelope (Figure 4e). Several small vesicles were closely associated with the outer nuclear membrane, suggesting their detachment from the nuclear envelope into the cytoplasm. The cytoplasm of the large cells was rich in various organelles. Particularly, the mitochondria tended to aggregate in a large mass resembling the mitochondria cloud of small oocytes (Figure 4f). The Golgi complex was prominent in the cytoplasm of large cells and typically adjacent to the plasma membrane and zona pellucida of early follicles. Darkly stained granules accumulated on the lamellar structure inside membrane-bound vesicles (Figure 4g).

A striking feature of this stage was the presence of numerous pinocytotic vesicles (PV) at the base of the microvilli (Figure 4h). Thus, as they developed, large granulosa cells increased in width and length to become flattened over the oocyte surface.

At the end of this stage, the granulosa layer was dominated by large cells which reached their maximum size (Figure 1h,i). The granulosa-oocyte interface increased in thickness because of the development of microvilli from both the oocyte and follicle cells. These microvilli from the oocyte were generally more prevalent and organized in shape compared to those in granulosa cells (Figure 4i).

During this transitional previtellogenic stage, the cytoplasmic contents of the oocyte were generally widely dispersed and more abundant than in the preceding stage. Particularly, darkly stained

FIGURE 1 *S. mitranus*, light microscopic view of ovarian sections stained in H&E. (a) Ovarian section through the germinal bed. The layer consists of an OOE and inner epithelium (IOE), which lines the ovarian cavity. The STR lies between the two epithelium layers. Note the oogonia with large round nucleus (arrowheads). (b) Germinal bed area: Mitotic oogonia (MOOG) which are intermixed with epithelia cells in the OOE. Note the interphase oogonia (IOOG) which have a large round nucleus. (c) Section though an oocyte at the early previtellogenic stage showing some migrated nuclei present in the ooplasm (arrows). (d) Detail of an oocyte at the early previtellogenic stage showing one of the migrated nuclei adjacent to the GV. A young diplotene oocyte approximately 50 μm in diameter is at the bottom of the germinal bed and clearly surrounded by a single layer of small epithelial cells. (e) Section through a follicle showing the granulosa layer (GL) consisting of small (SC), intermediate (INC), and large pyriform cells (LC). (f) Section through an oocyte at the transitional previtellogenic stage showing the granulosa layer containing small cells undergoing mitosis (large arrow). (g) Micrograph of a section showing an oocyte at the beginning of the transitional previtellogenic stage. Intermediate (INC) and large cells (LC) began to appear in the granulosa layer, whereas some parts of the granulosa layer are still occupied only by small cells (SC). The GV is eccentric and possesses two dense nucleoli (N). (h) Part of a follicle at the beginning of the late previtellogenic stage. Large cells surround the oocyte and cytoplasmic bridges (CB) connecting large cells to the oocyte are established. Note the numerous dark granules in the ooplasm. (i) Oocyte at the end of the transitional previtellogenic stage. Large cells (LC) occupy most of the granulosa layer. Scattered small cells (SC) among the large cells. (j): Ovarian section through the granulosa layer associated with an oocyte at the transitional previtellogenic stage. Small cells appear in apical (SA) and basal (SB) position as densely stained cells. Intermediate cells (INC) possess a less stained cytoplasm, whereas large cells possess slightly stained cytoplasm and relatively large nucleus (NU). (k) Part of a follicle at the end of the previtellogenic stage. The granulosa layer (GL) reverts to a single layer of small cells only. Note the presence of vitellogenic granules (VG) in the ooplasm. CB = Cytoplasmic bridge; COR = Cortical region; GL = Granulosa layer; GV = Germinal vesicle; INC = Intermediate granulose cells; IOE = Inner ovarian epithelium; IOOG = Interphase oogonia; IOS = Inner ovarian space; LC = Large granulosa cells; MOOG = Mitotic oogonia; N = Nucleolus: NU = Nucleus; OOC = Young oocyte; OOE = Outer ovarian epithelium; OOP = Ooplasm; SA = Apical small cells; SB = Basal small cells; SC = Small granulosa cells; STR = Stroma layer; TH:; VG = Vitellogenic granules; ZP = Zona pellucida

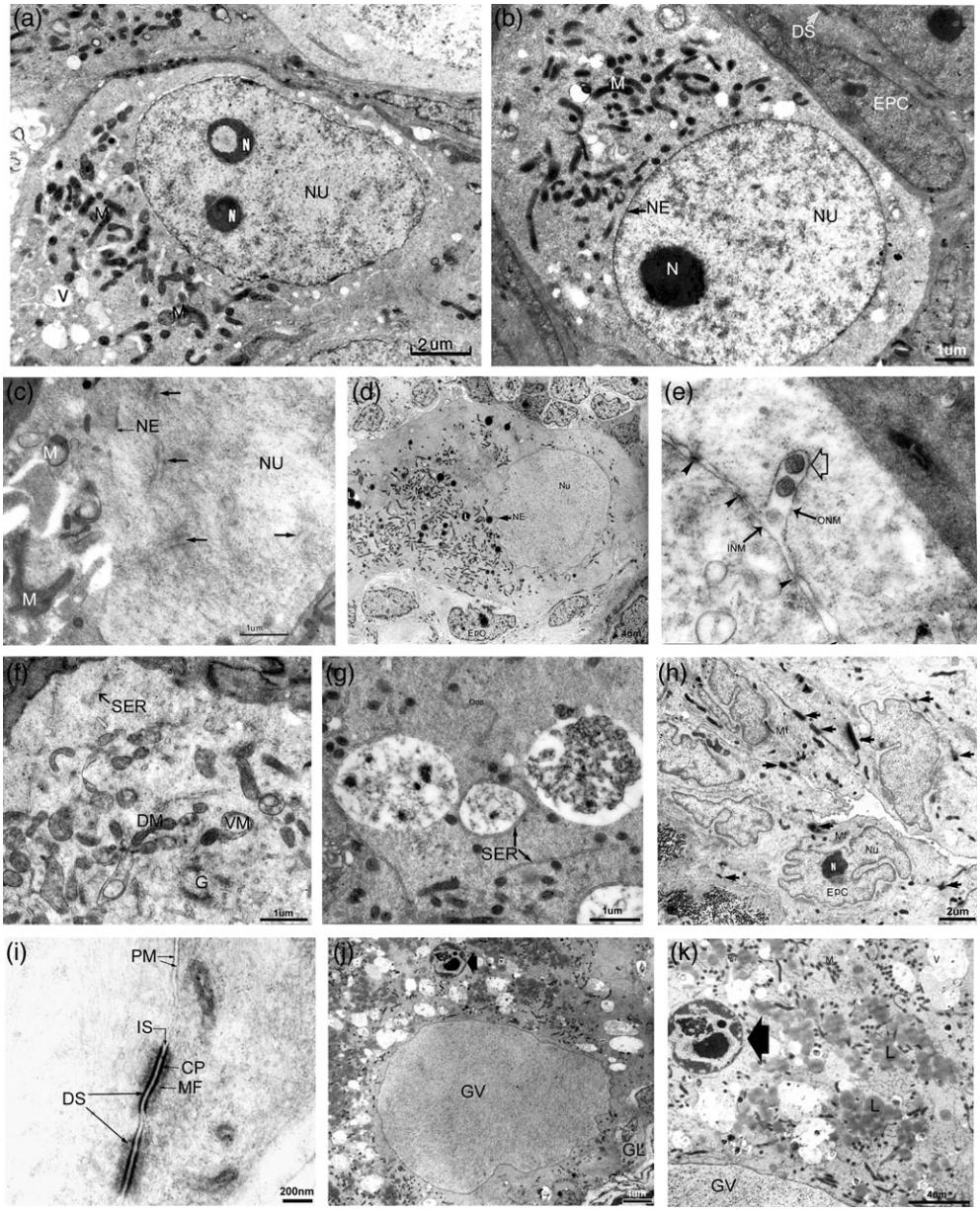

FIGURE 2 *S. mitranus*, electron micrographs of sections of the ovarian germinal bed. (a): Oogonium in an interphase stage containing a large nucleus with two large nucleoli, one of which is vacuolated. Note also the mitochondrial aggregation (M) in the cytoplasm and small dispersed vesicles (V). (b) Oogonium in preleptotene with a large round nucleus (NU) and large condensed nucleolus (N). The chromatin is scattered as small clumps throughout the nucleoplasm. (c) Oogonium in the zygotene-pachytene stage. Note the synaptonemal complexes (arrows) in the nucleoplasm. (d) Young diplotene oocyte containing a relatively large nucleus with an irregular surface of the nuclear envelope (NE) surrounding dispersed chromatin. Note also the epithelial cells (EPCs) which begin to organize around the oocyte. (e) Section through an early oocyte. The nuclear envelope possesses many nuclear pores (arrowheads). A perinuclear space separates the inner (INM) and outer (ONM) nuclear membranes, and contains large dense granules (large arrow). (f) Details of the cytoplasm of an oogonium in interphase stage showing various shapes of the mitochondrial profiles (elongated, vacuolated [VM], and dumb-bell [DM]). Some elements of Golgi bodies (G) and SER are also been observed at this stage. (g) Oocyte in the early previtellogenic stage showing various sizes of vesicles in the ooplasm. These vesicles contain fibrous and darkly stained materials. Some of these vesicles are surrounded by SER. (h) Abundant desmosomes (arrows) joint epithelial cells (EPC). The latter contain a nucleus with an irregular surface and large condensed nucleolus. (i) High magnification of two desmosomes (DS) in two adjacent cells in the OOE of the germinal bed. (j) Section through an oocyte at the early previtellogenic stage showing a migrated nucleus in the ooplasm (large arrow). The GV is large and has an irregular outline. The chromatin is dispersed in the nucleoplasm as dispersed granules. (k) Migrated nucleus at a higher magnification shows marginal clumps of chromatin and other large clumps resemble nucleoli. DM = Dumb-bell; DS = Desmosomes; CP = Cytoplasmic plaques; EPC = Epithelial cells; G = Golgi bodies; GL = Granulosa layer; GV = Germinal vesicle; INM = Inner nuclear membrane; IS = Inter cellular space; L = Lipids; M = Mitochondrial aggregation; MB = Multivesicular bodies; MF = Microfilaments; N = Nucleolus: NE = Nuclear envelope; NU = Nucleus; ONM = Outer nuclear membranes; SER = Smooth endoplasmic reticulum; V = Vesicles; VM = Vacuolated

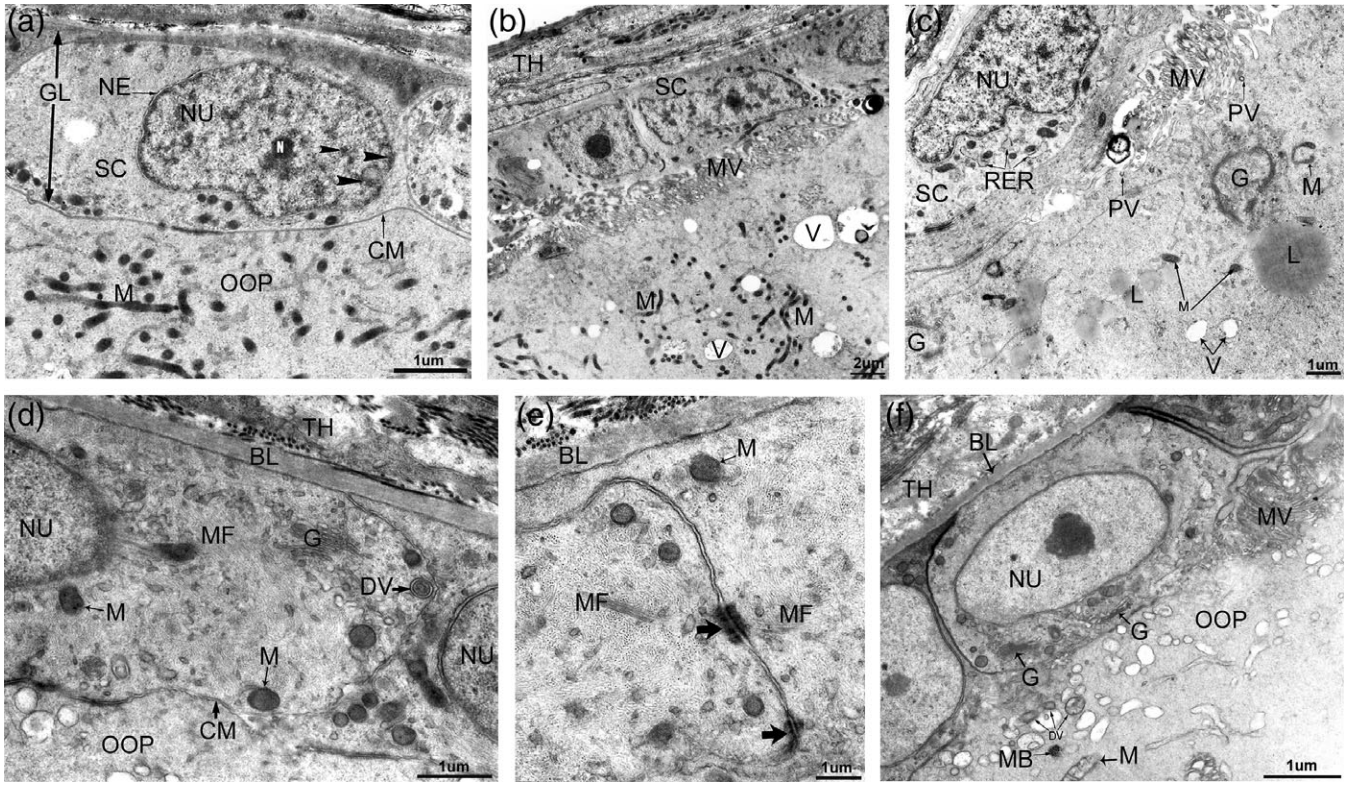

FIGURE 3 *S. mitranus*, electron micrographs of sections of follicles at early previtellogenic stage. (a) Plasma membranes (PM) of both the oocyte and granulosa cells are closely associated with a smooth interface. Note the chromatin clumps (arrowheads) in the nucleus of the small granulosa cell. (b) Mitochondria (M) are found in the cortical region associated with scattered vesicles (V). Microvilli (MV) start to appear in the zona pellucida region. (c) Many cytoplasmic organelles are observed in the cortical region of the ooplasm. Well-developed Golgi bodies (G) are typically found adjacent to the oolemma. Note the initial appearance of microvilli (MV) from both the oocyte and small cell. Some PV are present at the base of the microvilli. (d) The cytoplasm of the small cells is filled by numerous mircofilaments (MF). We also found well-developed Golgi bodies (G), mitochondria (M), and double-membrane vesicles (DV). (e): Two adjacent small cells connected by more than one desmosome (arrows). Note the microfilaments (MF) in the cytoplasm adjacent to the desmosome. (f) Golgi bodies (G) are found frequently in the cytoplasm of small granulosa cells. Note the initial appearance of the microvilli (MV) and abundance of the vesicles in the cortical region of the ooplasm. BL = Basal lamina; C = Centriole; DV = Double-membrane vesicles; G = Golgi bodies; GL = Granulosa layer; L = Lipids; M = Mitochondrial aggregation; MF = Microfilaments; MV = Microvilli; N = Nucleolus; NE = Nuclear envelope; NU = Nucleus; OOP = Ooplasm; PM = Plasma membrane; PV = Pinocytotic vesicles; RER = Rough endoplasmic reticulum; SC = Small cell; TH = Thecal layer; V = Vesicles

granules accumulated in small vesicles in the cortical region (Figure 4j) and then in larger vesicles deep in the ooplasm (Figure 4k). These granules were found singly in the zona pellucida, in contact with the microvilli surface, and aggregated in small vesicles at the base of the microvilli.

At the end of the transitional previtellogenic stage, the thecal layer which was consisting of one layer of longitudinal fibroblasts with numerous collagen fibers, differentiated into two layers, the theca interna, and theca externa.

3.4 | Late previtellogenic follicles

The oocyte continued developing and increased up to 2.5 mm in diameter. The GV of the oocyte was eccentric in position. The ooplasm was very rich in cytoplasmic organelles; particularly, mitochondria were abundant and many well-developed Golgi bodies were scattered in the ooplasm, mainly in the cortical region (Figure 5a). PV were abundant at the base of the microvilli and in the ooplasm (Figure 5b). Another prominent feature of the ooplasm at this stage was the presence of relatively large amorphous vesicles which were mostly filled with fibrous materials of low electron density (Figure 5c).

At this late previtellogenic stage, small and intermediate cells were not connected to the oocyte by cytoplasmic bridges. However, notably, large cells exhibited two distinct phases during this stage. In the first phase, these cells reached their greatest development (Figure 5d), whereas in the second phase, they regressed significantly in terms of size (Figure 5e). During both phases, the large cells maintained their connection with the oocyte through cytoplasmic bridges (Figure 5e–g). With respect to the greatest number of different cytoplasmic organelles (Figure 5h), large amounts of dark material, suggesting lipids, accumulated in the large cell cytoplasm and cytoplasmic bridges (Figure 5g), suggesting their transfer along with other materials from the large cell to the oocyte.

During the degeneration phase, the cytoplasmic bridges through the relatively thick zona pellucida through to the oocyte became long and narrow until they completely disappeared (Figure 5e). These bridges contained a large quantity of cytoplasmic material. Thus, the large

cells likely expelled their contents into the oocyte before they disappeared. The large cells decreased considerably in size and retracted from the oolemma surface and greyish droplets appeared in their cytoplasm (Figure 5i). At a later stage, the cytoplasm of large granulosa cells contained whitish lipid droplets and very few mitochondria (Figure 5j), which was later replaced with large amorphous lightly stained structures similar in appearance to lipids (Figure 5k). At the end of the late previtellogenic stage, the granulosa layer consisted only of small cells (Figure 5l).

The thickness of both the theca interna and theca externa was increased considerably compared to in the preceding stage (Figure 5m). The theca interna consisted of two to three layers of fibroblasts, whereas the theca externa was composed of numerous collagen fibers in which few scattered fibroblasts were present.

3.5 | Detection of cholesterol

The examination of follicles for cholesterol detection revealed two different configurations of cholesterol. The first type was fine electron-dense whorled structures, consisting of tightly packed fine spherical lamellae surrounding a central transparent core, found in relatively small vesicles in the ooplasm (Figure 6a,b). These whorled structures were not detected in granulosa cells associated with the relatively small oocytes.

The second type was represented by scattered small elongated cylindrical spicules or needle-like structures (Figures 6c,d). These small elongated cylindrical spicules, which were nearly absent in small cells, were abundant in larger oocytes. Abundant reaction products were scattered in the large granulosa cell cytoplasm and zona pellucida

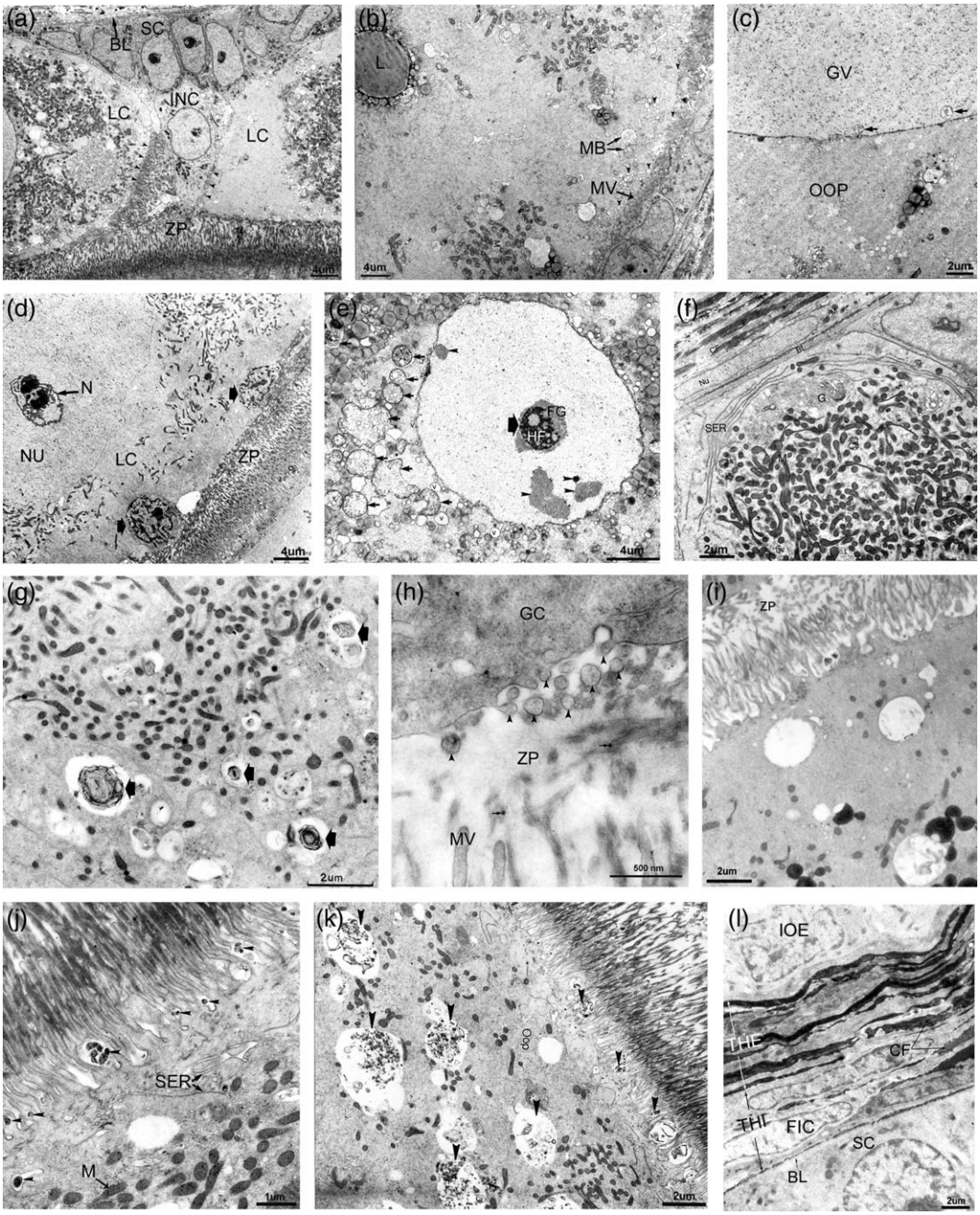

FIGURE 4. Legend on next page.

region just beneath the large cell surface. In large oocytes, digitonin-induced spicules accumulated at the base of microvilli, cytoplasmic bridges between the large cells and oocyte, and deep in the cortical region of the ooplasm. These digitonin-induced spicules accumulated in relatively large cytoplasmic vacuoles.

3.6 | Detection of phospholipids

In follicles of the transitional previtellogenic stage, a considerable number of densely stained phospholipids drops scattered randomly in the cytoplasm of large cells and cortical region of the oocyte (Figure 6e). They were present as tiny droplets up to relatively large drops. A strong positive reaction of phospholipid globules was observed in the large cells, whereas phospholipid droplets appeared to be mostly absent in the small cells. Additionally, few reactions were associated with the theca layer or granulosa layer-oocyte interface.

In control samples in which tissues were not treated with potassium ferricyanide and processed with absolute alcohol and propylene oxide, empty vacuoles were scattered in the cytoplasm, suggesting possible extraction of lipids materials (Figure 6f).

4 | DISCUSSION

The number of germinal beds is not related to the age of the animal or its reproductive status and therefore does not vary in the same species (Jones, Swain, Guillette, & Fitsgerald, 1982). This value may be constant among families; for example, *Gekkonidae* has one germinal bed, while *Lacertidae* and *Anguidae* have two germinal beds in the ovary (Jones et al., 1982; Klosterman, 1983). However, the number of germinal beds can vary in other families such as *Iguanidae* and *Scincidae* (Jones et al., 1982). In fact, Ibrahim (Ibrahim, 1977) observed only one germinal bed per ovary in the skink *Chalcides ocellatus*, whereas in the skinks *Eumeces copei* and *E. fasciatus*, the germinal beds were represented by several patches of cells scattered on the dorsal surface of each ovary (Jones et al., 1982). In *S. mitranus*, there are two germinal beds per ovary as in the skinks *Carlia (=Leiolopisma) rhomboidalis* (Wilhoft, 1963), *Scincella lateralis* (Jones, Gerrard, Roth, & Kiely, 1973), *Carinascincus metallicus*, and *Eutropis longicaudatus* (Jones et al., 1982).

The structure and development of follicles in *S. mitranus* has been reconstructed as shown in the Figure 7. In general, the structure of the germinal beds and some cytoplasmic and nuclear features of oogonia and early oocytes in *S. mitranus* are similar to those described for other lizards (Arronet, 1973; Bou-Resli, 1976; Filosa & Taddei, 1976; Hubert, 1970a, 1974, 1975; Hubert, 1970b; Hubert, 1985; Klosterman, 1982; Klosterman, 1983). However, in *L. sicula*, the presence of cytoplasmic bridges has been reported between the developing oogonia in the germinal bed (Filosa & Taddei, 1976). Such bridges were not detected in *S. mitranus* or other species such as *A. scutellatus* (Bou-Resli, 1976) and *G. coeruleus* (Klosterman, 1983). The paranucleolar mass is thought to be an important cytological marker of primordial germ cells and oogonia in lizards (Hubert, 1985, 1970a, 1970b, 1974, 1975), but was not detected in the oogonia of *S. mitranus* as in other lizards (Filosa & Taddei, 1976; Klosterman, 1982; Klosterman, 1983).

It is widely accepted that small granulosa cells are derived from epithelial cells of the germinal bed. Thus, small cells appear to serve as stem cells for the granulosa population. Hubert (1977) proposed a model for granulosa cell differentiation in *L. vivipara*, which was later applied to other lizard species (Hubert, 1985; Klosterman, 1982). In contrast to Bou-Resli (1976) who attributed mitotic activity to small and intermediate cells, most reports suggest that this is not the case and only small cells are observed in mitosis (Betz, 1963; Hubert, 1973; Hubert, 1985). In fact, it has been shown by measuring DNA content in the granulosa cells in follicles at different stages in the two species

FIGURE 4 *S. mitranus*, electron micrographs of sections of follicles at the transitional previtellogenic stage. (a) Section through the granulosa layer associated with an oocyte at the transitional previtellogenic stage, showing clusters of small cells (SC) adjacent to the basal lamina (BL), an intermediate cell (INC) between two large cells (LC) with a large nucleus (NU). Note the surface interaction between the three different granulosa cells and microvilli structure in the interface between the intermediate and large cells (arrowheads). (b) Pinocytosis becomes more intense during this stage, and PV (arrowheads) accumulate beneath the microvilli (MV) and in the ooplasm (OOP). Note the presence of numerous mitochondria (M), lipid droplets (L), and multivesicular bodies (MB). (c) Part of the GV and ooplasm (OOP): Membrane-bound vesicles (arrows) containing tubular and granular materials closely associated with the inner side of the nuclear membrane. (d) The apical small cells (large arrows) are typically trapped between the large cells (LC) and zona pellucida (ZP). Note the large nucleus (NU) and nucleolus (N) of the large cell. (e) Detail of a large cell associated with an oocyte showing a granulo-fibrillar nucleolus (large arrow). Note the patches of granular material (arrowheads) consisting of similar materials of the electron dense fine granular material of the nucleolus. These patches can be found in close association with the nuclear envelope. The prominent feature of this stage is the presence of multivesicular bodies (small arrows). Note the similarity between the numerous small vesicles (V) closely associated with the outer side of the nuclear envelope and those scattered in the cytoplasm and accumulated in multivesicular bodies. (f) The cytoplasm of the large cell is characterized by numerous mitochondria (M) showing various profiles, abundance of SER and Golgi bodies (G). Note the distinct basal lamina (BL) separating the theca from the granulosa layer. (g) Section though a large granulosa cell associated with an oocyte showing darkly stained granules which accumulate in the cytoplasm on the lamellar structure inside membrane-bound vesicles (large arrows). (h) Granulosa cell (GC) surface adjacent to the zona pellucida (ZP) showing single dark granules (arrowheads) present in the zona pellucida and likely originating from granulosa cell cytoplasm. (i) Various sizes of lipid droplets (L) occur in clusters in the ooplasm and cortical region. (j) Darkly stained granules accumulate on lamellar structure at the base of microvilli and down in the cortical region (arrowheads). (k) Darkly stained granules accumulate in larger vesicles (arrowheads) in the ooplasm. (l) The theca is differentiated into two layers, the theca interna (THI) and theca externa (THE). Note the abundance of collagen fibers (CF) in both thecal layers. BL = Basal lamina; CF = Collagen; FC = Highly dense fine fibrillar center; FIC = Fibroblast cells; G = Golgi bodies; GC = Granulosa cell; GV = Germinal vesicle; HF = Highly electron dense fibrillar material; IOE = Inner ovarian epithelium; INC = Intermediate granulose cells; L = Lipids; LC = Large granulosa cells; M = Mitochondrial aggregation; MB = Multivesicular bodies; MV = Microvilli; N = Nucleolus; NU = Nucleus; OOP = Ooplasm; SC = Small granulosa cells; SER = Smooth endoplasmic reticulum; THE = Theca externa; THI = Theca interna;V = Vesicles; ZP = Zona pellucida

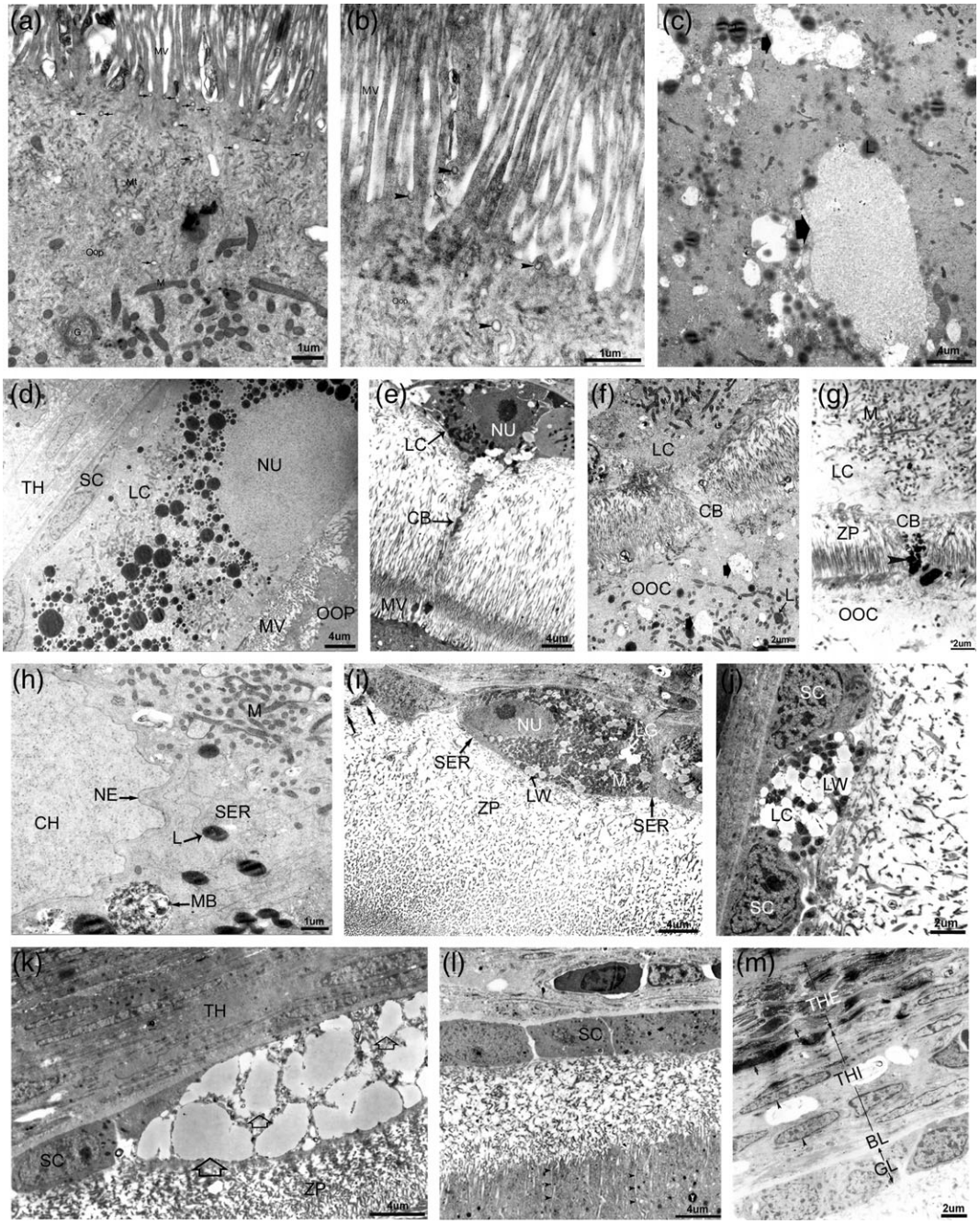

FIGURE 5 *S. mitranus*, electron micrographs of sections of follicles at the late previtellogenic stage. (a) Section through the cortical region showing the abundance of PV at the base of the microvilli (MV) and in the cortical region. (b) Region beneath the oolemma is characterized by the presence of coated vesicles (arrowheads). (c) Large amorphous vesicles (large arrows) filled with fibrous material of low electron density in the ooplasm. (d) Large granulosa cell in the first phase during the period of greatest development. (e) Regressing large granulosa cell connected to an oocyte through a relatively tight cytoplasmic bridge (CB). Note the regressed microvilli (MV). (f) Section showing a cytoplasmic bridge (CB) between a large cell (LC) and the oocyte (OOC). (g) Note the dark materials (arrowhead) in the cytoplasmic bridge (CB) between a large cell (LC) and oocyte. (h) Section of a large cell showing the undulating nuclear envelope (NE) and various cytoplasmic structures such as the mitochondria (M), SER, lipid droplets, and multivesicular bodies (MB). (i) Large granulosa cell in a regressing phase. The cytoplasm is filled with numerous mitochondria (M), some greyish lipid droplets (LG), and only few whitish lipid droplets (LW). Note the thick zona pellucida (ZP). (j) Part of a large cell (LC) between two small cells (SC) in the granulosa layer. The cytoplasm contains whitish lipid droplets (LW) and a few mitochondria. (k) Cytoplasmic contents of a large cell are replaced by large amorphous lightly stained structures (large arrows). The general appearance of these structures suggests the presence of lipids. (l) At the end of the previtellogenic stage-beginning of the vitellogenic stage, the granulosa layer consists only of small cells (SC). (m) The thecal layer is clearly separated into theca interna (THI) and theca externa (THE). Note the abundance of collagen fibers (arrows) in the theca externa and abundance of fibroblast cells (arrowheads) in the theca interna. BL = Basal lamina; CB = Cytoplasmic bridge; CH = Chromatin; GL = Granulosa layer; LC = Large granulosa cells; LG = Greyish lipid droplets; LW = Whitish lipid droplets; M = Mitochondrial aggregation; MB = Multivesicular bodies; MV = Microvilli; NE = Nuclear envelope; NU = Nucleus; OOC = Primary oocyte; OOP = Ooplasm; SC = Small granulosa cells; SER = Smooth endoplasmic reticulum; TH = Thecal layer; THE = Theca externa; THI = Theca interna; ZP = Zona pellucida

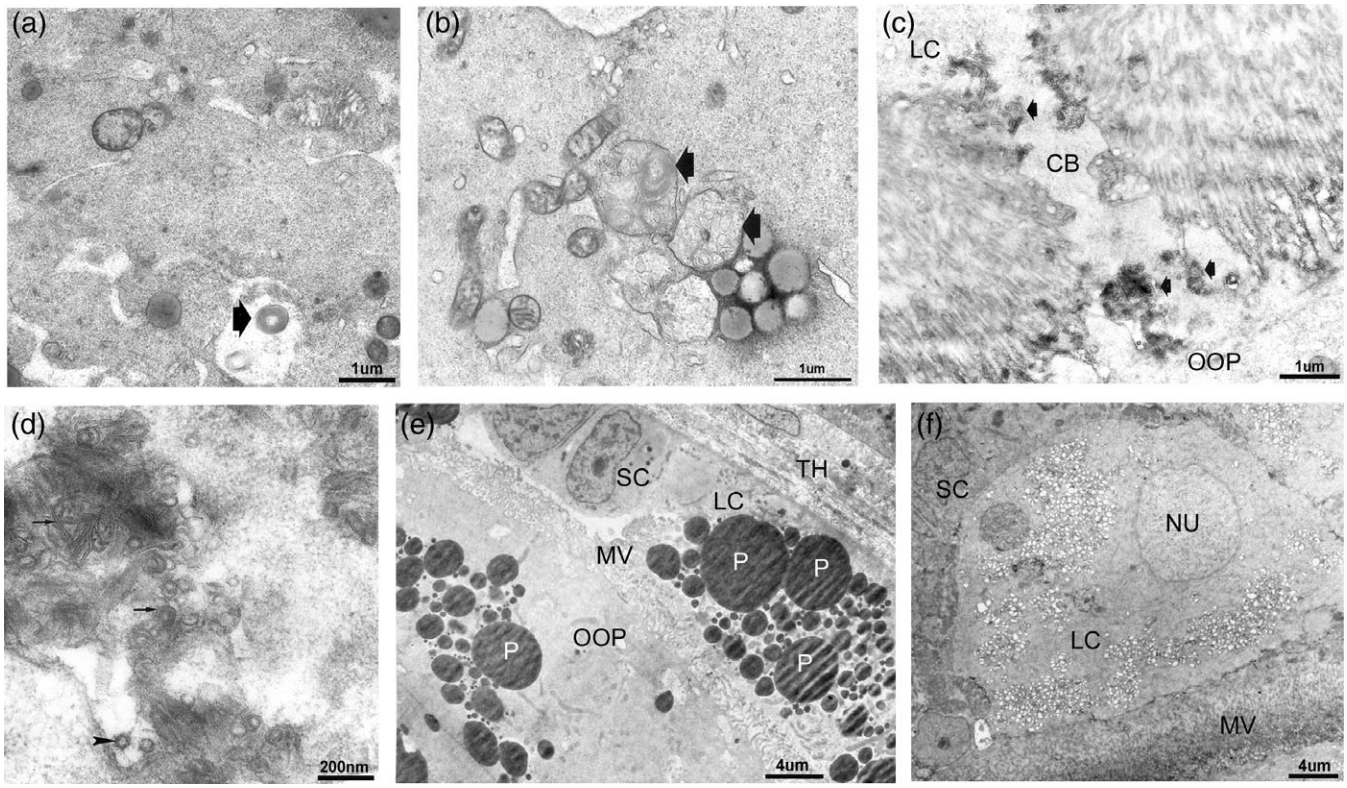

FIGURE 6 *S. mitranus*, histochemical detection and localization of cholesterol and phospholipids in the oocyte and granulosa layer at the electron microscope level. (a) The digitonin-cholesterol reaction product is present as a whorled structure of fine spherical lamellae found in vesicles in the ooplasm (large arrow). (b) Digitonin-induced whorled structures (large arrows) appear in vesicles in the ooplasm. (c) Digitonin-induced spicules (large arrows) in the region of the cytoplasmic bridge (CB) connecting a large cell (LC) to an oocyte. (d) Electron micrograph at a higher magnification from C showing the structure of the digitonin-induced spicules (large arrows). (e) Part of follicle showing the large granulosa cell (LC) studded with phospholipid drops (P). Note the similarity of phospholipid drops in both the large cell and ooplasm (OOP), while these phospholipid drops are absent from small cells (SC). No such reaction occurred in the thecal layer (TH). (f) Section of a follicle which not treated by potassium ferricyanide and treated with absolute alcohol and propylene oxide. Lipid materials were nearly absent from the cytoplasm of the large cell (LC) in the granulosa layer. Note the scattered empty vesicles which may represent the site of extracted lipid materials. CB = Cytoplasmic bridge; LC = Large granulosa cells; MV = Microvilli; NU = Nucleus; OOP = Ooplasm; P = Phospholipid drops; SC = Small granulosa cells; TH = Thecal layer

L. sicula and *G. coeruleus* that DNA was synthesized only in the small cells (Klosterman, 1982; Olmo & Taddei, 1974). These results suggested that only small apical cells adjacent to the oolemma lost the capacity to divide and could develop to intermediate cells, which then formed large cells, whereas small cells adjacent to the basal lamina were in mitosis (Betz, 1963; Hubert, 1973; Hubert, 1985).

The granulosa cells in squamata have the unique function of supplying the oocyte with DNA by engulfing whole nuclei. In fact, several studies detected nuclei in the cortical region of the ooplasm of small previtellogenic follicles (Betz, 1963; Bou-Resli, 1974, 1980; Bou-Resli, 1976; Boyd, 1940; Guraya, 1969). Bou-Resli (1976) and Guraya (1978) reported that these nuclei disintegrate during previtellogenesis. Laughran et al. (1981) confirmed their presence in *A. carolinensis*, but suggested that these nuclei were engulfed by the oocyte before it becomes surrounded by follicular cells rather than migrating later from the follicular cells. In all cases, these nuclei were reported as rare and their contribution was considered unimportant (Bou-Resli, 1980). In *S. mitranus*, such nuclei were observed in the cortical region of the ooplasm of follicles at the early previtellogenic stage. The follicle was either still in the germinal bed or just adjacent to the germinal bed. At this stage, the oocyte was surrounded by only one layer of small granulosa cells, and there were no intermediate or large cells. Such migrated nuclei were not observed in larger follicles, confirming the results of Laughran et al. (1981). The fate and function of these nuclei in lizards is still not completely understood. We found that the small cells remained relatively simple, suggesting low metabolic or synthetic activity during this stage, which agrees with previous results (Guraya, 1978). However, functional activity was indicated for the small granulosa cells in *L. vivipara*, as these cells incorporated tritiated uridine and leucine in vivo, revealing that these cells synthesize RNA and proteins (Neaves, 1971). Observations of the size and ultrastructure of intermediate cells in *S. mitranus* showed that the increase in the number of large cells closely followed the increase in the number of intermediate cells. Later, the number of intermediate cells decreased before that of large cells. This finding supports previous studies suggesting that intermediate cells are precursors of large cells (Andreuccetti, Taddei, & Filosa, 1978; Hubert, 1985, 1977; Neaves, 1971).

Since their discovery by Gegenbaur (1861), the function of large granulosa cells has been widely examined (Bou-Resli, 1974; Bou-Resli, 1976; Gabaewa, 1970; Goldberg, 1970; Guraya, 1974; Guraya, 1978; Ibrahim & Wilson, 1989; Klosterman, 1982; Klosterman, 1987).

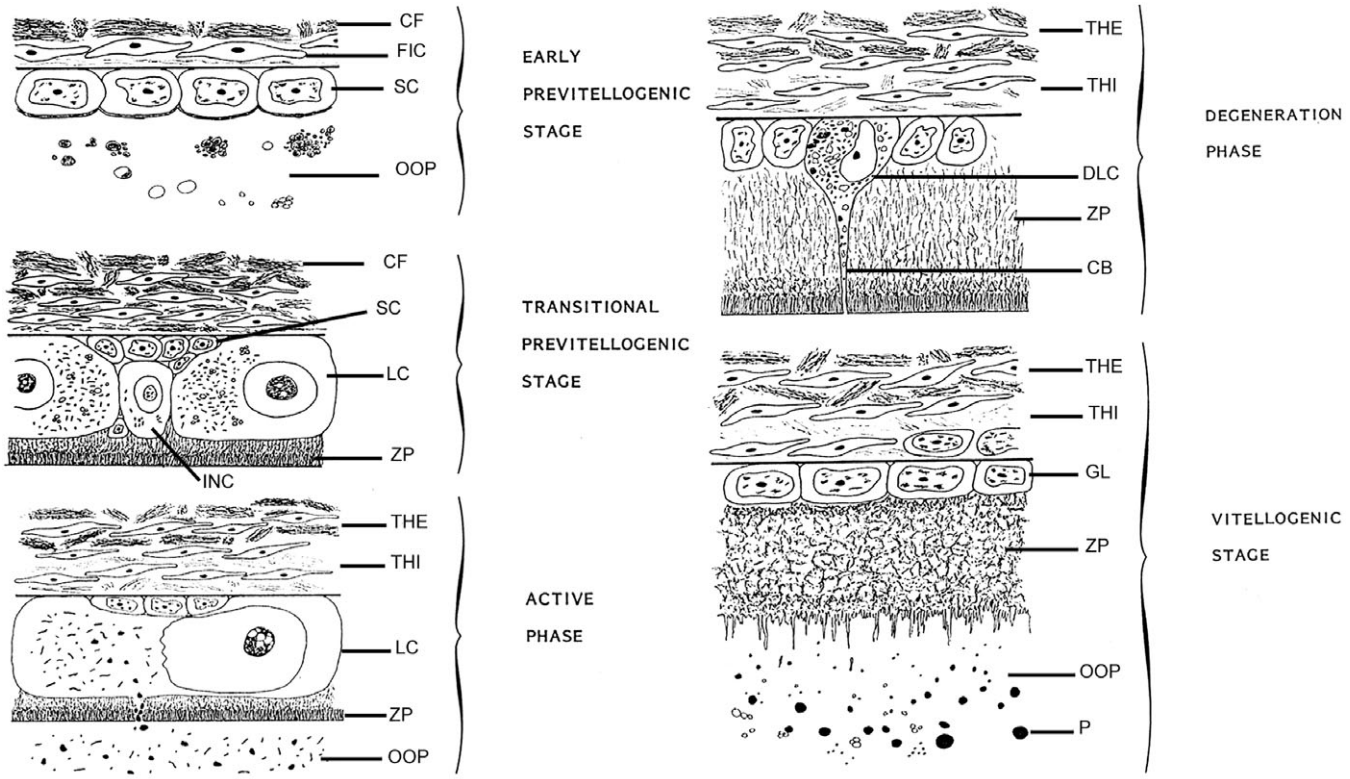

FIGURE 7 *S. mitranus*, schematic drawings of the structure and development of follicles. CB = Cytoplasmic bridge; CF = Collagen; FIC = Fibroblast cells; LC = Large granulosa cells; OOP = Ooplasm; SC = Small granulosa cells; P = Phospholipid drops; THE = Theca externa; THI = Theca interna; ZP = Zona pellucida

However, the precise function of these cells is unclear and requires further analysis. There are three main functions in which the large granulosa cells may be involved: vitellogenesis, steroidogenesis, or/and a nutritive role. These large granulosa cells in *S. mitranus* share some common features with steroid-producing cells as reported in previous studies (Dahl, 1971; Jones, Sedgley, Gerrard, & Roth, 1974; Morat, 1969). These include the presence of a large amount of lipid droplets, abundant SER, and mitochondria with complex internal cristae (Christensen & Gillim, 1969). It is well-established that steroidogenesis is restricted to the vitellogenic stage and only becomes extensive in preovulatory follicles (Guraya, 1978). Because large granulosa cells disappear before the beginning of the vitellogenesis stage, they are neither steroid-producing cells nor vitellogenesis-involved cells. However, we found that during the previtellogenic stage, a high metabolic rate and nuclear activity during large cell development and extensive development of microvilli occurred on both the oocyte and follicle cell surface. Histochemical analysis revealed cholesterol and phospholipid in the ooplasm of large cells, the zona pellucida, among the microvilli, in the bridges region, and finally accumulating in the cortical region of the oocyte ooplasm. This suggests that transfer of these materials occurs from large cells into the oocyte, confirming a nutritive role for large granulosa cells in *S. mitranus*. Several studies suggested that large pyriform cells play an important role in producing glycogen granules, lipids, and RNA (Betz, 1963; Guraya, 1969, 1978; Hubert, 1971a, 1971b; Klosterman, 1982; Klosterman, 1987; Laughran et al., 1981; Mosella, 1926; Neaves, 1971). Similar observations have been reported in various studies (Bou-Resli, 1974; Guraya, 1978; Hubert, 1985), and many cytoplasmic organelles such as the mitochondria, ribosomes, and Golgi bodies have been detected in cytoplasmic bridges (Ibrahim & Wilson, 1989; Laughran et al., 1981; Neaves, 1971; Taddei, 1972). The accumulation of a large number of lipid droplets by large cells in *S. mitranus* before degeneration also has been reported for the lizard *A. carolinensis* (Neaves, 1971) and for *G. coeruleus* (Klosterman, 1987). Large cells in the granulosa layer of *S. mitranus* may act in a holocrine manner at this stage, as these cells completely break down after depositing their contents as described previously (Betz, 1963; Gabaewa, 1970; Goldberg, 1970; Lance & Lofts, 1978).

5 | CONCLUSION

In general, the structure of the germinal beds and some cytoplasmic and nuclear features of oogonia and early oocytes in *S. mitranus* are similar to those described for other lizards. In the germinal bed, oogonia, and primary oocytes were identified and found to be intermixed with epithelial cells. At a later stage, the oogonia increase in size and became oocytes. At the diplotene stage, the oocytes become surrounded by a complete single layer of small cells and were considered as a young follicle. During previtellogenesis, primordial follicles undergo three stages: early, transitional, and late previtellogenic stages prior to vitellogenesis. However, some cytological features specific to the follicle development in *S. mitranus* have been found. In fact, the presence of cytoplasmic bridges that has been reported between the developing oogonia in the germinal bed and the paranucleolar mass which is thought to be an important cytological marker of

primordial germ cells and oogonia in lizards, were not detected in the oogonia of *S. mitranus*. Our observations on the size and ultrastructure of intermediate cells in *S. mitranus* agree with previous studies suggesting that intermediate cells are precursors of large cells.

The large granulosa cells in *S. mitranus* share some common features with steroid-producing cells as reported in previous studies. However, we found that during the previtellogenic stage, a high metabolic rate and nuclear activity during large cell development and extensive development of microvilli occurred on both the oocyte and follicle cell surface. Histochemical analysis revealed cholesterol and phospholipid transfer from large cells into the oocyte, confirming a nutritive role for the large granulosa cells in *S. mitranus*.

ACKNOWLEDGMENTS

The authors extend their appreciation to the Deanship of Scientific Research, King Saud University at King Saud University for funding the work through the research group project RG--164.

CONFLICT OF INTEREST

No conflict of interest.

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How to cite this article: Aldokhi OA, Alwasel S, Harrath AH. Ultrastructural and histochemical study of previtellogenic oogenesis in the desert lizard *Scincus mitranus* (Squamata, Sauropsida). *Journal of Morphology*. 2019;1–14. https://doi.org/10.1002/jmor.20950