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Original article

Comparison between oogenesis and related ovarian structures in a reptile, *Pseudemys scripta elegans* (turtle) and in a bird *Coturnix coturnix japonica* (quail)

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Summary — The aspect of the oogonia during their premitotic DNA synthesis and of the premeiocytes during their premeiotic DNA synthesis was studied in turtles by autoradiography, after injection of ^{3}H-thymidine. As in the adult laying quail, the intrafollicular oocytes of the adult turtle go through three successive stages: prelampbrush, lampbrush and postlampbrush. During the prelampbrush and lampbrush stage two kinds of nucleoli exist: peripheral and central. In contrast to avian yolk, during its final rapid growth, no polyhedric protein yolk units were found in turtle yolk. As in the yellow yolk of quail, highly osmiophilic alcohol insoluble satellite yolk (egg oil) accumulates between the protein globular yolk of *Pseudemys*. Turtle yolk globules increase in volume by fusion. The penetration of peripherally assembled yolk in the turtle germinal disc is analogous to what we have described in the quail. Also in postlampbrush germinal discs subcortical ooplasmic organelles are present. Below the turtle germinal disc no structure comparable to the avian nucleus of Pander could be observed. No pyriform cells (as in squamate reptiles) and no pyriform-like cells (as in birds: Callebaut, 1991b) were found in the chelonian ovarian granulosa layer. We could not demonstrate functional lacunoperitoneal communications via openings in the hilus ovarii of the turtle as is the case in birds.

ovary / turtle / oogenesis / avian oocyte

Résumé — Comparaison entre l'ovogenèse et structures de l'ovaire du reptile *Pseudemys scripta elegans* (tortue) et d'un oiseau (caille japonaise). L'aspect des ovogonies pendant leur synthèse d'ADN prémitotique et des prémeiocytes pendant leur synthèse d'ADN prémeiotique a été étudié chez *Pseudemys* par autoradiographie après injection de thymidine tritiée. Chez la tortue adulte, les ovocytes intrafolliculaires passent par trois stades successifs : avant le stade en écou-

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villon (prelampbrush), stade en écouvillon (lampbrush), stade après écouvillon (postlampbrush). Pendant la période de croissance rapide de l'ovocyte de tortue, on ne trouve pas de masses polyhédriques de vitellus comme chez l'oiseau. Pendant la période de croissance finale, on trouve des gouttes de lipides hautement osmiophiles, insolubles dans l'alcool entre les globules vitellins de tortue. Les globules vitellins de tortue grandissent par fusion. L'assemblage du vitellus qui participe à la formation du disque germinatif est analogue à ce que nous avons décrit chez la caille. Des organites ooplasmiques sous-corticaux sont également présents. Nous n'avons pas trouvé chez *Pseudemys* de structure analogue au noyau de Pander tel qu'il existe chez les oiseaux. Nous n'avons pas pu démontrer l'existence de communications entre les lacunes de l'ovaire de tortue et la cavité péritonéale.

ovaire / tortue / ovogenèse / ovocytes d'oiseau

INTRODUCTION

Only a small number of studies have been performed on oogenesis sensu latu and related ovarian structures in Chelonia: Munson (1904), Loyez (1906), Thing (1918), Bhattacharya (1925), Altland (1951), Guraya (1959) and R'ahil and Narbaitz (1973). In a previous study (Callebaut and Van Nassauw, 1987) some typical aspects of the ovary of the turtle *Pseudemys scripta elegans* were described. We have demonstrated the existence of a well developed smooth muscle-like layer (desmin immunoreactive) in the ovarian tunica albuginea, giving a particular aspect to the follicular stigma region. We have found that all the turtle oocytes with a diameter of 200 μm or more are surrounded by voluminous lacunae and are enclosed in a follicle with a superficial crater, bulging at the surface of the ovary. Also in the turtle ovary, smooth muscle-like cells were found in a suspensory apparatus formed by chordae, the tunica albuginea, and the theca externa of the ovarian follicles (Van Nassauw et al, 1991). An excellent comparative review of oogenesis and folliculogenesis in birds and reptiles has been published by Guraya (1989). In the present work, we have studied some aspects of oogenesis sensu latu and related ovarian structures in the turtle *Pseudemys scripta elegans*. This was performed at different ages, using histochemical and autoradiographic methods. Whenever possible a comparison was made with oogenesis and ovarian structures in birds.

MATERIALS AND METHODS

Female red-eared turtles (*Pseudemys scripta elegans*) of different ages, reared in our laboratory, were used in this study. The turtles were kept in tap water at 25–28 °C in 50 L containers under continuous infrared illumination. A stone emerging from the water in the centre of the container permitted the turtles to take a sunbath. The baby turtles were fed with small earth worms. After a few months they received quail embryos. Turtles older than 1 year received newly hatched quails. This procedure permitted us to study oogenesis without disturbing the turtles. Adult and prepuberal female turtles could be distinguished from the males by the presence of a shorter tail and the absence of long claws on their forelegs. Seven baby turtles (weight: 16–20 g) received an im injection of 100 μCi methyl ^{3}H-thymidine (Amersham; 82 Ci/mmol) in water, five 1-year-old turtles (weight: 130–150 g) received an im injection of 500 μCi methyl ^{3}H-thymidine (82 Ci/mmol). Six young adult female turtles (weight: 850–1 000 g) received im injections of 5 mCi L-[3,5–^{3}H] Tyrosine (Amersham, 51 Ci/mmol) during February or the beginning of March (just before or at the moment of egg laying under our experimental conditions). The turtles were killed 1 h to several days after the injection(s). Baby turtles or 1-year-old turtles still with a soft carapace were decapitated by a transverse section through the middle of their carapace. Older turtles with a hard carapace were, first anesthesized by an im injection of 2–6 mL Nembutal (60 mg/mL) solution in saline, before decapita-

tion. After removal of the ventral part of their carapace and the opening of the abdomen, the ovaries were fixed in situ (for baby turtles) or removed by sectioning through the hilus ovarii and then fixed. The volume of the ovaries varies enormously and depends on the age and physiological or hormonal state of the turtle. In the adult turtle both ovaries are functional in contrast to birds, and during the egg laying period they contain follicles of all sizes with a maximum diameter of approximately 20 mm. Several large follicles of the same diameter are seen. In contrast to birds both ovaries of turtles (and reptiles in general) contain permanent groups of extrafollicular germ cells (oogonia and oocytes) which are called germinal centres or beds. Using charcoal marks, the germinal disc of the largest oocytes was labelled. The oocytes of approximately the same diameter were fixed in different fixatives for comparison. Fixation was performed overnight at room temperature in Heidenhain's Susa without sublimate (Romeis, 1948), in calcium formalin for 1 night at room temperature or in a mixture of 2% paraformaldehyde and 0.1% glutaraldehyde in 1.0 mM phosphate buffered saline for 4 h at 4 °C. Other oocytes or part of the ovaries were fixed in Bouin for 1 night at room temperature followed by a postfixation in 70% alcohol (Callebaut et al, 1991) or rinsed directly in tap water to study the presence of lipids in the oocytes during vitellogenesis. Some of the oocytes, fixed in Bouin and postfixed in 70% alcohol or not, were rinsed in distilled water and placed in a 1% solution of osmium tetroxide in water for 1 h, followed by rinsing in tap water. The germinal disc of the largest oocytes was excised and pieces of ovary were dehydrated in the alcohol series, cleared in xylene and embedded in paraffin. After sectioning at a thickness of 8–10 μm, most of the slides were prepared for autoradiography.

Lipids were studied on the sections without deparaffination and without staining. Indeed during a study of lipids in avian follicles we observed that a second passage through xylene (for deparaffination) removes all lipids (Callebaut, 1988b). Some of the sections were Feulgen stained, according to Demalsy and Callebaut (1967). For the study of the labelling of cytogical details, photographs were take of some of the sections, before and after the autoradiographic processing (Callebaut and Demalsy, 1967). Comparable sections of similar oocytes of non-injected females were used as controls both before and after the autoradiographic procedure.

The radioactively labelled and some control sections (PAS or Feulgen stained or not) were dipped in nuclear emulsion L4 (Ilford, UK). After 5 weeks of exposure in the dark, the autoradiographs were developed according to Caro and Van Tubergen (1962). The unstained autoradiographs from calcium formalin fixed tissues were coloured with Unna. The tissue sections obtained after other fixations were stained with iron hematoxylin and eosin. Since Callebaut (1979a,b; 1988a) demonstrated the existence in birds of lacunoperitoneal communications, functional via the hilus ovarii we decided to investigate if this was also the case in *Pseudemys scripta elegans*. To do this, finely divided yolk of five unincubated quail eggs were suspended in 1.5 mL Ringer solution containing 1% trypan blue (Van Nassauw and Callebaut, 1991). From this suspension 0.3–0.5 mL were injected intraperitoneally ventrally from the hindleg via the opening in the carapace in four baby turtles, or 10 mL were injected in the same way into three adult turtles. Five to 16 hours later the turtles were killed and their ovaries fixed in Susa (Romeis, 1948). After fixation for 2 days at room temperature in this fixative, the ovaries were placed directly in 96% alcohol for 3 days. After further dehydration in absolute alcohol and clearing in xylene they were embedded in paraffin. After sectioning, the sections were deparaffinized and counterstained with Kernechtrot.

RESULTS

The general aspect of the baby or 1-year-old turtle ovary is shown in a transverse section (fig 1): larger oocytes bulge at the surface. At least on each side of the mesovarium a germinal bed is visible. However, by the growth of intra follicular oocytes in the neighbourhood, these germinal beds seem to become divided. A long, narrow mesovarium fixed to a narrow linear hilus ovarii is seen. Openings are not visible in the mesovarium, or in the hilus ovarii. After an intraperitoneal injection of trypan blue-labelled yolk, we found no yolk granules in the lacunae of the ovary, indicating that there is no functional communication between the peritoneal cavity and the ovarian lacunae.

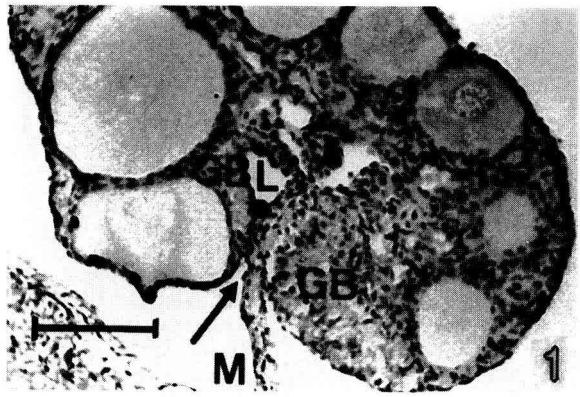


Fig 1. Perpendicular section through the ovary of a 3-month-old turtle. Note the large bulging follicles localized very close to the surface. Lacunae (L) begin to develop in the deeper part of the ovary, close to the linear hilus ovarii (arrow) fixed to a long narrow mesovarium (M); on either side of the mesovarium a germinal bed (GB) is seen; Susa without sublimate fixation, iron hematoxylin and eosin staining; bar: 100 μm.

After im injection of ^{3}H-thymidine in the baby or 1-year-old turtle

Shortly after injection (1 h), groups of oogonia localized in germinal centres directly below the ovarian surface epithelium (or sometimes on the surface) but superficially to the tunica albuginea, are seen to be labelled mainly on the peripheral part of their nuclei (containing a fine reticular Feulgen positive network with positive chromatin granules) surrounding a non-labelled central nucleolar area (compare fig 2A, before autoradiography and fig 2B, after autoradiography). This labelling of oogonia is due to premitotic DNA synthesis during their multiplication period. But also we presume that premeiotic DNA synthesis takes place in some larger germ cells that are already partially surrounded and separated from each other by prefollicular cells (fig 3). In these germ cells, which form a transition stage from oogonia to primary oocytes, relatively voluminous central nucleoli are seen. During the days following an injection of ^{3}H-thymidine, the number of labelled germ cells of the latter type increases in the germinal beds, because they differentiate after divisions from pre-existing labelled oogonia into primary oocytes (fig 4). The intensity of the labelling per nucleus, however, decreases but finally remains constant, since these divisions end with a final premeiotic division. The aspect of the nucleoli can most clearly be seen in sections through germinal beds of 1-year-old turtles after staining of the sections with iron hematoxylin and eosin before autoradiography (fig 5). We observed a typical chromosome-free clearer area of nucleoplasm surrounding each of the nucleoli. With this staining procedure two kinds of nucleoli can be discerned: (1) intensely red (eosinophilic) stained nucleoli, usually localized centrally in a chromosome-poor clear area, (2) dark stained nucleoli. This aspect of the nucleoli is even more pronounced when the oocyte grows: the central red stained (eosinophilic) nucleoli become surrounded by a large fine granular nucleoplasm, free from chromosomes, whilst the vacuolized dark stained nucleoli (only surrounded by

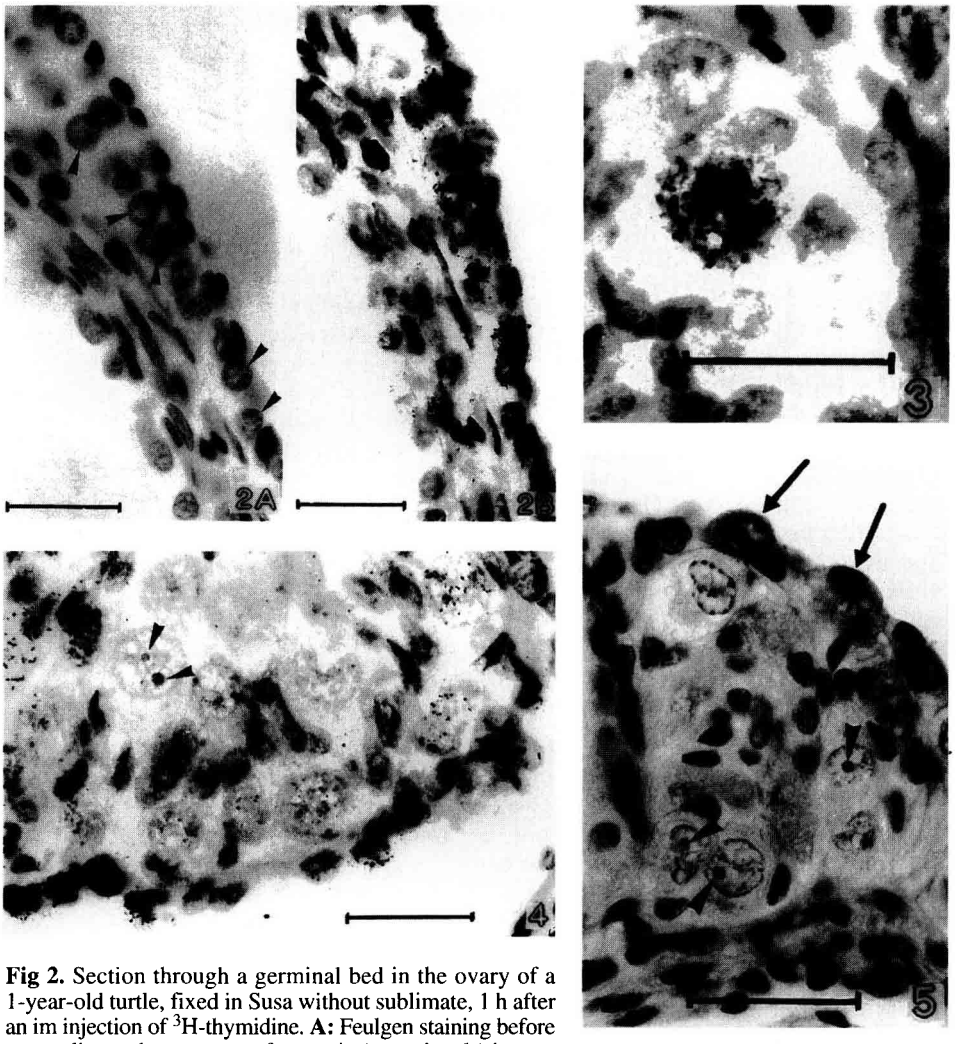


Fig 2. Section through a germinal bed in the ovary of a 1-year-old turtle, fixed in Susa without sublimate, 1 h after an im injection of ^{3}H-thymidine. **A:** Feulgen staining before autoradiography; a group of oogonia (arrowheads) is seen below the surface epithelium of the ovary; some oogonia are localized on the ovarian surface (superficial arrowheads). Note the aspect of the oogonia with a central Feulgen negative paranucleolar area surrounded by fine Feulgen positive chromosome threads and small chromatin clumps; no prefollicular cells are seen between the oogonia; bar: 20 μm. **B.** After autoradiography: the whole group of oogonia is labelled indicating premitotic DNA synthesis; bar: 20 μm.

Fig 3. Autoradiograph of a section through the same ovary as in figure 2: the labelled germ cell in the centre is larger than the oogonia of figure 2 and is partially separated from the neighbouring unlabelled germ cells by the surrounding prefollicular cells indicating premeiotic DNA synthesis; two nucleoli are visible surrounded by chromatin threads; bar: 20 μm.

Fig 4. Autoradiograph of section through the ovary of a baby turtle fixed 11 days after an im injection of ^{3}H-thymidine; several germ cell nuclei are faintly labelled; note the two nucleoli (arrowheads) surrounded by a chromatin-free area in the unlabelled meiocyte; the prefollicular cells are strongly labelled; iron hematoxylin and eosin staining; bar: 20 μm.

Fig 5. Section through a germinal bed of an ovary of a 1-year-old turtle, after iron hematoxylin and eosin staining. Note the intensely staining (eosinophilic) nucleoli (indicated by arrowheads), surrounded by a chromatin-free area in the meiocytes; note also the presence of germ cells (probably oogonia), indicated by arrows, bulging at the surface of the ovary and protruding in the peritoneal cavity. These oogonia can be distinguished from the prefollicular cells by their much larger ooplasm and nucleus with empty aspect; fixation: Susa without sublimate fixation; bar: 30 μm.

a clear halo) are localized at the inner side of the germinal vesicle membrane (fig 6).

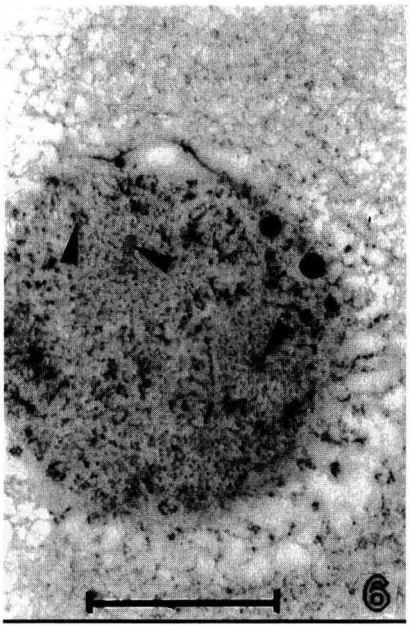
Cytology of the oocytes in the adult turtle ovary

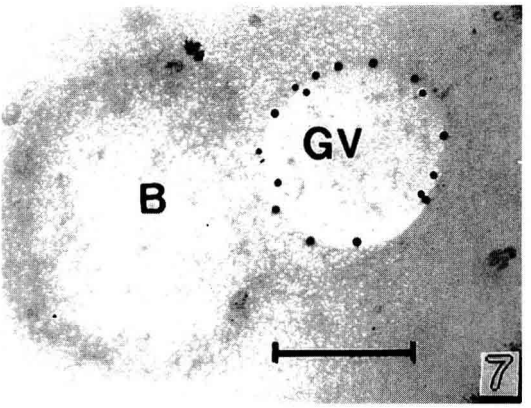
As in the oocyte of adult laying quail we can distinguish three successive developmental stages of intrafollicular oocytes in adult turtles before ovulation.

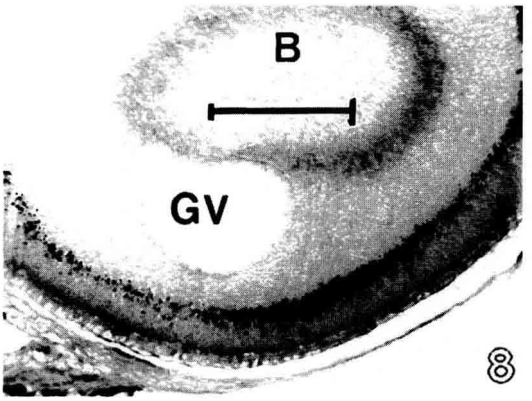
(1) The prelampbrush stage with chromosomes in diplotene without clearly defined lampbrush lateral loops and with individual nucleoli surrounded by a clearer chromosome-free halo (fig 7). In the ooplasm a voluminous paranuclear Balbiani complex close to an eccentric germinal vesicle could be seen (figs 7–9). At some distance from the surface a lipid granular layer can be observed, whilst at the surface of the Balbiani complex a lipid-containing layer is also seen. Between both lipid layers the intensely PAS staining ooplasm is visible (fig 9).

(2) The lampbrush stage with chromosomes presenting the lampbrush chromosome configuration with lateral loops as already described by Loyez (1906) in *Cistudo europaea* and with numerous voluminous pyroninophilic nucleoli against the inner side of the germinal vesicle wall (fig 10), often bulging at its surface. The germinal vesicle has a central or paracentral localization and increases strongly in volume. This stage begins when the oocyte has a diameter of 600–700 μm. During the lampbrush stage, two kinds of nucleoli can be distinguished (particularly obvious after Bouin fixation, alcohol postfixation, osmium tetroxide treatment and Unna staining) (fig 10): (a) the large peripheral pyroninophilic nucleoli, already visible at the beginning of the lampbrush stage; (b) the much smaller grey stained nucleoli close to or on the chromosomes, localized in the large central part of the germinal vesicle.

(3) The postlampbrush stage starts when the oocyte has a diameter of 3-4 mm and the germinal vesicle penetrates the deeper part of the cortex where the beginning of the assemblage of small yolk spheres takes place. At the beginning of the postlampbrush stage, the nucleoli are no longer localized at the inner side of the germinal vesicle wall but deeper at some distance on a sphere surrounding the shortened chromosomes in the central area (fig 11). When the germinal vesicle penetrates the cortex, a germinal disc forms. In this germinal disc we see the development of subcortical ooplasmic organelles (fig 12). These organelles are most clearly visible after Bouin fixation, osmium tetroxide treatment and Unna staining and are pressed between the yolk granules, as is also the case in the quail. In the 10-mm-diameter oocyte, the germinal vesicle has penetrated through the cortex (fig 13). Only a narrow cap of small yolk granules separates it from the vitelline membrane. The short chromosomes are hardly visible among the voluminous vacuolized nucleoli, aggregated in the centre of the germinal vesicle forming an oval caryosphere. At its maximum of development, the oocyte (at the end of the postlampbrush stage, just before maturation) reaches a diameter of 19–20 mm and the germinal vesicle is found flattened at the surface of the ooplasm (fig 14). However, the yolk cap above the germinal vesicle is still present. The smaller nucleoli are still visible as a flat group in the centre of the germinal vesicle. The prominent presence of nucleoli seems thus to be a characteristic feature of the chelonian oocyte during its whole intrafollicular period. This is not the case during the corresponding period in avian oocytes. In the intrafollicular *Pseudemys* oocytes, after Feulgen staining, the chromosomes are not or very faintly visible. Particularly during the postlampbrush stage no spherical contracted chromosomes are seen, as is the case in the quail (Callebaut, 1973a). Although at the end of their final growth period (end of the postlampbrush stage), both the quail and turtle oocyte reach the same maximal diam-







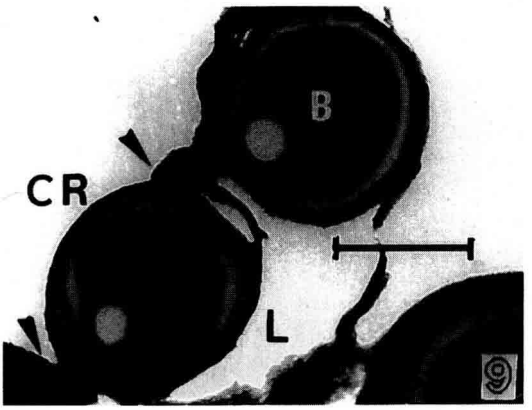


Fig 6. Section through the germinal vesicle of a larger oocyte in the ovary of a 1-year-old turtle (Susa without sublimate fixation and iron hematoxylin and eosin staining) to show the two kinds of nucleoli: (1) the central eosinophilic nucleoli (arrowheads) surrounded by a large finely granular nucleoplasm, in which no chromatin is seen, and (2) dark stained vacuolized peripheral nucleoli; bar: 30 µm.

Fig 7. Section through a prelampbrush oocyte of an adult turtle during the reproduction period; Bouin fixation, Unna staining: intensely staining (pyroninophilic) peripheral nucleoli in the germinal vesicle (GV); voluminous paranuclear Balbiani complex (B) is seen in the ooplasm; bar: 100 µm.

Fig 8. Undeparaffinized section through a prelampbrush oocyte of an adult turtle during the reproduction period, fixed in Bouin, postfixed in alcohol 70% after osmium tetroxide staining; two lipid layers are seen: a central lipid layer surrounds the Balbiani complex (B) and a more peripheral subcortical lipid layer; GV: unstained germinal vesicle; bar: 100 µm.

Fig 9. Section through prelampbrush oocytes of adult turtle during the reproduction period, fixed in Bouin, postfixed in alcohol 70% and stained with PAS; note the intensely stained voluminous central ooplasmic mass, localized between the Balbiani complex (B) and the cortex; L: lacuna, CR: cross section through a superficial crater-shaped stigma of the follicle (the arrowheads indicate the rim of the crater); bar: 200 µm.

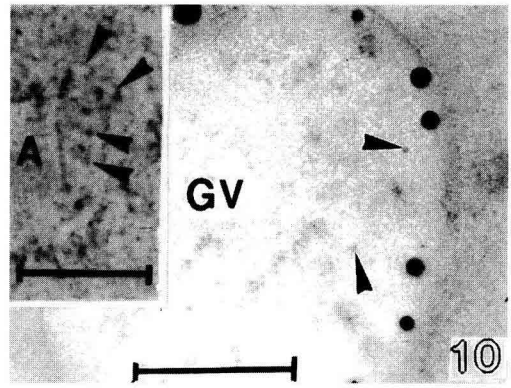


Fig 10. Section through germinal vesicle (GV) of lampbrush stage of an adult turtle during the reproduction period; Bouin fixation, Unna staining; note the larger peripheral pyroninophilic nucleoli and the smaller nucleoli (arrowheads) in the central nucleoplasm; bar: 50 μm. Inset: longitudinal section through the axis (A) of a lampbrush chromosome; lateral loops are indicated by arrowheads; iron hematoxylin and eosin staining; bar: 25 μm.

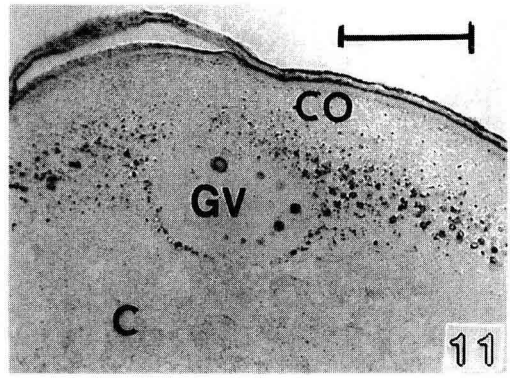


Fig 11. Section through early postlampbrush oocyte of an adult turtle during the reproduction period. Note the beginning penetration of the germinal vesicle (GV) in the subcortical granular yolk layer and cortex (CO); yolk granules slide laterally and below the germinal vesicle; the nucleoli start their centripetal movement and are no longer localized at the inner side of the germinal vesicle membrane; C: central ooplasmic mass; Susa without sublimate fixation, iron hematoxylin staining; bar: 200 μm.

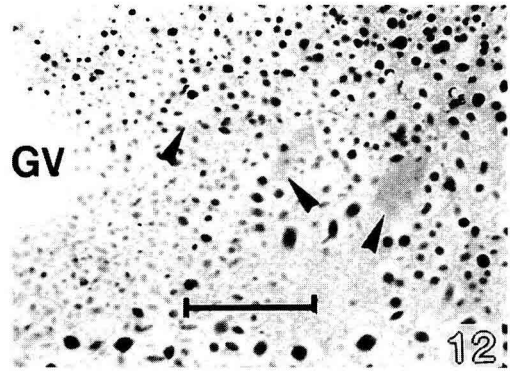


Fig 12. Section through germinal disc of 4-mm-diameter turtle postlampbrush oocyte fixed in Bouin and stained with Unna; GV: germinal vesicle; subcortical ooplasmic organelles (arrowheads) are visible pressed between yolk granules; bar: 100 μm.

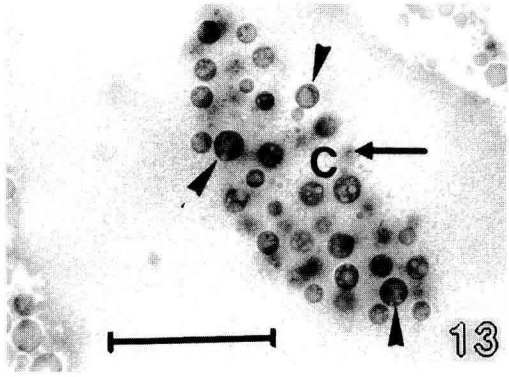


Fig 13. Section through germinal vesicle of 10-mm-turtle postlampbrush oocyte during the reproduction period: vacuolized nucleoli (some indicated by arrowheads) and hardly visible contracted chromosomes (arrow) gather together and form a central caryosphere (C); Susa without sublimate fixation and iron hematoxylin and eosin staining; bar: 50 μm.

eter (approximately 20 mm), the prelampbrush stage in the turtle develops to a greater diameter (600 µm), than in the quail (only 150 µm diameter: Callebaut, 1975). Also the lampbrush stage in the turtle becomes more voluminous (3 mm diameter) contra 1.5 mm in the quail. During the final growth period in the oocyte of *Pseudemys scripta elegans* alcohol insoluble satellite yolk or egg oil (as described in birds: Callebaut et al, 1991) localized between the protein yolk globules (fig 15), could be demonstrated by fixation in Bouin followed by postfixation in alcohol 70% and osmium tetroxide treatment. As in birds, lipid drops were also found in the theca interna. An accumulation of large clumps of intensely staining osmiophilic material was also locally observed, pressed against the outer side of the granulosa basement membrane. A paranuclear lipid drop could be seen in the granulosa cells and sometimes between the granulosa cells. After Bouin fixation and rinsing in water the satellite yolk is no longer visible. It disappears also from the sections after deparaffination by xylene (Callebaut, 1988b). This yellow stained satellite yolk seems to contribute to the yellow colour of the larger oocytes in *Pseudemys*. After ip injection of trypan blue-labelled yolk in adult turtles we found no yolk granules in the lacunae of the ovary, which indicates that also in the adult female no functional communications exist between the peritoneal cavity and the ovarian lacunae.

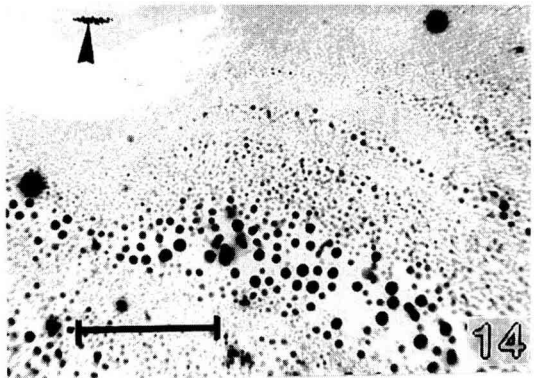


Fig 14. Section through the germinal disc of 20-mm-diameter postlampbrush turtle oocyte after Bouin fixation and Unna staining; the germinal vesicle is flattened at the surface of the ooplasm: the flat group of nucleoli in its central part is indicated by an arrowhead; note the two types of concentric alternating yolk layers: the largest yolk granules are pyroninophilic; bar: 200 µm.

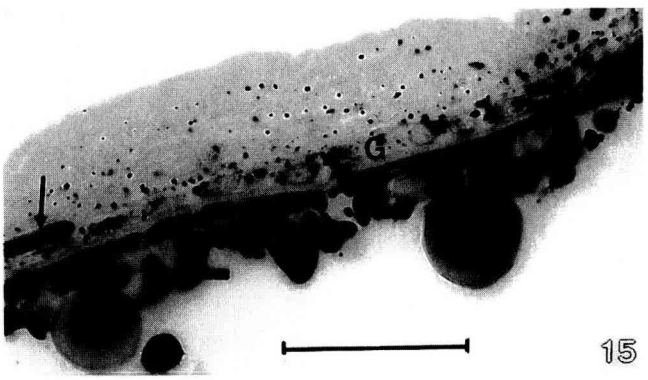


Fig 15. Undeparaffinized section through superficial ooplasm and follicle wall at the end of the postlampbrush stage after fixation in Bouin, postfixed with alcohol 70% and osmium tetroxide treatment: note the intensely black staining satellite yolk pressed between the proteid yolk globules (PG); paranuclear lipid drops in the granulosa cells (G) and lipids between the granulosa cells; large clumps (arrow) of lipids are pressed in the theca interna against the basement membrane; further small lipid drops in the remainder of the follicle wall; bar: 50 µm.

Adult turtle ovary, after an im injection of ^{3}H-tyrosine, 38 h before fixation

The labelling on the autoradiographs was most clearly seen after Bouin fixation and Feulgen staining before dipping. The radioactive labelling was, however, no longer visible in Feulgen stained sections after tissue fixation in Susa without sublimate, calcium formalin or paraformaldehyde and glutaraldehyde. After calcium formalin fixation, staining with Unna after the autoradiographic processing also gives a good contrast with the autoradiographic labelling.

Labelling of the nuclei of the extrafollicular germ cells (visible as clearer areas in the germinal beds) is seen (fig 16). The labelling of the germinal vesicle of the smallest intrafollicular oocytes (prelampbrush stage) is very intense and visible even at low magnification (fig 17). Also a less dense labelling is seen on the whole ooplasm at higher magnification (fig 16). In somewhat larger oocytes (lampbrush stage, eg, 700 μm diameter) the central ooplasmic mass presents a labelling whilst the peripheral cortex is not or poorly labelled (fig 18): this indicates a central, perinuclear metabolic activity (protein synthesis). In still larger oocytes (lampbrush chromosome stage), small yolk globules begin to accumulate below the cortex, and the labelling in the latter increases. From this moment on, there are clearly two kinds of labelling from different origins: (1) the central extravitelline labelling (most pronounced around the germinal vesicle), and (2) the peripheral labelling localized on the yolk globules forming a subcortical layer. Some larger more centrally localized yolk globules are not labelled and are obviously already formed before the injection. At this moment the germinal vesicle strongly increases in volume and the nucleoplasm and the large vacuolized peripheral nucleoli are intensely labelled (fig 19). In early postlampbrush oocytes (approximately 4 mm in diameter), a broad labelled cortex containing small labelled yolk granules is seen (fig 20). Below this cortex, larger unlabelled yolk spheres are seen. Small labelled granules adhere to the surface of this unlabelled yolk spheres, and seem to fuse with them after penetration through the cortex. In the neighbourhood of the germinal vesicle, which has penetrated the subcortical yolk layer, the first *Anlage* of the germinal disc forms (fig 21).

Laterally from and below the germinal vesicle, labelled yolk granules are visible (comparable with the first *Anlage* of the so-called polar granules in birds: Bartelmez, 1912, Callebaut, 1974) suggesting a sliding movement. In 8–10-mm-diameter postlampbrush oocytes (exterior to the germinal disc region) we see that the intensely labelled large yolk globules are localized below the less or non-labelled smaller yolk globules (fig 22). This indicates that the ^{3}H-tyrosine pool was already exhausted before fixation. In the germinal disc region of 8–10-mm-diameter postlampbrush oocytes, the penetration of the peripherally assembled labelled yolk granules seems to be impaired by the presence of weakly stained subcortical ooplasmic organelles (fig 23).

Sometimes the labelled penetrating yolk granules form a cap over these organelles, as was also seen in the quail germinal disc (Callebaut, 1974). In the germinal disc region of postlampbrush oocytes of approximately 15 mm in diameter, we see that the labelled yolk layer just reaches the angles of the germinal vesicle (fig 24) without sliding laterally from it. Above the germinal vesicle a narrow yolk cap is seen composed of two parts: the deepest part, close to the germinal vesicle is unlabelled (and thus formed first) whilst the superficial part is labelled and formed after the injection of ^{3}H-tyrosine. The penetration of yolk into this region is thus strongly reduced. In the largest postlampbrush oocytes, exterior to

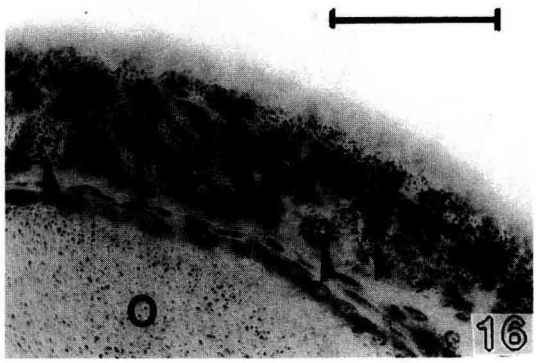


Fig 16. Autoradiograph of section through germinal bed of an adult turtle ovary during the reproduction period, 38 h after ^{3}H-tyrosine injection; the germ cell nuclei (arrowheads) are labelled; O: labelled ooplasm of neighbouring intrafollicular oocyte; Bouin fixation and iron hematoxylin and eosin staining; bar: 50 µm.

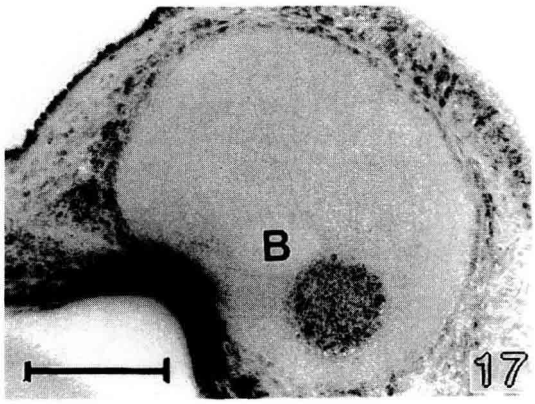


Fig 17. Autoradiograph of section through a turtle prelampbrush oocyte, 38 h after an im injection: intense labelling is seen in the germinal vesicle; B: paranuclear Balbiani complex visible as an unstained structure: the lipid layer is removed by the histological procedure; bar: 100 µm.

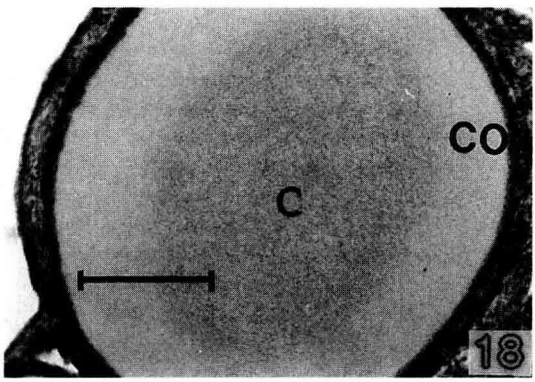


Fig 18. Autoradiograph of section through a beginning lampbrush stage turtle oocyte, 38 h after an im injection of ^{3}H-tyrosine: the massive central ooplasmic mass (C) presents a labelling, whilst the cortex (CO) is not labelled; fixation Susa without sublimate and iron hematoxylin and eosin staining; bar: 50 µm.

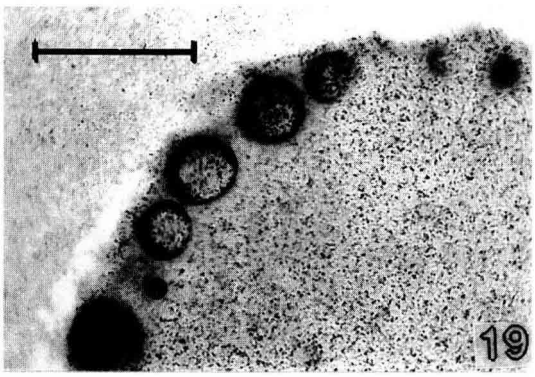


Fig 19. Autoradiograph of section through the germinal vesicle of a lampbrush stage turtle oocyte, 38 h after an im injection of ^{3}H-tyrosine; labelling of the nucleoplasm and of the vacuolized giant nucleoli, localized below the nuclear membrane is seen; fixation in Susa without sublimate and iron hematoxylin and eosin staining; bar: 50 µm.

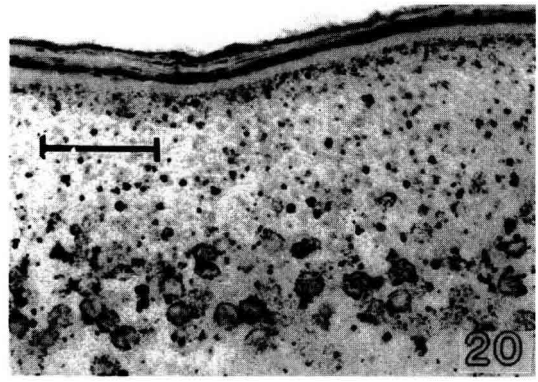


Fig 20. Autoradiograph of section through 4-mm-diameter turtle oocyte (beginning postlampbrush stage) exterior to the germinal disc region, after calcium formalin fixation and Unna staining, 38 h after an im injection of ^{3}H-tyrosine; note the fusion of small labelled yolk granules with the deeper larger unlabelled yolk spheres, below the cortex; bar: 50 µm.

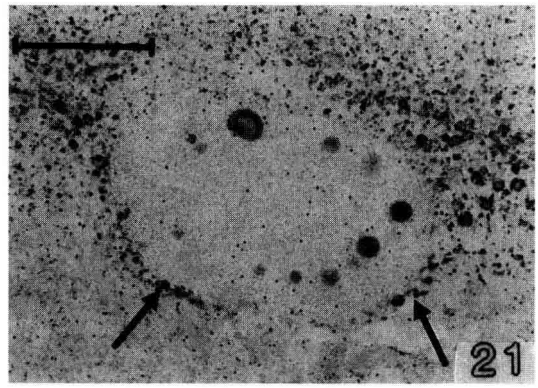


Fig 21. Autoradiograph of section through the germinal disc region of the same oocyte as in figure 20; fixation in calcium formalin and Unna staining; early formation of polar granules by the sliding movement of labelled yolk granules (arrows), below the germinal vesicle is seen; bar: 100 µm.

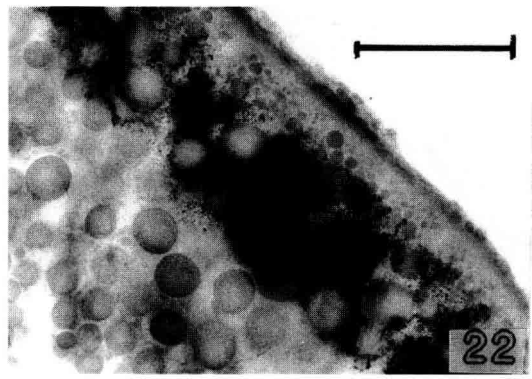


Fig 22. Autoradiograph of section through superficial ooplasm (exterior to the germinal disc region) of a 10-mm-diameter postlampbrush turtle oocyte 38 h after an im injection of ^{3}H-tyrosine: intense labelling of the protein yolk globules and intervitelline labelling is seen at some distance below the surface: the most superficial yolk globules are less or non-labelled, indicating exhaustion of the ^{3}H-tyrosine pool; Susa without sublimate fixation and iron hematoxylin staining; bar: 50 µm.

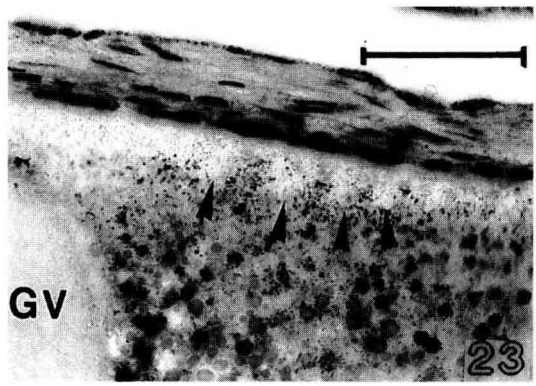
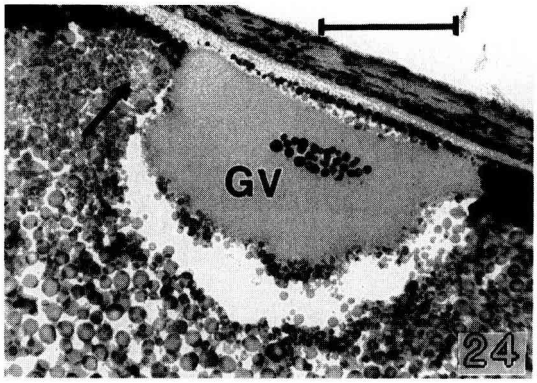


Fig 23. Autoradiograph of section through the germinal disc of a 9-mm-diameter postlampbrush turtle oocyte, 38 h after an im injection of ^{3}H-tyrosine. Note the penetration of labelled yolk between the pale staining subcortical ooplasmic organelles (indicated by arrowheads); GV: germinal vesicle; Susa without sublimate fixation and iron hematoxylin staining; bar: 50 µm.

the germinal disc, we can see that, at the boundary of labelled and unlabelled yolk, large yolk globules often have a partially polarized labelling pattern, ie, that they present a radioactively labelled cap directed towards the surface of the oocyte (fig 25). Sometimes large yolk spheres also contain one or more round central unlabelled parts, whilst the surrounding periphery of the yolk sphere is strongly labelled (fig 26). Even in the largest oocytes of *Pseudemys scripta elegans,* only round yolk units (yolk spheres) are assembled and no polyhedric yolk units, as has been described during the final rapid growth period in the oocytes of birds (Perry and Gilbert, 1985; Callebaut et al, 1991).

Therefore, we prefer not to speak of yolk platelets because the protein chelonian yolk is present in a globular form. This seems also to be the case in oocytes of other reptiles, eg, in the lizard *Sceloporus torquatus torquatus* (Uribe et al, 1995). In the largest postlampbrush oocytes the yolk cap above the germinal vesicle no longer contains labelled yolk, which indicates that here the penetration of yolk precursors is negligible



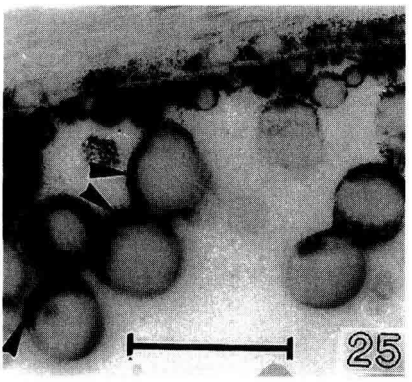
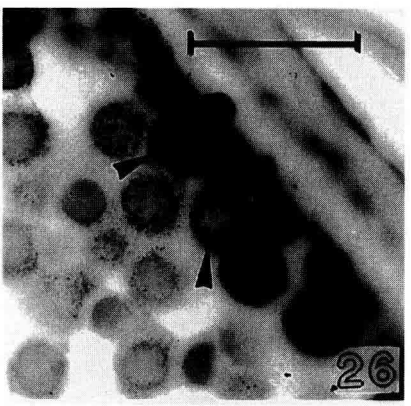


Fig 24. Autoradiograph of section through germinal disc of a 16-mm-diameter turtle postlampbrush oocyte, 38 h after an im injection of ^{3}H-tyrosine; Susa without sublimate fixation and iron hematoxylin and eosin staining; the radioactively labelled layer reaches the angles of the germinal vesicle (GV); above the germinal vesicle, deeper unlabelled and more superficial labelled yolk is seen; arrow indicates remnant of subcortical ooplasmic organelles; bar: 100 μm.

Fig 25. Autoradiograph of a section through large postlampbrush oocyte of adult turtle during the reproduction period; 38 h after an im injection of ^{3}H-tyrosine: note the radioactive cap on the large yolk globules (arrowheads) directed to the surface of the oocyte; Susa without sublimate fixation; bar: 50 μm.

Fig 26. Similar section as in figure 25 in another region; note the existence of large yolk globules with a round central unlabelled part, whilst the surrounding periphery of the yolk globule is strongly labelled (arrowheads); bar: 50 μm.



or absent (fig 27). The narrow labelled yolk layer in the germinal disc ends at the angles of the very flattened germinal vesicle. The granulosa cells above the germinal vesicle are very flat whilst more laterally in the immediate neighbourhood, they form a two nuclei thick layer (figs 27 and 28). This is exceptional since it is usually assumed that the turtle granulosa layer is only one cell thick. The labelling in the granulosa layer is extranuclear, both above the germinal disc region (fig 28) and exterior to this region (fig 25). This suggests that the transport of protein yolk precursors mainly occurs via the intercellular spaces as is the case in the quail (D'Herde and Vakaet, 1992) and eventually through the granulosa cytoplasm as also observed in the quail (Callebaut, 1991a). In contrast to the intense labelling found in the germinal vesicle of prelampbrush and lampbrush turtle oocytes, during the postlampbrush stage, no or very slight labelling is seen. During the latter period the germinal vesicle no longer increases in volume and the metabolic activity of the chromosomes and nucleoli decreases considerably.

Adult turtle ovary after two im injections of ^{3}H-tyrosine, 4 and 8 days before fixation

Labelling in the smaller turtle oocytes is similar to the autoradiographic observations after a single injection. In larger oocytes

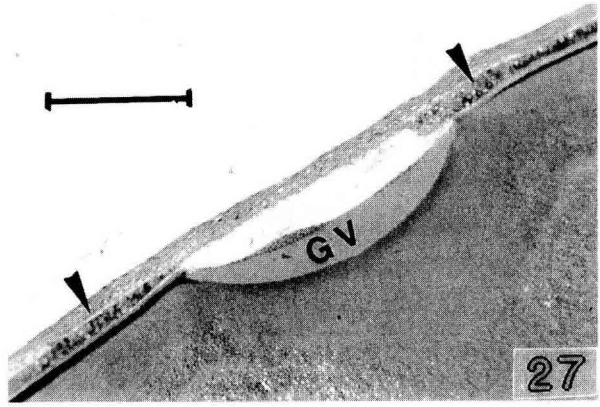


Fig 27. Autoradiograph of a section through the germinal disc of an 18-mm-diameter turtle postlampbrush oocyte, 38 h after an im injection of ^{3}H-tyrosine fixed in Susa without sublimate and stained with iron hematoxylin and eosin; the labelled superficial ooplasmic layer just reaches the angles of the germinal vesicle (GV); no labelled yolk is present above the germinal vesicle; note the thickening and labelling of the granulosa layer laterally from the germinal vesicle (arrowheads)by contrast to the very narrow granulosa layer above the germinal vesicle; bar: 200 μm.

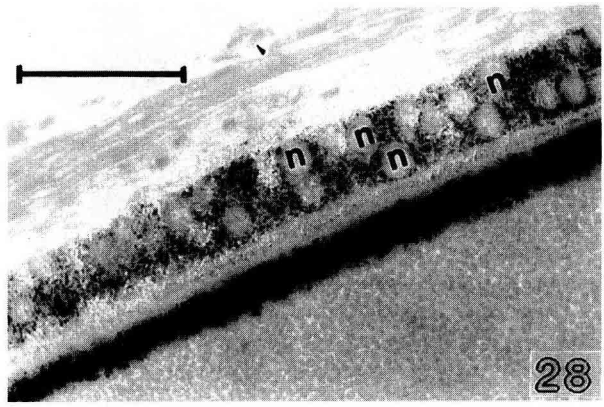
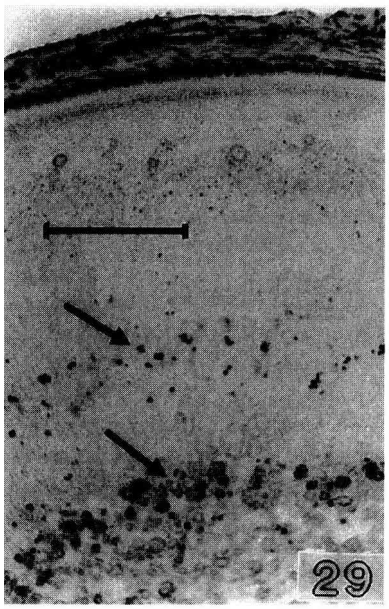


Fig 28. Enlarged view of the thickened granulosa layer of figure 27; note the extranuclear labelling in the granulosa cells; bar: 50 μm.

with the beginnings of peripheral protein yolk assemblage, two labelled layers can be recognized below the relatively broad cortex (fig 29). In still larger oocytes (beginning postlampbrush stage), the two labelled yolk layers become localized in a more superficial position (fig 30). Finally in the largest postlampbrush oocytes (where the cortex is absent) the last-formed labelled yolk layer is localized on the surface of the ooplasm (fig 31). Most obvious in the largest postlampbrush oocytes is the labelled yolk cap over the unlabelled yolk, localized just superficially to the central part of the germinal vesicle (fig 32). This again indicates that the penetration of yolk above the germinal vesicle is very slow (more than 8 days) and there is only one labelled layer visible above the germinal vesicle. The progressive sliding of the yolk layers below the rims of the germinal vesicle is also very slow (8 days or more). This sliding of the



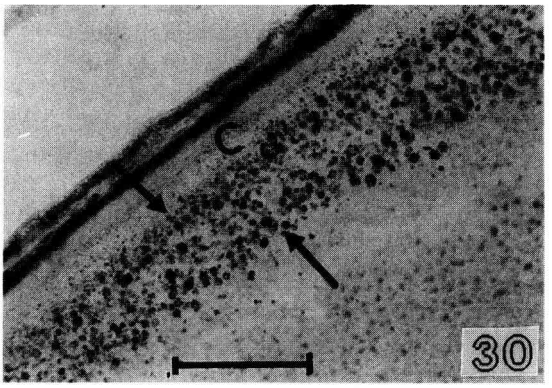


Fig 29. Autoradiograph of a section through a turtle oocyte at the end of the lampbrush stage after two injections of ^{3}H-tyrosine (4 and 8 days before fixation in paraformaldehyde and glutaraldehyde); iron hematoxylin and eosin staining; two labelled yolk layers (arrows) can be seen; bar: 100 μm.

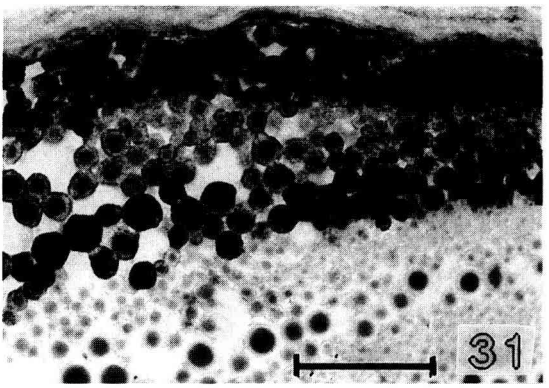


Fig 30. Autoradiograph of a section of an early postlampbrush turtle oocyte after the same procedure as in figure 29; the two labelled layers (arrows) are now localized in a more superficial position; C: cortex; bar: 100 μm.

Fig 31. Autoradiograph of a section of a 17-mm-diameter turtle oocyte after the same treatment as in figures 29 and 30; note that the labelled yolk layers are much broader and that the last-formed labelled layer is localized at the surface of the ooplasm; bar: 100 μm.

deep yolk layers below the germinal vesicle is the origin of the polar granules (fig 32) which are analogous to the polar granules in birds (Bartelmez, 1912; Callebaut, 1974). The formation and the structure of the germinal disc of *Pseudemys scripta elegans* seems to be approximately analogous to what has been described in the quail (Callebaut, 1974, 1975). The sliding is, however, slower than in the quail oocyte since in the latter, after 8 days, the labelled layer was found deep in the nucleus of Pander or in the latebra neck.

The nucleus of Pander, first described in birds by Pander (1817) as a large cushion-like mass of white yolk below the germinal vesicle, is however not clearly defined in the turtle. A large column extends from the centre of the oocyte to below the centre of the germinal vesicle (fig 33).

The alternating morphologically different yolk layers end perpendicularly to and laterally from this column. After fixation with calcium formalin and Unna staining of the sections after the autoradiographic processing, we could find at least 14–15 different layers alternatively composed of the two types of yolk and disposed in concentric layers encircling at a large distance the centre of the oocyte (figs 14 and 33). This ressembles the banding pattern described by Cuellar (1971) in the parthenogenetic lizard *Cnemidophorus uniparens* and by Uribe et al (1996) in *Ctenosaura pectinata.*

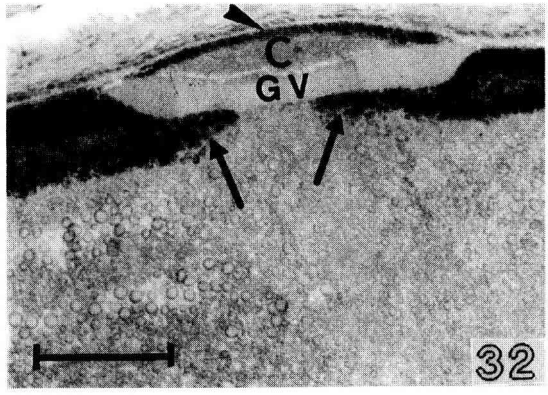


Fig 32. Autoradiograph of a section through the centre of the germinal disc of a 16-mm-diameter turtle oocyte after two im injections of ^{3}H-tyrosine (4 and 8 days before fixation in paraformaldehyde and glutaraldehyde); iron hematoxylin and eosin staining; note that the deeper labelled layer ends in the polar granules (arrows) below the germinal vesicle (GV); above the germinal vesicle a nuclear yolk cap (C) is seen; only its superficial part is labelled (arrowhead); bar: 200 μm.

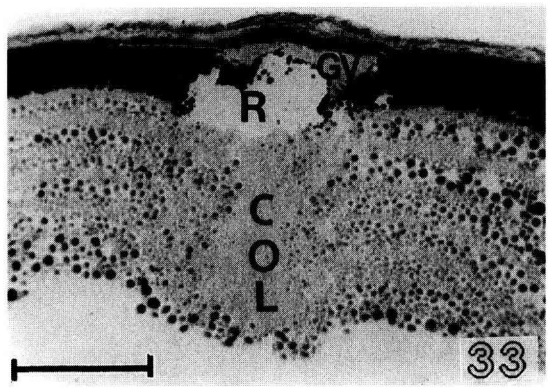


Fig 33. Autoradiograph of a section through the centre of the germinal disc of a 16-mm-diameter turtle oocyte after two im injections of ^{3}H-tyrosine; fixed in calcium formalin and stained with Unna; note the concentric banding of the yolk layers ending against a broad column (COL) going from below the germinal vesicle (GV) to the centre of the oocyte; no structure comparable to an avian nucleus of Pander is seen; R: retraction space below the germinal vesicle often seen after calcium formalin fixation; bar: 200 μm.

Comparison of the thickness and number of labelled yolk layers in the oocytes of different diameter suggests that 2–3 such layers are laid down in approximately 8 days.

Consequently, we may conclude that the period of relatively rapid growth in the studied chelonian oocyte lasts longer than in the quail oocyte, although both finally reach approximately the same volume.

DISCUSSION

By contrast to the presence of several germinal beds in the *Pseudemys* ovary, no germinal beds occur in the adult avian ovary. Indeed in the latter all the oogonia are transformed into primary oocytes at the end of embryonic and the beginning of postnatal life. In birds there are no stem germ cells for repeating the seasonal multiplication of oogonia by mitosis. The premitotic and premeiotic DNA synthesis in the chelonian oogonia and premeiocytes have some homology with what we have described in chicken and quail, respectively (Callebaut, 1967, 1973b). The labelling after ^{3}H-thymidine administration is localized on the Feulgen positive chromatin threads surrounding the central nucleoli. A difference with respect to the corresponding developmental stage in birds is that the germinal beds in the turtle are much less voluminous. In the turtle, oogonia or preoogonia are often not localized below the tunica albuginea of the ovary but still more superficially at the surface of the ovary in direct contact with the peritoneal cavity (fig 5). This very superficial position constitutes perhaps a migration path of undifferentiated female germ cells on the ovarian surface. Indeed since the studies of Waldeyer (1870) we know that in most female reptiles (in contrast to birds and mammals) oogonia persist until after sexual maturity and give rise to oocytes by mitotic divisions (Zuckerman, 1962). A follicular hierarchy with a limited number of follicles as in the avian ovary seems not to exist in the chelonian ovary. Our study also indicates that before the chelonian oocyte matures, it develops through three successive stages: prelampbrush, lampbrush and postlampbrush. As in the oocytes of birds (Callebaut, 1973a) there exists a correlation between the aspect and localization of the germinal vesicle and the ooplasm in the immediate neighbourhood. For instance in the prelampbrush stage, a voluminous paranuclear Balbiani complex is present. The postlampbrush stage begins exactly at the moment when the germinal vesicle migrates into the cortex. In the lampbrush stage, the germinal vesicle increases strongly in volume probably in relation to the lampbrush chromosome activity and the appearance of giant nucleoli. The labelling in the germinal vesicle after ^{3}H-tyrosine injection is most pronounced during the prelampbrush and lampbrush stages, whilst the labelling in the postlampbrush germinal vesicle is minimal or absent and its volume remains constant. Our study indicates that in the chelonian oocyte there also exist subcortical ooplasmic organelles distributed at the animal pole around the germinal vesicle. These organelles present homology (similar aspect, relationship with penetrating yolk and development at a comparable stage) with the geometrically distributed subcortical cytoplasmic organelles or ticos in the quail oocyte (Callebaut, 1972, 1983; D'Herde et al, 1995), and with the radially disposed mitochondrial clusters described by Tourte et al (1984) in the *Xenopus laevis* oocyte. In the present study, at the beginning of peripheral yolk formation, we demonstrated that after penetration through the oocytal cortex, granules adhere to the deeper, larger and earlier formed unlabelled yolk globules. This observation visualizes the fusion hypothesis of Ho (1987) and Guraya (1989). Moreover, the polarized or partially superficial radioactive labelling after ^{3}H-tyrosine administration often seen in the large yolk globules of the large postlampbrush oocytes also provides evidence

for this fusion hypothesis. In the largest *Pseudemys* postlampbrush oocyte, we found no particular yolk structure similar to the nucleus of Pander (1817), visible in birds. Even in the largest *Pseudemys* postlampbrush oocyte, we could (in contrast to the avian oocytes) distinguish morphologically the successive yolk layers in the deeper yolk mass below the germinal disc by the use of an appropriate fixative (calcium formalin) and Unna staining (fig 33). So it can be seen that the successive yolk layers in *Pseudemys* form only concentric spheres around the centre of the oocyte. These layers do not circumscribe a second centre (nucleus of Pander) localized below the germinal disc as we have demonstrated in the quail oocyte (Callebaut, 1974, 1975) by autoradiographic studies. In the parthenogenetic lizard, *Cnemidophorus uniparens*, Cuellar (1971) described that the germinal disc lies more or less isolated from the rest of the yolk. This does not seem to be the case in the chelonian oocyte where we observed in the present study that there is always continuity between the smaller radioactively labelled layers in the germinal disc and the broader superficial layers exterior to the germinal disc. Among Sauropsidia, a remarkable difference in the structure of the ovarian granulosa has long been recognized (Loyez, 1906). In turtles, it consists, throughout the follicle growth of a simple, homogenous cuboidal or columnar epithelium in which no pyriform cells were described (Munson, 1904; Loyez, 1906; Thing, 1918; Callebaut and Van Nassauw, 1987). In contrast, the granulosa of lizards and snakes (Squamata) develops into a complex layer composed of several cell types (Loyez, 1906; Betz, 1963; Varma, 1970; Uribe et al, 1995, 1996). The heterogeneity of the granulosa cell population in squamate reptiles is unlike that known for any other vertebrate: most notable is the transitory presence of very large clear pyriform cells. In the quail granulosa layer we have described pyriform-like cells (Callebaut, 1991b) with a structure and temporospatial distribution, that has much analogy with the pyriform cells described in squamate reptiles. We found no such pyriform-like cells in the chelonian granulosa layer. The peripheral arrangement of many nucleoli in the previtellogenic oocytes of fish, amphibia and turtles suggests some phylogenetic relationships among them (Guraya, 1989). By contrast the oocytes of squamates (Arronet, 1973) and of birds (Callebaut, 1975) have a few nucleoli that are localized in the central karyoplasm. This is a second similar aspect of the follicular and oocytal structures in squamate reptiles and birds. Moreover, the obvious and polymorphic development of the granulosa layer in squamates (with a.o. pyriform cells) and in birds (with pyriform-like cells: Callebaut, 1991b) seems to be inversely proportional to the feeble nucleolar development in the germinal vesicle of their oocytes. By contrast in Chelonians the granulosa remains monomorphic and mainly single-layered, whilst the nucleoli are very large and numerous and persist throughout the oocytal development. Perhaps only the central nucleoli observed during the prelampbrush and the lampbrush stage in *Pseudemys* are homologous to the nucleoli seen in aves and in squamate reptiles.

In the present study we demonstrate, by ip injection of trypan blue-labelled yolk, that no functional communications exist between the lacunae of the chelonian ovary and the peritoneal cavity. By contrast, in the quail it has been shown by different labelling procedures that lacuno peritoneal communications are present (Callebaut, 1979a,b, 1988a). The reason for the difference is not known. Between the hilus ovarii of both species there are indeed obvious differences. In birds the hilus ovarii is very irregular in form. Moreover, the avian ovary is fixed, without a mesovarium, directly to the dorsal wall of the peritoneal cavity in the immediate neighbourhood of the vena cava inferior. The eggs in birds are laid one by one, sometimes over long periods. The rapid

growth phase of the avian oocytes (end of the postlampbrush stage) is faster than in turtle oocytes. This requires perhaps the existence of an expansion space and escape route for the increasing pressure in the ovary.

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