NOTES

Histochemical identification of ovarian lipids during vitellogenesis in the lizard *Lacerta vivipara*

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Histochemical identification of lipids was performed on frozen sections of ovary and liver throughout thermal-induced vitellogenesis in the lizard *Lacerta vivipara*. Two classes of lipids were identified in both organs: triglycerides and phospholipids. The former are in a fluid state (stained by Sudan Black B), neutral (Nile Blue method), and unsaturated (reduce OsO_4). The latter react to a dichromate–hematoxylin method and are acidic (Nile Blue method). Throughout vitellogenic growth, oocytes simultaneously accumulate triglyceride and phospholipids linked to polypeptide granules. Both types of lipid inclusions always remain distinct.

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L'identification histochimique de lipides a été réalisée sur coupes au cryostat d'ovaire et de foie pendant la vitellogenèse du lézard *Lacerta vivipara*. Deux catégories de lipides ont été identifiées : des triglycérides et des phospholipides. Les premiers sont en phase liquide (colorés par le Noir Soudan B), neutres (méthode au Bleu de Nil) et insaturés (réduisent OsO_4). Les seconds sont spécifiquement révélés par une méthode au bichromate–hématoxyline et sont acides (méthode au Bleu de Nil). Pendant toute la croissance vitellogénique, les oocytes incorporent simultanément des triglycérides et des phospholipides liés aux granules polypeptidiques. Ces deux types d'inclusions lipidiques restent toujours distinctes.

Introduction

In nonmammalian vertebrates, the yolk (or vitellus) stored in the oocyte prior to ovulation supplies all the material required for development and growth of the embryo. The general pattern for yolk formation is quite well known and similar in all classes of nonmammalian vertebrates (reviewed by Wallace and Selman 1981; Ho et al. 1982; Wallace 1985; Ho 1987). The yolk precursor, vitellogenin, is synthesized by the liver under the main control of estradiol. This phosphorylated polypeptide–Ca^{2+} complex associated with phospholipids is thereafter incorporated by the oocytes. Vitellogenin is cleaved into lipovitellin and phosvitin fractions that form, yolk platelets or fluid-filled yolk globules, depending on the species. The lipid moiety of yolk, considered to be distinct yolk component, has received less attention, particularly in reptiles (reviewed by Wallace and Selman 1981; Ho et al. 1982; Hubert 1985; Wallace 1985). Therefore this question was addressed in a lizard, *Lacerta vivipara*, whose vitellogenesis was studied (Hubert 1970; Gavaud 1986*a*, 1986*b*). Technical difficulties had to be overcome because lipids are not covalently bound to yolk polypeptides, and large yolk-filled oocytes are brittle and difficult to section. Therefore, frozen sections were used for histochemical identification, and the biochemical nature of lipids in liver cells and in ovarian oocytes throughout vitellogenic growth could be investigated *in situ*.

Materials and methods

Animals and housing conditions

Adult females (50–60 mm in snout–vent length) were collected in the Massif Central region in August. Under laboratory conditions lizards were housed in 40 × 60 × 45 cm terraria containing soil, a shelter, and an incandescent lamp (60 W Mazda) providing heat between 10:00 and 16:00. Temperatures were 19–21°C with lamps off. Lizards were given water, crickets, and mealworms *ad libitum*. Starting in October, females were transferred to a cold room (3–7°C; 0 h L : 24 h D) for an artificial hibernation. After 4 months they were returned to warm conditions with a natural photoperiod. This thermal treatment elicits the occurrence and completion of vitellogenesis after 1 week of warming (Gavaud 1983).

Autopsy and organ processing

Lizards were sacrificed by decapitation at the end of artificial hibernation and throughout subsequent vitellogenesis. The diameter of ovarian follicles was measured to the nearest 0.1 mm using an ocular micrometer. The left ovary was fixed in Bouin's fluid and embedded in Paraplast. Serial 5-μm sections were stained with Azan's trichrome for histological examination. The right ovary and part of the liver were frozen and cut into 10-μm sections with a cryostat.

Histochemical procedures

Detailed histochemical procedures and their specificity may be found in Gomori (1952), McManus and Mowry (1963), and Wigglesworth (1988). Frozen sections were fixed for 1 h at 4°C in Baker's fixative (90 mM $CaCl_2$ in 10% neutral formalin). Lipids in a fluid state were detected according to Lison (1934), using an oil-soluble dye. Sections were stained for 45 min in a saturated solution of Sudan Black B in 50% diacetate glycerol. Unsaturated lipids were identified by their ability to reduce OsO_4 after 24 h incubation in a 0.25% OsO_4 and 0.75% $KClO_3$ solution (Adams 1959). The Nile Blue method (Cain 1947) was used to distinguish neutral lipids in a fluid state (pink staining) from acidic lipids (dark blue staining). The dichromate and hematoxylin procedure described by Bourgeois and Hubbard (1965) was chosen to detect phospholipids, whether linked to proteins or not. The specificity of all reactions was systematically controlled by extracting all lipids over a 2-h incubation period at 60°C in pyridine and extracting all lipids except phospholipids for 2 h at room temperature in acetone. Nuclear counterstaining (Nuclear Red) was used before the sections were mounted in a water-soluble medium.

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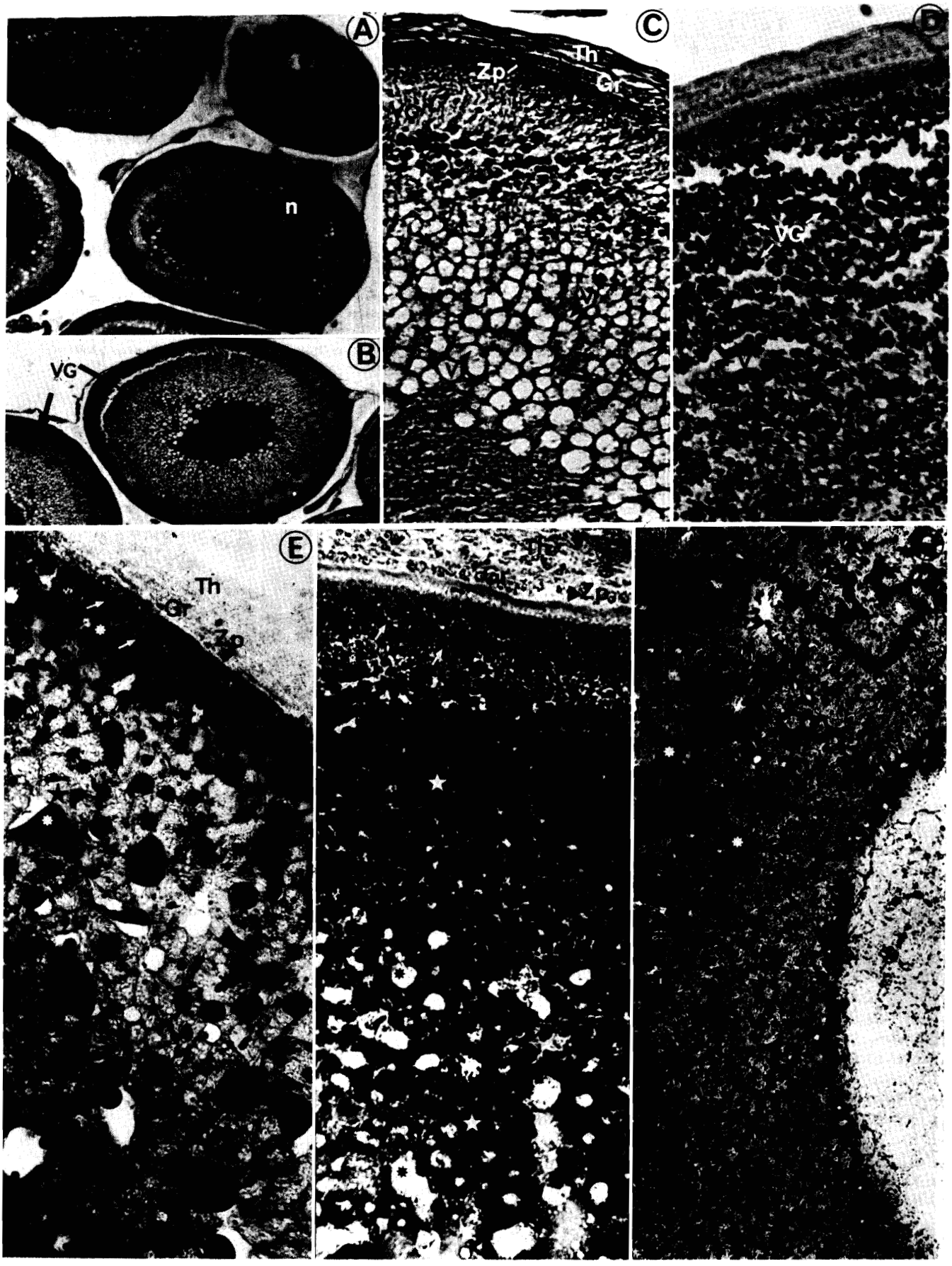

Results and discussions

Lizard hepatocytes and ovarian oocytes contain the same two classes of lipids throughout vitellogenesis. Based on four criteria, the first one is identified as a triglyceride. These lipids are in a fluid state because they stain with Sudan Black B (Fig. 1E); neutral because they stain pink with the Nile Blue method; unsaturated because they reduce OsO_4 (Fig. 1G); and extracted by both pyridine and acetone. The second class corresponds to phospholipids for three reasons: they strongly react to the dichromate-hematoxylin method (Fig. 1F); they are acidic as revealed by the Nile Blue method; and they can be extracted by pyridine only. Triglycerides and phospholipids have also been identified in hepatocytes, in blood during vitellogenesis, and in the yolk in all classes of nonmammalian vertebrates (Hahn 1967; Marshall and Gist 1973; Khoo 1979; Garstka et al. 1982; reviewed by Derickson 1976; Ho et al. 1982; Wallace 1985).

At the end of hibernation the lizard ovary contains follicles at all stages of development up to the previtellogenic stage (Panigel 1956; Hubert 1985). The previtellogenic follicles are the largest, 1.2–1.5 mm in diameter, and opalescent white. This opacificaton is enhanced during the onset of vitellogenesis while the follicles reach about 1.8–2 mm in diameter. Thereafter and until ovulation (5–8 mm), the vitellogenic follicles remain yellow. Histological and histochemical approaches allow the distinction and localization of different yolk components within the growing oocytes (Fig. 1). Small vesicles first form a crown at the periphery of previtellogenic oocytes (Fig. 1A), and then occupy most of the cytoplasm during the onset of vitellogenesis (Figs. 1B and 1C). These vesicles appear to be vacuoles when processed for histological examination; in fact they are artefacts resulting from the embedding procedure. Indeed, on frozen sections these vesicles are always filled with triglycerides. Throughout their growth, oocytes keep accumulating large amounts of triglycerides (Figs. 1E and 1G). A second type of inclusion is first observed at the onset of vitellogenesis as a peripheral crown of small granules (Figs. 1B and 1C). After the onset of vitellogenesis these vitellin granules fill the growing oocyte (Fig. 1D). They are stained red by Azocarmine G, thus they are polypeptidic in nature. Moreover, they always satisfy all criteria for phospholipid identification (see above, Fig. 1F). It can be concluded that the polypeptide granules carry the phospholipid moiety. Triglycerides remain between the phospholipid and polypeptide material (Figs. 1E, 1F, and 1G). Both types of inclusions tend to be larger towards the interior of the oocyte, droplets forming drops and granules, platelet, (Figs. 1E and 1G). These observations could indicate a centripetal migration as generally described in nonmammalian vertebrates (reviewed by Wallace and Selman 1981; Wallace 1985). Incorporation of extraovarian vitellogenin (Gavaud 1986*a*) by means of typical micropinocytosis (Hubert 1970) was clearly proven in *L. vivipara*. However, the origin of the ovarian triglycerides is unknown. These fluid lipids are most likely mobilized form the fat bodies and incorporated by the oocytes rather than synthesized *in situ* (Hahn 1967; Marshall and Gist 1973; Derickson 1976; Garstka et al. 1982).

The hepatic changes in the amount of triglycerides and phospholipids are well correlated with the oocyte-related events during *L. vivipara* vitellogenesis. At the end of hibernation only a few triglyceride droplets are seen in the hepatocytes, then the amount displays a transitory increase while oocytes incorporate only triglycerides. Thereafter and until ovulation, triglycerides are far less abundant in the hepatocytes than phospholipids are. This massive presence of phospholipids in the hepatic cells and their association with polypeptide granules within the oocytes could indicate that vitellogenin lipid formation occurs in the liver. This has been demonstrated in several nonmammalian species (reviewed by Wallace and Selman 1981; Ho et al. 1982; Wallace 1985).

The present study emphasizes that vitellogenesis in the lizard *L. vivipara* is characterized by the simultaneous accumulation of triglyceride between the vitellin granules, and phospholipids linked to yolk polypeptides (Figs. 1E, 1F, and 1G). This is the first description of a significant accumulation of triglycerides as a distinct yolk component in a reptile. Triglycerides have also been localized in vitellogenic oocytes in fishes. However, these fluid lipids are always associated with proteins and phospholipids within the vitellin vesicles (Khoo 1979; reviewed by Wallace and Selman 1981).

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FIG. 1. Photomicrographs of ovarian sections in the lizard *Lacerta vivipara* at the the previtellogenic stage (A), at the onset of vitellogenesis (B and C), at a later stage, 3 mm in diameter (D, E, and F), and before ovulation (G). (A) Previtellogenic oocytes stained with Azan's trichrome. They contain a fine peripheral layer of vesicles (V) emptied by the embedded procedure (×25). (B) Vitellogenic oocytes are first filled, with emptied vesicles well individualized (V). Vitellin granules (VG) form a narrow peripheral crown. Azan's trichrome (×25). (C) The section shown in B at a higher magnification (×400). (D) Later, vitellogenic oocytes are filled with vitellin granules (VG) and emptied vesicles (V). Azan's trichrome (×400). (E) Cryostat section of a vitellogenic oocyte, showing that the vesicles emptied by the histological procedure are in fact filled with lipids stained with Sudan Black B. Arrows indicate peripheral droplets and white asterisks, drops of triglycerides. The material between the triglyceride drops (black star) is not stained with this method. (×600). (F) Cryostat section of a vitellogenic oocyte stained with the bichromate–hematoxylin method (×600). Phospholipids are dark stained. Arrows indicate peripheral granules and white stars, platelets. Triglycerides did not react (black stars). (G) Cryostat section of a vitellogenic oocyte stained with the OsO_4 method (×600). Triglycerides in a fluid state reduced OsO_4 (white asterisks), whereas the vitellin platelets did not react. *n*, nucleus; Th, theca; Gr, granulosa; Zp, zona pellucida.

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Immunoreactivity to the antiserum to the mammalian neuropeptide substance P in the central nervous system of the house fly, *Musca domestica*

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Localization of immunoreactivity to the antiserum to the mammalian neuropeptide substance P in the central nervous system of the house fly, *Musca domestica*, was investigated by immunocytochemical methods. In both the larva and the adult a total of 16 neurons reacted positively against substance P antiserum. Of these, 10 were in the brain and 6 in the thoracic ganglia. The neurons were arranged in bilateral pairs. In the larval nervous system each brain lobe contained a cluster of four pairs of immunoreactive neurons in the dorsal protocerebrum, and the subesophageal ganglion and each of the thoracic neuromeres contained one pair each. The adult nervous system possessed the same number of imunoreactive neurons with identical distribution within the subesophageal and thoracic ganglia. However, the position of the protocerebral neurons was slightly altered. The pattern of immunoreactive axonal processes as well as the very high immunoreactivity observed in the dorsal neural sheath of the adult thoracic ganglion suggest a neurohormonal–neuromodulator role for substance P in this insect.

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Des méthodes immunocytochimiques ont été utilisées pour identifier l'origine de l'immunoréactivité d'une substance semblable au neuropeptide « facteur P » des mammifères dans le système nerveux central de la Mouche domestique, *Musca domestica*. Chez les larves aussi bien que chez les adultes, 16 neurones au total ont une réaction positive à l'antisérum du facteur P. Parmi ceux-ci, 10 sont situés dans le cerveau, et 6 dans le ganglion thoracique. Les neurones forment des paires bilatérales. Dans le système nerveux central de la larve, chaque lobe du cerveau contient un faisceau de quatre paires de neurones immunoréactifs dans le protocérébrum dense, alors que le ganglion sous-oesophagien et les neuromères thoraciques en contiennent chacun une paire. Chez l'adulte, le système nerveux est constitué du même nombre de neurones immunoréactifs, et leur répartition dans les ganglions sous-oesophagien et thoracique est identique. Cependant, la disposition des neurones protocérébraux est légèrement modifiée. La disposition des processus immunoréactifs des axones et l'immunoréactivité très élevée enregistrée dans le manchon neural dorsal du ganglion thoracique de l'adulte permettent de supposer que le facteur P joue un rôle neurohormonal–neuromodulateur chez la Mouche domestique.

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