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OVARIAN ECDYSTEROID LEVELS AND BASAL OÖCYTE DEVELOPMENT DURING MATURATION IN THE COCKROACH *BLATTELLA GERMANICA* (L.)

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Abstract—By using enzyme immunoassay techniques we have quantified the ovarian ecdysteroids during the 7-day cycle of oöcyte maturation in *Blattella germanica*. Immunoreactive ecdysteroid levels were low on days 0–2, increased to higher values on days 3–6, and peaked on day 7 (about 5 ng of 20-hydroxyecdysone equivalents per pair of ovaries). The predominant non-conjugated ovarian ecdysteroid detected by HPLC analysis was 20-hydroxyecdysone. During oöcyte maturation, the dynamics of patency, vitellogenin uptake, increase in follicle cells diameter (suggested to be due to polyploidy), and choriogenesis was studied. A comparison of the ovarian ecdysteroid profile with the succession of physiological processes occurring within the oöcyte maturation cycle suggests that these hormones could be directly or indirectly involved in the control of patency, polyploidy and choriogenesis in the follicle cells.

Key Word Index: Immunoreactive ecdysteroids; 20-hydroxyecdysone; juvenile hormones; developing oöcyte; follicle cells; vitellogenesis; choriogenesis; cockroach

INTRODUCTION

The presence of ecdysteroids in the ovaries of reproductively competent female insects has been reported in a variety of species (for reviews see Hagedorn, 1985; Lanot *et al.*, 1989) and it is generally accepted that insect ovary, and more specifically the follicular epithelium (see Zhu *et al.*, 1983), is able to synthesize ecdysteroids at some stage of the maturation cycle.

Concerning the functions exerted by ovarian ecdysteroids, and according to the studies initiated by Hagedorn and co-workers, it now seems clear that in mosquitoes a brain hormone induces the synthesis of ovarian ecdysteroids and these, in turn, trigger the synthesis of vitellogenin in the fat body. In other insects in which vitellogenin synthesis is induced by juvenile hormone, different functions have been proposed for ovarian ecdysteroids. These include control of oöcyte meiotic reinitiation, supply of a source of ecdysteroids to the embryo, and inhibition of juvenile hormone production (for reviews see Hagedorn, 1985; Lanot *et al.*, 1989).

Nevertheless, possible functions of ecdysteroids in oöcyte development during maturation are still conjectural. Therefore, we studied the ovarian ecdysteroid contents during the first cycle of oöcyte maturation, bearing in mind the idea of correlating the obtained profile with the chain of cellular changes occurring in the follicular epithelium during this cycle. As an experimental insect species, we used the cockroach *Blattella germanica*. For this species many data are available concerning the role of juvenile hormone and the regulation of the corpora allata during oöcyte maturation (Bellés and Piulachs, 1983; Bellés *et al.*, 1987, Bellés and Piulachs, 1989; Piulachs, 1988; Gadot *et al.*, 1989). This study could plausibly allow stimulating comparisons between the two endocrinological systems, juvenile hormone and ecdysteroids.

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MATERIALS AND METHODS

Insects

Colonies of *B. germanica* were reared in complete darkness at 30 ± 1°C and 60–70% r.h. Freshly ecdysed females were isolated in absence of males and maintained in the same conditions as the colony until use.

Ovarian extracts for ecdysteroid determinations

Both ovaries were individually dissected under Ringer solution and processed by extraction with methanol (200 μl), followed by homogenization and centrifugation (13,000 *g*, 5 min). The pellet was resuspended in methanol (200 μl) in an ultrasonic bath and centrifugated again (13,000 *g*, 5 min). Then the supernatants of both centrifugations were joined, evaporated under nitrogen and stored at -20°C until use. Enzyme immunoassay determinations were carried out with extracts of individual ovaries. For high performance liquid chromatography determinations, extracts of a pool containing 20–30 ovary pairs were used for each analysis.

Enzyme immunoassays (EIA)

Solid-phase EIA were performed according to the method described by Porcheron *et al.* (1989) with slight modifications (see Marco *et al.*, 1990), by using a Multiskan MC spectrophotometer (Flow Laboratories, Helsinki, Finland) set at 405 nm. Microtitre plates were from NUNC (Model 96F, Denmark). The main antiserum (AS4919) used was supplied by Professor P. Porcheron (École Normale Supérieure, Paris, France). The enzymatic tracer (20-hydroxyecdysone-carboxymethoxime-acetylcholin-esterase conjugate) was supplied by Dr P. Pradelles (Service de Pharmacologie et d'Immunologie, CEA/Saclay, France). All other reagents, including pure goat antirabbit immunoglobulin monoclonal antibody, were from Sigma, St Louis, MO, U.S.A. Samples were redissolved in the EIA buffer and determinations were carried out in duplicate. Since the main antiserum used has the same affinity for ecdysone and 20-hydroxyecdysone, and the standard curve was obtained with calibrated solutions of the latter compound, results are expressed as nanograms of 20-hydroxyecdysone equivalents per ovary pair. For each batch of microtitre plates, coating with the second antibody was performed by simultaneous addition of glutaraldehyde to stick the antibody to the plate (see Porcheron *et al.*, 1989). The efficiency of the coating was optimized following the procedure described by Muñoz *et al.* (1986), which is based on a horseradish peroxidase saturation technique. In our conditions, the optimal concentration for second antibody coating was 20 μg/ml.

High performance liquid chromatography (HPLC) analysis

Chemicals. Standard ecdysteroids were obtained from the plants *Ajuga reptans* (ajugalactone) and *Polypodium vulgare* (ecdysone and 20-hydroxyecdysone). Extraction and purification were basically as described elsewhere (Jizba *et al.*, 1967; Camps *et al.*, 1982). HPLC grade solvents were obtained from Merck (Darmstadt, Germany) or Scharlau (Barcelona, Spain) and reversed phase Sep-pak C_{18} cartridges were from Merck. Water for HPLC was purified by a Milli-Q system and, prior to use, filtered through 0.45 μm Millipore filters (type HA, for aqueous solvents) and degassed in ultrasonic bath under vacuum.

HPLC system. Two Applied Biosystems model 400 pumps were used for solvent delivery. Samples were introduced via an AB-491 dynamic mixer/injector. Detection of compounds and acquisition of u.v. spectra were performed with an AB-1000S diode-array detector, chromatograms were monitored at 242 and 330 nm and recorded with a Hewlett-Packard 3396A integrator or a Waters-740 data module. Temperature was regulated with a Spark Holland SPH-99 column thermostat. The analyses were carried out on a LiChroCART 12.5 × 0.4 cm (5 μm) LiChrospher 100 RP-18 (Merck) reversed phase column under two different isocratic conditions. Samples were eluted using isopropanol/water (9.75:90.25) at 45°C or isopropanol/water (7:93) at 55°C as mobile phase, in both cases at a flow-rate of 1.2 ml/min. 20-hydroxyecdysone and ecdysone were identified by comparison of their retention times and u.v. spectra with those of authentic samples. In these systems ecdysone and 20-hydroxyecdysone show a u.v. maximum at 247 nm.

Preparation of samples. Ovarian extracts were resuspended in 7 ml of water in an ultrasonic bath and a solution of ajugalactone (840 ng in 40 μl) was added as internal standard. The solution was partitioned with Cl_3CH (2 × 3 ml) and centrifugated (1500 *g*, 10 min) to remove apolar products and precipitate proteins. The organic layers were washed with water (3 ml) and the mixed aqueous solutions (10 ml) were filtered through a reversed phase C_{18} Sep-pak cartridge. The more polar substances were removed by elution with 10 ml of 15% aqueous methanol. Then, ecdysteroids were recovered with 4.5 ml of 85% aqueous methanol (substances less polar than ecdysone were not eluted under these conditions), the first 0.5 ml were discarded and the next 4 ml were collected, dried under a current of nitrogen and redissolved in 150 μl of a methanol/water (50:50) mixture. This solution was injected into the HPLC column for quantitative analysis.

Quantification. Ecdysteroid amounts were estimated by interpolation in the calibration curves obtained for 20-hydroxyecdysone, ecdysone and ajugalactone. Ajugalactone was used as an internal standard to calculate the recovery factor. This compound elutes between 20-hydroxyecdysone and

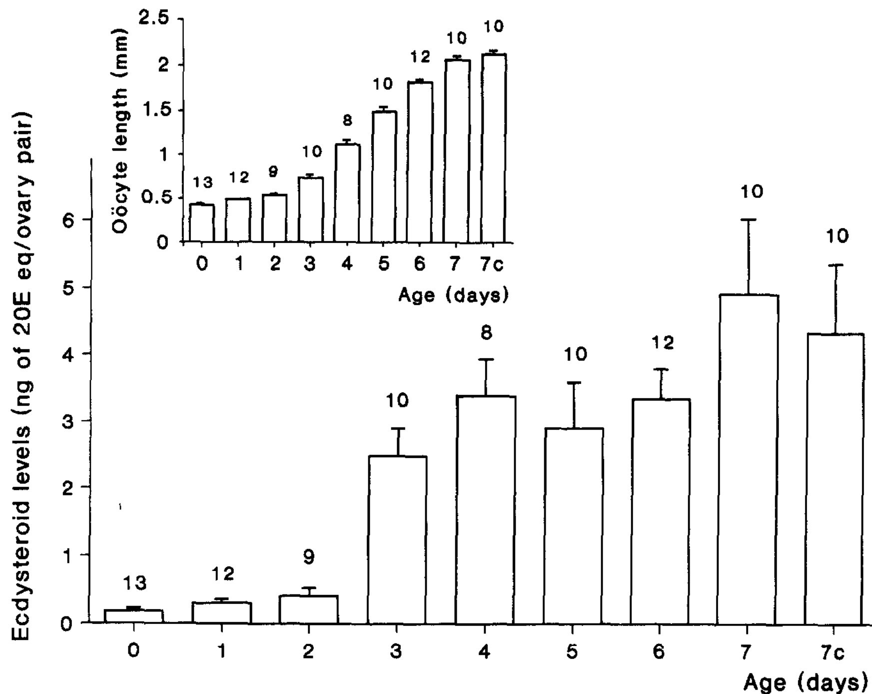

Fig. 1. Ovarian immunoreactive ecdysteroid levels as a function of age during the first gonadotrophic cycle in *B. germanica*. Results are expressed as ng of 20-hydroxyecdysone equivalents (20 E eq) per pair of ovaries. The inset shows the basal oöcyte length of the extracted ovaries. Vertical bars represent the standard error of the means (SEM) and the number of experimental specimens is indicated at the top of each column. The age 7c means ovaries from 7-day-old females with the basal oöcytes chorionated.

ecdysone and does not interfere with other products under our analytical conditions.

Oöcyte and follicle cell biometry

Basal oöcyte length was measured using an ocular micrometer adapted to a stereomicroscope. Three basal oöcytes selected at random in each ovary per specimen were measured and averaged.

To estimate the total number of follicle cells in basal oöcytes of different ages, the cells contained in a photograph covering an actual surface of 0.03 mm^2 in the equatorial zone of the oöcyte were counted (C value). Then, the oöcyte surface (OS) was calculated according to the formula $OS = 4\pi\ (D \times d/2)$, where D and d are the two diameters (in mm) of the oöcyte. Finally, the total number of follicle cells (TN) was estimated according to the expression $TN = C \times OS/0.03$.

Follicle cell diameter was measured on the photographs obtained from the follicular epithelium of each specimen and using the appopriate conversion scale to real size in μm. Ten cells selected at random were measured and averaged.

Use of trypan blue to show vitellogenin uptake

The vital dye trypan blue was used to demonstrate vitellogenin incorporation into the oöplasm, as it is an effective mimic of vitellogenin in its ability to be taken by oöcytes (Ramamurty, 1964; Telfer and Anderson, 1968; see also Yonge and Hagedorn, 1977). Trypan blue (Merck 11732) was dialysed against distilled water for at least 24 h and was then oven-dried for 24 h. With double distilled water as solvent, a fresh 2% solution was made for each experiment. A 2 μl injection of the solution was given to each female between two abdominal sternites. The experimental specimens were dissected 24 h after the injection, and the incorporation of the colourant into the oöplasm was checked under a stereomicroscope.

Patency measurements

To study the patency (degree of enlargement of the intercellular spaces in the follicular epithelium, see Davey, 1981), ovaries were dissected and placed on a microscope slide in a drop of 1% Evans' blue in Ringer solution. After about 10 min, the excess dye was drained off and the preparation observed and photographed in a light microscope, without the use of a cover slip and using reflected light. With the photographs obtained we calculated the patency index (PI), $PI = SE/SC$, where SE is the surface of the intercellular spaces and SC the surface of the follicle cells. These surfaces were estimated on the photographs by using a digital system of image processing "Microm Image Processing 1.4" (Microm, Barcelona, Spain).

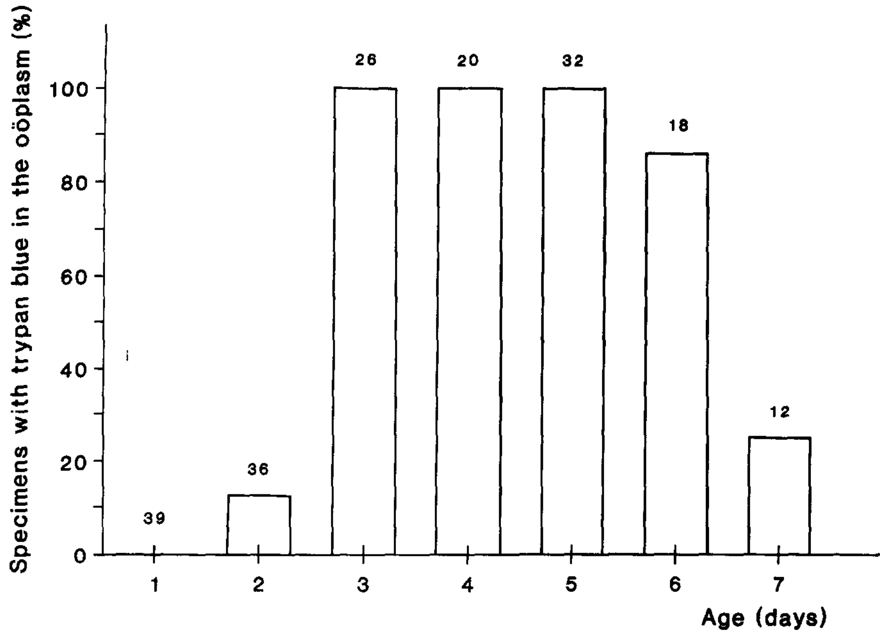

Fig. 2. Percentage of specimens of *B. germanica* adult females which incorporated trypan blue dye into the oöplasm in the different days within the first gonadotrophic cycle. The number of experimental specimens is indicated at the top of each column.

Structural study of choriogenesis

Conventional light microscopy techniques were used to study the choriogenesis process. Dissected ovaries were fixed for 24 h in Bouin's mixture and, after dehydration through alcohol steps, samples were embedded in paraffin wax, sectioned at 7 microns and processed for haematoxylin-eosin staining. Previous to the histological preparation, the shape of the oöcyte apical pole, where the micropile

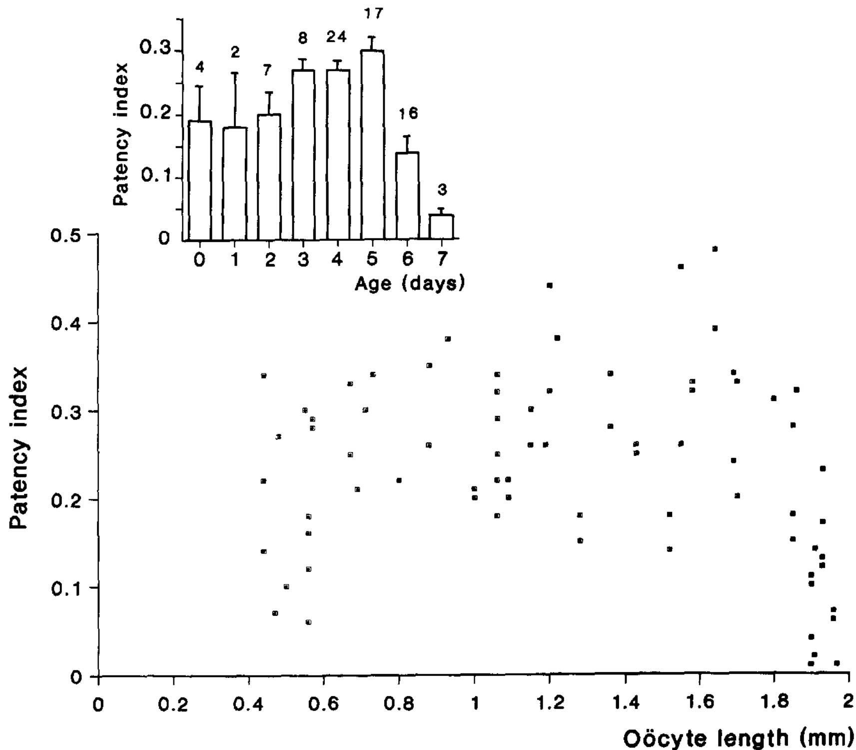

Fig. 3. Relationship between patency index and basal oöcyte length in individual adult female *B. germanica* in the first gonadotrophic cycle. The inset shows the patency index as a function of age. Vertical bars represent the SEM, and the number of experimental specimens is indicated at the top of each column.

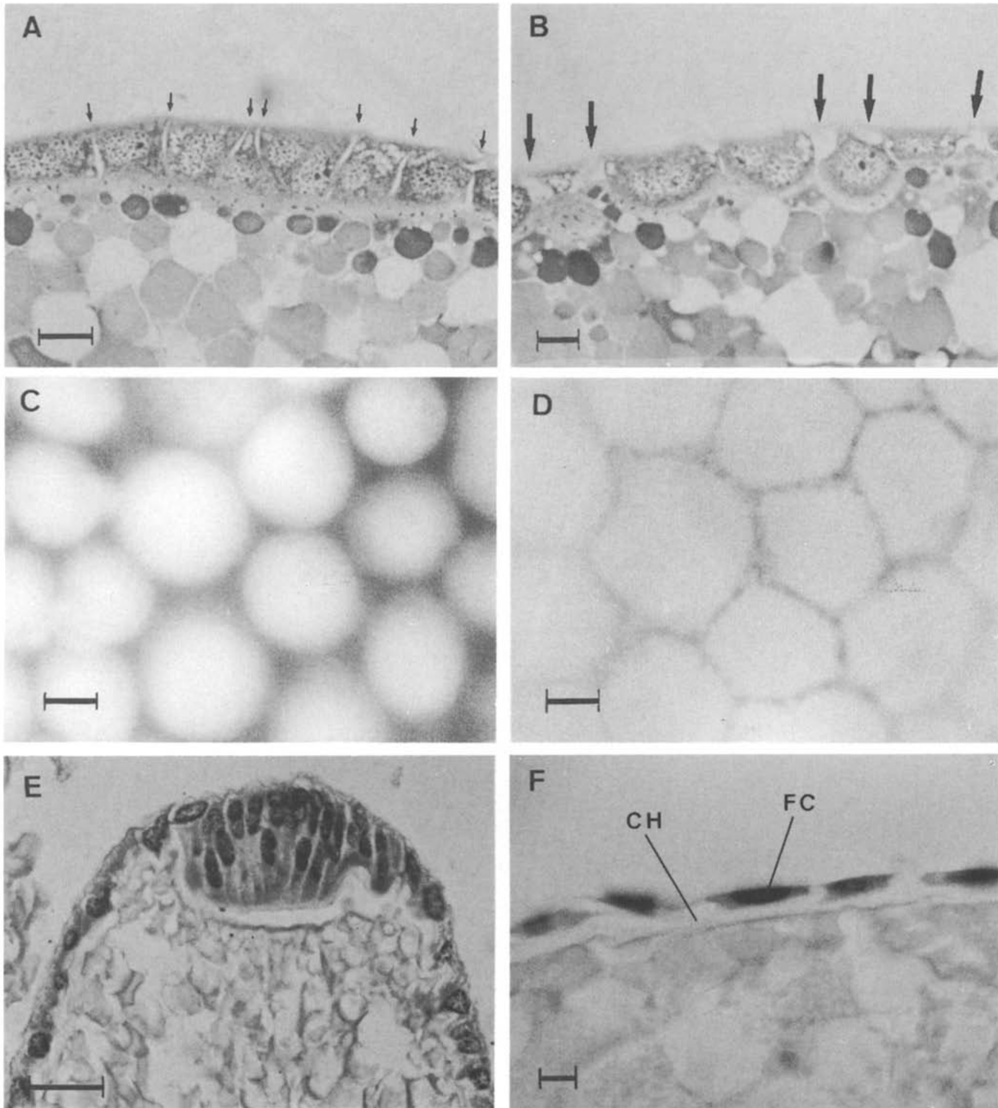

Fig. 4. Follicular epithelium in *B. germanica* oöcytes. (A) and (B) Sections at the equatorial zone in oöcytes from 3-day-old (A) and 5-day-old (B) females; note that intercellular spaces (arrows) are larger in (B). (C) and (D) Photographs of the surface of a vitellogenic oöcyte from a 6-day-old female (C) and from an oöcyte just before choriogenesis from a 7-day-old female (D). (E) and (F) Sections at the apical pole of an oöcyte at about mid-chorion formation (E) and at the equatorial zone of an oöcyte at late-chorion formation (F); note (in F) the chorion layers (CH) and the degenerated follicle cells (FC). Scale lines: 10 μm (A, B, C, D, F) and 100 μm (E).

is formed in the chorion, was recorded in order to correlate this feature with the level of chorion deposition.

RESULTS

Immunoreactive ecdysteroid levels

Immunoreactive ecdysteroid levels in ovaries throughout the first maturation cycle are shown in Fig. 1 plotted against age. The basal oöcyte length profile of the extracted ovaries is also shown (Fig. 1, inset). Ecdysteroid levels are relatively low on days 0–2, increase to quite high values on days 3–6 and peak on day 7.

In addition, supplementary measurements of immunoreactive ecdysteroid levels in freshly formed oötheca (less than 24 h after their complete formation) were carried out. These measurements gave 4.175 ± 0.484 ng ($n = 4$) of 20-hydroxyecdysone equivalents per oötheca, a value similar to that obtained in ovaries from 7-day-old females with chorionated oöcytes (cf. Fig. 1).

HPLC quantification of 20-hydroxyecdysone

To assess the identity of the main free ecdysteroids measured by the EIA, HPLC analyses of ovarian extracts were carried out.

By comparison of retention time and u.v. spectrum with standard compounds, the main non-conjugated ovarian ecdysteroid detected was 20-hydroxyecdysone, which was shown to be present at quantifiable levels in all ages studied. In addition, trace amounts (not quantifiable) of ecdysone were present in some samples. Using the phytoecdysteroid ajugalactone as an internal standard, we were able to quantify the levels of 20-hydroxyecdysone by interpolation with previously established calibration lines. The results, expressed as ng of 20-hydroxyecdysone ($\bar{x} \pm$ SEM corresponding to the number of analyses carried out with the same extract), were as follows: day 3, 1.2 ± 0.1 ($n = 4$); day 4, 1.5 ± 0.1 ($n = 3$); day 5, 1.4 ± 0.4 ($n = 3$); day 6, 1.9 ± 0.2 ($n = 4$); day 7, 4.0 ± 0.2 ($n = 2$). These results are in good agreement with those obtained with the EIA, since the differences between the titres measured by the two techniques at the corresponding ages are not statistically significant (Mann–Whitney U-test).

Number and diameter of follicle cells

Prior to study the increase in size of the follicle cells, an estimation of the cell number in the follicular epithelium of basal oöcytes from females of different ages was carried out. The results obtained ($\bar{x} \pm$ SEM, $n = 5$ in all cases) were as follows: day 1, 3152 ± 80; day 3, 3224 ± 91; day 5, 3230 ± 124; day 6, 3226 ± 182. Differences between the total number of follicle cells in different ages were not statistically significant (Mann–Whitney U-test) which suggest that there is no cellular division during the process of ovarian maturation.

Conversely, the follicle cell size shows a steady increase during the ovarian maturation process (graphic not shown). The cell diameter increases from 5–7 μm (previtellogenic oöcytes, about 0.5 mm in length) to 30–40 μm (oöcytes just before chorionation, about 2 mm in length). In other species of cockroaches (for example, in *Leucophaea maderae*, see LaPointe *et al.*, 1985) the increase in size of the follicle cells during the maturation cycle has been demonstrated to be due to polyploidy. It is reasonable, therefore, to hypothesize that this might also be the case in *B. germanica*.

Dynamics of vitellogenin uptake as showed by trypan blue

Figure 2 shows the percentage of specimens which incorporated trypan blue dye into the oöplasm on the different days of the first gonadotrophic cycle. From these results, it can be inferred that vitellogenin uptake effectively begins on day 3, continues on days 4 and 5, slightly diminishes on day 6 and shows a remarkable decrease on day 7.

The low percentage observed in 2-day-old specimens seems not to be significant. From a sample of 36 specimens, only five of them showed symptoms of colourant incorporation, their basal oöcyte length being at the upper limit of that age (0.54–0.55 mm).

It is also worth mentioning that two out of 18 and five out of 12 specimens of the 6- and 7-day age groups, respectively, showed signs of colourant in the follicular epithelium, but that no incorporation into the oöplasm was observed. These specimens were not considered as positive cases of uptake in Fig. 2.

Patency

Values obtained for the patency index plotted against the basal oöcyte length of the corresponding specimen are represented in Fig. 3. Although there is a remarkable dispersion of values, the general tendency is that oöcytes between 0 and 2 days. [Fig. 4(A)] have low patency index which then increases on day 3, maintains quite high values on days 4 and 5 [Figs 4(B) and (C)], and decreases on day 6. On day 7 only three specimens which had not began the choriogenesis (thus with intact follicle cells) were found. These three specimens showed a very low patency index [Fig. 4(D)]. When patency index values

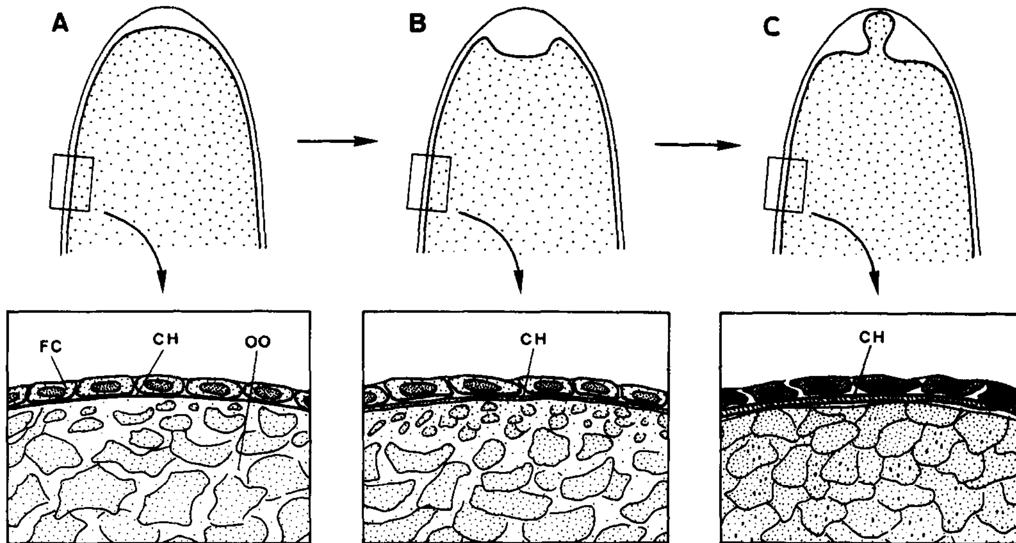

Fig. 5. Changes undergone by the apical pole of *B. germanica* oöcytes during choriogenesis. (A) Early chorion formation, (B) mid chorion formation and (C) late chorion formation CH, chorion; FC, follicle cells; OO, oöplasm.

are plotted against the follicle cell diameter of the corresponding specimen (graphic not shown), a picture similar to Fig. 3 is obtained.

Choriogenesis

Choriogenesis begins on the 6th day, the basal oöcyte length measuring between 1.8 and 2 mm, and continues until completion, when the oöcyte reaches 2 to 2.4 mm in length. The whole process is completed in a period of 24 h or less, during which the apical pole of the oöcyte undergoes discrete changes that can be correlated with the process of chorion deposition (Fig. 5). The first structural signs of chorion secretion were observed when the shape of the oöcyte apical pole showed the basement membrane slightly pulled away from the follicle cells [Fig. 5(A)]. In the stage designated here as mid-chorion formation, these anterior follicle cells become markedly taller [Figs 4(E) and 5(B)], and at late chorion formation [Fig. 4(F)], the apical pole of the oöcyte has a claviform aspect [Fig. 5(C)].

DISCUSSION

Ovarian ecdysteroids

The ovarian ecdysteroid levels during the first maturation cycle of *B. germanica* are low on days 0–2, suddenly increase on day 3, maintain quite high values until day 6 and peak on day 7 (Fig. 1). In other species of cockroaches, like *Blaberus craniifer* (Bullière *et al.*, 1979), *Leucophaea maderae* (Koeppe, 1981), *Nauphoeta cinerea* (Zhu *et al.*, 1983; Lanzrein *et al.*, 1985) and *Diploptera punctata* (Stay *et al.*, 1984), the highest levels of ovarian ecdysteroids are also found towards the end of the maturation cycle, around the choriogenesis stage. Yet, in adult female of *Periplaneta americana*, titres of haemolymph ecdysteroids (which apparently come mainly from the ovaries) also peak when the basal oöcytes are chorionating (Weaver *et al.*, 1984).

The predominant non-conjugated ecdysteroid detected by HPLC analysis of ovarian extracts from *B. germanica* was 20-hydroxyecdysone. In addition, results obtained by the quantification of 20-hydroxyecdysone levels (from days 3 to 7) by using this technique are in good agreement with data resulting from EIA determinations. Thus, it appears that 20-hydroxyecdysone is responsible for most of the EIA activity observed. The predominance of 20-hydroxyecdysone in ovarian tissues has also been shown by physico-chemical methods in *N. cinerea* (Zhu *et al.*, 1983; Zhu and Lanzrein, 1984) as well as in *D. punctata* (Stay *et al.*, 1984). Conversely, Koeppe (1981) reported the ovarian ecdysteroid in *L. maderae* to be ecdysone (analytical methodology not detailed).

In *P. americana*, the haemolymph ecdysteroid, apparently coming mainly from the ovary, was identified (HPLC-RIA) as 20-hydroxyecdysone (Weaver *et al.*, 1984). However, the predominant ecdysteroids in *P. americana* eggs are 2-deoxyecdysone, followed by ecdysone and 20-hydroxyecdysone, as shown by HPLC and RIA (Slinger and Isaac, 1988). In freshly laid eggs of *L. maderae*, ecdysone detected by TLC-RIA was reported as the predominant ecdysteroid (Matz, 1980). However, Imboden and Lanzrein (1982), using TLC, HPLC

and RIA, gave 20-hydroxyecdysone as the predominant one in freshly ecdysed eggs of this species as well as for *N. cinerea*.

Although it seems premature to generalize, at least in oöcyte tissues, 20-hydroxyecdysone appears to be predominant in most of the cockroach species so far studied.

Basal oöcyte development

Under our rearing conditions, the basal oöcytes mature and chorionate within 7 days following the imaginal ecdysis. The basal oöcyte length of the ovaries used for EIA determination of ecdysteroids gave a profile (Fig. 1, inset) which is similar to that previously reported (Bellés and Piulachs, 1983).

Between imaginal ecdysis and day 3, the basal oöcytes are previtellogenic. Intercellular spaces in the follicular epithelium are narrow and no vitellogenin is incorporated into the oöplasm. The length of the oöcyte and the size of the follicle cells have a slight tendency to increase.

Day 3 marks the transition between previtellogenic oöcytes and vitellogenic ones. Large intercellular spaces appear in the follicular epithelium, and vitellogenin begins to be taken by the oöcyte, as shown by trypan blue experiments. Apparently due to polyploidy, the follicle cell diameter increases more or less in parallel with the increase in oöcyte length. Large intercellular spaces in the follicular epithelium [Figs 4(B) and (C)] are maintained from days 3 to 5, and decrease on days 6 and 7.

On day 7 choriogenesis takes place; its progress can be followed through discrete morphological changes experienced by the oöcyte apical pole (Fig. 5). These changes can be correlated structurally with the increase in thickness observed in the chorion layers. Choriogenesis is immediately followed by ovulation and oviposition.

Possible roles of ovarian ecdysteroids during oöcyte maturation

It seems clear that in mosquitoes the ovarian ecdysteroids induce the synthesis of vitellogenins in the fat body. In other insects in which vitellogenin synthesis is induced by juvenile hormone, functions proposed for ovarian ecdysteroids include control of meiotic reinitiation, supply of a source of ecdysteroids to the embryo and inhibition of juvenile hormone production (for reviews see Hagedorn, 1985; Lanot *et al.*, 1989).

The detection of immunoreactive ecdysteroids in the freshly formed oötheca of *B. germanica* suggests that the function of an ecdysteroid supply to the embryo could be reasonably postulated in this species as well. In addition, the comparison of the profile of ovarian ecdysteroid levels (Fig. 1) with the succession of cellular changes occurring in the follicular epithelium during oöcyte maturation (Figs 3–5) allows us to see that important changes occurring on day 3 (opening of intercellular spaces, thus allowing vitellogenin uptake) and on day 7 (secretion of chorion materials) are coincident with significant increases of ecdysteroid levels. It is also interesting to note that the cell diameter increase, possibly due to polyploidy, parallels the maintenance of quite high levels of ovarian ecdysteroids. These coincidences suggest that ecdysteroids could play some role in the induction of patency, polyploidy and choriogenesis.

Taking into consideration the practical co-occurrence of choriogenesis and the ecdysteroid peak appearing at the end of the maturation cycle, the possible role of ovarian ecdysteroids in such a process has been suggested in other cockroaches (Zhu *et al.*, 1983; see, however, Stay *et al.*, 1984).

Conversely, induction of patency and polyploidy are functions classically assigned to juvenile hormone (for review see Koeppe *et al.*, 1985). However, it may also be envisaged that these juvenile hormone effects could be mediated by ovarian ecdysteroids, since juvenile hormone effectively stimulates ecdysteroid synthesis in cockroach ovaries (Koeppe, 1981).

Further studies involving the use of exogenous hormones and the induction of hormonal depletions appear essential to assess whether or not the ovarian ecdysteroids are involved in the control of patency, polyploidy and choriogenesis in the follicle cells. This is the subject of current experimental work in our laboratory.

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REFERENCES

Bellés X. and Piulachs M. D. (1983) Desarrollo de los corpora allata, oocitos y glándulas colaterales durante el primer ciclo gonotrófico de *Blattella germanica*. *Revta esp. Fisiol.* **39**, 149–154.

Bellés X. and Piulachs M. D. (1989) Occurrence of brain allatostatic factors in the cockroach *Blattella germanica*. *Acta ent. Bohemoslov.* **86**, 161–166.

Bellés X., Casas J., Messeguer A. and Piulachs M. D. (1987) *In vitro* biosynthesis of JH III by the corpora allata of adult females of *Blattella germanica* (L). *Insect Biochem.* **17**, 1007–1010.

Bullière D., Bullière F. and de Reggi M. (1979) Ecdysteroid titres during ovarian and embryonic development in *Blaberus craniifer*. *Wilhelm Roux Arch. Devl Biol.* **186**, 103–144.

Camps F., Coll J. and Cortel A. (1982) 29-Norsengosterone and 29-norcyasterone, new C-28 phytoecdysteroids from *Ajuga reptans* (Labiatae). *Chem. Lett.* pp. 1313–1316.

Davey K. G. (1981) Regulation of cell volume in follicle cells of *Rhodnius* by JH. In *Juvenile Hormone Biochemistry. Action, Agonism and Antagonism* (Eds Pratt G. E. and Brooks G. T.), pp. 233–240. Elsevier/North Holland, Amsterdam.

Gadot M., Burns E. and Schal C. (1989) Juvenile hormone biosynthesis and oocyte development in adult female *Blattella germanica*: effects of grouping and mating. *Archs Insect Biochem. Physiol.* **11**, 189–200.

Hagedorn H. H. (1985) The role of ecdysteroids in reproduction. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Eds Kerkut G. A. and Gilbert L. I.), Vol. 8, pp. 205–262. Pergamon Press, Oxford.

Imboden H. and Lanzrein B. (1982) Investigations on ecdysteroids and juvenile hormones and on morphological aspects during early embryogenesis in the ovoviviparous cockroach *Nauphoeta cinerea*. *J. Insect Physiol.* **26**, 37–46.

Jizba J., Herout V. and Sorm F. (1967) Isolation of ecdysterone (crustecdysone) from *Polypodium vulgare* L. rhizomes. *Tetrahedron Lett.* pp. 1689–1691.

Koeppe J. K. (1981) Juvenile hormone regulation of ovarian maturation in *Leucophaea maderae*. In *Regulation of Insect Development and Behavior* (Eds Sehnal F., Zabza A., Menn J. J. and Cymborowski B.), Vol. 2, pp. 505–522. Wroclaw Technical Univ. Press, Wroclaw, Poland.

Koeppe J. K., Fuchs M., Chen T. T., Hunt L.-M., Kovalick, G. E. and Briers T. (1985) The role of juvenile hormone in reproduction. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Eds Kerkut G. A. and Gilbert L. I.), Vol. 8, pp. 165–203. Pergamon Press, Oxford.

Lanot R., Dorn A., Günster B., Thiebold J., Lagueux M. and Hoffman J. A. (1989) Functions of ecdysteroids in oocyte maturation and embryonic development of insects. In *Ecdysone. From Chemistry to Mode of Action* (Ed. Koolman J.), pp. 262–270. Georg Thieme, Stuttgart.

Lanzrein B., Gentinetta V., Abegglen H., Baker F. C., Miller C. A. and Schooley D. A. (1985) Titers of ecdysone, 20-hydroxyecdysone and juvenile hormone III throughout the life cycle of a hemimetabolous insect, the ovoviviparous cockroach *Nauphoeta cinerea*. *Experientia* **41**, 913–917.

LaPointe M. C., Koeppe J. K. and Nair K. K. (1985) Follicle cell polyploidy in *Leucophaea maderae*: regulation by juvenile hormone. *J. Insect Physiol.* **31**, 187–193.

Marco M. P., Pascual N., Bellés X., Camps F. and Messeguer A. (1990) Ecdysteroid depletion by azadirachtin in *Tenebrio molitor* pupae. *Pest. Biochem. Physiol.* **38**, 60–65.

Matz G. (1980) Variations du taux des ecdystéroides au cours de l'embryogenèse de *Leucophaea maderae* Fabr. (Insecte, Dictyoptère). *C. r. hebd. Séanc. Acad. Sci., Paris* **291**, 501–504.

Muñoz C., Nieto A., Gayá J., Martinez J. and Vives J. (1986) New experimental criteria for optimization of solid-phase antigen concentration and stability in ELISA. *J. Immun. Methods* **94**, 137–144.

Piulachs M. D. (1988) Acción gonadotrófica de la hormona juvenil en hembras de *Blattella germanica* alatectomizadas o sometidas a ayuno prolongado. *Miscelánea zool., Barcelona* **12**, 121–124.

Porcheron P., Morinière M., Grassi J. and Pradelles P. (1989) Development of an enzyme immunoassay for ecdysteroids using acetylcholinesterase as label. *Insect Biochem.* **19**, 117–122.

Ramamurty P. S. (1964) On the contribution of the follicle epithelium to the deposition of yolk in the oöcyte of *Panorpa communis* (Mecoptera). *Expl Cell Res.* **33**, 601–605.

Slinger A. J. and Isaac R. E. (1988) Ecdysteroid titres during embryogenesis of the cockroach, *Periplaneta americana*. *J. Insect Physiol.* **34**, 1119–1125.

Stay B., Ostedgaard L. S., Tobe S. S., Strambi A. and Spaziani E. (1984) Ovarian and hemolymph titres of ecdysteroid during the gonadotrophic cycle in *Diploptera punctata*. *J. Insect Physiol.* **30**, 643–651.

Telfer W. H. and Anderson L. M. (1968) Functional transformation accompanying the initiation of a terminal growth phase in the Cecropia moth oocyte. *Devl Biol.* **17**, 512–535.

Weaver R. J., Strambi A. and Strambi C. (1984) The significance of free ecdysteroids in the haemolymph of adult cockroaches. *J. Insect Physiol.* **30**, 705–711.

Yonge C. and Hagedorn H. H. (1977) Dynamics of vitellogenin uptake in *Aedes aegypti* as demonstrated by trypan blue. *J. Insect Physiol.* **23**, 1199–1203.

Zhu X. X. and Lanzrein B. (1984) Distribution of free and conjugated ecdysteroids between follicle cell sheath and ooplasm in oocytes of the cockroach *Nauphoeta cinerea*. *Experientia* **40**, 496–498.

Zhu X. X., Gfeller H. and Lanzrein B. (1983) Ecdysteroids during oögenesis in the ovoviviparous cockroach *Nauphoeta cinerea*. *J. Insect Physiol.* **29**, 225–235.