The ovary is a source of circulating ecdysteroids in *Blattella germanica* (Dictyoptera: Blattellidae)

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**Ecdysteroids, ovary, haemolymph volume, prothoracic gland, *Blattella germanica***

**Abstract.** The developmental fluctuations in immunoreactive ecdysteroids in the haemolymph of adult female *Blattella germanica* (L.) are reported. There is a relatively low titre in freshly ecylosed females, an increase during vitellogenesis to a peak at chorionogenesis, and a decrease after ovulation. These fluctuations parallel those of ecdysteroid content in the ovary. In addition, haemolymph ecdysteroid levels are reduced following ovariectomy, and single ovarioles incubated in vitro effectively release ecdysteroids into the medium. The data strongly suggest that the ovaries are a major source of circulating ecdysteroids in adult females of this species. Since the prothoracic gland degenerates shortly after the imaginal moult, the presence of ecdysteroids in the haemolymph of ovariectomized females suggests that there are other tissues that also serve as a source of these circulating hormones during the reproductive cycle of *B. germanica*.

**INTRODUCTION**

The occurrence of circulating ecdysteroids in the adult female has been reported in many insect species, but data concerning the source and functions of such compounds remain largely controversial (see Hagedorn, 1985; Lanot et al., 1989). In cockroaches, as in many other insects, the ovary synthesizes and accumulates large quantities of ecdysteroids (Bullière et al., 1979; Zhu et al., 1983; Stay et al., 1984; Pascual et al., 1992) and, in addition, the oocyte follicle cells have been identified as the site of ecdysteroid synthesis (Zhu & Lanzrein, 1984). Ecdysteroids have also been detected in the haemolymph of adult females (Bullière et al., 1979; Lanzrein et al., 1981; Stay et al., 1984; Weaver et al., 1984), which has raised the question of whether the ovary could be the source of these circulating hormones. However, where ecdysteroids have been measured in the ovaries as well as in the haemolymph, data obtained show clear differences between different species. Whereas in *Blaberus craniifer* (Bullière et al., 1979) and *Nauphoeta cinerea* (Zhu et al., 1983) the ecdysteroid titres in the ovaries and haemolymph fluctuate in parallel, in *Diploptera punctata* the high ecdysteroid levels found in the ovary contrast with the low titres measured in the haemolymph (Stay et al., 1984).

The use of ovariectomy to elucidate the possible role of the ovary as a source of circulating ecdysteroids has been reported in *D. punctata* (Stay et al., 1984) and *Periplaneta americana* (Weaver et al., 1984). However, *D. punctata* is of limited interest as a case study because this species shows very low levels of circulating ecdysteroids, both in intact and in ovariectomized specimens (Stay et al., 1984). In *P. americana*, the circulating

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ecdysteroid levels are reduced after ovariectomy (Weaver et al., 1984), but there are no data available on the developmental fluctuations in ovarian ecdysteroid content.

For the present study, we used the cockroach Blattella germanica, an ooviviparous species with discrete gonadotrophic cycles, mainly regulated by juvenile hormone (JH) (Bellés et al., 1987; Gadot et al., 1989). These cycles are separated by periods of ootheca transport, during which development of the new batch of basal oocytes remains arrested, in a state that is functionally equivalent to pregnancy. A peculiar feature of B. germanica is that virgin females are able to produce JH through a cyclical pattern and complete oocyte maturation and oviposition in a period of time similar to mated females (Bellés et al., 1987; Gadot et al., 1989). In this species we have reported the developmental fluctuations in ovarian ecdysteroids throughout the gonadotrophic cycle (Pascual et al., 1992) and the choriogenetic activity induced by 20-hydroxyecdysone (Bellés et al., 1993). Our aim has been to investigate the levels of immunoreactive ecdysteroids in the haemolymph of adult females during the gonadotrophic cycle, in order to correlate them with those of the ovary. In addition, we have studied the process of degeneration of the prothoracic gland (PG) and the effects of ovariectomy upon circulating ecdysteroid levels. The ovary appears to be a major ecdysteroid-releasing organ in the adult female of this species.

MATERIALS AND METHODS

Insects
Virgin females of B. germanica of different ages were used in all experiments. They were obtained from a colony fed on dog chow (Panlab 125, Barcelona, Spain) and water, and reared in the dark at 30 ± 1°C and 60–70% r.h.

Ovariectomy
Ovariectomy was performed in the first days of last larval instar. Specimens were immobilized on ice and ovaries were removed through an incision that was made in the intersegmental membrane between the fourth and fifth tergite. Only those females which underwent a normal imaginal moult (approximately 90% of the specimens) were used in the experiments. Complete absence of ovaries in these specimens was assessed by dissection after PG or haemolymph sampling (see below).

Prothoracic gland studies
PG morphology was studied throughout the last larval instar and during the first days of adult life. Glands were carefully explanted from insects that had been anesthetized with CO₂, stained with methylene blue, and examined under an optical microscope (Labophot, Nikon, Tokyo, Japan). The width of the 4 arms of the PG at the medial part was measured with an ocular micrometer, and the mean of the four measurements was recorded. This biometrical parameter is a useful criterion for the functional state of the glands (Lanzaire, 1975).

Haemolymph volume determination
The isotope dilution method was utilized to determine haemolymph volume (Ebler et al., 1986). At selected ages, specimens were injected with 1 μl of a solution of [¹³C] carboxyl insulin (specific activity: 0.11 GBq/g) (New England Nuclear, Berlin, Germany) in Ringer such that each insect received 22,000 dpm. Twenty minutes after injection (see below) a haemolymph sample of 2 μl was collected with a micropipette from an incision made in the mesosoma. The sample was transferred to a vial containing 10 ml of scintillation cocktail (Optiphase HiSafe II, Pharmacia, Uppsala, Sweden) and then counted in a Kontron (Betamatic V, Montigny le Bretonneux, France) apparatus. Preliminary tests performed to estimate the apparent volume of haemolymph at 5, 10, 20, 40 and 60 minutes after the injection of [¹³C] carboxyl insulin revealed that in 20 min the compound was uniformly distributed in the haemolymph, both in intact and ovariectomized specimens. Since the ovaries are active in taking up circulating large molecules during vitellogenesis, we studied the possible incorporation of [¹³C] carboxyl insulin into the ovaries of 6-day-old females, an age at which the incorporation rate of circulating vitellogenin is 56 μg/h

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(unpublished data). Thus, we injected the equivalent of 22,000 dpm of [14C] carboxyl insulin into intact 6-day-old females, and 20 min later the ovaries were dissected and thoroughly rinsed. The ovaries contained 259 ± 48 dpm (n = 7), which represented a “loss” of ca. 1% of the [14C] carboxyl insulin administered, a percentage that was considered insignificant for our estimations of total haemolymph volume.

In vitro incubation of ovarioles

Incubations were carried out in Grace’s medium, with L-glutamine and without insect haemolymph (Whitaker Bioproducts, Inc., Walkersville, MD, USA), to which Streptomycin (100 µg/ml) and Penicillin G (30 µg/ml) had been added. Single ovarioles from 5-day-old females were incubated for 4, 8 or 16 h in 400 µl of medium, at 30°C in the dark and with gentle shaking. Ecdysteroid content of the medium and of the ovariole was measured at the end of the incubation.

Ecdysteroid extraction for ELISA determinations

Freshly explanted ovarioles and single ovarioles were extracted with 100% methanol (200 µl). Tissues were homogenized and centrifugated at 13,000 g for 5 min, while haemolymph was centrifuged only. The pellet was resuspended in 100% methanol (200 µl) using an ultrasonic bath and centrifugation was repeated. The supernatants were combined, evaporated under nitrogen, stored at −20°C and later assayed for ecdysteroid (ELISA, see below). Since, in our in vitro system, the control medium used for incubating the ovarioles interfered with the ELISA, the ecdysteroids contained in the medium were purified by Sep-Pak according to Lafont et al. (1982). Experimental or control medium (400 µl) was loaded onto a conditioned C18 Sep-Pak cartridge (Waters Assoc., Milford, Mass., USA) previously washed with 10 ml water. Elution was performed first with 25% aqueous methanol, then 65% aqueous methanol and finally 100% aqueous methanol. The 65% aqueous methanol fraction (containing the free ecdysteroids) was dried and assayed for ecdysteroid by ELISA.

ELISA determination of ecdysteroids

Solid-phase ELISA was performed basically as described in Porcheron et al. (1989) and Marco et al. (1990). Color was read on a Multiscan MC spectrophotometer (Flow Laboratories, Helsinki, Finland) set at 405 nm. Microtitre plates were from Nunc (Model 96F, Roskilde, Denmark). The antiserum (AS4919) was supplied by Prof. P. Porcheron (École Normale Supérieure, Paris, France). The enzymatic tracer (20-hydroxyecdysone-carboxymethoxime-acetylcholinesterase) was from Cayman Chemical Company (SpiBio, Saclay, France). All the other reagents, including goat antirabbit immunoglobulin antibody, were from Sigma (Madrid, Spain). The ecdysteroid antiserum used has the same affinity for ecdysone and 20-hydroxyecdysone. Since the standard curve was obtained with the latter compound, results are expressed as pg or ng of 20-hydroxyecdysone equivalents.

RESULTS

Haemolymph ecdysteroids

The concentration of immunoreactive ecdysteroids was measured in haemolymph from intact females sampled daily during the first gonadotrophic cycle, i.e., from freshly ecdysed (5 and 12 h after the imaginal moult) to ovipositing females (day 7) (Fig. 1). Two additional measurements were performed in ootheca-carrying females 1 and 5 days after oviposition (8- and 12-day-old females, respectively, Fig. 1). In order to study specimens at the beginning of the second gonadotrophic cycle (14-day-old females), the ootheca was experimentally removed in 12-day-old females and haemolymph ecdysteroid levels were measured 2 days later (Fig. 1). The amount of ecdysteroid in ovaries, as well as haemolymph ecdysteroid levels in ovarioctomized specimens, were also determined.

Haemolymph ecdysteroid concentration in intact females was relatively low until day 3, at which time increased steadily. A peak occurred on day 7, the time of chorionogenesis and ovulation. After oviposition, circulating ecdysteroids decreased sharply and reached the lowest value in ootheca-carrying day-12 females. On day 14, 2 days after ootheca removal, haemolymph ecdysteroid levels were still low, the concentration being similar to that
found at the beginning of the first gonadotrophic cycle (Fig. 1). Interestingly, the fluctuations of haemolymph ecdysteroid levels parallel those of ovarian content, which were measured independently at the same ages (Fig. 1, upper graph). Conversely, haemolymph ecdysteroid levels in ovariectomized females were low, fluctuating only slightly during the period of study. There was no increase to that observed in intact females 3 to 7 days old (Fig. 1).

**Haemolymph volume and total circulating ecdysteroids**

In order to determine whether the differences in circulating ecdysteroids between intact and ovariectomized females were dependent on haemolymph volume, we studied this latter parameter at the ages used for ecdysteroid measurements (Fig. 2). In intact females haemolymph volume did not show great fluctuations. It was low—(around 20 μl per specimen) in freshly ecdysed specimens, then increased slightly at the beginning of vitellogenesis to maximal values on day 5 (around 30 μl per specimen). It decreased towards the end of vitellogenesis and in oviposition and post-oviposition stages, when the values were similar to those observed in freshly ecdysed specimens (Fig. 2). In ovariectomized females the fluctuations were similar until day 5, but instead of decreasing thereafter, the volume
increased to maintain quite high values (around 40 µl per specimen) during the last days of the period studied. These results, combined with those of haemolymph ecdysteroid concentration (Fig. 1), allowed an estimation of total haemolymph ecdysteroid content per specimen at the ages studied (Fig. 2, upper graph). With this approach, differences between intact and ovarioctomized specimens were less striking than those observed when measuring ecdysteroid concentrations (Fig. 1). However, ovarioctomized specimens showed quite low ecdysteroid levels, whereas the fluctuations in intact females were similar to those based on concentration values, i.e. showed a peak at the time of choriongenesis.

Prothoracic gland degeneration

Although at low levels, the presence of ecdysteroids in ovarioctomized females suggests that there are ecdysteroid sources other than the ovary in the adult female. A candidate for this alternative source could be the PG, the major ecdysteroid-producing gland in pre-imaginal stages. Therefore, we studied PG morphology in intact specimens during the last larval instar and the process of gland degeneration which followed the imaginal molt, in intact as well as in ovarioctomized females.

The PG of B. germanica, like that of other cockroach species, has an X-shaped morphology, with a thin muscular strand along each band. Around the bands, intermingled with tracheae and nerve terminals, lie more or less densely packed glandular cells. The entire PG is covered by a thin sheath. In the last larval instar, the PG has a turgid and lobulated aspect, the bands are large (between 100 and 150 µm width), and the glandular cells are densely packed, with few, narrow intercellular spaces (Fig. 3). In freshly emerged females (either intact or ovarioctomized), the PG still show densely packed glandular cells (Fig. 4), but in 72 h, the gland clearly undergoes a process of regression. There is a rapid loss of turgidity, a sharp decrease in cell density (the nuclei in great part are pycnotic) which parallels the increase in intercellular spaces, and the bands become progressively narrower (from 100 to 60 µm width). On day 3, the glands are reduced to the central axis.
Fig. 3–6. Prothoracic glands of female *Blattella germanica*. 3 – Normal anatomical appearance in last larval instar, showing the bands crossed and the respective muscular axes superimposed. 4 – From a freshly emerged adult, with the bands artificially uncrossed to show that both bands are fused and that each one has an independent muscular axis. 5 – From a 3-day-old adult (bands uncrossed) showing a practically complete regression. 6 – From a 2-day-old adult showing asynchronic degeneration in the four arms. Scale bar: 100 μm.
of muscle, trachea and nerve surrounded by the sheath, while the bands have become very narrow (≤ 50 μm width) (Fig. 5). The degenerative process is sometimes asynchronous in the four arms of the gland (Fig. 6).

A graph illustrating the dynamics of this degenerative process can be obtained by plotting the width of the arms against time (Fig. 7). Fluctuations in PG size during the last larval instar shows a characteristic peak preceding the imaginal moult. In the imaginal stage, both in intact and in ovarietomized specimens, there is a sharp decrease in the size of the PG, a decrease which reflects the degenerative process. Although we did not carry out direct functional measurements, the cytomorphological features of glands from day 3 of the adult stage onwards suggest that they are no longer functional. Equivalent observations carried out on adult intact males (not shown) reveal a pattern of degeneration similar to that found in females.

Release of ecdysteroids by ovarioles in vitro

Since the differences in circulating ecdysteroids observed between intact and ovarietomized females (Figs. 1 and 2) suggest that the ovary is a source of haemolymph ecdysteroids, we studied the release of ecdysteroids by single ovarioles incubated in vitro.
Ecdysteroid contents in the medium were measured at 0, 4, 8 and 16 h after incubation. Results (Fig. 8) showed that ecdysteroid release into the medium increased with time. Simultaneous measurements of ecdysteroid levels in the ovariole showed that ecdysteroid content apparently increased with time, although the differences observed were not statistically significant. The control medium did not result in ELISA activity.

DISCUSSION

In intact females of *B. germanica*, haemolymph ecdysteroid levels are low during previtellogenesis, increase during vitellogenesis to a peak at the time of choriongenesis, and decrease after ovulation. Concerning the presence of circulating ecdysteroids in cockroaches, the first unequivocal data were reported by King & Marks (1974) for *Leucophaea maderae*. These authors using HPLC reported that there are 100 ng/ml 20-hydroxyecdysone in the haemolymph of 6–7-month adult females. Later, detailed developmental changes in hormonal levels were reported for other cockroaches, including *B. craniifer* (Bullière et al., 1979; Goudey-Petrièr et al., 1992), *N. cinerea* (Lanzrein et al., 1981), *P. americana* (Weaver et al., 1984) and *D. punctata* (Stay et al., 1984). As in *B. germanica*, a peak in hormonal titer at the time of choriongenesis has been described in *B. craniifer, N. cinerea* and *P. americana*. In *P. americana*, high levels of circulating ecdysteroids were also found in freshly ecdysed adult females (Weaver et al., 1984). These ecdysteroids appear to have originated from PG, which in this species does not degenerate until some 12 days after the imaginal moult (Bodenstein, 1953). The low levels measured in freshly ecdysed specimens of *B. craniifer* and *N. cinerea* (Bullière et al., 1979; Lanzrein et al., 1981) are consistent with the faster degeneration of PG in the adults of these species: 5 days in *B. craniifer* (Scharer, 1966) and 3 days in *N. cinerea* (Lanzrein, 1975). This more rapid degeneration is also characteristic of *B. germanica* (see below). A dissonant note to the pattern of these developmental fluctuations is the case of *D. punctata*. In this cockroach, the highest titres of ecdysteroids in the haemolymph were found shortly after the imaginal moult. The levels decline during the next 3 days and remain low throughout the gonadotrophic cycle (Stay et al., 1984). As is the case for *P. americana*, the ecdysteroids present in the haemolymph just after the imaginal moult may have originated from the PG. In *D. punctata*, however, this gland appears to degenerate by the third day of adult life (Stay et al., 1984).

In general, the developmental fluctuations of circulating ecdysteroids in intact females parallel those of ovarian ecdysteroid content, the latter being in close agreement with those previously reported by Pascual et al. (1992). This suggests that the ovary could release ecdysteroids into the haemolymph. However, the sharp peak observed on day 7 in the ovaries has no parallel in the haemolymph levels observed between days 6 and 7. This suggests that the hormone released between days 6 and 7 is metabolized faster or, more likely, that the ovary does not release the hormone during this period. The latter possibility is suggested by the fact that choriongenesis starts in this period, and is accompanied by a sealing of intercellular spaces followed by deep changes in the follicle cells, which ultimately degenerate to be replaced by chorion layers (Pascual et al., 1992; Bellès et al., 1993). In addition, experiments carried out on *L. maderae* seem to indicate that ovarian ecdysteroid release ceases at the end of vitellogenesis (Koepppe, 1981).
Similar correspondences between haemolymph and ovarian ecdysteroids have been described in other species of cockroaches like B. cranifer (Bullière et al., 1979) and N. cinerea (Lanzrein et al., 1981; Zhu et al., 1983). In D. punctata, ecdysteroid titres in the ovary are very high around choriogenesis, but this is not paralleled in the haemolymph where, as seen before, ecdysteroid levels remain low during the vitellogenic cycle (Stay et al., 1984).

Ecdysteroid levels measured in the haemolymph of ovariecotomized specimens are clearly lower than those observed in intact females, whether the results are expressed as pg/μl or as total contents (ng/specimen) estimated on the basis of haemolymph volume. This again suggests the ovary as a source of circulating ecdysteroids in the adult female of B. germanica. Reduced levels of haemolymph ecdysteroids following ovariecotony have also been observed in other orthopteroid species, such as the locust Locusta migratoria (Lagueux et al., 1977), the house cricket Acheta domestica (Renucci & Strambi, 1981), and the cockroach P. americana (Weaver et al., 1984).

Although present at reduced levels, the occurrence of circulating ecdysteroids in ovariecotomized females raises the question of the source of these ecdysteroids. It cannot be the PG, since this gland degenerates in the first three days of adult life. Indeed, the most reasonable candidate may be the epidermis, which has been reported to actively produce and release ecdysteroids in several insect species (see Delbecque et al., 1990), including B. germanica (Skol & Hoffmann, 1993).

Our results showed that single 5-day-old ovarioles released ecdysteroids in vitro. These in vitro experiments allowed us to generate an additional hypothesis concerning the release of ovarian ecdysteroids. Our measurements of the total ecdysteroid content in the haemolymph indicate that the absolute hormonal increase from day 5 to 6 is approximately 1 ng, whereas the average release rate from a single 5-day-old ovariole after 4 h of incubation is approximately 8 pg/h (300 pg/h per ovary pair), which represent ca. 7 ng released by an ovary pair in 24 h. If there is a similar release rate in vivo, one could hypothesize that approximately 85% of the ecdysteroids released by the ovary are quickly metabolized in the internal milieu of the insect. Release of ecdysteroids by ovarian tissue in vitro has been demonstrated in insect species of different orders including the locust L. migratoria, the wax moth Galleria mellonella, the mosquitoes Aedes aegypti and A. atropalpus (see Hagedorn, 1985), the blowfly Phormia terraenovae (Wilps & Zöller, 1989) and the cricket Gryllus bimaculatus (Hoffmann et al., 1992; Weidner et al., 1992).

In summary, the information available strongly suggests that the ovary is a major source of circulating ecdysteroids in the adult female of B. germanica, although other tissues, such as the epidermis, may also be a source of these hormones.

Finally, the release of ovarian ecdysteroids into the haemolymph leads one to question the function of these circulating hormones in the adult female. Their possible role as inhibitors of JH production has been hypothesized by several authors. As early as 1959, Engelmann reported that injections of extracts containing ecdysteroids, or PG implantations, suppress ovarian development in L. maderae, possibly by reducing CA activity. When direct measurement of ecdysteroids became available, it was soon apparent in a number of cockroach species that the peak of haemolymph ecdysteroids, which occurs at choriogenesis, is almost coincident with the decline in JH production preceding ovulation. These observations led to the hypothesis that ovarian ecdysteroids could trigger the inhibition of JH
synthesis (Stay et al., 1980, 1984; Lanzrein et al., 1981; see, however, Weaver et al., 1984). Ecdysteroids administered to adult cockroaches in vivo inhibit CA activity in D. punctata (Friedel et al., 1980; Stay et al., 1980) and B. germanica (Chiang et al., 1991). However, at least in D. punctata, the effect seems to be indirect since 20-hydroxyecdysone did not inhibit JH production in CA of this species incubated in vitro (Friedel et al., 1980).

Paradoxically, a great deal of data concerning the role of ecdysteroids in inhibiting JH production has been reported for D. punctata (Friedel et al., 1980; Stay et al., 1980), the only species in which there are very low amounts of free ecdysteroids in the haemolymph at the time of the JH decline (Stay et al., 1984). Thus, the hypothesis of the possible inhibitory role of circulating ecdysteroids upon the CA remains open. Clearly, a more appropriate insect species should be chosen to test this hypothesis, and work along this line, including a chemical characterisation of the ecdysteroids involved, is currently in progress in our laboratory using B. germanica as a model.

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