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Ecdysteroid release and ecdysteroid titer during larval-adult development of the Mediterranean field cricket, *Gryllus bimaculatus* (Ensifera: Gryllidae)

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Ecdysteroid biosynthesis, ecdysteroid titer, prothoracic gland, ovary, integument, moulting, radioimmunoassay, *Gryllus bimaculatus*

Abstract. Female penultimate instar larvae of *Gryllus bimaculatus* show a maximum in haemolymph ecdysteroid titer as well as in hormone release in vitro by prothoracic glands on day 4, two days before final larval moult. Ovary and abdominal integument of the larvae contain considerable amounts of ecdysteroids, but no significant hormone release is detectable during a 16 h in vitro incubation of these tissues.

In last instar larvae, the prothoracic glands reach their maximal ecdysteroid release on day 5. This peak is followed by a maximal release of hormones by the ovary as well as by the abdominal integument on day 6. The highest titer of ecdysteroids in haemolymph is found on day 7, two days before imaginal moult. The haemolymph titer is governed only by free ecdysteroids. The prothoracic glands and the abdominal integument release free ecdysteroids, whereas from the ovary considerable quantities of apolar conjugates appear. The abdominal integument of day 6 last instar larvae converts [^{3}H]5β-ketodiol into [^{3}H]ecdysone in vitro.

From these results it can be concluded that in last instar larvae of *G. bimaculatus* the prothoracic gland as well as the ovary and the abdominal integument are involved in the changes of haemolymph ecdysteroid titer in relation to imaginal moulting. The data indicate an ecdysteroid synthesis outside the prothoracic gland in *G. bimaculatus* last instar larvae.

INTRODUCTION

The roles of ecdysteroids in insect development are well documented (for review see Lanot et al., 1989; Sehnal, 1989; Steel & Vafopoulou, 1989). Remarkable changes are seen in the haemolymph ecdysteroid titer throughout the life of an insect, which indicate a temporal correlation between moults and peaks in ecdysteroid titers. Larval moults generally display a single massive peak prior to moulting, and in hemimetabolous insects the larval-adult moult likewise displays a single peak.

The haemolymph ecdysteroid titer at any instant reflects the balance between the entry of ecdysteroids into the haemolymph due to synthesis or release from storage, and their removal from the haemolymph by excretion, sequestration or uptake into storage. Such changes may also lead to changes in the relative proportions of different ecdysteroid molecules in the haemolymph.

In the majority of insects, the prothoracic glands (ecdysial glands) are the major sources of ecdysteroids during larval development and the ecdysone-secretory activity of the glands is regulated in relation to moulting cycle. The involvement of alternative sites of ecdysteroid production seems to be limited to the imago or to the pupal stage, which means that reproduction is concerned (Redfern, 1989; Delbecque et al., 1990). Several

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arguments, e.g. in *Tenebrio molitor*, suggest that alternative sources are probably absent or inhibited in larvae (Soltani et al., 1989; Delbecque, 1990). For example, diflubenzuron which inhibits the alternative production in pupae, does not have any effect on ecdysteroid titers during larval-larval moults.

However, tissues in isolated larval abdomens of *T. molitor*, *Bombyx mori*, *Musca domestica*, *Leptinotarsa decemlineata* and *Periplaneta americana* can convert [^{14}C]cholesterol to ecdysone and 20-hydroxyecdysone (see Gersch, 1978 for review). In *P. americana*, radiolabelled 20-hydroxyecdysone and small amounts of ecdysone were synthesized after injection of [^{14}C]cholesterol into larvae without prothoracic glands (Gersch & Eibisch, 1977). In *Tenebrio*, oenocytes may be a source of hormone, and in *Heliothis virescens* the larval testes have the capacity to synthesize ecdysteroids (Loeb et al., 1982). Very recently, Jarvis et al. (1994) have presented significant supporting evidence that testes of the last instar larvae of *Spodoptera littoralis* synthesize ecdysteroids.

However, the relevance of abdominal sources of ecdysteroids during normal development is not certain. In fourth instar *Aedes aegypti*, the thorax, without the prothoracic gland, revealed the same amount of ecdysteroids in vitro as the intact thorax. Ecdysteroids were also released in vitro from the abdomen, and the haemolymph ecdysteroid titer correlated with thoracic and abdominal release of ecdysteroids in vitro (Jenkins et al., 1992). The tissue identified as the prothoracic gland (PG) complex in fourth instar mosquito larvae did not release radioimmuno-positive substances. The authors also could not find any indication to a release of 3-dehydroecdysone by the PG complex which would have not been recognized by the antiserum used in these studies.

In *Gryllus bimaculatus*, ecdysteroid titers have been determined in whole bodies of penultimate and last instar larvae by Maas (1974) (in Wildmann & Romer, 1977) and Gnatzy & Romer (1980). Besides prothoracic glands, the larval oenocytes have been proposed as a source of ecdysteroids. This suggestion is mainly based on the fact that oenocyte ultrastructure is similar to that of vertebrate steroid producing glands (Romer et al., 1974). In adult crickets, we found ecdysteroids in haemolymph and tissues of both females and males (Hoffmann et al., 1981; Hoffmann & Behrens, 1982; Espig et al., 1989; Hoffmann & Wagemann, 1994). Recently, we have shown that the ovary and the abdominal integument together with the adjacent segmental fat body represent alternative sites of ecdysteroid production in adult females (Hoffmann et al., 1992). The rates of ecdysteroid synthesis, as measured in vitro, change during vitellogenesis in a characteristic way, reaching peak values when animals start to deposit eggs (Weidner et al., 1992). Both ecdysteroid sources represent primary sources of ecdysteroids in adult female crickets.

In the present study we report on the net release in vitro of ecdysteroids by the ovary and abdominal integument of *G. bimaculatus* last instar larvae, at a time when prothoracic glands are still active. All three tissues clearly contribute to changes in ecdysteroid titer of the haemolymph relative to moulting. In penultimate instar larvae, the ovary and the abdominal integument contain considerable amounts of ecdysteroids, but no significant hormone release was detected.

MATERIAL AND METHODS

Insects

Gryllus bimaculatus were reared at 27°C and 60% relative humidity on dog flakes (Bonzo komplett, Quaker Latz GmbH, Euskirchen, Germany), Altromin rabbit (2021) and rat/mouse (1311) mixed standard

diet (Altromin GmbH, Lage, Germany) and water in a 16 h light/8 h dark photoperiod. Under these conditions the penultimate larval stadium lasts 6 days and the last larval stadium 9 days.

Tissue preparation and in vitro incubation

All dissections and in vitro incubations were performed during the daytime (2 to 4 p.m.) and under sterile conditions. Appropriately aged female larvae were briefly dipped in liquid nitrogen, decapitated, and an incision was made along the midventral line. After removing the intestinal tract, ovaries were excised, cleaned of extraneous tissue and rinsed three times in sterile Grace's medium (GIBCO, Paiseley, Great Britain), pH 6.2, containing 50 IU penicillin G/ml and 69 IU streptomycin sulfate/ml (Sigma, Deisenhofen, Germany). The abdominal integument was divided in half by a sagittal cut (Hoffmann et al., 1992). Intact prothoracic glands (PG) were extirpated from CO_2-anaesthetized larvae under cricket ringer (Behrens & Hoffmann, 1983) and placed into Grace's medium on ice.

For each incubation, one of each pair of ovaries, one-half of the abdominal integument plus segmental fat body and an individual prothoracic gland (PG), respectively, were washed in sterile Grace's medium to remove adhering haemolymph and placed in one well of a 24-well tissue culture plate (Costar, Cambridge, USA) that contained 1 ml of sterile Grace's medium. The contralateral organ/tissue and the prothoracic gland from a larva of the same age, respectively, served as 0-time control allowing a determination of ecdysteroid content prior to incubation. The multi-well plates were placed in the dark for 16 h at 27°C in a water-saturated atmosphere. During incubation, samples were continuously bubbled with O_2 and gently shaken (150 rpm) with a motorized rotating table (IKA Vibrax VXR; Jahnke and Kunkel, Staufen, Germany).

Haemolymph collection

Haemolymph was collected from larvae of appropriate age with a glass microliter pipet (Transferpettor; Brand, Wertheim, Germany) by puncturing the neck membrane.

Ecdysteroid analysis

After incubation, tissues were briefly washed with 2 ml methanol to remove attached incubation medium (the methanolic wash was added to the incubation medium), lyophylized (only abdominal integument), homogenized in 800 µl 100% methanol and centrifuged (8,000 g, 2 min). The pellets were reextracted twice with 100% methanol and 70% (v/v) methanol in water, respectively, and all supernatants were combined, resulting in an 88% methanol solution. The methanolic extracts were partitioned against an equal volume of n-hexane before being concentrated to a very small volume (ca. 200 µl) and filled up to 4 ml with distilled water (final methanol concentration < 5%). Haemoylmph samples and the incubation media were extracted with 5 ml 100% methanol and partitioned against the same volume of n-hexane.

Fractionation of ecdysteroids was carried out with the Sep-Pak C18 purification procedure as previously described (Bulenda et al., 1986; Espig et al., 1989). The ecdysteroid fractions were eluted from the Sep-Pak cartridges as follows: 4 ml water; 4 ml 25% (v/v) methanol in water- polar ecdysteroid conjugates; 4 ml 60% (v/v) methanol in water- free ecdysteroids; 4 ml 100% methanol- apolar ecdysteroid conjugates. Polar and apolar ecdysteroid conjugates were completely hydrolyzed during a 16 h treatment with porcine liver esterase at 37°C prior to quantification by a radioimmunoassay (RIA) (Thiry & Hoffmann, 1991). The antiserum used (Hoffmann et al., 1981) had been prepared in our laboratory according to the method of Soumoff et al. (1981). Values for cross-reactions of the antiserum were ecdysone = 1.0; 20-hydroxyecdysone, makisterone and inokosterone = 0.9; 3-dehydroecdysone $\leq$ 0.1; cholesterol = 0.

To calculate the net amount of RIA-positive ecdysteroids released, the amount of ecdysteroids present in the 0-time control tissues was subtracted from post-incubation values (tissues plus incubation media). Grace's control medium did not contain significant amounts of RIA-positive material.

In vitro incubation in the presence of [^{3}H]5β-ketodiol

The radioactive 2,22,25-trideoxyecdysone ([^{3}H]5β-ketodiol; initial specific activity 107 µCi/mmol; kindly provided by Dr R. Lafont, Paris) was purified on a RP-HPLC column as described recently (Hoffmann et al., 1992). Abdominal integument preparations were incubated in the presence of 0.7 µCi [^{3}H]5β-ketodiol for 16 h and the radioactive compounds formed during incubation were extracted from tissues and incubation media as described above. The combined Sep-Pak 60% methanol fractions which contained the free ecdysteroids were further fractionated by RP-HPLC. The HPLC analysis used a

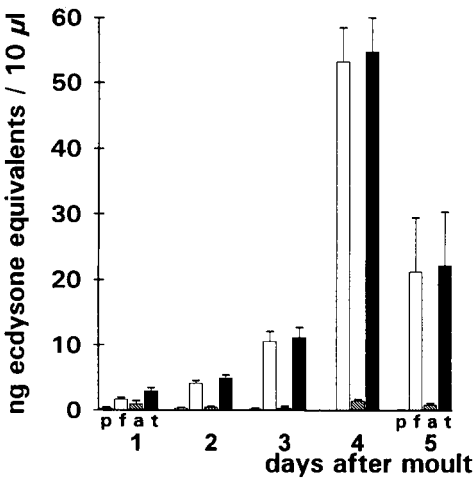


Fig. 1. Titers of ecdysteroids in the haemolymph of female penultimate instar larvae. p – immunopositive substances released from polar conjugates by esterase treatment; f – free ecdysteroids; a – immunopositive substances released from apolar conjugates by esterase treatment; t – total ecdysteroids. Bars represent SEM; n = 5–6.

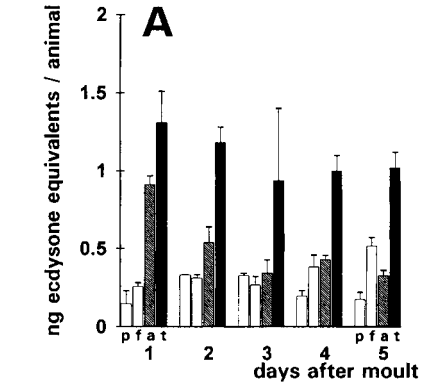
Superspher 100 RP-18 column (125 × 4 mm; Merck, Darmstadt, Germany) eluted with 16% (v/v) acetonitrile in water for 10 min, a 16–40% acetonitrile linear gradient over 25 min, followed by a 15 min purge with 100% acetonitrile. Radioactivity was determined by liquid scintillation counting (LSC) of the HPLC fractions (Rothiszint 2211; Roth, Karlsruhe, Germany; Packard Tri-Carb 1500 LSC System). The counting efficiency in LSC was determined to be ca. 33%.

RESULTS

Ecdysteroid titers and ecdysteroid release in penultimate instar larvae

In the early part of the penultimate larval stadium, haemolymph ecdysteroid titer increased slightly from about 3 ng/10 µl at day 1 to 11.2 ng/10 µl at day 3. A single peak in ecdysteroid concentration (54.7 ng/10 µl) appeared on day 4 (Fig. 1). After this peak, the titer dropped prior to the final larval moult. The major haemolymph ecdysteroids present during the peak on day 4 eluted with the 60% methanol fraction from the Sep-Pak cartridge (free ecdysteroids), along with some higly apolar conjugated material. The pattern of in vitro PG ecdysteroid release was similar to the changes in haemolymph ecdysteroid titer (Fig. 2B), starting at a low rate of 0.07 ng/16 h·animal on day 1, peaking at 0.69 ng/16 h·animal on day 4 and dropping again prior to moulting. A kinetic view of the changes in PG ecdysteroids during the 16 h in vitro incubation revealed an initial turnover of apolar ecdysteroid conjugates into polar conjugates and free ecdysteroids (days 1–2 of penultimate larval stage), but a net release of free and conjugated ecdysteroids over the next two days (Fig. 2B). These results tally with data for the ecdysteroid concentration in PG of penultimate instar larvae. PG from day 1 larvae contained about 1 ng of apolar ecdysteroid conjugates and this value dropped to 0.3 ng on day 3 (Fig. 2A).

The ecdysteroid level in the larval integument also showed a peak value on day 4 (33 ng/animal) and dropped prior to the final larval moult (Fig. 3A). The integument mainly contained free ecdysteroids. A 16 h in vitro incubation of the abdominal integument resulted in a constant low rate of net release of free and apolar conjugated ecdysteroids (2.9 to 5.8 ng/16 h·animal) on all days of the penultimate larval stage (Table 1). No peak value in ecdysteroid release was found. Ovaries from penultimate instar larvae contained 2.5 to



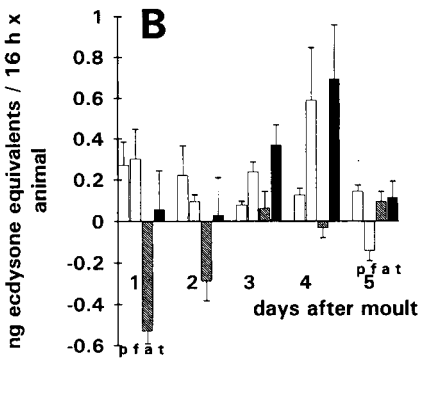
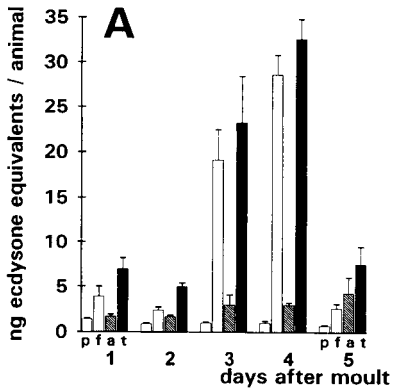


Fig. 2. A – Content of ecdysteroids in pre-incubation prothoracic glands from female penultimate instar larvae. Bars represent SEM; n = 6. Other details as in Fig. 1. B – Net release of ecdysteroids by the prothoracic gland from penultimate instar larvae during a 16 h incubation in Grace's medium. Bars represent SEM; n = 6. Other details as in Fig. 1.



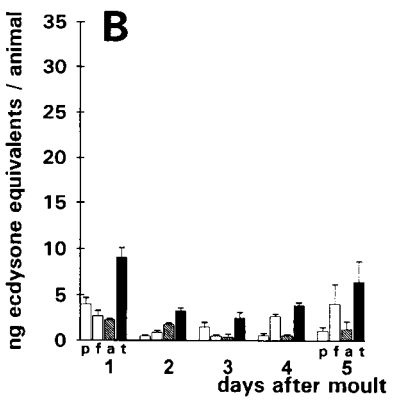


Fig. 3. A – Content of ecdysteroids in the abdominal integument from female penultimate instar larvae. Bars represent SEM; n = 5–6. Other details as in Fig. 1. B – Content of ecdysteroids in the ovary from penultimate instar larvae. Bars represent SEM; n = 5–7. Other details as in Fig. 1.

9 ng of ecdysteroids per animal with highest values at the beginning and at the end of the stadium (Fig. 3B). During a 16 h incubation in vitro of the ovaries no significant net release of ecdysteroids was observed (Table 1).

Ecdysteroid titers and ecdysteroid synthesis in last instar larvae

In last instar larvae, the level of ecdysteroids in the haemolymph varied as shown in Fig. 4. The haemolymph showed a small peak of ecdysteroids on day 1 (6.6 ng/10 µl) and a large peak on day 7 (35.2 ng/10 µl), After this large peak, the titer declined drastically prior to the imaginal moult. The haemolymph mainly contained free ecdysteroids. The rate of ecdysteroid release from prothoracic glands ranged from 0.34 ng/16 h·animal to 2.26 ng/ 16 h·animal (Fig. 5). PG of young (days 1–2) and older (days 7–8) larvae showed low

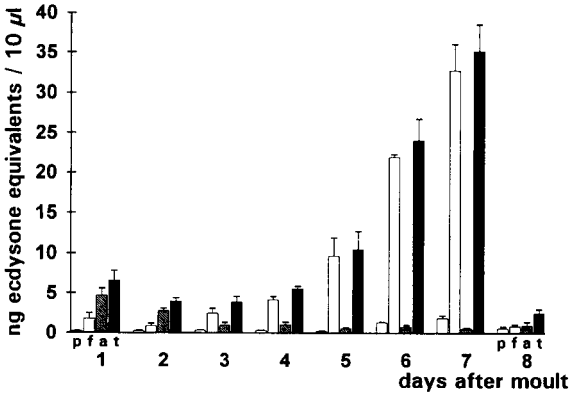


Fig. 4. Titers of ecdysteroids in the haemoylmph of female last instar larvae. Bars represent SEM; n = 6–8. Other details as in Fig. 1.

of release while the highest secretory activity was detected with PG from day 5 larvae. The 0-time control PG did not contain measurable amounts of ecdysteroids, suggesting that during in vitro incubation new ecdysteroids were formed and secreted immediately.

TABLE 1. Net release of ecdysteroids by the abdominal integument and by the ovary from female penultimate instar larvae during a 16 h incubation in Grace's medium. Mean values of 5–7 determinations ± SEM.

	Abdominal integument (ng ecdysone equivalents/16 h·animal)				
	day 1	day 2	day 3	day 4	day 5
Polar conjugates	1.39 ± 0.16	1.06 ± 0.21	1.10 ± 0.19	0.94 ± 0.29	0
Free ecdysteroids	1.25 ± 0.86	2.35 ± 0.54	0.85 ± 1.65	–0.94 ± 4.50	0.74 ± 0.16
Apolar conjugates	3.20 ± 0.48	1.57 ± 0.30	1.58 ± 0.81	2.93 ± 0.33	2.91 ± 0.30
Total ecdysteroids	5.84 ± 1.04	4.98 ± 0.67	3.53 ± 2.15	2.93 ± 2.24	3.65 ± 1.05
	Ovary (ng ecdysone equivalents/ 16 h·animal)				
	day 1	day 2	day 3	day 4	day 5
Polar conjugates	–1.07 ± 0.81	0.34 ± 0.25	–0.58 ± 0.81	0.34 ± 0.13	0.18 ± 0.14
Free ecdysteroids	–0.85 ± 0.62	–0.14 ± 0.39	0.45 ± 0.30	0.61 ± 0.37	–1.70 ± 0.83
Apolar conjugates	0.46 ± 0.44	0.30 ± 0.53	1.41 ± 0.48	–0.06 ± 0.11	0.62 ± 0.50
Total ecdysteroids	–1.46 ± 1.10	0.50 ± 0.72	1.28 ± 0.89	0.89 ± 0.39	–0.90 ± 0.99

Negative values in the net release of total ecdysteroids indicate a loss of immunopositive substances during the 16 h incubation in vitro.

Ecdysteroid levels in the abdominal integument paralleled the rate of ecdysteroid release by PG, with low values (4.7 to 11.3 ng/animal) during early and late larval stadium and a maximum amount of mainly free ecdysteroids on day 5 (47.8 ng/animal; Fig. 6A). A 16 h in vitro incubation of the abdominal integument resulted in a constant low rate of net release of free and conjugated ecdysteroids up to day 5 (4.16 to 9.12 ng/16 h·animal), followed by a peak value of mainly free ecdysteroids on day 6 (Fig. 6B; Table 2). Net release of ecdysteroids then dropped prior to imaginal moult.

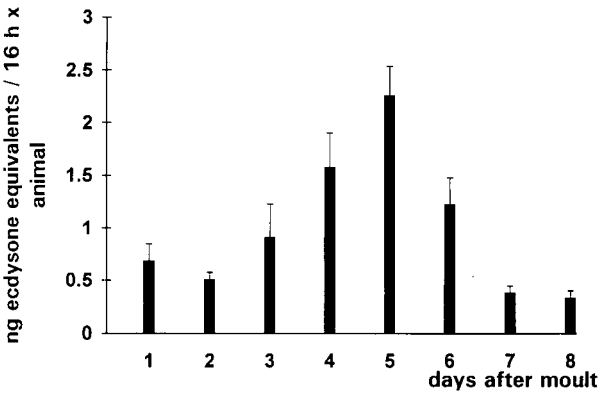


Fig. 5. Ecdysteroid secretion by prothoracic glands from female last instar larvae during a 16 h incubation in Grace's medium. Preincubation glands did not contain measurable quantities of ecdysteroids. During the 16 h incubation in vitro, only free ecdysteroids were released into the medium. Bars represent SEM; n = 4–5.

TABLE 2. Mean ng ecdysone equivalents per abdominal integument of day 6 last instar larvae before and after a 16 h in vitro incubation. Mean values of 13 determinations ± SEM.

	Polar conjugates	Free ecdysteroids	Apolar conjugates
0-time control integument (prior to incubation)	2.5 ± 0.5	30.4 ± 7.7	2.2 ± 0.4
Release into medium	1.7 ± 0.2	47.3 ± 5.5	4.5 ± 1.3
Integument post incubation	1.2 ± 0.5	18.1 ± 7.0	1.9 ± 0.3

Larval ovaries contained low amounts of ecdysteroids up to day 5 of the stadium (8.6 to 14.8 ng/animal), followed by high hormone levels from day 6 to moulting (71.7 to 77.7 ng/animal; Fig. 7A). The increase in ecdysteroid titer on day 6 goes along with an increase in the net release in vitro of free and apolar conjugated ecdysteroids, whereas no hormone release was measurable in ovaries from younger larvae (Fig. 7B). A kinetic view of the changes in ovarian ecdysteroids during the 16 h in vitro incubation revealed a turnover of free ecdysteroids into apolar conjugates on day 7 (see Fig. 7B) which was confirmed by the increase in the titer of apolar ecdysteroids but a decrease of free ecdysteroids (at a constant level of total ecdysteroids) from day 7 to day 8 (see Fig. 7A).

Conversion of [^{3}H]5β-ketodiol into ecdysone

Abdominal integument from six day-6 last instar larvae were separately incubated in the presence of tritium-labelled 5β-ketodiol for 16 h. The labelled ecdysteroids were extracted from tissues and incubation media and the radioactive molecules from the Sep-Pak 60% methanol fractions were then separated by RP-HPLC (Fig. 8). The radioactive precursor was converted into several tritiated metabolites, one of which co-migrated with reference ecdysone. Significant amounts of radiolabelled 20-hydroxyecdysone were not found. Control incubations were performed under the same incubation conditions but in the absence of a biological sample. The resulting radiochromatograms never showed peaks in the region corresponding to the migration zone of ecdysone (Hoffmann et al., 1992).

DISCUSSION

The major peak in the haemolymph ecdysteroid titer during larval-larval and larval-adult development (in hemimetabolous insects) is usually present in the latter half of each

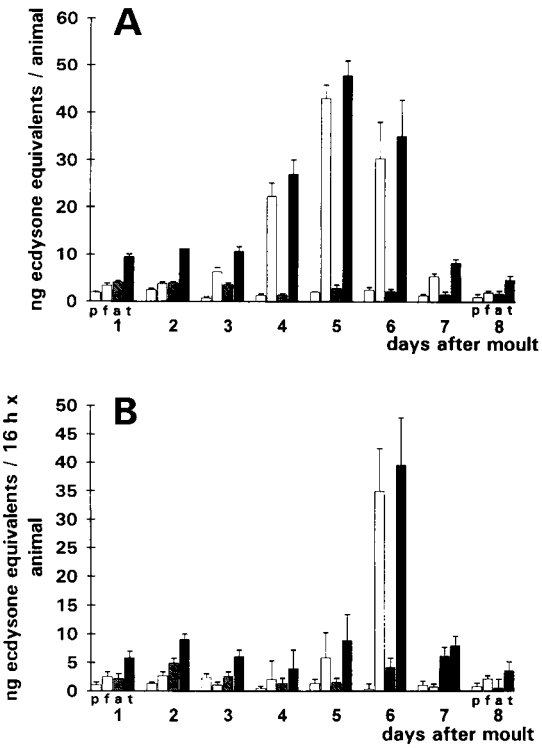


Fig. 6. A – Content of ecdysteroids in the abdominal integument from female last instar larvae. Bars represent SEM; n = 6–13. Other details as in Fig. 1. B – Net release of ecdysteroids by the abdominal integument from last instar larvae during a 16 h incubation in Grace's medium. Bars represent SEM; n = 6–13. Other details as in Fig. 1.

stadium. Temporally and quantitatively, this peak is characterized by a rapid rise from basal levels before apolysis; maximal levels either slightly before, at, or slightly after apolysis; and a rapid drop back to low or basal levels by the time of ecdysis (Smith, 1985). In most insect species, 20-hydroxyecdysone is predominant in the moulting peak. It is generally suggested that the main regulation of haemolymph moulting hormone titer is achieved through variations in prothoracic gland activity, but changes in the half-life of ecdysteroids and changes in the catabolic rate of hormones may also be important in the regulation of hormone titer.

In the present study we determined ecdysteroid titers in the haemoylmph during the last two larval stages of *G. bimaculatus* as well as the rates of ecdysteroid release by PG in vitro. In female penultimate instar larvae, one distinct peak of ecdysteroid release from PG was observed which occurred on the same day as the peak of moulting hormone concentration in the blood. Therefore, the source of the haemolymph ecdysteroids is presumably the moulting gland. The ovaries and the abdominal integument did not show a distinct peak of ecdysteroid release in vitro during the penultimate larval stage. The maximal haemolymph hormone level was similar to that in locusts (*Locusta migratoria*) (Baehr et al., 1979), whereas migratory grasshoppers, *Melanoplus sanguinipes*, have a 10-fold lower concentration of hormone (Ismail & Gillott, 1993). At the end of the penultimate larval stage, the ecdysteroid titer was reduced, but had not yet fallen to the basal level observed at the beginning of this larval stage. It seems to be a general feature of the haemolymph ecdysteroid titer that it never reaches zero during the last two larval instars. Thus,

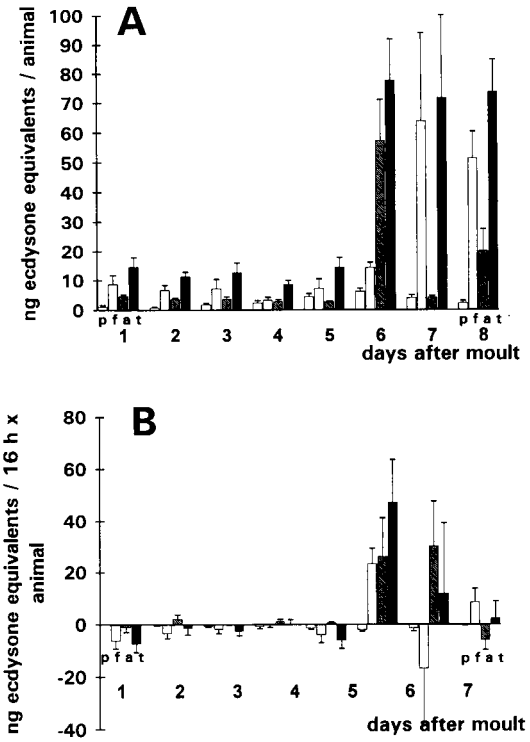


Fig. 7. A – Content of ecdysteroids in the ovary from female last instar larvae. Bars represent SEM; n = 6. Other details as in Fig. 1. B – Net release of ecdysteroids by the ovary from last instar larvae during a 16 h incubation in Grace's medium. Bars represent SEM; n = 6. Other details as in Fig. 1.

morphogenetic events cannot be regarded as activated by the hormone unless the titer exceeds a critical value of 5–10 ng/10 µl.

A different relationship between ecdysteroid release by PG and haemolymph ecdysteroid titer was found in last instar larvae, where the maximum ecdysteroid secretion by the prothoracic gland preceded the haemolymph ecdysteroid peak by two days. Ecdysteroids released from PG during days 4–5 seem to be stored, at least partly, in the abdominal integument, where they may induce ecdysteroid release on day 6. The transitory storage of ecdysteroids from PG in the abdominal integument is deduced from the parallel increase in ecdysteroid secretion by PG and hormone concentration in the abdominal integument during days 3 to 5. Ovarian ecdysteroid content as well as ovarian ecdysteroid release in vitro did not increase before day 6. The present results suggest a haemolymph ecdysteroid composition resulting from at least three hormone sources with temporally different maxima in hormone synthesis and release.

In *G. bimaculatus*, the rates of ecdysteroid release in vitro by alternative larval sources were similar to those in ovaries and abdominal integument of female adults at the time when ovaries reached maturity and animals deposited their first eggs (Weidner et al., 1992). The observation that ovaries and abdominal integument accumulated and released specific amounts of ecdysteroids at particular times during the final larval stage suggests that developmental events occurring in these organs have a specific period of sensitivity as well as a specific titer requirement. Indeed, studies performed with *M. sanguinipes* fifth (last) instar male larvae showed that peaks of ecdysteroids in the testes and male accessory

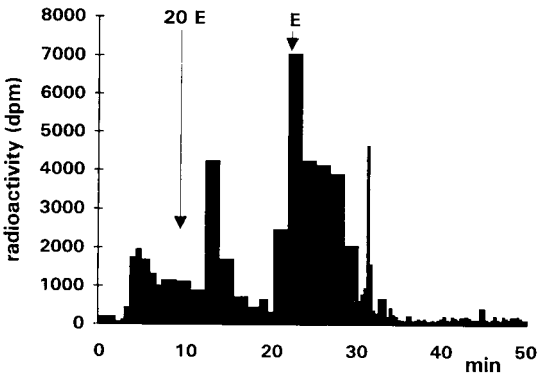


Fig. 8. Conversion of tritiated 2,22,25-trideoxyecdysone (5β-ketodiol) by the abdominal integument from day 6 female last instar larvae during a 16 h incubation in Grace's medium. RP-HPLC elution profile of tritiated compounds (combined Sep-Pak 60% methanol fractions) extracted from the tissues (n = 6). Arrows indicate retention times of ecdysone (E) and 20-hydroxyecdysone (20E).

reproductive glands (due to accumulation from the haemoylmph or to synthesis by the organs themselves) marked the beginning of their growth and differentiation (Ismail & Gillott, 1993). In *Periplaneta americana*, the development of the collaterial glands, which occurs at the end of the last larval instar, also requires ecdysone (Dixon & Blaine, 1973). In the same species, 20-hydroxyecdysone was shown to enable ovarian follicle cells to stimulate their DNA synthesis during the larval-adult transition (Bell & Sams, 1975). Transplantation of the male accessory reproductive gland anlage from 4th or 5th instar larvae of the field cricket *Plebeiogryllus guttiventris* into last instar larvae or adults showed that the differentiation of the accessory gland tubules only takes place in the last stadium, when the ecdysone titer is high and little or no juvenile hormone is present (Raabe, 1986).

In this study, we determined ecdysteroid release and hormone titers only in female larvae. Ismail & Gillott (1993) showed peaks in female and male *M. sanguinipes* larval haemolymph ecdysteroids on the same day, but there was a slight difference in the amounts present. Gnatzy & Romer (1980) did not find pronounced differences between the modulation of hormone level in the male and the female of last instar larvae of *G. bimaculatus*.

5β-ketodiol is a precursor of ecdysone in phylogenetically distant insect orders (Meister et al., 1987). Our experiment clearly showed that last larval abdominal integument is able to convert 5β-ketodiol into ecdysone. This, however, does not necessarily mean that the larval integument represents a primary source of ecdysteroids. Enzymatic systems which hydroxylate the 5β-ketodiol at C-2, C-22 and C-25 are present in many insect tissues (Meister et al., 1987). In female adults of *G. bimaculatus*, ovaries as well as the abdominal integument are able to convert 4-^{14}C[cholesterol] into ecdysone and were referred to as primary sources (Hoffmann et al., 1992). Of course, it is also necessary to demonstrate formation of ecdysteroids in ovaries and abdominal integument of last instar larvae from a distal precursor, such as cholesterol or 7-dehydrocholesterol. Such experiments are in progress.

The rates of ecdysteroid release as measured in this study were low when compared with hormone titers. These low rates can be attributed to cell injuries which may have occurred during dissection (PG), and to the lack of stimulating factors (ecdysiotropins) from the brain. We also used the same incubation medium for all tissues tested, which may not have been the optimal composition for PG. In contrast to last instar ovaries and abdominal integument, which released free and (mainly apolar) conjugated ecdysteroids, PG from

these larvae secreted only free ecdysteroids. We have not determined the nature of the intrinsic products of PG but, so far, there is no evidence in literature that moulting glands from orthopteroid insects synthesize and release others than ecdysone. Surprisingly, PG of the cockroach *P. americana* synthesized a 1 : 1 ratio of 3-dehydroecdysone and ecdysone, but the animals had negligible 3β-oxoecdysteroid 3β-reductase activity in their haemolymph (Kiriishi et al., 1990).

In conclusion, we did not demonstrate unequivocally that ovaries and abdominal integument of last instar larvae of *G. bimaculatus* synthesize ecdysteroids, but significant supporting evidence has been obtained. It has been shown that the ecdysteroid titers of haemolymph, ovaries and abdominal integument are different both in the timing of their fluctuations and in their content. We also found that the abdominal integument (day 6 last instar larvae) can release more ecdysteroids in vitro than are present in the tissue prior to or after incubation, therefore eliminating just a release of ecdysteroids from storage. As to the physiological function of ecdysteroids released from non-prothoracic gland cells, Jenkins et al. (1992) suggested that ecdysteroid release from dispersed cells throughout the thorax and abdomen of larvae or pupae may have paracrine effects by which moulting and developmental events are more efficiently synchronized and accelerated as compared to regulation via the PG alone.

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