Ecdysteroid titers during larval reproduction of the dipteran insect *Heteropeza pygmaea*[1]

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Summary. Ecdysteroid titers were measured in whole-body extracts of pedogenetically reproducing larvae of the dipteran insect *Heteropeza pygmaea* and in the dietary fungus. The titers are very low in the first 3 days of larval growth, but increase during the last 2 days. The level of 20-hydroxyecdysone is then significantly higher than that of ecdysone. Measurements of the titers in the fungus gave no conclusive results.
Key words. Ecdysteroids; pedogenesis; *Heteropeza pygmaea*, mycelium.

Heteropeza pygmaea, like many other gall midges, is phytophagous and feeds on fungus. Fresh (3-day-old) mycelium causes the *H. pygmaea* larvae to reproduce by pedogenesis (= parthenogenetic reproduction in the larval stage[3,4]). In pedogenetic reproduction the ovaries will develop prematurely and produce follicles already in the young larvae within the first 24 h of larval development[5]. When *H. pygmaea* larvae are set on aged (7-day-old) mycelium, the mode of reproduction is altered; ovarian development is retarded and follicle formation only takes place after several days[3,5].

The effect of 20-hydroxyecdysone on ovarian development of *H. pygmaea* was examined in an earlier investigation[6]. The test system consisted of larval ovaries cultured in hemolymph drops. In this in vitro system the hormone was shown to stimulate strongly formation and separation of pedogenetically developing follicles. The same result was obtained when high doses of 20-hydroxyecdysone were applied topically to the larvae[7]. In 3 insect species with imaginal reproduction[8-11] 20-hydroxyecdysone was found to have an almost identical effect on the ovary, i.e. to induce the organization of follicles and their separation.

In order to find out whether the stage of follicle formation in the ovary of the *H. pygmaea* larva is characterized by an increase of ecdysteroids in situ, we measured the amounts of 20-hydroxyecdysone and ecdysone in the pedogenetically reproducing larvae throughout the life cycle, by using thin layer chromatography (TLC) in combination with a radioimmunoassay (RIA). Since fresh fungus fed to the larvae has an effect on the ovaries similar to that of 20-hydroxyecdysone on ovaries cultured in vitro (i.e. it stimulates follicle formation), we also examined the fungus for the presence of ecdysteroids using TLC and high pressure liquid chromatography (HPLC) combined with a RIA.

Material and methods. Larvae of *Heteropeza pygmaea* (Cecidomyiidae, Dipt.) were kept under conditions whereby they reproduce by thelytokous (female producing) pedogenesis only[12]. When fed on fresh fungus (the Basidiomycete *Peniophora albula*, cultured on agar-malt substrate), the larva mainly consists of a body wall filled with female offspring at the end of the reproductive cycle. Young female larvae were collected at the moment of hatching from their mother larvae and then either homogenized and stored for further treatment (see below) or placed on food. A heap of 150 larvae was placed in the center of a Petri dish with fresh (3-day-old) fungus and then kept in the dark at 25 °C. Generally, 10 Petri dishes with growing larvae were used for each experiment. At varying times (½, 1, 2, 3, 4, 5 days; variation in time ± 5 min at most) after the start of feeding, a number of larvae were collected, homogenized in 70% methanol and stored at −20 °C; to obtain 50–60 mg fresh weight of each stage, between 55 and 3000 larvae had to be collected. Because of the small size of the larvae, only whole-body extracts were examined.

We also tested 3- and 7-day-old mycelium for the possible presence of ecdysteroids (the age refers to the number of days after inoculation of the agar-malt plates). For this purpose most of the fungus in the Petri dish was scraped off from the agar-malt substrate. In addition blocks of the agar-malt substrate were examined. The subsequent procedures for homogenizing, storage, extraction, purification, etc., were the same as for the larvae.

Extraction and purifications were carried out as described previously[13]. Some of the fungus samples were, in addition, purified by using C-18 Sep-pak cartridges (Waters and Associates)[14]. After TLC purification or HPLC separation the different zones or fractions were assayed in a RIA[15,16]. This RIA is 5–7 times more sensitive to ecdysone than to 20-hydroxyecdysone. Ecdysone and 20-hydroxyecdysone used as standards in each assay were purchased from Simes (Milan), antibodies were a generous gift from J.D. O'Connor (Los Angeles) and 23,24-[$^3H_2$]-ecdysone from J. Koolman (Marburg).

Results and discussion. In order to characterize the pedogenetic larvae according to stage we first measured the weight increase and related it to the developmental stages of the progeny floating in the hemocoele of the larvae (table). Young larvae developing into mother larvae rapidly increase in weight, about 5-fold within the first day of development. The total weight increase during larval reproduction is about 40-fold. After 4 days of larval growth the larvae stop feeding and the peritrophic membrane is expelled. This may cause the weight decrease of the larvae recorded for the last day.

Analysis of ecdysteroids in whole-body extracts of developing mother larvae including progeny revealed the presence of RIA-positive material in the TLC zones of reference ecdysone and 20-hydroxyecdysone. Titer variations are given in the figure. In the first 3 days of larval growth, the concentrations recorded are very low. Similar low titers of ecdysteroids have also been found in larval stages of other insects, e.g. for *Drosophila melanogaster*[17-19], *Sarcophaga bullata*[20] and *Galleria mellonella*[21].

Weight increase of the *Heteropeza pygmaea* larva related to its age and to the developmental stage of its progeny during pedogenetic reproduction

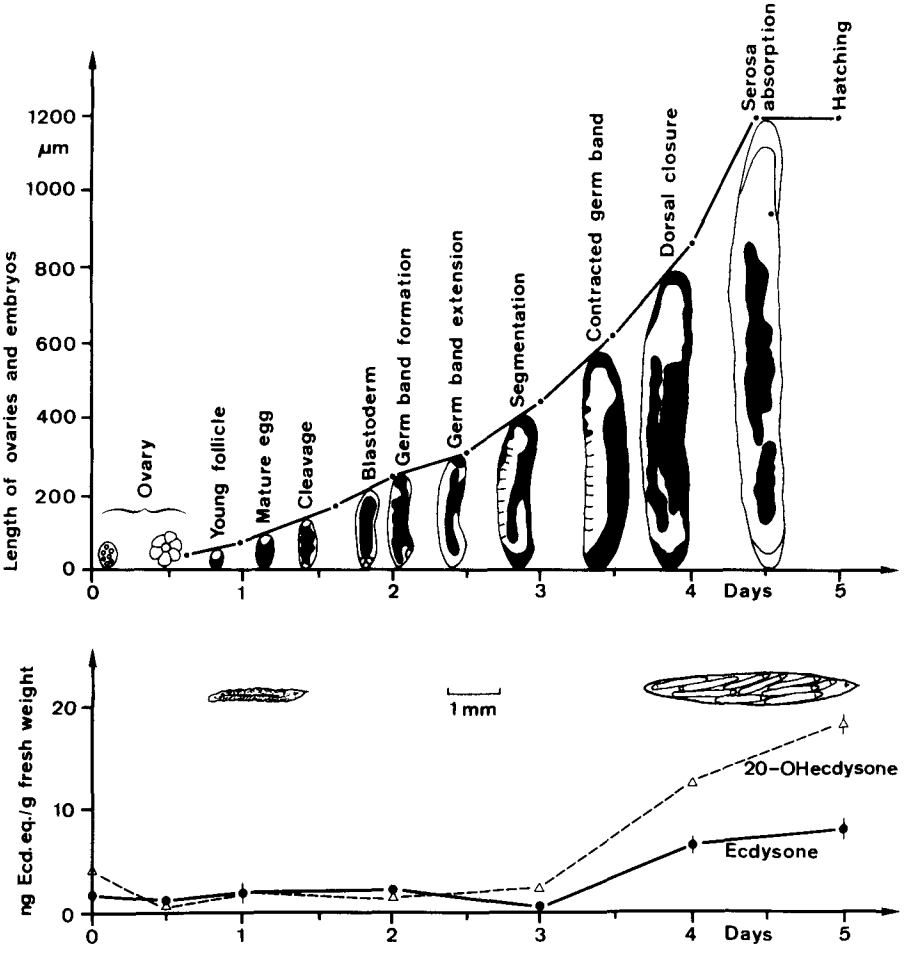
	Age in days after hatching of the larva from its mother larva						
	0	½	1	2	3	4	5
Weight in µg* (± SEM)	22.2 ± 0.3	48.7 ± 0.4	106 ± 1	341 ± 2	603 ± 10	891 ± 12	833 ± 10
Developmental stage and length of the larval progeny	Ovary prior to follicle formation	Follicle formation in the ovary almost completed	Young follicles, 40–60 µm	Cleavage and blastoderm stages, 160–240 µm	Germ band extension-retraction, 400–500 µm	Contracted germ band, gut anlage visible, 600–900 µm	Shortly before hatching of the young larvae

* Larvae used for weighing, homogenizing and further treatment represented the fast-growing and well-fed fraction (circa 75%) of the total number of larvae in a Petri dish. They were not weighed individually but in groups of 55–3500 larvae; between 3 and 11 such groups were weighed for each developmental stage.

During the last 2 days of larval reproduction of *H.pygmaea*, the levels of 20-hydroxyecdysone and ecdysone increase significantly although they are still relatively low. The activity in the 20-hydroxyecdysone zone is now significantly higher than that in the ecdysone zone. The increase in ecdysteroid titers coincides with the strong increase in size of the prothoracic glands at the end of larval development; according to Kaiser[22] these glands are the last tissues of the mother larva to be histolyzed. The elevated levels of ecdysteroids may also be related to molting of both the mother larva as well as its enclosed progeny on the last day of larval development[23].

Because follicle formation in the ovary seems to be 20-hydroxyecdysone-dependent[6,7], we had presumed that the titer of ecdysteroids might be elevated in the young larva during follicle formation. Assuming that we did not miss a peak, this seems not to be the case. On the other hand, it has been shown for female *Aedes aegypti* imagos that very small amounts of 20-hydroxyecdysone (2 consecutive injections of 10 and 25 pg per animal) cause organization and separation of follicles in this insect[10]. Since the unfed female adult of *A.aegypti* weighs about 2–3 mg, effective concentrations of 20-hydroxyecdysone would be as low as 4–10 ng/g fresh weight and perhaps even lower[10]. Taking this into account together with the data mentioned in the introduction[6–11], it still seems possible that in the *H.pygmaea* larva ecdysteroids, in spite of their low level, induce follicle formation in situ. However, the results of this investigation do not give any evidence for this assumption.

Another question is concerned with the possible source of the ecdysteroids. The prothoracic glands are only small at the beginning of larval development[22] and they do not seem to be required for follicle formation[7]. As the developmental fate of the larvae – and thus of the ovaries and their products – is determined ultimately by their food[12], we speculated that the fungus *Peniophora albula* might be a source of ecdysteroids for the larvae. Analysis of 3- and 7-day-old mycelium of the fungus for the presence of ecdysteroids using crude extracts or TLC and HPLC combined with RIA gave no definite answers. No RIA-positive material, or only traces, were detected in 7-day-old mycelium (11 extracts were examined; all values were below 1.3 ng ecdysone equivalents/g fresh weight) and in the agar-malt substrate (12 extracts). Since aged mycelium offered to the larvae inhibits ovarian development and follicle formation for some days[3,5], the finding that 7-day-old mycelium contains no ecdysteroids does not contradict our speculation. The values for 3-day-old mycelium (which is known to induce rapid follicle formation[5]) varied between 0 and 10.4 ng ecdysone



Stages of embryonic development (top) and titers of ecdysone and 20-hydroxyecdysone (bottom) in the female *Heteropeza pygmaea* larva during pedogenetic reproduction. The pedogenetically developing larva is most suitably characterized by its progeny which develops in the hemocoele. Therefore, the upper part of the diagram gives some stages of oogenesis and embryogenesis which take place within the growing larva. The lower part of the diagram shows the titers of ecdysteroids in whole-body extracts of developing larvae as determined by TLC combined with RIA; 2 larvae, a 1-day-old larva and a 4–5-day-old larva filled with offspring, are also shown. Zero time on the abscissa is the time when the young larva has just hatched from its mother larva and starts feeding. The results are expressed in ng ecdysone equivalents for the ecdysone zone (●) and in ng 20-hydroxyecdysone equivalents for the 20-hydroxyecdysone zone (△). Each point represents the mean value from individual measurements of 2–11 different extracts, 4 extracts on average. For each extract, an average titer was obtained from 2 determinations. SEM (bars) are indicated only when they are greater than ± 1 ng ecdysteroid equivalent/g fresh weight.

equivalents/g fresh weight (12 extracts). In addition, analysis of HPLC fractions covering a wide range of polarity did not show an accumulation of RIA-positive material in any particular fraction. The difficulty of determining the exact titer of ecdysteroids in the fresh fungus may be explained by the assumption that ecdysone is present in a conjugated form or in a precursor form. It may be speculated that these precursor forms are then converted into ecdysteroids inside the larvae, where they finally determine the reproductive mode.

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Persistent inverse maternal effect on corticosterone production in vitro

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Summary. Adrenal cells from C57BL/Tb mice produced more steroid than those from DBA/2J mice. Reciprocal differences between both backcross and F1 hybrids showed a persistent maternal effect. Mothers with high output produce offspring with reduced hormone production when adult. Corticosterone output thus depends on maternal phenotype as well as on the genotype of the isolated cells.
Key words. Adrenals; corticosterone; maternal effects; stress; inbred strains; mice.

C57BL/10J mice show a much greater rise in plasma corticosterone following stress than DBA/2J mice and have 7-fold greater stores of esterified cholesterol in their adrenals[2]. We have used enzymically dissociated suspensions of adrenal cells to investigate the synthesis of corticosterone so that factors intrinsic to the steroidogenic cells could be separated from genetic differences acting elsewhere[3]. To reveal their biosynthetic potential adrenal cells isolated from individual mice of the C57BL/Tb and DBA/2J strains and their hybrids were maximally stimulated with ACTH. All the mice were young adult males, as developmental changes in the juxtamedullary X-zone complicate adrenal structure in females[4], and were given the same standard diet[5,6].

Cells suspensions were made from the paired adrenals of individual mice by a modification of the method of Barofsky et al.[7]. Cells were dissociated enzymically first with 0.35% trypsin in Krebs-Ringer bicarbonate buffer containing 0.2% glucose (1 ml KRBG per adrenal pair) and then with 0.04% chromatographically purified collagenase in KRBG containing 4% bovine serum albumin (0.25 ml KRBG-BSA per adrenal pair). Both enzyme incubations also contained 12.5 µg/ml deoxyribonuclease and 50 µg/ml ribonuclease. The cells were mechanically dispersed by repeatedly drawing them into a Pasteur pipette, washed and aliquots counted and assayed for viability. Steroid production was stimulated by incubating about $3 \cdot 10^5$ viable cells in 0.605 ml of KRBG-BSA containing 17 ng (2.5 m units/ml) of porcine ACTH. All 3 incubation steps were carried out at 37 °C for 1 h in an atmosphere of 95% O_2 and 5% CO_2. All glassware was siliconized. Steroid production was stopped by adding 0.2 ml of cold 39% ethanol to each incubation and steroids were extracted with methylene chloride. Corticosterone was measured fluorimetrically in duplicate samples for each incubation[8]. The identity of corticosterone was established by chromatography in 3 thin-layer systems, before and after acetylation.

Cells from C57 mice produced significantly more ($p = 0.02$, 2-tailed Kolmogorov-Smirnov test) corticosterone that those from DBA males (table). Differences in overall mean between the backcross to DBA and the backcross to C57 were expected on the basis of segregation at a locus resembling *ald*, which controls cholesterol ester levels in crosses with AKR mice[9]. The striking reciprocal differences in all 3 hybrid generations were completely unexpected. The significant ($p = 0.02$, 2-tailed K-S test) difference between the reciprocal F1 males could have been caused by differences in either their sex-chromosome complement or by other differences in their parents[10]. Measurements on adrenal cells from individual mice showed significant differences ($p = 0.04$, 2-tailed K-S test) between the backcrosses of reciprocal F1 females to males of the C57 strain. Only maternal effects differed between these 2 backcrosses. Such effects were the only difference between the reciprocal backcrosses to DBA, whose adrenal cells also differed in their output of corticosterone. The maternal effect on the backcrosses cannot have been due to differences in their nuclear genotype for reciprocal F1 females have identical sets of autosomes and X-chromosomes. The difference between the 2 C57 backcrosses was 28% of their joint mean. The maternal effect could thus account for most or all of the difference be-