Ecdysteroids and control of embryonic diapause: changes in ecdysteroid levels and exogenous hormone effects in the eggs of cochineal *Lepidosaphes*

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Summary. Embryos of the diapausing strain of *Lepidosaphes ulmi* showed a low ecdysteroid level at the onset of diapause whereas an ecdysteroid peak occurred at the same stage in the non-diapausing strain. This observation, together with the effects of 20-OH-ecdysone on diapausing eggs, strongly suggests that this hormone inhibits diapause in embryos.

Numerous workers have demonstrated that diapause of insect larvae and pupae can be terminated by injecting ecdysone[4]. However, there is a lack of information concerning diapause in the embryo. This is partly due to the smallness of the egg and its tough and impervious shell, which renders such experimentation difficult. The cochineal *Lepidosaphes ulmi* is especially suitable for investigating this problem because it has both a diapausing and a non-diapausing strain. We determined the changes in the ecdysteroid level during embryonic development as well as at the beginning and end of diapause. Furthermore, we determined the effects of ecdysone and of 20-OH-ecdysone on diapausing eggs.

Materials and methods. Lepidosaphes ulmi: Diaspine scales were reared in the laboratory on watermelon, at 24 °C and 70% relative humidity. When they are laid, multi-voltine eggs contain embryos in the germ band stage located in the posterior pole. Development starts immediately and lasts without interruption for 14 days[5]. In the uni-voltine strain, however, newly laid eggs contain embryos which have already begun to develop and have reached the metamerisation stage, exhibiting 3 thoracic and 7 abdominal segments. These embryos remain unchanged from July/August, when they are laid, to January, when development resumes. However, their embryonic diapause can be terminated artificially by exposure to a temperature of 13 °C for 8 weeks, followed by incubation at 24 °C. In this case, complete development takes 4 weeks (Gharib, unpublished).

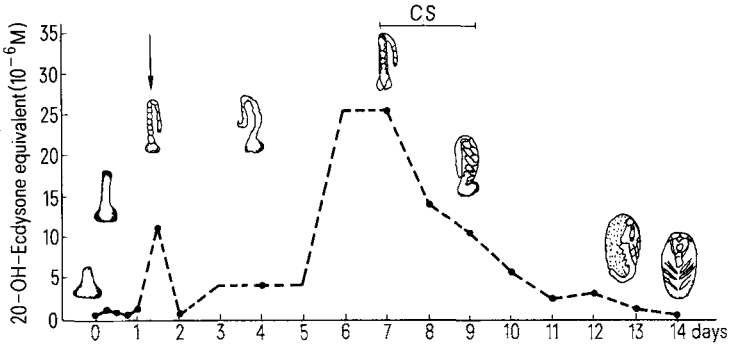
Ecdysteroid determinations. The ecdysteroid content of developing multi-voltine eggs was measured daily; that of the uni-voltine strain was checked only at the beginning and end of diapause. Each assay required 450 eggs (about 1 mg total) which were sonicated in 200 µl of 1 N $HClO_4$ and assayed according to the radioimmunoassay method (RIA) of De Reggi et al.[6]. Generally speaking, developing eggs of Arthropods contain a wide range of ecdysteroids, including ecdysone and 20-OH-ecdysone, as well as highly or slightly polar products[7]. In these experiments, we did not separate these compounds. The RIA activity recorded is expressed as µmoles of 20-OH-ecdysone-equivalent per mg of fresh eggs (this corresponds to a molar concentration).

Hormonal treatment. Exogenous ecdysones were administered to eggs of the uni-voltine strain as follows. The chorion was first removed, according to Zalokar[8] and the vitellin membrane was carefully pierced with a small needle. The eggs were then immersed in an isotonic solution of ecdysone or of 20-OH-ecdysone 10^{-4} M. Sham-treated eggs received the same treatment, except that they were dipped in a solution of only NaCl. All eggs were soaked for 48 h. One treatment or the other was applied to the following sample pools (each containing 30 eggs):

- sample 1: eggs removed from the female before being laid;
- sample 2: newly laid eggs;
- samples 3-5: eggs chilled at 13 °C for 1, 2 or 3 weeks respectively.

After being treated or sham-treated, eggs were allowed to develop at 24 °C for 4 weeks. The percentage of hatching was calculated at the end of the incubation.

Results. A. Variations of ecdysteroid level during embryonic development. The figure illustrates the changes in ecdysteroid content recorded from non-diapausing eggs during embryonic development. The hormonal level was generally high, i.e. often equal to or greater than 4×10^{-6} µmoles/mg. Moreover, it rose markedly on 2 occasions. The largest peak occurred between days 5 and 10; it reached 25×10^{-6} µmoles/mg on days 6 and 7. At that time, the embryos were fully segmented and had differentiated appendages. Attention should also be called to an earlier peak which took place 36 h after the eggs were laid. It lasted only a few hours but reached a high level (12×10^{-6} µmoles/mg). At the time of this peak, young embryos had already begun segmentation and had 3 thoracic and 7 abdominal segments. In the uni-voltine strain, newly laid eggs contained embryos which have reached this characteristic stage; however, their ecdysteroid content was low. A hormonal rise, similar to that observed in the multi-voltine strain occurred, but it was after the termination of diapause, i.e. on the 1st day of incubation at 24 °C, after hibernation; the



Variations of ecdysteroid level during the embryonic development of non-diapausing eggs (RIA activity, µmoles of 20-OH-ecdysoneequivalent per mg of fresh eggs). The different developmental stages are schematized. The arrow indicates the time at which embryos of the uni-voltine strain enter diapause; CS, cuticular secretion.

Percentage hatching in eggs of the diapausing strain of *Lepidosaphes ulmi* after hormonal treatment. After being treated or sham-treated, eggs were allowed to develop at 24 °C for 4 weeks. Controls remained at 24 °C, without any treatment

	Sample number	Untreated	Sham-treated	Treated by Ecdysone	20-OH-ecdysone
Unchilled eggs, taken					
Before laying	1	0	0	15	58
After laying	2	0	0	0	0
Eggs chilled at 13 °C for					
1 week	3	0	0	6	6
2 weeks	4	10	0	3	6
3 weeks	5	10	0	12	65

ecdysteroid content of the eggs rose to 10×10^{-6} µmoles/mg.

B. Termination of diapause by hormonal treatment. The percentage of hatching in eggs which were treated, sham-treated or untreated and then transferred to 24 °C, are summarized in the table.

Control and sham-treated eggs: unchilled eggs, taken either before or after laying, or eggs chilled for not more than 3 weeks, did not hatch (only a few hatchings were observed in untreated eggs which were chilled for 2 or 3 weeks). This was true for untreated eggs as well as for those which were soaked in a saline solution without hormone.

Treated eggs: the consequence of hormonal treatment in unchilled eggs depended on whether they were removed before being laid, or not. Nearly 60% of those in the first group hatched after being treated with 20-OH-ecdysone, whereas the same hormone was totally ineffective for the others. The susceptibility of the eggs to the hormone was restored by chilling: after 3 weeks at 13 °C, over 60% of the eggs hatched. Ecdysone was observed to produce the same effect as 20-OH-ecdysone, although to a much lesser extent. Only a little more than 10% of the eggs of sample 1 (eggs taken before laying) and of sample 5 (eggs chilled for 3 weeks) hatched.

Discussion. The high concentration of ecdysteroids observed in the embryos of *Lepidosaphes ulmi* is a frequent occurrence since it has been found in all species yet studied: *Bombyx*[9], *Blaberus*[10], *Locusta*[11], *Carausius* and *Clitumnus*[12], *Schistocerca*[13]. This statement is also valid for the high hormonal peak which preceded the synthesis of the embryonic cuticle. Conversely, the peak which appeared 36 h after laying is more unusual. It coincided with the metamerisation of the embryo as was described in *Blaberus*[10] and in the crab *Acanthonyx lunulatus* (Chaix and de Reggi, unpublished). Moreover, and most importantly, this peak also coincided with the beginning of diapause in the uni-voltine strain. Insects of this strain did not exhibit a peak at this time: ecdysteroids were low at the onset of diapause. The same phenomenon has already been reported in late larvae of 2 species with diapausing pupae, *Pimpla*[14] and *Philosamia*[15].

It is tempting to believe that this hormonal peak is responsible for preventing the multi-voltine strain from diapausing. And this is in good agreement with the observed effects of 20-OH-ecdysone on diapausing eggs. If administered before laying, the hormone permits the eggs to develop. It is highly probable that at this stage diapause is not firmly established and can still be reversed by the hormone. It does however become irreversible following laying. Thereafter it requires 3 weeks of chilling for the eggs to recover their responsiveness to the hormone. This strongly suggests that the latter inhibits diapause in embryos of *Lepidosaphes*. The phenomenon was also observed in *Bombyx mori* eggs[16].

Something should be said of the fact that the hormonal treatment never led to 100% hatching. This is because of the high ecdysteroid concentration (up to 10^{-4} M) which had to be used. At this level, the exogenous hormone allows the embryos to develop, but it further drastically unbalances hormonal control of development.

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4 R. Sieber and G. Benz, J. Insect Physiol. *26*, 213 (1980).
5 B. Gharib, C.r. Acad. Sci. Paris *286*, 1313 (1978).
6 M. De Reggi, M. Hirn and M. Delaage, Biochem. biophys. Res. Commun. *66*, 1307 (1975).
7 C. Hetru, M. Lagueux, M. Luubang and J. Hoffman, Life Sci. *22*, 2141 (1978).
8 M. Zalokar, Drosoph. Inf. Serv. *47*, 128 (1971).
9 M. Hirn, M. Coulon and M. De Reggi, C.r. Acad. Sci. Paris *284*, 2147 (1977).
10 D. Bullière, F. Bullière and M. De Reggi, Roux's Arch. *186*, 103 (1979).
11 M. Lagueux, C. Hetru, F. Goltzene, C. Kappler and J.A. Hoffman, J. Insect. Physiol. *25*, 709 (1979).
12 F.P. Louvet, B. Fournier, M. Cavallin, F. Delachambre, F.P. Delbecque and J. Gautier, IVth Eur. Symp. on Ecdysone, Strasbourg 1979.
13 A.R. Gande and E.D. Morgan, J. Insect Physiol. *25*, 289 (1979).
14 J. Claret, P. Porcheron and F. Dray, C.r. Acad. Sci. Paris *286*, 639 (1978).
15 B. Calvez, M. Hirn and M. De Reggi, FEBS Letters *71*, 57 (1976).
16 B. Gharib, J.M. Legay and M. De Reggi, J. Insect Physiol. *27*, in press (1981).