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Morphoanatomic evolution and ecdysteroid variation during the metamorphosis of *Neodiprion sertifer*

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Abstract

Previous studies have emphasized that, in sawfly metamorphosis, most of morphogenesis and hormonal activity occurs during the pronymphal phase in the prepupal stage. The titres of ecdysteroids in haemolymph and ovaries were measured in selected stages of development of the sawfly *Neodiprion sertifer* (Hymenoptera Diprionidae), during the metamorphosis and in the adult. The hormonal levels detected were compared with the main morphological and anatomical differentiations observed. The last larval instar showed a peak of ecdysteroid secretion about two days before the apolysis. During the prepupal stage there were two peaks of ecdysteroid secretion with the second much higher. At the pupal stage a sudden increase of the hormone titre was found. The sawfly therefore showed a variation of the molting hormone titre comparable with that observed for other holometabolous insects. Moreover an intense secretive activity of the ovaries from the late pupal stage to the adult stage was clearly detected. The dose-response hypothesis of ecdysteroid action is discussed.

Key words: *Neodiprion sertifer*; metamorphosis; ovaries; ecdysteroids; 20E.

Introduction

The sawfly *Neodiprion sertifer* (Geoffroy) (Hymenoptera Diprionidae) is a feared pest of pine in Europe, Asia, and North America. The trees are defoliated in spring by the larvae leading to a progressive weakening of the plant, which often turn in reduced growth (Baronio *et al.*, 1988) and death in few years (Baronio *et al*., 1997).

The insect has one generation per year except for high altitudes and northern territories where it has a 2-year life cycle with a summer aestivation accomplished by the eonymph (Pschorn-Walcher, 1965; 1970; Morimoto and Nakamura, 1989) and a slowed down development of the embryo during the winter (Martini *et al.*, 2006). The fully grown larva moults to a prepupa that distinguishes an eonymphal phase followed by a pronymphal phase, a period of intensive morphogenesis (Sláma, 1964).

The peculiarity of the sawfly metamorphosis is the presence of a stage of prepupa during which the morphogenesis is transferred from the pupal stage to the previous (Sláma, 1964). For *N. sertifer* this was also shown by the histological studies of Fogal and Kwain (1974), who considered the pronymphal phase at *stage 3* as a "*pharate pupa*". Sláma (1964) found that in sawflies the eonymphal phase represents a phase of hormonal inactivity whereas the pronymphal phase is a period of intensive morphogenesis and hormonal activity. The same author demonstrated that in sawflies the critical period in which the moulting hormone (i.e. ecdysteroids) is required occurs around most of the pronymphal phase and in the beginning of the pupal stage.

In this study we analyzed the variations of ecdysteroids in haemolymph and ovaries in relation to morphological and anatomical modification in *N. sertifer* females.

Materials and methods

The insects

The larvae, prepupae and pupae used in the experiments were obtained from a mass rearing conducted as described by Baldassari *et al.* (2003) except for a photoperiod of 15 h of light which allows a complete inhibition of the eonymphal diapause (Sullivan and Wallace, 1964).

From the first instar, newly moulted larvae were daily isolated and put together, in order to obtain female larvae of the fifth (the last one). This could be easily done, because the just ecdysed larvae are well recognisable due to their yellow head, which maintains this colour for about two hours. In this way last instar female larvae could be isolated since male larvae complete their development through four instars.

Female prepupae were obtained from cocoons. To allow the hardening of the cocoon and its manipulation, the prepupae were removed two days after the cocoon spinning. They were then placed singly in small glass tubes (8 x 40 mm), sealed with absorbent cotton which was maintained moist. This small apparatus was placed in a polystyrene stand maintained at 20 °C and 15:9 photoperiod (L:D) and allowed us to follow the progress of metamorphosis in each specimen daily.

In order to have well recognisable developmental characters to be related to hormonal variations, a staging of prepupae and pupae was elaborated. The prepupal development was divided into eonymphal and pronymphal phase according to the appearance of the underlying pupal eye in the pronymph. Moreover, for this last phase eight different recognisable stages were described, based on the development of the pupal eye (figure 1). The pupal development was divided into nine different stages (table 1). For each stage specimens were dissected under saline (169.4 mM NaCl/2.7 mM KCl/1.8 mM $CaCl_2$) in order to emphasise the anatomical development.

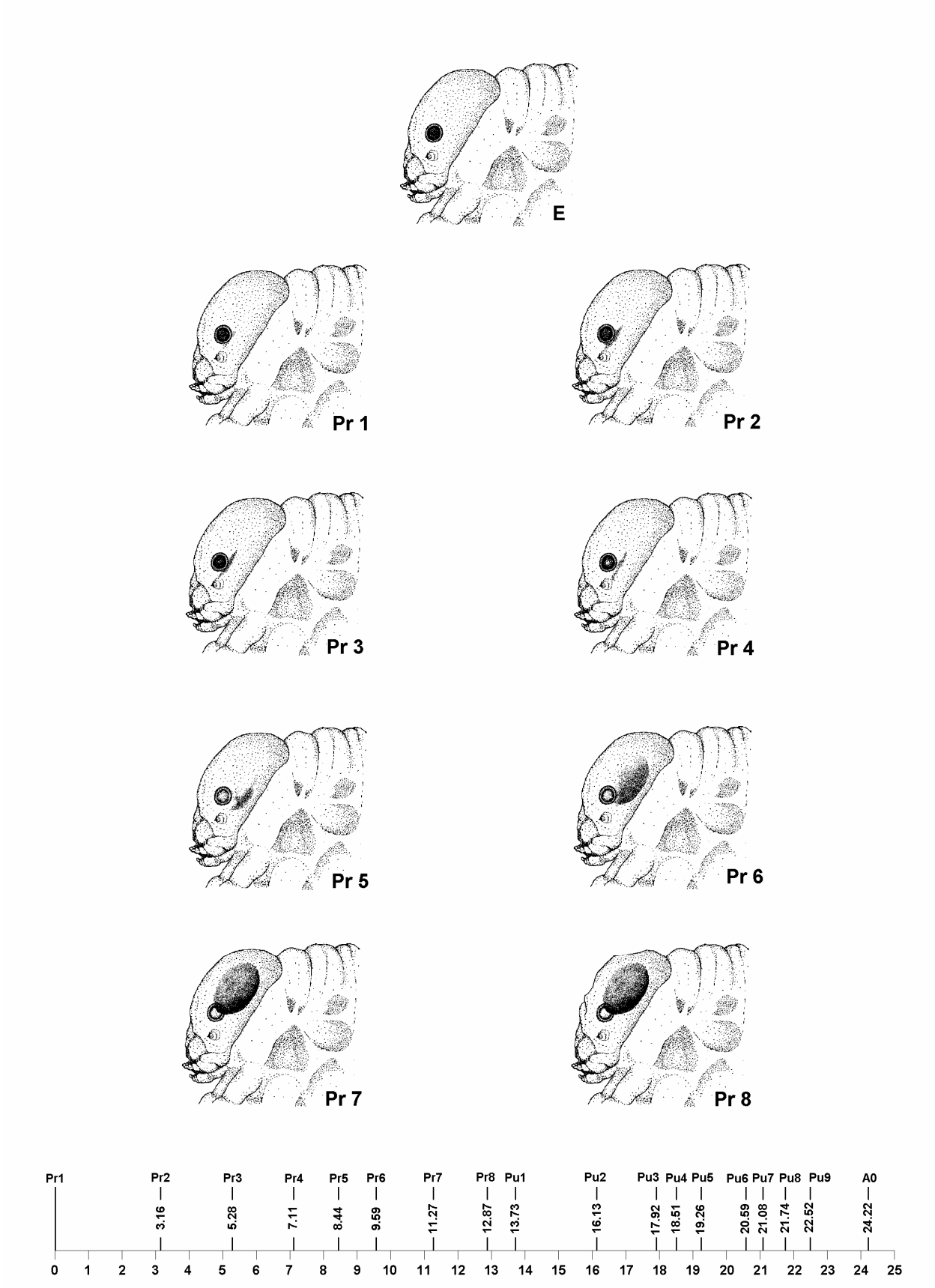

Figure 1. The stages of the prepupal development of *N. sertifer* established according to the progressive differentiation of the underlying pupal eye. E = eonymph; Pr = pronymphal phases; Pu = pupal phases; A = adult. At the bottom a diagram showing the days of development from Pr 1 to adult.

Table 1. Morphological and anatomical characters of different developmental stages of prepupae and pupae of *N. sertifer* and their duration (E = eonymph; Pr = pronymphal phases; Pu = pupal phases; A = adult).

Stage/Phase	Morphological characters	Anatomical characters	Mean lenght (days)	n	SE
E	Eonymph: larval eye	Like a larva.	20.86	33	0.818
Pr 1	Pronymph: the posterior ridge of the stemma is enlarging	Ovaries primordia are still small and adhere tightly to the fat body; salivary glands absent; foregut and hindgut empty, foregut diverticula are still full but some material is pouring into the foregut; malpighian tubes absent.	3.16	19	0.520
Pr 2	Pronymph: two tail spot at the posterior ridge of the stemma	Ovaries primordia less adherent to fat body; salivary glands absent; diverticula contents is pouring into the foregut, midgut threadlike and white; hindgut threadlike but hyaline.	2.12	17	0.270
Pr 3	Pronymph: the tails of the spot are elongated	Fat body less consistent; salivary gland absent; foregut diverticula empty; midgut larger than in the previous phase and cylindrical; malpighian tubes thin and hyaline.	1.83	12	0.441
Pr 4	Pronymph: pigment retraction on the stemma	Ovaries detached from the fat body; salivary glands absent; midgut and malpighian tubes as above; hindgut thin and hyaline.	1.33	9	0.236
Pr 5	Pronymph: the spot is well separated from the stemma	Ovaries as above; apolysis still not evident; midgut cylindrical with a narrowing at his midpoint and without peristalsis; malpighian tubes small, hyaline and motionless.	1.14	7	0.143
Pr 6	Pronymph: the spot is very enlarged	Apolysis almost complete at dorsal level; midgut very enlarged; pupal fore- and hindgut formed; malpighian tubes elongated and motile; podotechae and pterotechae formed (pupal discs evaginated).	1.69	16	0.176
Pr 7	Pronymph: pupal eye well formed	Ovaries still small; fat body less but still consistent; apolysis completed at pleural level and as far as the 2nd thoracic segment; malpighian tubes still hyaline but motile; midgut without peristalsis.	1.60	10	0.296
Pr 8	Pronymph: head collapsed	Head collapsed; apolysis completed at the level of lateral longitudinal trunk; midgut larger than in the previous phase; apolysis of the foregut intima completed; fat globules adherent to the piloric valve.	0.86	7	0.092
Pu 1	Just ecdysed pupa, red eye	Ovaries developed; midgut enlarged and with intense peristaltic activity, peritrophic envelop completely developed; malpighian tubes in motility.	2.40	10	0.163
Pu 2	Pupa with black eye	Well developed ovaries; foregut hyaline, midgut enlarged, pear-shaped, without peristaltic activity; thoracic muscles well developed.	1.79	12	0.278
Pu 3	Start of darkening of mandibulae	Sperm gland of the spermatheca well developed; no oocytes in vitellogenic phase; midgut with peristaltic activity; malpighian tubes in motility; wings almost completely developed within pterotechae.	0.58	6	0.083
Pu 4	Mandibulae completely darkened	As above but with midgut showing no peristaltic activity.	0.75	9	0.102
Pu 5	Spurs of tibiae darkened, antennae grey	As above.	1.33	20	0.108
Pu 6	Antennae black	Ovaries with some oocytes showing vitellogenesis; apolysis of the pupal cuticle almost completed, adult cuticle formed; foregut threadlike; midgut without peristaltic activity; malpighian tubes in motility; fat body with fat globules detached from each other.	0.49	11	0.081
Pu 7	Pterothecae grey	Almost every ovariole shows one vitellogenic oocyte; melanization of the adult cuticle at the thoracic level; the midgut shows a non-peristatltic contraction; malpighian tubes in motility.	0.66	14	0.083
Pu 8	Start of darkening of adult integument (pharate adult)	Every ovariole shows at least one vitellogenic oocyte; sclerotization of the cuticle at the metathoracic level; the abdomen shows intense motion.	0.78	9	0.088
Pu 9	Adult integument well darkened	As above but with motion of legs.	1.71	29	0.183
A 0		The oocytes are turned in light pink (may be vitellogenesis completed), no oocyte shows choriogenesis, spermatheca swollen; midgut full of meconium; malpighian tubes in motility.			
A 2		Intense contraction of the ovaries; most of ovarioles show 2 chorionated oocytes; nurse cells atrophic; the meconium is poured into the hindgut.			
A 6		Almost every oocyte is chorionated with the exception of 4-5 oocytes per ovary, arrested at an early vitellogenic phase.			

Haemolymph and ovaries sampling

Haemolymph and ovaries samples were daily made with specimens from the penultimate moult to the last larval apolysis, which was evident from the outside of the larva as two longitudinal white strips in correspondence with the tracheal longitudinal trunks. Samples were taken from prepupae and pupae according to their morphologically determined degree of development. Ovaries and haemolymph were collected every two days from adult females from the day of eclosion to day 12.

The haemolymph was extracted from larvae and prepupae (by cutting a prothoracic leg) and collected with a pipette. The amount collected was then measured by a graduated capillary, dissolved in 50 µl of a 70% methanol solution and maintained at -18 °C in PE vials until analysis. From the same specimens utilized for the haemolymph extraction, the ovaries or their *primordia* were removed under saline solution and preserved in 500 µl of 70% methanol at -18 °C. For each insect age/phase 3-7 specimens were tested.

Ecdysteroid measurement

Just before the measurement the ovaries were squeezed with a pestle and sonicated for few seconds. Haemolymph and ovaries solutions were then centrifuged at 15000 rpm for 10'. Supernatants were evaporated in vacuum and known amount of AB/BSA was added to the pellet. AB/BSA was 25 mM sodium phosphate/0.15 M NaCl/1M Na_2EDTA, pH 7.5 containing 0.1% BSA.

Free ecdysteroids were measured by EIA according to the method of Kingan (1989). Primary antibody rabbit anti-ecdysone (polyclonal antiserum) and enzymatic tracer 20E-HRP (20 hydroxyecdysione conjugate to horse radish peroxidase) were purchased from Timoty Kingan. The secondary antibody was goat anti-rabbit whole serum (Sigma R-4751). The standard was 20E purchased by Sigma. The substrate was ODP (orthophenilendiamine - Sigma P-9029)/0.1M citric acid/0.2M Na_2HPO_4/H_2O_2. Microtitration plates were Costar® 3369 and absorbance was detected at 450 nm by a Spectra Max 340 PC by Molecular Devices. Each sample was measured twice.

Results

The main morphological and anatomical features of the identified prepupal and pupal phases and their mean duration in days (± standard error) are displayed in table 1.

The results of the ecdysteroid measurement are displayed in table 2. No more than 4 microliters of haemolymph could be collected from larvae and prepupae. Moreover, from the phase Pr 5 to Pr 8 the haemolymph didn't flow from the insect leg. The same happened with pupae from the phase Pu 3 to Pu 9 and adult from the 4th day after ecdysis (A 4).

During the penultimate larval stage, i.e. LV, the ecdysteroid levels in the haemolymph remained quite low until the 2nd day, showed a sudden increase from the 4th (Mann-Whitney comparison LV 4 *vs* LV 2; U = 1, p = 0.032) and reached a peak on the 6th day, approximately two days before the apolysis (Mann-Whitney comparison LV 6 *vs* LV 4; U = 0, p = 0.036). On the 7th day the title of hormone dropped significantly (Mann-Whitney comparison LV 7 *vs* LV 6; U = 0, p = 0.036) but showed a little increase during the moult (LV-apo). During the prepupal stage there were apparently two peaks of the hormone, at the phase E 6 (Mann-Whitney comparison E 6 *vs* E 4; U = 0, p = 0.008), i.e. 27.73 days before the moult, and at the phase Pr 4, about 9 days before the moult.

After the pupal moult a rapid increase of the ecdysteroid level in the haemolymph was measured and a peak of hormone titre was detected at the phase Pu 2 (Mann-Whitney comparison Pu 2 *vs* Pu 1; U = 0, p = 0.036), i.e. about 12 hrs before a complete differentiation of wings was observed in the *pterotechae*. Only from one specimen at the phase Pu 6 a sample of haemolymph could be measured. For the same reasons the haemolymph samples could be collected only during the first 4 days of the adult stage, where large amounts of ecdysteroids were found.

The ovaries of *N. sertifer* produced ecdysteroids in detectable amounts in the late pupal stage and throughout the whole adult life, but with large variations among specimens. The larger amount could however be detected after the stage A 2, when the ovaries were fully grown and choriogenesis had started. A significant increase of the hormone titre was also detected at the phase Pu 2 (Mann-Whitney comparison Pu 2 *vs* Pu 1; U = 0, p = 0.008) in correspondence with the peak measured in the haemolymph. Their primordia during the last larval instar or prepupa showed only traces (0.01 to 0.4 ng 20E-eq/pairs of ovaries) of the hormone.

Discussion and conclusions

By comparing the ecdysteroid amounts found in haemolymph samples, in selected steps of the development during the metamorphosis of non diapausing eonymphs of *N. sertifer*, it was found that the hormone is always present. Conversely, it was absent in wild diapausing ones (Sláma, 1964). Moreover Sláma (1964) emphasized that the eonymphal phase is the diapausing stage and for this reason it is lacking in ecdysone. This should be possible, as in lepidopterans an inhibition of ecdysone secretion is responsible for larval diapause (Nijhout, 1998). As a proof, Hamel *et al.* (1998) obtained the resumption of the development in diapausing eonymphs of *Diprion pini* (L.) (Hymenoptera Diprionidae) by injection of large doses of 20-hydroxyecdysone. But how can the eonymphs resume their development becoming the pronymphal and morphogenetic active phase, when the hormonal stimulus is certainly present? No indications are reported by Sláma (1964), because the eonymphal phase of diapausing specimens is a phase of hormonal inactivity (is therefore ecdysone lacking?). On the contrary, in the present work it was found that the hormone is present also in that phase in non diapausing specimens. Therefore it appears that the stimulus for the resumption of the development in diapausing forms is acting on the ecdysone secretion by the eonymph, as

Table 2. Ecdysteroid contents in the haemolymph and ovaries of *N. sertifer* females during metamorphosis.

Stage/Phase	Description	Haemolymph			Ovaries		
		n	20E equiv. ng/ml	SE	n	20E equiv. ng/pairs of ovaries	SE
LV 0	fifth instar female larva, just ecdysed	5	5.02	1.083	5	0.02	0.007
LV 1	fifth instar female larva, 1 day old	2	6.76	4.603		not extracted	
LV 2	fifth instar female larva, 2 day old	4	6.25	1.102	5	0.02	0.006
LV 4	fifth instar female larva, 4 day old	5	107.36	53.539	5	0.01	0.002
LV 6	fifth instar female larva, 6 day old	3	795.16	220.719	3	0.02	0.004
LV 7	fifth instar female larva, 7 day old	5	24.55	8.967		not extracted	-
LV apo	fifth instar female larva, at apolysis	5	40.58	26.701	5	0.01	0.001
E 0	eonymph, just ecdysed	5	16.57	2.714	5	0.01	0.001
E 4	eonymph, 4 days from edysis	5	27.09	4.997		not extracted	-
E 6	eonymph, 6 days from edysis	5	105.70	34.297	5	0.01	0.002
E 12	eonymph, 12 days from ecdysis	7	39.69	5.482	2	0.01	0.004
E 20	eonymph, 20 days from ecdysis	5	63.63	15.122		not extracted	-
Pr 1	pronymph, phase 1	5	91.90	11.522	5	0.02	0.003
Pr 2	pronymph, phase 2	5	91.83	23.230	5	0.02	0.004
Pr 3	pronymph, phase 3	5	292.88	84.398	5	0.02	0.003
Pr 4	pronymph, phase 4	1	2096.22	-	5	0.06	0.004
Pr 5	pronymph, phase 5	-	not collectable	-	5	0.25	0.066
Pr 6	pronymph, phase 6	-	not collectable	-	5	0.11	0.013
Pr 7	pronymph, phase 7	-	not collectable	-	5	0.08	0.028
Pr 8	pronymph, phase 8	-	not collectable	-	5	0.04	0.014
Pu 1	Pupa, just ecdysed: red eye	5	621.01	69.578	5	0.06	0.012
Pu 2	Pupa, black eye	3	5407.54	223.572	5	0.37	0.131
Pu 3	Pupa, start of darkening of mandibulae	-	not collectable	-	5	0.13	0.018
Pu 4	Pupa, mandibulae completely darkened	-	not collectable	-	4	0.05	0.015
Pu 5	Pupa, tarsal claws darkened, antennae grey	-	not collectable	-	5	0.10	0.015
Pu 6	Pupa, antennae black	1	917.79	-	5	1.01	0.273
Pu 7	Pupa, pterotechae grey	-	not collectable	-	5	1.88	0.472
Pu 8	Pupa, beginnig of darkening	-	not collectable	-	5	4.01	0.787
Pu 9	Pupa, adult integument darkened	-	not collectable	-	5	9.80	2.322
A 0	just eclosed female	5	196.88	66.296	5	13.79	3.776
A 2	2 days eclosed female	5	311.06	189.980	5	12.48	1.095
A 4	4 days eclosed female	1	50.19	-	5	32.90	13.869
A 6	6 days eclosed female	-	not collectable	-	5	40.37	14.988
A 8	8 days eclosed female	-	not collectable	-	5	19.66	0.800
A 10	10 days eclosed female	-	not collectable	-	5	25.86	8.202
A 12	12 days eclosed female	-	not collectable	-	5	36.18	16.656

it always happens in the non diapausing insects, despite the lack of macroscopic morphological or anatomical changes. Moreover, the finding of a correspondence between specific morphogenetic events and peculiar hormone levels supports the hypothesis of a dose-response relationship (Sláma, 1980).

The two peaks of ecdysteroids found during the prepupal phase of *N. sertifer* are similar to the findings in other studies dealing with holometabolous insects. In particular, it was found that the second peak of ecdysteroid secretion is followed by the beginning of apolysis and the evagination of imaginal discs. Also the anatomical differentiation is consistent with the dose-response hypothesis of Sláma (1980). As a proof, in *N. sertifer* females the pouring of the *meconium* into the hindgut occurred in correspondence with a small peak of ecdysteroids in haemolymph. This was observed also in the pupae of *Bombyx mori* L. (Suzuki *et al.*, 2009).

The hormonal activity of the ovaries of *N. sertifer*, that started secreting ecdysteroids at the beginning of vitellogenesis, suggests that the hormone can trigger a feed-back mechanism allowing the growth of the ovaries themselves with a character of homeostasis. The stimulation of vitellogenesis by ecdysteroids was found also in many dipterans (Nijhout, 1998). Moreover, the maternal ecdysteroids could be incorporated into the eggs as inactive conjugates in order to control embryonic moults, as already shown for other insects (Nijhout, 1998). This hypothesis is supported by the results of the HPLC analysis of chorionated eggs (Martini, 2001). It is still to be explained why the ovaries keep secreting ecdysteroids when all oocytes are already chorionated.

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