

The embryonic development of *Diprion pini* and the related ecdysteroid levels

Antonio MARTINI¹, Camilla CHIECO², Maria Luisa DINDO¹, Piero BARONIO¹

¹Dipartimento di Scienze e Tecnologie Agroambientali - Entomologia, Università di Bologna, Italy

²Consiglio Nazionale delle Ricerche, Istituto di Biometeorologia, sezione di Bologna, Italy

Abstract

The embryonic development of pine sawfly *Diprion pini* (L.) was studied in relation to ecdysteroid levels by histological analysis with light microscopy and immunoenzymatic assay. The progressive differentiation of cells, tissues and organs, including the hypocerebral ganglion, the corpora cardiaca, the corpora allata and the prothoracic glands, was described. Only two germ layers, i.e. the ectoderm and the mesendoderm, were detected. It was also highlighted and defined the deposition of three embryonic cuticles, of which the first secreted by the serosa and the other two by the ectoderm and epidermis, respectively. The deposition of the first and of the last cuticle occurred after a peak of ecdysteroid secretion.

Key words: embryogenesis, sawflies, *Diprion pini*, ecdysteroids, 20-hydroxyecdysone.

Introduction

The pine sawfly *Diprion pini* (L.) (Hymenoptera Diprionidae) feeds on needles of various pine species, with preference to *Pinus sylvestris* L. (Eliescu, 1932; Escherich, 1942; Francke-Grosman, 1953; Géri, 1988; Turrisi and Bella, 1999). The insect is univoltine in high altitude and in the north of Europe while is bivoltine in central and southern European regions (Géri and Gousard, 1988).

The female is oviparous and can reproduce by amphigony or arrenothocous parthenogenesis. All eggs are laid in a row in the needles, generally of one twig, and covered with a brownish glandular secretion (Escherich, 1942).

Once the larva has reached maturity it moults to prepupa, which provides two phases, namely an eonymph, which spins the cocoon, followed by a pronymph. During the eonymphal phase, most individuals undergo diapause, for a variable period of time, while some others don't. There are no clear explanations for this variability, although Eichhorn (1976) has shown that diapause is prevented at 20 °C with a photoperiod of 17L:7D.

The ectoblastic embryo develops in a meroblastic egg without a true diapause, but with different growth times depending on temperature, namely 11 days at 25 °C, 14 days at 20 °C and 28 days at 15 °C (Eichhorn, 1976).

In the present work the histological and anatomical development of the *D. pini* embryo was studied in relation to ecdysteroid levels measured, in order to improve the understanding of the embryo differentiation in sawflies. This approach was suggested as different concentrations of ecdysteroids of maternal and embryonic origin were detected during the different phases of the embryo development in the confamilial species *Neodiprion sertifer* (Geoffroy) (Hymenoptera Diprionidae) (Martini, 2001).

The histological analysis and immunoenzymatic assays were conducted exclusively on male eggs, laid by non inseminated females, in order to avoid potential differences related to the sex of the embryo.

Materials and methods

The insects

The eggs used in the experiment were laid by *D. pini* females raised in the laboratory. The insect breeding started from larvae of the third instar onwards, which were collected in the forest at S. Martino in Monte, near Bressanone (Bolzano province, Italy) and S. Dorlingo della Valle (Trieste province, Italy). For the continuous breeding the larvae were maintained on *P. sylvestris* twigs, in plastic boxes (28 × 18 × 12 cm) with a hole in the cover closed by a metal mesh. The boxes with larvae were in turn placed in a rearing chamber under conditions preventing eonymphal diapause according to Eichhorn (1976), namely 20 °C, 60% RH and 17L:7D photoperiod. Cocoons were maintained under the same conditions. Since in small spaces adults mutilate each other (Knerer, 1984), each cocoon was isolated in a 4 cm-diameter plastic Petri dish so as to avoid this phenomenon.

The newly-emerged females were placed to oviposit on 2 year-old *P. sylvestris* plants, grown in pots in turn placed in transparent polycarbonate cylinders (130 cm high and 40 cm diameter). To prevent escape the cylinders were closed at the top with gauze.

After oviposition, the plants with eggs were removed from the cylinders and kept under the same environmental conditions until hatching. The larvae were transferred into the plastic boxes when they reached the third instar. They were fed *ad libitum* with fresh *P. sylvestris* twigs until the cocoon formation.

The eggs for histological analysis and immunoenzymatic assay were laid by virgin females kept individually on *P. sylvestris* twigs, which were treated as in Eichhorn (1976), in order to avoid infections and maintain them alive. Namely, the twig base was washed and brushed with soapy water and then sealed within a tube filled with water. Moreover, every two days the branch base was cut and the water changed. This procedure was adopted so as to avoid the twig and needle dieback which eventually negatively affects the egg viability.

Histology

The samples consisted of eggs of different ages, namely from newly-laid eggs (0-6 hrs old) to the 16th day from oviposition, fixed daily. To avoid damage to eggs during the preparation, entire needles containing eggs were fixed soon after collection for 24 hours in 10% buffered formalin. The eggs were then carefully removed from the needle, dehydrated by serial transfer through graded ethanol solutions, and embedded in paraffin. Sagittal or frontal sections of 4 µm were cut with a rotary microtome (Leica RM 2125 RT). Sections were stained with hematoxylin and eosin and mounted with DPX mountant (BDH Chemical, Poole, UK).

The serial sections were observed with a Carl Zeiss Axioskop light microscope equipped with AxioCam digital camera to observe and detect the tissues and organs at different times of the embryonic development.

Ecdysteroid assessment

The ecdysteroid assessment in developing embryos was carried out by removing eggs from the pine needles at 24 hrs intervals, from 1 day until 17 days after oviposition. This procedure was performed while keeping the pine needle dipped in saline solution.

For all the ages analyzed, the ecdysteroid levels were measured in 10 replicates, each consisting of groups of 10 eggs of the same age collected from egg clusters laid by 10 different females. Each group of eggs was placed in a vial (1.5 ml capacity) containing 500 µl of 70% methanol and stored at -18 °C.

For the ecdysteroid extraction, the egg samples were first crushed and then subjected to sonication for 20 seconds. Subsequently, the material was centrifuged at 15000 rpm for 10 minutes at 2 °C. The supernatant was extracted and then subjected to complete evaporation under vacuum. The resulting pellet was added with 500 µl solution of 70% methanol. Final samples were stored until analysis at -18 °C.

A known volume (two aliquots of 50 µl) of appropriate dilutions of the solutions obtained from each sample was analyzed. Free ecdysteroids were measured by competitive enzyme-linked immunosorbent assay (EIA) according to the method of Kingan (1989). Primary antibody rabbit anti-ecdysone (polyclonal antiserum) and enzymatic tracer 20E-HRP (20-hydroxyecdysone conjugate to horse-radish peroxidase) were purchased from Timothy Kingan, University of California, Riverside. The secondary antibody was goat anti-rabbit whole serum (Sigma R4751). The standard was 20E (20-hydroxyecdysone) (Sigma H5142). The substrate was Tetramethylbenzidine (Sigma T0440). 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.

The concentration values for each age were obtained as mean ± standard error of the 10 replicates.

Results

Histology

In the eggs of 0-6 h the blastoderm is already formed and consists of a single layer of blastomeres. It is sepa-

rated from the yolk mass by a basement membrane on which vitellophages accumulated while in endocytosis activity (figure 1A). The germ cells are located in the posterior pole outside the blastoderm (figure 1B). Other yolk cells are evident in the yolk mass.

In the eggs of 1 day, the germ band is differentiated on the ventral side of the embryo. The protocephalic lobes are already developed, the amnion is developing (figure 1C) while the serosa, consisting of flattened cells, is complete (figure 1D). The germ band has already undergone the gastrulation and differentiated the ectoderm. This consists of a pseudostratified layer of lanceolate-shaped cells.

In the eggs of 2 days, the amniotic membrane limiting the homonymous cavity, differentiated along the whole length of the embryo, is formed by elongated cells and is extremely flattened. The ectoderm has evolved into a multistratified layer with elongated cells, on which the inner layer is very distinct with globose cells with larger nuclei (figure 1E).

In the eggs of 3 days the segmentation begins: the ectoderm is crossed by transverse furrows limiting the different segments (figure 1F) and differentiates polygonal neuroblasts, with clear edges, and neuritic projections (figure 2A).

In the egg of 4 days the caudal area is surrounded by the amnioproctodeal cavity bounded by amnion, and the invagination which will originate the proctodeum is evident, along with the two coelomic cavities behind which the germ cells are clustered (figure 2B). In the protocephalon there are two coelomic cavities. The amniotic cavity has enlarged and the amnion surrounds the embryo and the yolk, with the exception of a small fraction which remains enclosed in the amnion and the serosa. The latter has secreted a distinct cuticular layer (figure 2C). The segmentation is more advanced.

In the egg of 5 days the differentiation of the malpighian tubules has begun (figure 2D).

In the egg of 6 days the differentiation of the labrum occurs in the protocephalon, over which the yolk is still present. In the protocorm, the buds of the gnathal appendages, of the three pairs of legs and of the ten abdominal segments are evident. In the first abdominal segment there is the *pleuropodium*, conical shaped and small, as described in *Athalia rosae* (L.) (Hymenoptera Tenthredinidae) (Yamamoto *et al.*, 2004). In the following seven segments and in the tenth there are the proleg buds. The ventral nerve cord is well defined and the ganglia, in which the perikarial region is distinguishable from the neuropile, are linked by connectives (figures 2E and 2F). The epineural sinus bounded by the mesoderm can also be detected (figure 3A). The mesoderm consists of two layers of cells, over which several yolk nuclei are arranged in syncytia with yolk spherules (figure 3B). Along the lateral area ectoderm invaginations, leading to the formation of the tracheal system, appear (figure 3C). The salivary glands begin to differentiate (figure 3D). The dorsal closure of the embryo by the ectoderm has started in the head, where the musculature is also evident (figure 3E).

In the egg of 7 days the dorsal closure in the head is complete. The proleg buds have differentiated at the

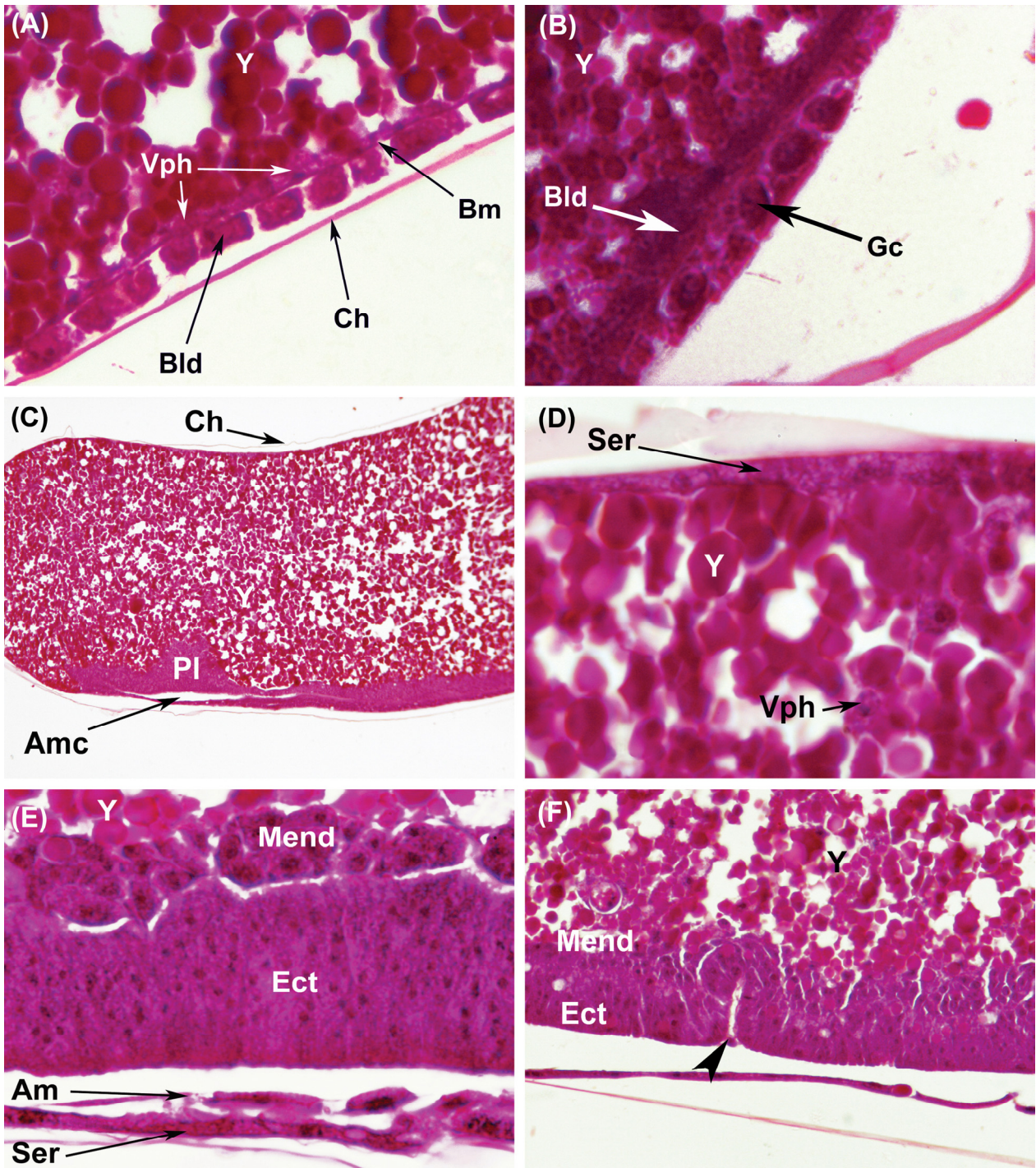


Figure 1. **A:** *six-hour egg*: yolk (Y), chorion (Ch), blastoderm (Bld) and basement membrane (Bm) on which vitelophages (Vph) accumulate (sagittal section). **B:** *six-hour egg*: yolk (Y) and germ cells (Gc) external to the blastoderm (Bld) layer (sagittal section). **C:** *one-day egg*: germ band with differentiated protocephalic lobes (Pl) and amniotic cavity (Amc) (sagittal section). **D:** *one-day egg*: complete serosa (Ser) and active vitelophages (Vph) in the yolk (Y) (sagittal section). **E:** *two-day egg*: amnion (Am), serosa (Ser) ectoderm (Ect) and mesendoderm (Mend) (sagittal section). **F:** *three-day egg*: the beginning of the embryo segmentation (arrow); yolk (Y), mesendoderm (Mend) and ectoderm (Ect) (sagittal section).

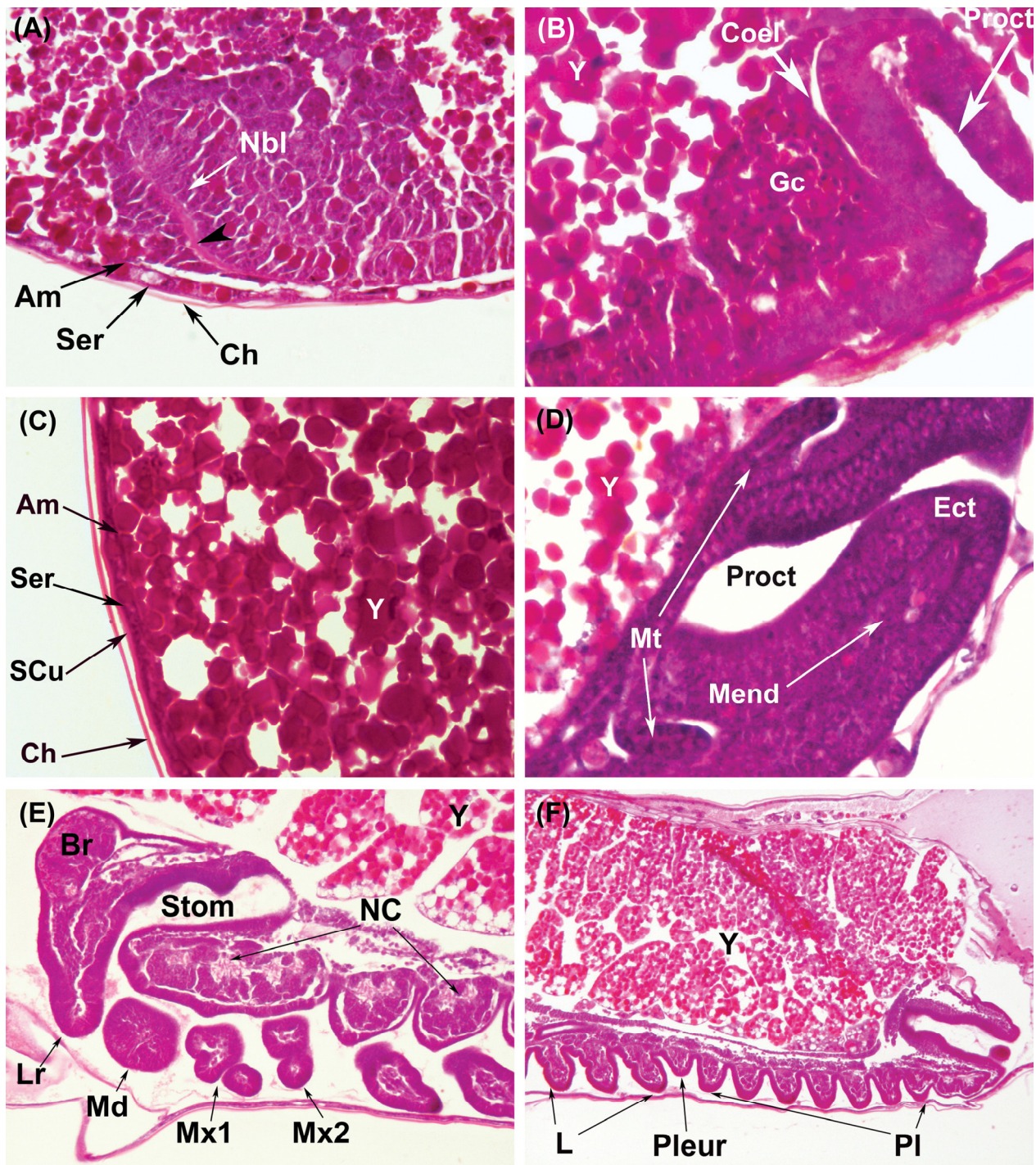


Figure 2. **A:** *three-day egg*: protocephalic lobes with evident neuroblasts (**Nbl**) neuritic projections (**arrow**); amnion (**Am**), serosa (**Ser**) and chorion (**Ch**) (sagittal section). **B:** *four-day egg*: caudal region of the embryo with the invagination of the proctodeum (**Proct**), the celomatic cavity (**Coel**) and germ cells (**Gc**) (sagittal section). **C:** *four-day egg*: cuticular layer (**SCu**) secreted by the serosa (**Ser**); amnion (**Am**) and chorion (**Ch**) (sagittal section). **D:** *five-day egg*: the beginning of differentiation of the malpighian tubules (**Mt**); proctodeum (**Proct**), mesoderm (**Mend**) and ectoderm (**Ect**) (sagittal section). **E:** *six-day egg*: differentiation of the labrum (**Lr**), of the bud of the mandibles (**Md**), of the maxillae (**Mx1**), and of the labium (**Mx2**) and ventral nerve cord (**NC**); yolk (**Y**), brain (**Br**) and stomodeum (**Stom**) (sagittal section). **F:** *six-day egg*: buds of the legs (**L**), of the pleuropodium (**Pleur**) and of the prolegs (**PI**); yolk (**Y**) (sagittal section).

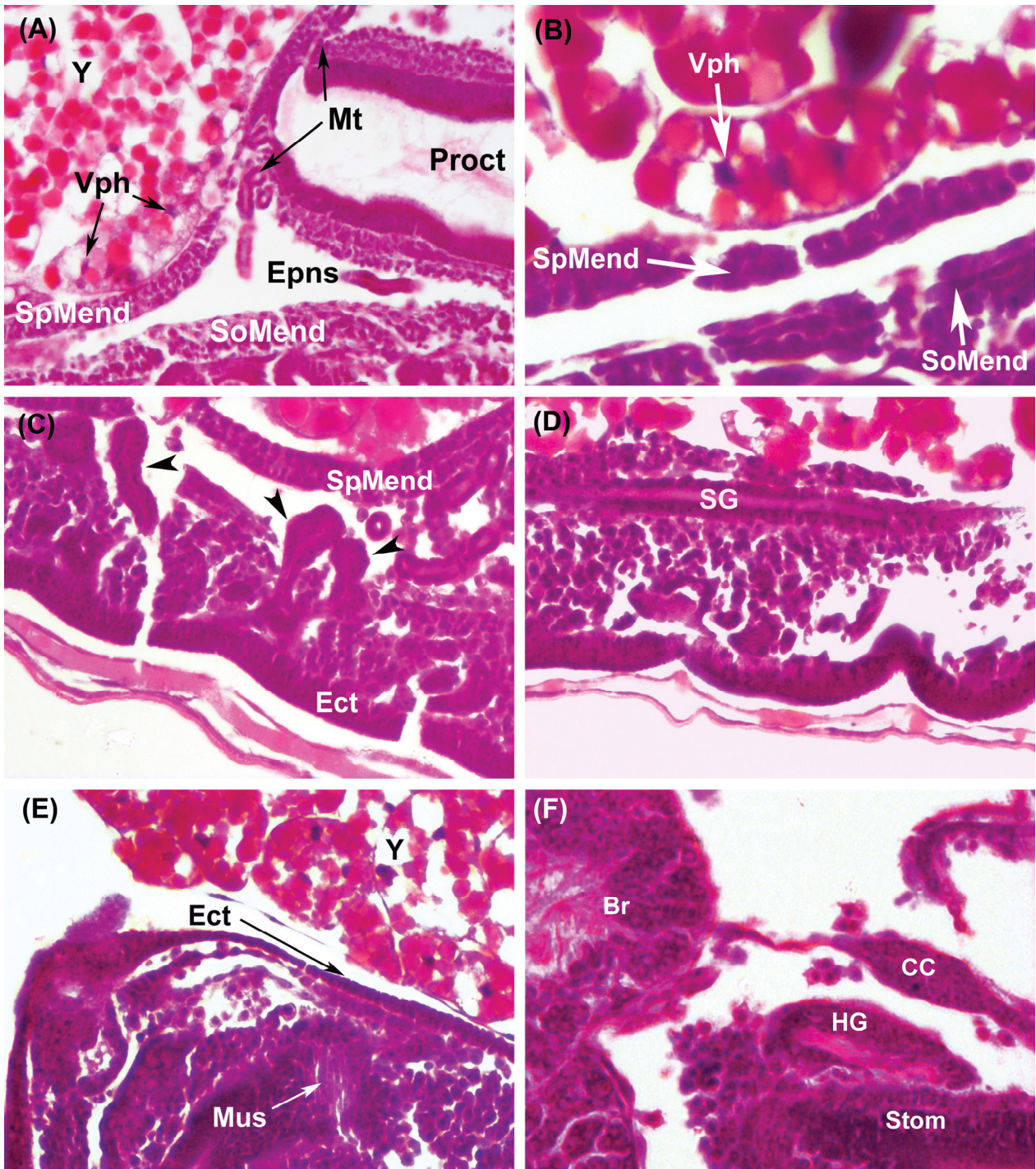


Figure 3. **A:** *six-day egg*: epineural sinus limited by the somatic (**SoMend**) and splanchnic (**SpMend**) mesoendoderm; yolk (**Y**), vitellophages (**Vph**), malpighian tubules (**Mt**) and proctodeum (**Proct**) (sagittal section). **B:** *six-day egg*: detail of the syncytium of vitellophages (**Vph**) and yolk (**Y**) spherules; somatic (**SoMend**) and splanchnic (**SpMend**) mesoendoderm (sagittal section). **C:** *six-day egg*: invaginating ectoderm (**Ect**) that differentiates tracheae (**arrows**); splanchnic mesoendoderm (**SpMend**) (sagittal section). **D:** *six-day egg*: salivary gland (**SG**) (sagittal section). **E:** *six-day egg*: beginning of the dorsal closure of the head by the ectoderm (**Ect**); muscles (**Mu**) and yolk (**Y**) (sagittal section). **F:** *seven-day egg*: the hypocerebral ganglion (**HG**) and the corpora cardiaca (**CC**); brain (**Br**) and stomodeum (**Stom**) (sagittal section).

base both their own and common muscles. The frontal and hypocerebral ganglia and the corpora cardiaca are differentiated (figure 3F). The last two organs have been identified through the description and iconography performed by L'Helias (1952) in the larvae of the same insect. The posterior end of the embryo begins to turn on the ventral side (figure 4A). The splancnic muscles of the proctodeum begin to differentiate with internal circular and external longitudinal fibres.

In the egg of 8-9 days the ectoderm starts growing dorsally and covering the yolk mass. The tentorium branches, with a strong attachment of the cephalic musculature, appear clearly in the head (figure 4B). An epithelium of cuboid cells envelops the entire yolk mass (figure 4C).

In the egg of 10 days a deposition of a cuticle by the ectoderm can be observed for the first time (figure 4D). The mesenteron is differentiated with an epithelium which, in the posterior end, consists of columnar cells with an evident secretory activity (figure 4E). Conversely, the anterior end shows a cuboid epithelium in which many yolk cells seem to incorporate. The musculature of the stomodeum and mesenteron is well formed. As in the larvae of others sawflies (Formigoni, 1955), the prothoracic glands are differentiated on a region of the lateral trunks which enter the head from the prothoracic stigma (figure 4F). Longitudinal tracheal trunks in the abdomen are being completed. In the head the differentiation of the stemmata is being completed (figure 5A).

In the egg of 11 days the dorsal closure of the embryo is completed and the first cuticle covers it entirely.

In the egg of 12 days the caudal part of the abdomen is elongated while remaining completely folded ventrally. The yolk that was between the amnion and the serosa is distributed outside the embryo around the head and at the point where the abdomen bends ventrally.

In the egg of 13 days, the apolysis of the first cuticle begins.

In the egg of 14 days there is a progressive reduction of the yolk mass within the mesenteron (figure 5B). The development of the corpora allata occurs (figure 5C). Their identification was achieved following the description and the iconography of Heymons (1895) in the embryo of *Forficula* sp. (Dermaptera Forficulidae) as well as that of Nelson (1915) in the embryo of *Apis* sp. (Hymenoptera Apidae).

In the egg of 15 days the apolysis of the first cuticle is complete (figure 5D).

In the egg of 16 days the stomodeum and mesenteron are now connected (figure 5E), whereas a separation remains between the mesenteron and the proctodeum. A new cuticle, which differentiates hairs and setae, is secreted under the first, without ecdysis (figure 5F). The epidermal cells in intense activity of protein synthesis have rounded nuclei.

Egg hatching occurs between the 16th and the 17th day.

Ecdysteroid assessment

The amount of ecdysteroids detected in the eggs collected from the plant day by day is shown in table 1 and figure 6 where two peaks of ecdysteroid titre are evident, the first on the 3rd day and the second between the 12th and 13th day of the embryonic development.

Discussion and conclusions

The comparison between the ecdysteroid level found at different ages and the embryo differentiation shows clearly that the deposition of the three cuticles occurs after the serosa, the ectoderm and the epidermis were subjected to an increase of hormone titre. The deposition of several cuticles in the embryo related to ecdysteroid peaks has already been shown in other insects, as reported by Lagueux *et al.* (1979). The same authors have also reported that when the embryo of *Locusta migratoria* (L.) (Orthoptera Acrididae) is producing a new cuticle it undergoes apolysis without ecdysis, similarly to our observations on *D. pini*.

In *D. pini* an ectodermal cuticle is laid down over the head once dorsal closure has occurred in this anterior region. The ectodermal cuticulogenesis proceeds at the thorax and abdomen when the dorsal closure is complete at that level. Therefore it appears that the embryo of *D. pini* produces only two embryonic cuticles in addition to that secreted by the serosa, like in *Pontonia capreae* [= *Nematus (Pteronidea) salicis* (L.)] (Hymenoptera Tenthredinidae) (Ivanova-Kasas, 1959). Conversely, in another sawfly *Pteronidea ribesii* [= *Nematus (Pteronidea) ribesii* (Scopoli)] (Hymenoptera Tenthredinidae) only the serosal and one epidermal cuticles, were reported (Shafiq, 1954). The deposition of only two embryonic cuticles, the ectodermal and the epidermal one in addition to the extraembryonic serosal cuticle, occurs also in higher flies, i.e. *Drosophila melanogaster* Meigen (Diptera Drosophilidae) (Poulson, 1950) and *Calliphora erythrocephala* Meigen (Diptera Calliphoridae) (Bordes-Allèaume and Sami, 1986).

The differentiation of the mesenteron, with regard to its derivation from the germ layers, has different interpretations (Schwalm, 1988). Based on our observations, in *D. pini* it appears that the mesenteron is formed from the mesoendoderm, as claimed by Henson (1932) for

Table 1. Mean values (\pm SE) of the ecdysteroid titre expressed in 20E equivalent ng/egg, in the different days of the embryonic development.

days	n.	mean	SE
1	10	0.25	0.021
2	9	0.25	0.016
3	10	0.30	0.014
4	10	0.25	0.028
5	10	0.22	0.008
6	10	0.21	0.022
7	10	0.26	0.011
8	10	0.34	0.029
9	10	0.38	0.042
10	10	0.42	0.023
11	10	0.52	0.036
12	10	0.72	0.126
13	10	0.76	0.091
14	10	0.52	0.027
15	9	0.58	0.060
16	9	0.46	0.038
17	7	0.50	0.063

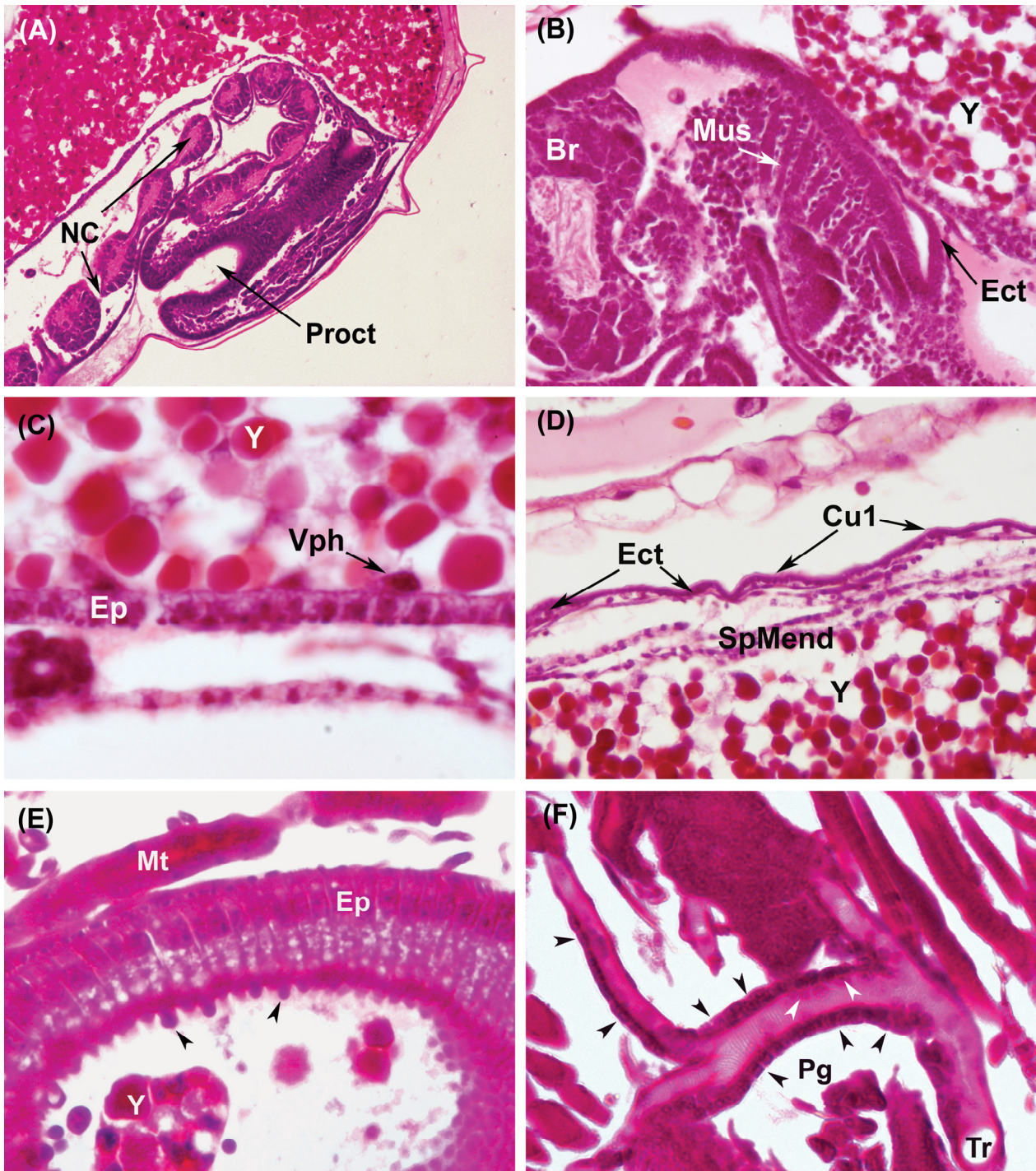


Figure 4. **A:** *seven-day egg*: the posterior end of the embryo has begun to turn on the ventral side; ventral nerve cord (NC) and proctodeum (Proct) (sagittal section). **B:** *eighth-day egg*: the ectoderm (Ect) has begun to grow over the yolk (Y) mass; brain (Br) and cephalic muscles (Mus) (sagittal section). **C:** *ten-day egg*: the mesenteron epithelium (Ep) formed by cuboid cells over which vitellophages (Vph) accumulate Yolk (Y) (sagittal section). **D:** *ten-day egg*: first embryonic cuticle (Cu1) secreted by the ectoderm (Ect); splanchnic mesendoderm (SpMend) and yolk (Y) (sagittal section). **E:** *ten-day egg*: posterior end of the mesenteron with a columnar epithelium (Ep) and secretory activity (arrows); yolk (Y) and malpighian tubules (Mt) (frontal section). **F:** *ten-day egg*: prothoracic gland (Pg) with its cells (arrows) developed on a tracheal trunk (Tr) (sagittal section).

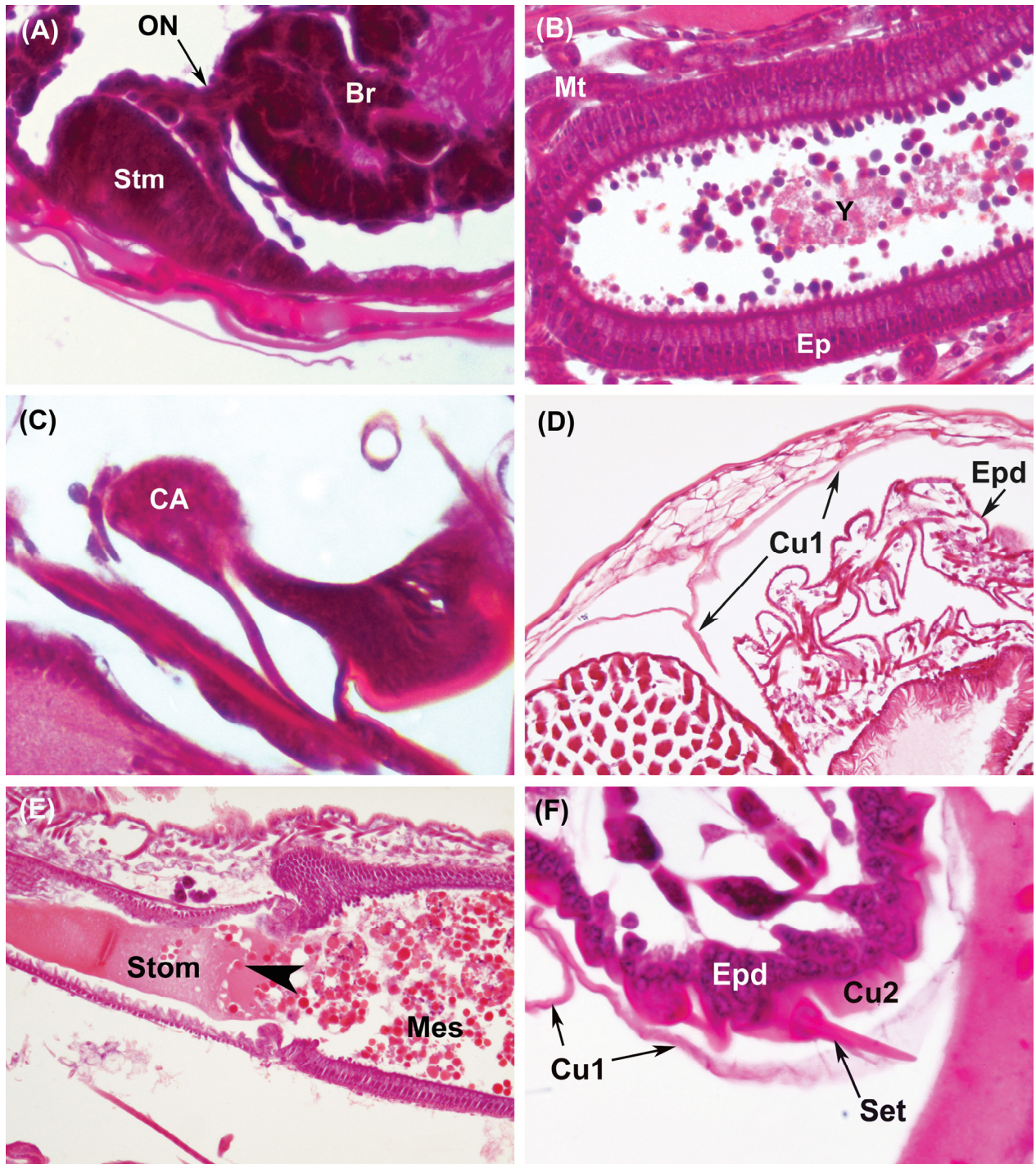


Figure 5. **A:** *ten-day egg*: stemma (**Stm**), ocellar nerve (**ON**) and brain (**Br**) (frontal section). **B:** *fourteen-day egg*: posterior end of the mesenteron with a reduced yolk (**Y**) mass; epithelium (**Ep**) and malpighian tubules (**Mt**) (sagittal section). **C:** *fourteen-day egg*: corpora allata (**CA**) (frontal section). **D:** *fifteen-day egg*: apolysis of the first embryonic cuticle (**Cu1**); epidermis (**Epd**) (sagittal section). **E:** *sixteen-day egg*: the connection (**arrow**) realized between the stomodeum (**Stom**) and the mesenteron (**Mes**) (sagittal section). **F:** *sixteen-day egg*: the last embryonic cuticle (epidermal) (**Cu2**) with setae (**Set**); first embryonic cuticle (**Cu1**) and epidermis (**Epd**) with rounded nuclei (sagittal section).

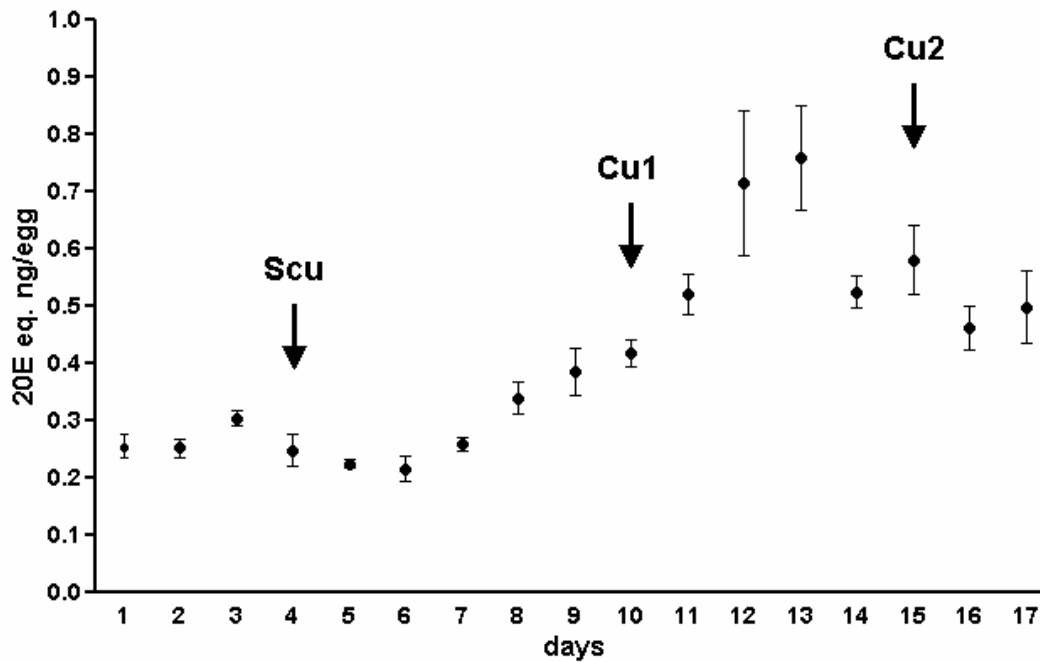


Figure 6. Variations of the ecdysteroid titre, expressed in 20E equivalent ng/egg (mean \pm SE), during the embryogenesis of *D. pini* and the deposition of the serosal (Scu), ectodermal (Cu1) and epidermal (Cu2) cuticles.

Pieris brassicae (L.) (Lepidoptera Pieridae) and Shafiq (1954) for *N. ribesii*. Nevertheless, a contribution of other cells to the construction of the mesenteron epithelium can be suggested because several yolk cells arranged in syncytia were observed over the mesendodermal layer.

The quantitative ecdysteroid variations during the embryogenesis of *D. pini* suggest the need for different amounts of these hormones for the embryo differentiation and growth. A correspondence between specific morphogenetic events and peculiar hormone levels was also shown during the metamorphosis of this insect. For this correspondence Martini *et al.* (2010) accepted the dose-response hypothesis of Sláma (1980), which states that the effects of ecdysteroids have quantitative character and the velocity of their biological response increases with the increase of their concentration. A similar hypothesis could also be proposed for embryonic development.

The analysis performed show that the newly-laid eggs of *D. pini* were provided with ecdysteroids of maternal origin. In fact it is well known that ecdysteroids are secreted by the follicle cells of the ovaries (Glass *et al.*, 1978; Goltzené *et al.*, 1978), converted to conjugates Gande *et al.*, 1979; Dinan and Rees, 1981) and then linked to the yolk proteins (Lagueux *et al.*, 1981). The conjugated ecdysteroids, present in the yolk, are hydrolyzed by phosphatases during the embryonic development (Tawfik *et al.*, 1999). This could partly explain the rise of the free ecdysteroids detected in the eggs of *D. pini* during embryogenesis. Otherwise, a *de novo* synthesis of ecdysteroids by the embryo could also be suggested because, like in the embryo of *L. migratoria* and of *Blaberus craniifer* Burmeister (Blattaria Blaberidae)

(Lagueux *et al.*, 1979; 1981; Bullière *et al.*, 1979), a substantial peak of hormone is evident after the corpora cardiaca (7th day) and the prothoracic glands (10th day) have differentiated.

In *D. pini* the ecdysteroid concentration per egg tripled from 0.25 ng for newly-laid eggs to a maximum of 0.76 ng at the 13th day. The same increase in the hormone titre was observed in embryos of *Schistocerca gregaria* (Forsk.) (Orthoptera Acrididae) in which the 4 ng per newly-laid egg rose to 13 ng per egg at the time of maximum concentration (Scalia *et al.*, 1987). In the confamiliar species, *N. sertifer*, the ecdysteroids in the eggs increased 17-fold from 2.82 ng per newly-laid egg to 48.14 ng per egg at the end of the embryonic development (Martini, 2001). The different ratios of increase of ecdysteroids in the developing embryo of *N. sertifer* and *D. pini* may be related to the different duration of the embryo development in the two diprionids. In fact, under optimal rearing conditions, the embryogenesis of *D. pini* occurs in 17 days, whereas that of *N. sertifer* requires 90 days at least (Martini, 2001; Martini *et al.*, 2006).

Acknowledgements

This research was supported by the *Alma Mater Studiorum* Università di Bologna (RFO ex 60% and PhD program in Agricultural Entomology, which provided a 3-year fellowship for Camilla Chieco). The histological samples were prepared in the Center for Applied Biomedical Research (CRBA), S. Orsola-Malpighi University Hospital of Bologna, and our special thanks are due to Milena Pariali for the excellent technical support.

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Authors' addresses: Antonio MARTINI (corresponding author: antonio.martini@unibo.it), Maria Luisa DINDO, Piero BARONIO, DiSTA - Entomologia, *Alma Mater Studiorum Università di Bologna*, viale G. Fanin, 42, 40127 Bologna, Italy; Camilla CHIECO, Consiglio Nazionale delle Ricerche, Istituto di Biometeorologia, sezione di Bologna, via P. Gobetti 101, 40129 Bologna, Italy.

Received February 17, 2011. Accepted September 21, 2011.