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Temporal correlation between ecdysteroids and mitotic activity in larval tissues of *Clitumnus extradentatus* (Phasmoptera: Phasmatidae: Lonchodinae)

MADELEINE SAUVE-GUILLAUME[1], PATRICK PORCHERON[2] and GINETTE LAUGE[1]

[1] Laboratoire de Reproduction, Développement de l'Insecte, Université de Paris-Sud, 91405 Orsay cedex, France

[2] Laboratoire de Physiologie Cellulaire des Invertébrés, Université Pierre et Marie Curie, 12, rue Cuvier, 75005 Paris, France

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Abstract. A comparative study of mitotic activities and haemolymph ecdysteroid levels was performed in the phasmid *Clitumnus extradentatus*. Temporal correlation was found between increases in mitotic frequency in mandibular and general epidermis, and variations of ecdysteroid levels in the haemolymph of the insects. Whereas, mitotic waves occurring in the fat body cells or in the basal cells of the midgut did not appear to be strictly correlated with these hormonal variations. During the fourth larval instar of this phasma, an accurate study of mitotic figures, monitored from histological sections, indicated a time-lag in their stimulation according to the studied area, with a peak on day 2 in the mandible tips, on day 5 in the mandible bases and on day 7 in the head capsule, thorax and abdomen epidermis: namely a five-day delay with respect to the 12 days of the fourth instar. Simultaneously, the evolution of ecdysteroid levels in the haemolymph showed three increases of different importance. Each hormonal increase occurred 24 h before the triggering of each increase in the mitotic activity, whereas a fourth and very high peak, occurring on day 8, corresponded to the sudden fall in the number of epidermal mitoses.

INTRODUCTION

The post-embryonic development of insects is periodically punctuated by moultings. The new cuticle produced is the result of the activity of individual differentiated epidermal cells. Epidermal growth of the animal during the moulting cycle occurs through cell hypertrophy and hyperplasia.

The early triggering of epidermal mitoses in specific abdominal areas was demonstrated in *Rhodnius prolixus* (Hemiptera: Reduviidae), and *Tenebrio molitor* (Coleoptera: Tenebrionidae) (Wigglesworth, 1940, 1948). Subsequently, the need for a moulting hormone as a trigger of epidermal cell activation was shown (Wigglesworth, 1963). In *Calpodes ethlius* (Lepidoptera: Hesperiidae) (Locke, 1970) the presence of DNA synthesis peaks before occurrence of mitotic clones in the epidermis or polyploidization of the fat body cells was shown. Later, through hormonal titrations (Dean et al., 1980), a correlation was shown between an increase of haemolymph ecdysteroid levels, synthesis of DNA and mitoses in the epidermis of *C. ethlius*. Similar results were given for *T. molitor* epidermis (Delachambre et al., 1980). Several papers led to the same conclusion, namely, that ecdysteroid hormones control cell cycles. For example, in vitro cultures confirmed the importance of ecdysteroid levels to obtain mitotic induction or cell polyploidization in the epidermis of *Manduca sexta* (Lepidoptera: Sphingidae) (Kato & Riddiford, 1987); moreover, the study indicated that the in vitro level required was similar to that found in vivo. More recently,

Kawasaki (1995) claimed that medium containing 0.02 µg/ml 20-hydroxyecdysone increased the number of mitoses in wing disc cells of *Bombyx mori* (Lepidoptera: Bombycidae); these discs developed the same in vitro as in vivo.

Taking into account the above results and a previous study on post-embryonic development of *Clitumnus extradentatus* (Phasmoptera: Phasmatidae), a possible relationship between the evolution of circulating ecdysteroids and changes in mitotic activity in various tissues during the moulting cycle of that phasmid was investigated.

MATERIAL AND METHODS

Breeding

Individuals of *Clitumnus extradentatus* were bred at 25°C and fed with bramble *Rubus fruticosus* L. leaves. Each animal was individually followed from hatching, with the aid of nail varnish markings. Females with a 12 day duration for the third instar and a weight of 50 mg on the first day of the fourth instar were the only insects selected for experimentation. Under these conditions, the fourth instar lasted 12 days, and we ended with sets of animals accurately synchronized.

Determination of mitotic index

In order to appreciate mitotic activity, the animals were injected with colchicin 24 h before the killing of each animal. A dose of 0.05 mg of colchicin for one gram of phasma was injected in Ringer solution modified according to Ephrussi & Beadle (1936). At this concentration, all mitoses were blocked and the mitotic index in treated animals was three times higher than in controls without any increase in lethality.

Once anaesthetized by diethyl-ether, animals were dissected and fixed with A.F.A. (alcohol 95°/formaldehyde/acetic acid, 6/3/1, v/v/v) for 24 h. They were treated by salycilate-celloidin (5%) for a week in order to soften the cuticle before embedding into cyto-paraffin. Sections (7.5 µm) were submitted to Feulgen nucleal reaction and stained with light green.

On each day of the instar, mitosis numbering was performed in different tissues (general epidermis of the body and mandibular epidermis, fat body cells and midgut basal cells) belonging to the same individual. Mitotic percentages were calculated from sets of 500 or 1,000 cells in each observed area. The mitotic index was expressed in percent of metaphasic plates compared to the total number of counted cells.

Titration of ecdysteroids in the haemolymph

Haemolymph samples (1 µl) were taken from animals without any colchicin treatment, using a microcapillary tube. Each sample was dropped into 1 ml of pure methanol for analysis and stored in a deep-freezer at –20°C before titration.

Enzymo-immunological assays (E.I.A.) were performed according to Porcheron et al. (1989). Alcoholic supernatants were pooled, evaporated to dryness and then diluted in phosphate buffer (0.1 M, pH 7.4, 0.4 M NaCl, 1 mM EDTA, 0.1% bovine serum albumin, 0.01% sodium azide). Assays were performed on a microtitre plate in a total volume of 150 µl per lane composed of 50 µl of ecdysteroid standard or biological extract, 50 µl of diluted enzymatic tracer and 50 µl of an appropriate dilution of specific anti-20-hydroxyecdysone antiserum raised in rabbit. In this assay, 20-hydroxyecdysone and ecdysone cross-reacted equally with the antiserum but other ecdysteroids were less reactive (Porcheron et al., 1989). 20-hydroxyecdysone was used as a standard. Therefore, results were expressed in picograms of ecdysteroids per microlitre of haemolymph (pg/µl). Under our conditions, the detection limit of the assay was 3.5 pg/µl.

As far as is known, the precise ecdysteroid composition of phasmid haemolymph has not been established.

RESULTS

This study deals with the comparison of the mitotic activity between different epidermal regions, fat body and midgut cells. This activity was then correlated to changes observed in haemolymph ecdysteroid titres.

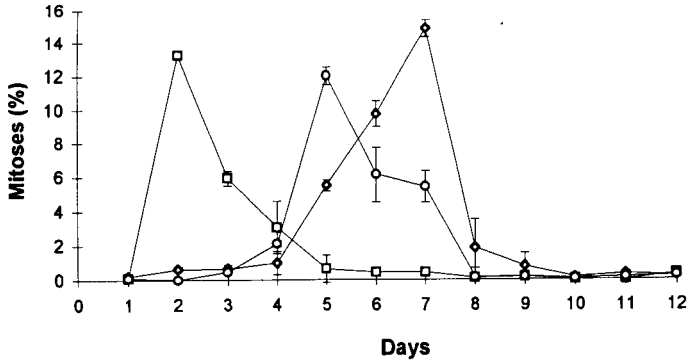

Fig. 1. Evolution of the epidermis mitotic index during the fourth instar of *C. extradentatus*: in mandible tips (□); mandible basis (○); in head capsule, thorax, abdomen (◇) (means ± standard error).

Differential mitotic activity

GENERAL EPIDERMIS. The epidermal cells of the head capsule, as well as those of the thorax and abdomen, started dividing on day 4 of the instar. Mitotic index increased gradually and reached its maximum on day 7 (15%). Then, it decreased slowly to 2% on day 8 (Fig. 1).

No antero-posterior gradient has been revealed in the mitoses of general epidermis, i.e., head capsule, thorax and abdomen epidermis.

MANDIBULAR EPIDERMIS. The mandibles are strongly sclerotinized, with tips coming from a pseudo-stratified epidermis as shown by an electron microscopy study (Sauve-Guillaume, 1995, and Sauve, pers. obs). In the bases, the epidermis comprises one layer of cells.

On day 1 of the instar, the first mitoses appeared in the mandible tips. A maximal mitotic activity (13%) was observed in that area on day 2. Then, a slow decrease in the

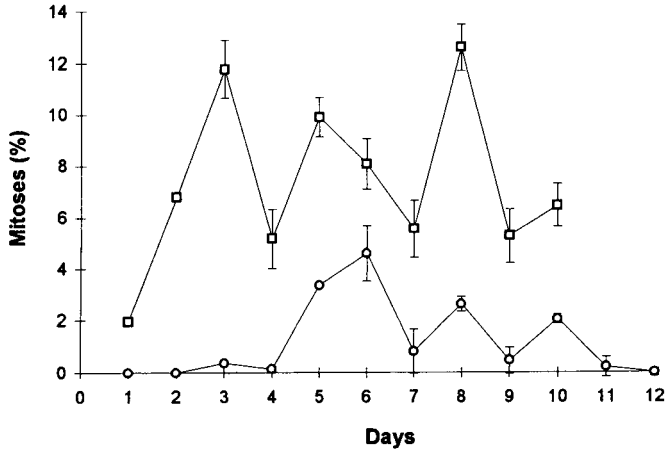

Fig. 2. Evolution of the mitotic index during the fourth instar of *C. extradentatus*: in the fat body cells (○); in midgut basal stem cells (□) (means ± standard error).

mitotic index occurred until day 5; afterwards, the mitotic index stayed close to 0% until the end of the instar.

The mitotic activity in the mandible basis reached its maximum on day 5 (12%) and, therefore, showed a three day delay compared to the maximum observed in the mandible tips, but still occurred 2 days before the maximal mitotic activity observed in the general epidermis (Fig. 1).

FAT BODY CELLS. Mitotic activity in the fat body was lower than that observed in the epidermis. The mitotic activity of the fat body cells showed 3 peaks occurring on days 6, 8 and 10 (Fig. 2). Many giant cells were observed in the fat body, which are 2n, 4n or 8n cells (Bergerard, 1958).

MIDGUT BASAL CELLS. The functional cells of the midgut were terminally differentiated and no longer divided, as in other insects such as *Gryllus bimaculatus* (Orthoptera: Gryllidae) (Romer & Eisenbeis, 1983). However, basal stem cells still divided. The basal cell mitotic index never fell below 5% during the instar. However, three increases of mitotic activity were observed on days 3, 5 and 8 (Fig. 2).

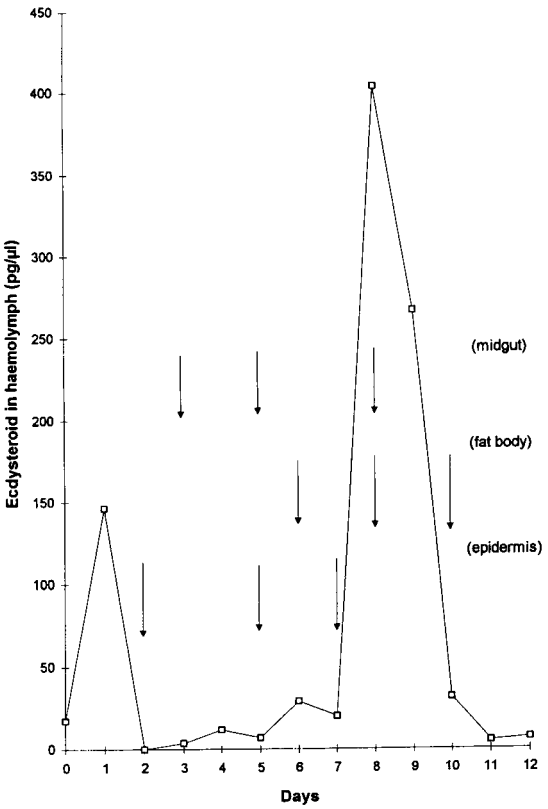

Fig. 3. Typical curve of evolution of haemolymphatic ecdysteroid levels during the fourth instar of *C. extradentatus*. Arrows indicate positions of mitotic peaks, in various observed tissues: epidermis, fat body and midgut basal stem cells.

Evolution of circulating ecdysteroid levels

Fig. 3 shows a typical curve of the evolution of ecdysteroid levels in the haemolymph during the fourth instar of *C. extradentatus*. Different experiments were performed, but they were not numerous enough to get an accurate statistical analysis of the results. However, even the smallest peak, on day 4 (11 pg/µl), is three times above the detection limit of the method used (3.5 pg/µl). So it may be concluded that even the minor peaks are significant.

Two obvious peaks, of different values, occurred during this instar. The first occurred on day 1 (146 pg/µl), the second on day 8, when hormonal concentration reached a maximum value (400 pg/µl). This second peak was preceeded by two slight increases on day 4 (11 pg/µl) and on day 6 (29 pg/µl).

DISCUSSION

Our results show that mitotic patterns are different in various tissues studied during postembryonic development of *C. extradentatus.*

In the epidermis, the mitotic activity was restricted to an unique period that presents a huge peak of mitoses. While all the mitoses occurred simultaneously in the general epidermis of the whole body, a distinct situation was observed in the mandibular territories with a gap of 3 days between tips and basis of these appendages. Thus, a five day time-lag separates the peak in mandibular tips from the peak in general epidermis. Such a spatio-temporal gap has seldom been indicated. In *Aeschna cyanea* (Odonata: Aeschnidae) (Schaller, 1960) the early triggering of mitoses in pterotheca, in comparison with the mitotic peak in the general epidermis was observed. When describing mitoses in the abdominal epidermis of *Rhodnius,* Wigglesworth (1940) explained the early mitotic triggering in those areas by mechanical stretching of tissues after a blood meal. Such an explanation is not valid for the strongly sclerotinized mandibles in *Clitumnus.* It is noteworthy that the anticipated mitotic peak in mandibular epidermis led to an anticipated apolysis (Sauve-Guillaume, 1995), certainly affecting the larva phasma feeding during the greater part of the instar, as noted from weight daily controled on the animals, showing fluctuations every two or three days (Sauve-Guillaume, pers. obs.).

In contrast in the basal stem cells of the midgut and in the fat body cells, several peaks of mitotic activity, that may be considered to be mitotic waves, were observed within the same tissue. Mitotic activity in midgut was restricted to basal stem cells in which a basal activity was found during the whole instar, allowing a continuous renewing of functional cells. However, the mitotic index showed oscillations of upper values that might be connected to periods of greater need for replacement of functional cells injured by the alimentary ingesta following periodical feeding, as mentioned above. In the fat body of *Rhodnius*, Wigglesworth (1967) noticed the presence of a 7 day mitotic activity, 3 days after the blood meal. Following this, it is assumed that the oscillations observed in the mitotic activity of fat body cells of *Clitumnus* may be considered as being related to the supply of nutrients in the larvae. That situation differs from that observed in *B. mori,* in which the fat body displays a mitotic peak, close to ecdysis and super-added to basal activity during the whole instar (Kato & Oba, 1977).

Ecdysteroids are well known for their role in promoting and/or in controlling insect development, the success of which depends on the presence or absence of the hormone, at critical times and at critical levels. Several studies showed ecdysteroid peaks of different values during one larval or during the pupal stage, as in L5 of *Locusta migratoria* (Orthoptera: Acrididae) (Cassier et al., 1980), in L3 and pupa of *Sarcophaga argyrostoma* (Diptera: Calliphoridae) (Richard et al., 1987), or in pupa of *Ceratitis capitata* (Diptera: Trypetidae) (Vafopoulou et al., 1993). Different authors have tried to relate these peaks to biological (DNA synthesis, mitoses, polyploidy, cuticular material synthesis) or behavioural events, looking at parallelism, even establishing a causal relationship between them. Further, studying DNA synthesis, mitoses and polyploidy of the cells, most of the authors concluded that low titres stimulate DNA synthesis and that high titres inhibit [(Wielgus et al., 1979 and Kato & Riddiford, 1987 in *M. sexta*, Delachambre et al., 1980 in *T. molitor,* Blais & Lafont, 1980 in *Pieris brassicae* (Lepidoptera: Pieridae), Kawasaki et al., 1986

and Kawasaki, 1995 in *B. mori*)]. In that case, the cells most of the time are blocked in G2 phase (Besson-Lavoignet & Delachambre, 1981, for example).

Strict synchronisation of animals and their slow development permitted the identification of several changes in ecdysteroid levels during the fourth instar of *C. extradentatus*, as did Vafopoulou et al. (1993) with *C. capitata.* The comparison between changes in mitotic activity of epidermal cells and changes in ecdysteroid levels in *C. extradentatus* displays a clear parallelism between the latter (on days 1, 4, 6, 8 of the instar) and the mitotic rises observed in the different epidermic regions (on days 2, 5, 7 of the instar). Each mitotic peak occurred 24 h after each of the first three ecdysteroid increases. It should be noted that Truman et al. (1974) claimed that different parts of the epidermis in *M. sexta* vary in the length of time exposure to ecdysteroids, required for the summation of the effects.

As other papers (see above), the present results for *C. extradentatus* indicate that the high peak of ecdysteroids on day 8 correlates with disappearance of mitoses in general epidermis.

Concerning the fat body cells and the midgut basal cells, the present findings indicate that there is no evident correlation between the ecdysteroid level and the mitotic activity. This interpretation is in contrast with Dean et al.'s (1980) results, which assumed that the events in the fat body cells of *C. ethlius* are correlated with ecdysteroid peaks. Therefore, more data are needed to clarify further this situation.

As other authors' results which did not demonstrate a causal relation but rather a parallelism of two events (e.g., Romer & Eisenbeis, 1983, in *G. bimaculatus*, or Vafopoulou et al., 1993, in *C. capitata*), our present data show only a temporal correlation between mitotic activity in the epidermis and ecdysteroid concentrations in the haemolymph of *C. extradentatus*.

Several questions have to be answered to conclude further: in particular, demonstration of a causal relationship between ecdysteroid peaks and mitoses, knowledge of hormonal metabolism and of relations between the brain and the prothoracic gland. Different techniques may be used: (i) in vitro culture of integument and fat body, with different exposures and hormonal concentrations [e.g., Kato & Riddiford in *M. sexta* (1987); Kawasaki in *B. mori* (1995)] and/or (ii) in vivo, through prothoracic gland ablation or, alternatively, hormonal injections.

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REFERENCES

Bergerard J. 1958: La polyploïdie somatique chez les animaux. *L'Année Biol.* **34**: 119–132.

Besson-Lavoignet M.T. & Delachambre J. 1981: The epidermal cell cycle during the metamorphosis of Tenebrio molitor L. (Insecta, Coleoptera). *Dev. Biol.* **83**: 255–265.

Blais C. & Lafont R. 1980: In vitro differentiation of Pieris brassicae imaginal wing discs: effects and metabolism of ecdysone and ecdysterone. *Wilhelm Roux Arch.* **188**: 27–36.

Cassier P., Porcheron P., Papillon M. & Lensky Y. 1980: Contribution à l'étude des protéines cuticulaires du criquet migrateur, Locusta migratoria migratorioïdes (R. et F.), données quantitatives. *Ann. Sci. Nat. (Zool., 14-ème Sér.)* **2**: 51–65.

DEAN R.L., BOLLENBACHER W.E., LOCKE M., SMITH S.L. & GILBERT L.I. 1980: Haemolymph ecdysteroid levels, and cellular events in the intermoult/moult sequence of Calpodes ethlius. *J. Insect Physiol.* **26**: 267–280.

DELACHAMBRE J., BESSON M.T., CONNAT J.L. & DELBECQUE J.P. 1980: Ecdysteroid titres and integumental events during the metamorphosis of Tenebrio molitor. In Hoffman J.A. (ed.): *Progress in Ecdysone Research.* Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 211–234.

EPHRUSSI B. & BEADLE G.W. 1936: A technique of transplantation for Drosophila. *Am. Nat.* **70**: 218–225.

KATO Y. & OBA T. 1977: Temporal pattern of changes in mitotic frequency in the epidermis and other larval tissues of Bombyx mori. *J. Insect Physiol.* **23**: 1095–1098.

KATO Y. & RIDDIFORD L.M. 1987: The role of 20-hydroxyecdysone in stimulating epidermal mitoses during the larval-pupal transformation of the tobacco hornworm, Manduca sexta. *Development* **100**: 227–236.

KAWASAKI H. 1995: Ecdysteroid concentration inducing cell proliferation brings about the imaginal differentiation in the wing disc of Bombyx mori in vitro. *Dev. Growth Differ.* **37**: 575–580.

KAWASAKI H., KIGUCHI K., AGUI N. & IWASHITA Y. 1986: Ecdysteroid titer and wing development during the pupal-adult tranformation of Bombyx mori. *Zool. Sci.* **3**: 301–308.

LOCKE M. 1970: The molt/intermolt cycle in the epidermis and other tissues of an insect Calpodes ethlius (Lepidoptera, Hesperiidae). *Tissue Cell* **2**: 197–223.

PORCHERON P., MORINIÈRE M., GRASSI J. & PRADELLES P. 1989: Development of an enzyme immunoassay for ecdysteroids using acetylcholinesterase as label. *Insect Biochem.* **19**: 117–122.

RICHARD D.S., WARREN J.T., SAUNDERS D.S. & GILBERT L.I. 1987: Haemolymph ecdysteroid titres in diapause- and non-diapause-destined larvae and pupae of Sarcophaga argyrostoma. *J. Insect Physiol.* **33**: 115–122.

ROMER F. & EISENBEIS I. 1983: DNA content and synthesis in several tissues and variation of moulting hormone-level in Gryllus bimaculatus DEG. (Ensifera, Insecta). *Z. Naturforsch. (C)* **38**: 112–125.

SAUVE-GUILLAUME M. 1995: *Crises de mitoses au cours de la croissance larvaire d'un insecte Phasmide. Originalité des territoires mandibulaires.* Thesis, Université de Paris-Sud, Paris, 130 pp.

SCHALLER F. 1960: Etude du développement post-embryonnaire d'Aeschna cyanea Mull. *Ann. Sci. Nat. (Zool., 12-ème Sér.)* **11**: 751–868.

TRUMAN J.W., RIDDIFORD L.M. & SAFRANEK L. 1974: Temporal patterns of response to ecdysone and juvenile hormone in the epidermis of the tobacco hornworm, Manduca sexta. *Dev. Biol.* **39**: 247–262.

VAFOPOULOU X., STEEL C.G.H., ALEPOROU-MARINOU V. & PATARYAS T. 1993: Haemolymph ecdysteroid titres at metamorphosis in the fruit fly Ceratitis capitata: multiple peaks not apparent in whole body extracts. *Physiol. Entomol.* **18**: 87–92.

WIELGUS J.J., BOLLENBACHER W.E. & GILBERT L.I. 1979: Correlations between epidermal DNA synthesis and haemolymph ecdysteroid titre during the last larval instar of the tobacco hornworm, Manduca sexta . *J. Insect Physiol.* **25**: 9–16.

WIGGLESWORTH V.B. 1940: The determination of characters at metamorphosis in Rhodnius prolixus (Hemiptera). *J. Exp. Biol.* **17**: 201–222.

WIGGLESWORTH V.B. 1948: The structure and deposition of the cuticle in the adult of Tenebrio molitor L. (Coleoptera). *Quart. J. Micr. Sci.* **89**: 197–217.

WIGGLESWORTH V.B. 1963: The action of moulting hormone and juvenile hormone at the cellular level in Rhodnius prolixus. *J. Exp. Biol.* **40**: 231–245.

WIGGLESWORTH V.B. 1967: Polyploidy and nuclear fusion in the fat body of Rhodnius (Hemiptera). *J. Cell Sci.* **2**: 603–616.

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