Belg. J. Zool. — Volume 121 (1991) — issue 1 — pages 27-37 — Brussels 1991

(Manuscript received on 22 March 1990)

STRUCTURE AND FUNCTION OF THE EXOCRINE GLANDS OF THE GENITALIA OF FEMALES OF THE TWO-SPOT LADYBIRD, *ADALIA BIPUNCTATA* (LINNAEUS, 1758) (COLEOPTERA : COCCINELLIDAE)

by

JEAN-LOUIS HEMPTINNE (*), MICHÈLE LECLERCQ-SMEKENS (**) and JACQUELINE NAISSE (†)

Université Libre de Bruxelles, Dept. de Biologie Animale, CP 160, avenue Roosevelt 50, B-1050 Bruxelles, Belgique

(*) *Adresse actuelle :* Zoologie Générale et Appliquée, Faculté des Sciences Agronomiques, B-5030 Gembloux (Belgique)

(**) Facultés Universitaires Notre-Dame de la Paix, Dépt. Histologie et Embryologie, rue de Bruxelles 65, B-5000 Namur (Belgique)

ABSTRACT

In females of *Adalia bipunctata* (LINNAEUS, 1758), there are exocrine glands associated with the coxites that form part of the genitalia. The coxites are extended anteriorly in the form of a gutter with the concavity orientated towards the vagina. The edges are continuous with a shiny membrane that forms a coxal reservoir. The concave side of this gutter-like extension is lined by a layer of glandular cells. Their ultrastructure is typical of the type 1 insect epidermal cells of NOIROT and QUENNEDEY (1974). The coxal cells undergo a three stage activity cycle that is synchronised with the ovarial activity and correlated with ladybird age. The huge development of the smooth endoplasmic reticulum in the third stage of the activity cycle indicates that terpenoid substances are produced. On the basis of behavioural observations we suggest that these exocrine glands have a role in the sexual behaviour of *A. bipunctata* although we cannot exclude a role in the chemical protection of eggs.

Key-words : Adalia bipunctata, exocrine glands, ultrastructure.

INTRODUCTION

In the course of a histological study of the genital tube in the female *Adalia bipunctata* (LINNAEUS, 1758) to determine the effect of climatic and trophic conditions on reproductive activity, our attention was attracted by a shiny, kidney-shaped organ on either side of the vagina. Preliminary observations indicated that these organs are exocrine glands associated with the genitalia (HEMPTINNE, 1989).

In this paper we describe the morphology of the genitalia, the ultrastructure of the exocrine glands and discuss their role in ladybird reproduction.

This is the first report of exocrine glands in Coccinellidae ; a family for which no pheromone has been reported (BLUM, 1985 ; HABORNE, 1988). A few papers suggest that chemical mediators could play a role in the sexual behaviour of these beetles. RICHARDS (1980) recorded that adult males of *Leptothea galbula* (MULSANT, 1850) guard female pupae and wait for the females to emerge. This guarding behaviour is thought to be linked with the production of a sex pheromone by both the pupal and teneral adult stages of the females. OBATA (1987) suggested that in *Harmonia axyridis* PALLAS, 1773, mating is triggered by chemicals secreted by the females.

MATERIALS AND METHODS

Experimental insects

Observations were made in the laboratory on adults of *A. bipunctata* fed a mixture of instars of the pea aphid (*Acyrthosiphon pisum* HARRIS, 1776). The beetles were housed in an environmental chamber at 21 +/− 1°C and a 16L:8D photoperiod. Fourth instar larvae were isolated in Petri dishes and their sex when teneral adults was determined by checking the shape of their last abdominal segment. Beetles were mated when 24 h old.

HEMPTINNE (1989) has established that there are three main phases of reproductive activity in this ladybird and studied the exocrine cells associated with the genitalia in individuals in each of these phases. In the first phase young adults (age : 1-4 days) did not display any sexual activity ; in the second phase adults (age : 5-10 days) began to copulate and in the third phase adult females (age : 11-21 days) began egg laying.

Light microscopy

General staining method

The abdomens of the female beetles were cut off, fixed in Bouin-Hollande solution and embedded in paraffin wax. After embedding, the specimens were softened in Mollifex® (Gurr) for 24 h and serial sections were cut at a thickness of 7 µm. These sections were successively stained with Heidenhain's iron hematoxylin, phloxine and light green. This technique was used to determine the relationship between the activity of the exocrine cells and the reproductive activity of the females.

Specific staining methods

Lipids : the abdomens of female beetles were cut off, dipped in liquid nitrogen and sectioned in a cold-chamber cryostat. The sections were stained with Sudan

black B. In addition, the genitalia of some females were dissected, pared of fat and directly treated with the same stain.

Mucopolysaccharides : PAS, Alcian blue (pH 1 and pH 2,5) and 7 biotinylated lectins, provided in an ABC kit by Vector Lab. (Burlingame, CA, USA), were applied to serial sections prepared as above. The staining technique based on the glucidic specificities of the lectins involved three steps : first, the sections are incubated with biotinylated lectins that bind to their target glucoside ; secondly, an avidin-biotin-peroxidase complex reacts with the biotin attached to the lectin and thirdly, the peroxydase radicals appear as brown dots after treatment with a mixture of 3-3'-diaminobenzidine (3-3'-DAB) and H_2O_2 (SHARON and LIZ, 1989).

Electron microscopy

The beetles were dissected in Ringer solution and their genitalia prepared for transmission microscopy. The specimens were fixed for 24 h at 4°C in a solution of 2.25 % paraformaldehyde and 2 % glutaraldehyde in a cacodylate buffer (0.1 M ; pH 7.5). They were subsequently rinsed in the buffer before being post-fixed for 1 h at 4°C in a solution of 2 % OsO_4 in the same buffer. Dehydration and embedding in EPON 812 preceded staining with uranyl acetate and lead citrate. The grids were examined with a Philips EM 202 microscope.

RESULTS

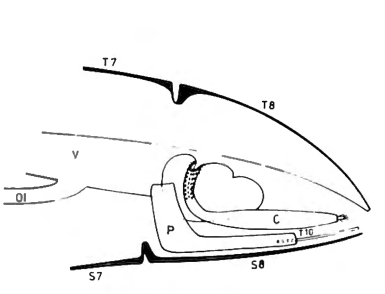
General description

The genitalia of female *A. bipunctata* are made of one coxite and one pleurite on either side of the vagina with the 10^{th} arc-shaped tergite positioned between them. The coxites which are roughly discoidal in shape, extend anteriorly in the form of a gutter, which runs in a plane at right angle to the main part of the coxite. The concavity of this gutter-like extension is orientated towards the vagina and its edges continue as a shiny membrane that forms a coxal reservoir, which encloses the convex side of the gutter. This reservoir has an aperture at the point where it meets the main part of the coxite. The concave side of the gutter is lined by a layer of gland cells. Posteriorly each coxite bears a group of chemosensory setae (PL. IA and IB).

Ultrastructure of the gland cells

Whatever the age of the females, the gland cells show the following characters. The apical surface is invaginated to form a prominent cavity lined with microvilli. This cavity is connected to a spherical hollow located inside the 4 µm thick cuticular intima and filled with entangled filaments. These two cavities are joined by a thin neck. The cells have an approximately ellipsoidal nucleus with

euchromatine regularly dispersed in the nucleoplasm. Their lateral membranes are thrown into a series of folds and adjacent cells are attached to each other by junctional complexes (apical septate desmosomes and zona occludens) (Pl. IC). The microvilli, each about 100 nm in diameter, contain microfibrils around a tubular structure (Pl. IIA). The electron dense entangled filaments inside the extracellular reservoir have a diameter of about 16 nm. In the vicinity of the cuticle, they seem



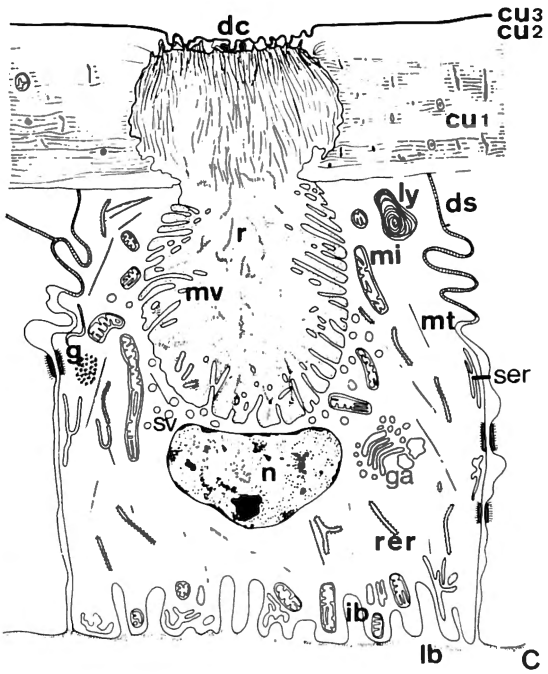


PLATE I

General morphology of the genitalia and associated gland cells in female *Adalia bipunctata*.

A : Diagram of a sagittal section through the abdomen showing the position and the relationship between the different parts of the genitalia.

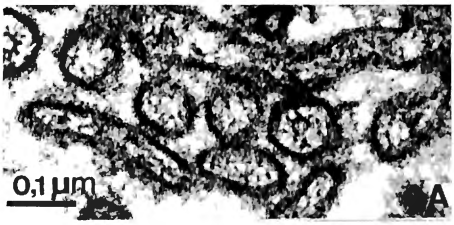
B : In toto preparation of the genitalia with the coxal reservoirs (cr) stained with Sudan black.

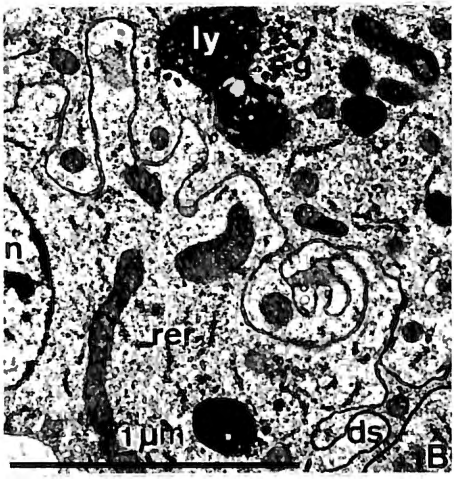
C : Diagrammatic representation of a typical gland cell associated with the genitalia.

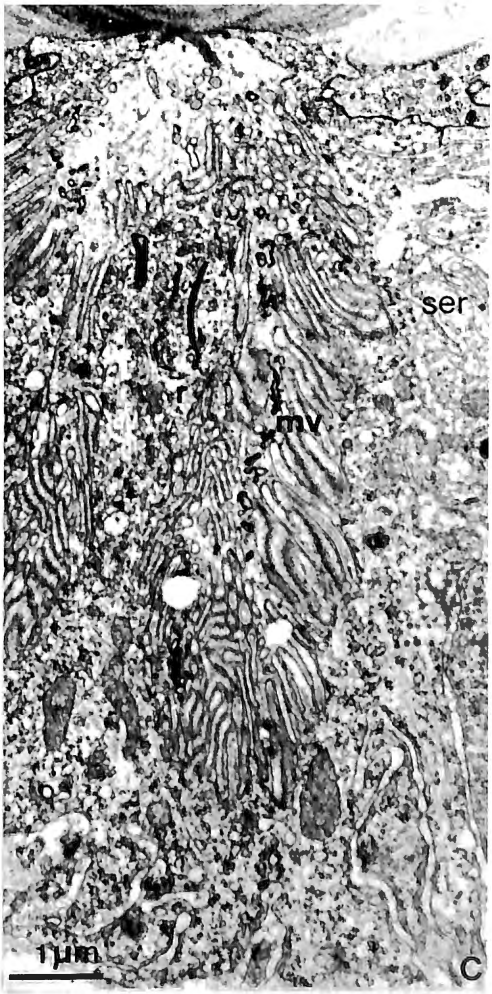
Legend of the symbols

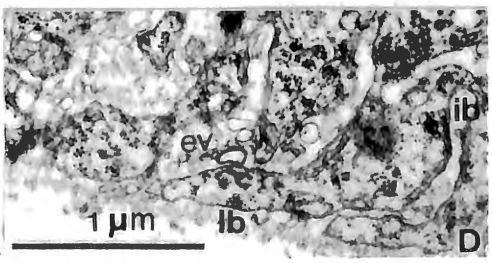
c : coxite ; cr : coxal reservoir ; cu 1, cu 2 and cu 3 : respectively endo-, exo- and epicuticle ; dc : cribellarium ; ds : septate desmosome ; ev : endocytosis vesicle ; g : glycogen ; ga : Golgi apparatus ; ib : infolding of the basal membrane ; lb : basement membrane ; ly : lysosome ; mi : mitochondrion ; mv : microvillus ; mt : microtubule ; n : nucleus ; ol : lateral oviduct ; p : pleurite ; r : extracellular reservoir ; rer : granular endoplasmic reticulum ; ser : smooth endoplasmic reticulum ; sv : secretory vesicle ; s7 and s8 : 7th and 8th sternite ; t7, t8 and t10 : respectively 7th, 8th and 10th tergite ; v : vagina.

PLATE II









more ordered, converging towards the extremities of little tubular infoldings, each about 130 nm in diameter. There are at least 10 of these infoldings forming a permeable area or cribellarium at the outer cuticular surface (Pl. IC and Pl. IIE).

At the base of the epithelium, there are numerous haemocytes, probably spherulocytes in a very active condition with well developed granular endoplasmic reticulum (RER), large Golgi apparatus and many secretory vesicles that are released into the haemolymph inside the coxite gutter.

The general morphology of the secretory cells of the coxites changes with the age of ladybirds.

Stage 1

The cytoplasm has a low electron density and contains the following organelles : elongated mitochondria with a dense matrix, mainly situated close to the extracellular reservoir as well as inside the spaces between the infoldings of the basal membrane. There are many free ribosomes. The RER is wide spread in the cytoplasm whereas the poorly developed smooth endoplasmic reticulum (SER) is located in the vicinity of the basal and lateral membranes. The Golgi apparatus releases some small vesicles containing a flaky electron dense substance. There are also lysosomes and multivesicular bodies, numerous microtubules and granules of stored glycogen (Pl. IIB).

The extracellular reservoir is regularly ovoid. The presence of mitochondria between the infoldings of the basal membrane, evidence of basal endocytosis, and the vesicles from the Golgi apparatus all indicate that the cells are becoming active. In light microscopy, the cells display a basophilic cytoplasm and a small transparent ovoid reservoir.

Stage 2

The cytoplasm has a higher electron density than in the previous stage. The infoldings of the basal membrane are more prominent (Pl. IIC) and the external

---

PLATE II

Electron micrographs of gland cells at different stages of maturation.

A : Detail of a cross section of the microvilli lining the extracellular reservoir.
B : Cytoplasm of a gland cell at stage 1.
C : Cytoplasm of a gland cell at stage 2 ; the microvilli (mv) are packed in the extracellular reservoir.
D : Detail of the basal membrane of a gland cell at stage 2. The infoldings of the membrane (ib) are prominent and the external space between them contain vesicles of endocytosis (ev).
E : Cytoplasm of a gland cell at stage 3.

Legends of the symbols as in Plate I.

spaces between them contain small vesicles (Pl. IID) that apparently resulted from the fragmentation of bigger ones produced by the spherulocytes. The intensity of the basal endocytosis is greater and the SER is more extensive. The small vesicles released by the Golgi apparatus (see stage 1) are more abundant and aggregate at the base of the microvilli. These are longer than in the first stage and are tightly packed in the extracellular reservoir into which they extend (Pl. IIC). Under a light microscope, the cells have a basophilic cytoplasm but the extracellular reservoirs are no longer visible. The cellular secretion begins to reach the coxal reservoir through the cribellarium.

Stage 3

During this stage, the cells reach their maximum electron density and the extracellular reservoirs again become visible because they are dilated by the secretion (Pl. IIE). As a consequence of the increase in volume, the microvilli become separated. The basal endocytosis remains intense but more striking is the extensive SER and large lysosomes filled with lamellar structures. Under a light microscope, the extracellular reservoirs are visible and full of secretion but the cytoplasm is no longer basophilic.

The extensive development of both the SER and RER suggests that the coxal cells produce lipid-like substances, proteins or glycoconjugates. The specific dyes used strengthened this hypothesis. The cytoplasm reacted positively to the PAS test and to tests with Alcian blue at pH 1 and 2.5 indicating the presence of neutral and acid mucopolysaccharides. Among the lectins, only Con-A and SBA reacted with the cells. The former, which reveals the presence of α-D-mannose or α-D-glucose, was strictly located near the basal membrane infoldings while the latter which reveals α-D-galactose or N-acetylgalactosamine groups, was associated with the filaments inside the extracellular reservoir. The coxal cells of complete genitalia stained strongly with Sudan black (Pl. IB). Frozen sections always shrank badly probably because we did not find the equivalent of Mollifex® for cuticle softening for cryostat. These sections were unsuitable for pinpointing cells with an affinity for the dye.

Correlation between the morphology of the glands and the reproductive activity of the females

Young females (1 to 4 days old) do not show any sexual activity and their coxal cells are mainly at stage 1. Females from 5 tot 10 days old mainly have stage 2 cells while older ones (11 to 21 days old) are at stage 3 (Tab. 1 ; contingency coefficient $c = 0.63$, $P < 0.001$). HEMPTINNE (1989) also found that ovarial activity also showed three stages. In the first the gonads had differentiated or growing oocytes, the second, oocytes engaged in vitellogenesis and in the third chorionated oocytes in the oviducts. As for the coxal glands, ovary maturation is positively correlated with female age ; the third stage is significantly more frequent in 12-21 days old beetles (Tab. 1 ; contingency coefficient $c = 0.56$, $P < 0.001$). In addition, the fre-

quencies of beetles with immature ovaries and stage 1 coxal cells in the three age groups of ladybirds are identical as are those with mature ovaries and stage 3 coxal cells (Tab. 1, $P > 0.05$). This highlights synchronisation between ovarial and coxal gland activity.

TABLE 1

Frequencies (in %) of the maturation stages (1, 2 and 3) of the ovaries and the coxal glands in females of A. bipunctata 1-4, 5-10 and 11-21 days old

Age group (in days)	Ovaries (%)				Coxal glands (%)			
	n	1	2	3	*n*	1	2	3
1-4	15	66.7	26.6	6.7	15	80.0	13.3	6.7
5-10	43	16.3	32.5	51.2	37	13.6	48.6	37.8
11-21	50	2.0	10.0	88.0	43	4.7	9.3	86.0

Legend : n = number of coccinellid beetles observed ;
maturation stage : see text for description ;
frequencies of ovary and coxal gland stage 1, 2 and 3 do not differ significantly in each age group (two by two proportion comparisons, $P > 0.05$).

DISCUSSION

The morphology of the gland cells associated with the coxites in female *A. bipunctata* is characteristic of the type 1 insect epidermal cell of NOIROT and QUENNEDEY (1974). They release their secretion into an extracellular reservoir which does not communicate directly with the outside. The integument above this reservoir is, however, modified into a thin permeable area (the cribellarium), which facilitates the export of the cells' products into the coxal reservoir. This latter encloses the convex side of the coxal gutter.

Three stages of cell activity were recognised. In the first stage, the cells displayed a low level of activity. During the second stage, the microvilli became so prominent that they completely invaded the extracellular reservoir. This resulted in a further increase in the surface area of the reservoir in order to accommodate the secretion released during the third stage. A similar concomitant development of microvilli and reservoir has also been described in *Periplaneta americana* (LINNAEUS, 1758) (GUPTA and SMITH, 1969) and *Schistocerca paranensis* (BURMEISTER, 1861) (HAWKES *et al.*, 1987). The most important organelle in the gland cells in the third stage is the SER. This feature generally indicates the production of terpenic or steroidic substance as has been observed in the *corpora allata* (SMITH, 1968), the Leydig cells, the hepatocytes and the yellow bodies (ALBERTS *et al.*, 1983 ; PORTER and BONNEVILLE, 1969 ; FAWCETT, 1966 ; SCHOENMAKERS *et al.*, 1977 ; THREADGOLD, 1976) as well as oenocytes that secrete cuticular lipids (LOCKE, 1969). Moreover, the SER is also well developed in the defensive cells of *Ocypus olens* MÜLLER, 1764 and

Drusilla canaliculata (FABRICIUS, 1787), where it is involved in the synthesis of hydrocarbons and aldehydes (ARAUJO, 1978). The activity of the coxal cells and of the ovaries is synchronized and correlated with ladybird age.

The morphology of the genitalia is used as a taxonomic character in coccinellid beetles (SASAJI, 1968) but there is no report of exocrine glands associated with the genitalia in any other species than *A. bipunctata.* However we have observed similar structures in *Coccinella septempunctata* LINNAEUS, 1758 and *Propylea quatuordecimpunctata* (LINNAEUS, 1758).

Our observations combined with information on ladybird behaviour suggest two possible roles for the coxal glands : they could be involved, firstly in egg laying, and secondly in mating behaviour.

Coxal glands and egg laying

The eggs of *A. bipunctata* are bright yellow and laid in batches on plants where aphids are thriving (HODEK, 1973). Thus, the eggs are exposed to predators that forage on the leaves as do the ladybirds (PRICE *et al.,* 1980 ; CARTER *et al.,* 1984). In such circumstances the evolution of a chemical protection for the eggs would be favoured. This kind of defence has been widely recorded in the chrysomelid beetles (PASTEELS *et al.,* 1986, 1988) and the seven-spot ladybird (TURSCH *et al.,* 1971 ; PASTEELS *et al.,* 1973) where the eggs are defended by a maternal allomone produced and incorporated into the yolk during oogenesis (PASTEELS *et al.,* 1986). This however does not rule out the possibility of there being a protective coating in some species (HINTON, 1981). Thus the coxal gland secretion in *A. bipunctata* may serve as glue to attach the eggs to plants and/or as a defensive coating. Of these two hypotheses, the second is more likely because terpenoid substances are better suited to a protective function than to an adhesive one (HARBORNE, 1988 ; PASTEELS *et al.,* 1986, 1988). Moreover, the bursa copulatrix epithelium secretes huge quantities of mucopolysaccharides (HEMPTINNE, 1989) which could act as glue.

Coxal glands and mating behaviour

The description of the mating behaviour of *H. axyridis* by OBATA (1987) suggests that males recognize females by mean of a chemical cue. Similarly during the courtship of *A. bipunctata,* the male generally palpates the apex of the abdomen of its mate before attempting copulation. Palpation occurs less frequently when males are offered freshly killed males (3 h in a freezer) or freshly killed females washed in ethanol for 24 h than with freshly killed females. The application of an ethanol extract of the coxal glands to the females previously washed in ethanol restored their attractiveness to males (HEMPTINNE and MABILDE, own unpublished observations). Although this needs to be confirmed, it is put forward as evidence that coxal cells may synthesize a sexual pheromone. In *A. bipunctata,* these cells commence their secretory activity when the females are approximately 5 to 10 days

old (Table 1), at the time when the first copulations are recorded (HEMPTINNE, 1989).

Further research is needed to discriminate between these two hypotheses, i.e. whether the coxal glands are involved in egg protection or in the production of a sex pheromone. Nevertheless, our observations indicate that various chemicals are important in ladybird ecology and behaviour. An accurate knowledge of the nature of these signals and of their role will improve our understanding of ladybird population ecology and, hopefully, have a positive effect on the design of aphid biological control programs.

ACKNOWLEDGMENTS

We thank J. Rhodes, G. Kearn and A.F.G. Dixon (University of East Anglia) for comments and reviewing the manuscript.

REFERENCES

ALBERTS B., D. BRAY, J. LEVIS, M. RAF, K. ROBERTS and J.D. WATSON (1983) — *Molecular biology of the cell.* Garland Publishing, New York, 1146 pp.

ARAUJO, J. (1978) — *Anatomie comparée et ultrastructure des systèmes de défense chimique des Staphylinidae (Insectes, Coléoptères).* PhD Thesis, Université Libre de Bruxelles, 139 pp.

BLUM, M.S. (1985) — Exocrine systems. In : BLUM, M.S. (ed.) *Fundamentals of insect physiology,* pp. 535-579, J. WILEY.

CARTER, M.C., D. SUTHERLAND and A.F.G. DIXON (1984) — Plant structure and the searching efficiency of coccinellid larvae. *Oecologia,* **63** : 394-397.

FAWCETT, D.W. (1966) — *The cell. Its organelles and inclusions. An atlas of fine structure.* W.B. Saunders Company, Philadelphia and London.

GUPTA, B.L. and D.S. SMITH (1969) — Fine structural organization of the spermatheca in the cockroach, *Periplaneta americana. Tissue & Cell,* **1** : 295-324.

HARBORNE, J.B. (1988) — *Introduction to ecological biochemistry.* Academic Press, 356 pp.

HAWKES, F., J. RZEPKA and G. GONDRAND (1987) — The scent gland of the male South American *Schistocera cancellata,* an electron microscopy study. *Tissue & Cell,* **19** : 687-703.

HEMPTINNE, J.-L. (1989) — *Ecophysiologie d'Adalia bipunctata (L.) (Coleoptera Coccinellidae).* PhD Thesis, Université Libre de Bruxelles, 156 pp.

HINTON, H.E. (1981) — *Biology of insect eggs.* Vol. 1, Pergamon Press, Oxford.

HODEK, I. (1973) — *Biology of Coccinellidae.* Academia, Praha and Junk N.V., The Hague, 260 pp.

LOCKE, M. (1969) — The ultrastructure of the oenocytes in the molt/intermolt cycle of an insect. *Tissue & Cell,* **1** : 103-154.

NOIROT, C. and A. QUENNEDEY (1974) — Fine structure of insect epidermal glands. *Annu. Rev. Ent.,* **19** : 61-80.

OBATA, S. (1987) — Mating behaviour and sperm transfer in the ladybird beetle, *Harmonia axyridis* PALLAS (Coleoptera, Coccinellidae). *Appl. Ent. Zool.,* **22** : 434-442.

PASTEELS, J.M., C. DEROE, J.C. BRAEKMAN, D. DALOZE and C. HOOTELE (1973) — Distribution et activités des alcaloïdes défensifs des Coccinellidae. *J. Insect Physiol.*, **19** : 1771-1784.

PASTEELS, J.M., D. DALOZE and M. ROWELL-RAHIER (1986) — Chemical defence in chrysomelid eggs and neonate larvae. *Physiol. Entomol.*, **11** : 29-37.

PASTEELS, J.M., J.C. BRAEKMAN and D. DALOZE (1988) — Chemical defence in the Chrysomelidae. In : JOLIVET, P., E. PETITPIERRE and T.H. HSIAO (eds.) *Biology of Chrysomelidae,* pp. 233-252, Kluwer Academic Publishers, Dordrecht.

PORTER, K.R. & M.A. BONNEVILLE (1969) — *Structure fine des cellules et des tissus.* Ediscience, Paris.

PRICE, P.W., C.E. BOUTON, P. GROSS, B.A. MC PHERON, J.N. THOMPSON and A.E. WEISS 1980) — Interactions among three trophic levels : influence of plants on interactions between insect herbivores and natural enemies. *Annu. Rev. Ecol. Syst.*, **11** : 41-65.

RICHARDS, A.M. (1980) — Sexual selection, guarding and sexual conflict in a species of Coccinellidae (Coleoptera). *J. Aust. ent. Soc.*, **19** : 26.

SASAJI, H. (1968) — Phylogeny of the family *Coccinellidae (Col.). Etizenia,* **35 :** 1-37.

SCHOENMAKERS, H.J.N., P.H.J.M. COLENBRANDER and J. PEUTE (1977) — Ultrastructure evidence for the existence of steroid synthesising cells in the ovary of the starfish, *Asterias rubens* (Echinodermata). *Cell Tiss. Res.*, **182** : 275-279.

SHARON, N. and H. LIZ (1989) — *Lectins.* Chapman and Hall, 127 pp.

SMITH, D.S. (1968) — *Insect cells : their structure and function.* Oliver & Boyd, Edinburgh, 372 pp.

THREADGOLD, L.T. (1976) — *The ultrastructure of the animal cell.* Pergamon International Library.

TURSCH, B., D. DALOZE, M. DUPONT, J.M. PASTEELS and M.C. TRICOT (1971) — A defensive alkaloid in a carnivorous beetle. *Experientia,* **27** : 1380.