A peptide from the male accessory glands of the Colorado potato beetle.

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Stellingen

1 Uit de waarneming dat de proteolytische omzetting van het eiwit Acp26Aa, een product van mannelijke accessorisch geslachtsklieren van *Drosophila*, naar de actieve vorm plaatsvindt in het vrouwelijk voortplantingskanaal, kan niet worden geconcludeerd dat het wijfje in dit proces samenwerkt met het mannetje.

M. Park and M.F. Wolfner (1995) Male and female cooperate in the prohormone-like processing of a *Drosophila melanogaster* seminal fluid protein. Dev. Biol. 171, 694-702.

2 Het feit dat in *Melanoplus sanguinipes* de zich ontwikkelende oöcyten eiwitten opnemen die oorspronkelijk afkomstig zijn uit mannelijke accessorische geslachtsklieren, wil niet zeggen dat er sprake is van "cryptic paternal investment", aangezien de hoeveelheid eiwit die per paring naar het wijfje wordt overgedragen daarvoor te gering is.

M.T. Cheeseman & C. Gillot (1989) Long hyaline gland discharge and multiple spermatophore formation by male grasshopper. Physiol. Entomol. 14, 257-264.

3 De waarneming van De Loof en Lagasse (1972), dat het meerlagig epitheel van de mannelijke accessorische geslachtsklieren van de Coloradokever, na allatotectomie, degenereert tot eenlagig epitheel, berust op een onjuiste interpretatie van histologische coupes. Deze klier heeft altijd één laag epitheelcellen.

A. de Loof & A. Lagasse (1972) The ultrastructure of the male accessory reproductive glands of the Colorado potato beetle. Z. Zellforsch. 130, 545-552.
Dit proefschrift.

4 De benaming "tokens of love" voor mannelijke accessorische geslachtsklierproducten kan beter worden vervangen door "tokens of male selfishness", gezien de giftigheid van sommige van deze producten voor het wijfje.

M.F. Wolfner (1997) Tokens of love: Functions and regulation of Drosophila male accessory gland products. Insect Biochem. Molec. Biol. 27, 179-192.
Dit proefschrift.

5 Het effect van hoge concentraties kopertoevoegingen aan veevoer op het ontstaan van prion-gerelateerde ziekten als BSE en Scrapie dient nader onderzocht te worden nu blijkt dat het prion-eiwit specifiek bindt aan koper-ionen en onder invloed van deze binding van conformatie verandert.

M.P. Hornshaw, J.R. McDermott, J.M. Candy & J.H. Lakey (1995) Copper binding to the N-terminal tandem repeat region of mammalian and avian prion protein: structural studies using synthetic peptides. Biochem. Biophys. Res. Com. 214, 993-999.

6 De in "Sexual selection" (J.L. Gould and C. Grant Gould) vermelde kwalificaties "dirty, noisy and unrewarding" ten aanzien van kinderen zegt meer over de aandachtsverdeling werk-gezin bij de familie Gould dan over kinderen.

J.L Gould & C. Grant Gould (1989) Sexual selection. Scientific American Library, New York.

7 Het feit dat het op straat parkeren van een hondendrol ongeveer 400 keer zo duur is als het parkeren van een auto (gebaseerd op houderschapsbelasting; gecorrigeerd voor gewicht), relativeert de veelgehoorde klacht dat de automobilist door de staat als melkkoe wordt gebruikt.

8 De elasticiteit van de grenzen aan de groei van Schiphol is grenzeloos.

9 Aan "de vier jaargetijden" van Vivaldi valt te beluisteren dat het verval van de herfs meer inspiratie oplevert dan de bloei van de lente.

10 De jaarlijkse economische groeicijfers zijn te onnauwkeurig om er beleid op te baseren.
Zvi. Griliches (1994) Productivity, R&D and the data constraint. American Economic Review 84, 1-23

11 De enige essentiële verbetering in gebruikersvriendelijkheid van de personal computer in de afgelopen 10 jaar is de verplaatsing van de uitschakelaar van de achterzijde naar de voorzijde van het apparaat.

12 De coloradokever komt niet uit Colorado.

Stellingen behorende bij het proefschrift: A peptide from the male accessory glands of the Colorado potato beetle.

H. M. Smid, Wageningen, 22 april 1998

Contents

1

General introduction

Endocrine research on the Colorado potato beetle.

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is an attractive model for studies on insect endocrinology. It belongs to the family of Chrysomelidae (leaf beetles) of the order Coleoptera. This insect is an economically important species, in that it is a severe pest on potato in the Northern hemisphere. The extensive use of pesticides for its control has led to the development of resistance to most insecticides in several parts of the world (Casagrande, 1987). Research that may lead to alternative control strategies is therefore required. The aim of the present study is to obtain new fundamental knowledge of physiological processes that may be helpful in designing such strategies in the not-too-distant future.

L. decemlineata exhibits seasonal adaptation to changes in daylength. In temperate climates, it responds to short daylengths by a phenomenon called diapause. This means that the beetles do no longer invest energy in reproduction, but build up reserves and then enter the soil to survive winter. In spring, the beetle emerges as a consequence of elevated soil temperature and starts to reproduce when potato plants are available. Thus, depending on the photoperiod, the beetle will either reproduce or enter diapause (De Wilde, 1955). The phenomenon of diapause has been the subject of extensive research at our department and most questions have focused on how the environmental information, through the action of the endocrine system, is translated into diapause (see de Kort, 1990 for review).

The key factor regulating seasonal adaptation proved to be juvenile hormone (JH). High JH titres in the beetle's hemolymph induce reproduction, while diapause is induced by a low JH titre. The JH titre, in turn, is regulated by a complex process which involves the rate of production of JH by the corpora allata (CA), and the rate of breakdown of JH by JH-specific esterases. The production of JH by the CA is regulated, at least partly, by a small group of neurons, the lateral neurosecretory cells (L-NSC) in the protocerebrum (Schooneveld, 1970, 1974; Khan *et al.*, 1983). These neurons innervate the CA and produce peptides which regulate CA activity. Such peptides inhibit or stimulate CA activity and are hence called allatostatins or allatotropins, respectively. The chemical characterization of allatostatins or allatotropins seemed desirable, and part of the endocrine research at the department has so far been focused on the isolation of allotostatins or allatotropins from *L. decemlineata*.

For the isolation of such bio-active peptides, a reliable and simple bioassay is essential to demonstrate the presence of active peptides in fractions that arise from the purification process. In the case of a peptide that regulates CA activity, an *in vitro* bioassay must be developed to measure CA activity before and after addition of peptides. The usefulness of such an assay in *L. decemlineata* was severely limited by the large individual fluctuations in CA activity, due to pulsatic release of JH (Khan *et al.*, 1982). For that reason, the assay could not be used routinely during a purification program. The problem of how to design a reliable and sensitive bioassay is not unique in insect endocrinological research. In fact, few

bioassays are available at present to assist in the purification of peptide hormones. One often has to rely on the use of heterologous bioassays, that is employing responses of an assay organ from a different species to peptide preparations of the species of interest. The drawback of the use of heterologous assays is the risk of physiological irrelevancy of the assay data obtained. For instance, neuropeptides from *Calliphora vomitoria* were isolated that had an allatostatic effect on CA of *Diploptera punctata*, and were called allatostatins. The isolated peptides were, however, inactive when tested on CA from *C. vomitoria* itself (Duve *et al.*, 1993). Their true function, therefore, remains to be resolved and depends on the development of appropriate assays.

Concept of reversed endocrinology using monoclonal antibodies.

An alternative approach of extraction and identification of peptides is based on the availability of antibodies, rather than that of a bioassay. In this concept of 'reversed endocrinology' (Lafont, 1991), one first isolates a peptide, e.g. by using a specific antibody, followed by its functional characterization. The need for a bioassay during purification is then bypassed, which enormously expands the possibilities to isolate neuropeptides. On the other hand, establishing the biological function of an isolated peptide remains required.

At our department the concept of reversed endocrinology has been applied to the isolation of neuropeptides that control CA activity. Previous work demonstrated that the L-NSC innervate the CA (Khan *et al.*, 1983), and probably release a CA inhibiting factor. For the detection of the neuropeptides in these L-NSC, specific monoclonal antibodies were prepared. This was accomplished by immunization of mice with crude brain extracts. Hybridoma cell lines obtained from these immunized mice were assayed on microscopical brain sections, to select those cell lines that specifically recognize neuropeptides in the L-NSC. In this way, three different monoclonal antibodies were selected that specifically labelled the L-NSC in brain sections. On the electron-microscopical level, it was shown that indeed the contents of secretory granules in the L-NSC pericarya as well as at the nerve endings in the CA were recognized by the antibody. These antibodies, designated MAC (Monoclonal Anti Colorado potato beetle) nrs. 8, 9 and 18 were candidates to be used for isolation of their antigens in the L-NSC (Schooneveld *et al.*, 1989).

The male accessory gland as an "endocrine" organ.

Other organs than the brain of *L. decemlineata* were examined for the possible presence of antigens immuno-reactive to MAC-18, to identify new targets or alternative sources of peptide. For instance, allatostatic peptides had in the meantime been localized not only in the brain of insects, but also in the hindgut and the male accessory glands (for review, see Stay, 1994). We screened serially sectioned entire bodies of *L. decemlineata* with MAC-18 and indeed found immuno-reactivity in the male accessory glands. The presence of MAC-18 immuno-reactivity in these glands was intriguing, since they are known to produce peptides that are added to the ejaculate during mating, and enter the female hemolymph to exhibit a hormonal function (see below). For instance, such peptides stimulate oviposition and reduce female receptivity for further matings in *Drosophila* species (Chen, 1991). This effect may well be accomplished by peptides acting on the female's JH level, by stimulation of CA activity (Kelly *et al.*, 1994). Thus, the question arose whether the

antigens recognized by MAC-18 in the L-NSC were identical in structure and function to the antigens in the male accessory glands.

On the basis of this observation, we regarded the male accessory glands as an ideal source of peptides. The glands are of the exocrine type and are relatively large, so that they could provide us which large amounts of peptide. This would greatly simplify the design of an isolation protocol. The isolation procedure itself would further provide knowledge about a straightforward purification protocol of the brain peptide, that is present in so much smaller quantities.

Male accessory glands and their role in reproductive strategies.

The insect's male accessory glands (MAGs) are highly variable among different species, in morphology as well as in function. The simplest design comprises two tubular glands, which end into the lateral ejaculatory duct. This situation is found, for instance, in *L. decemlineata* and in *Drosophila*. Variations on this basic design are found in other species and MAGs may contain additional numbers of tubules, and bean-shaped glands. The variable morphology is perhaps related to the different functions of MAGs (for review, see Chen, 1984). One important function is the production of proteins used for the formation of the spermatophore, a capsule used by some insect species to encapsulate and transfer sperm to the female. The glands may also secrete nutritive proteins, that are sequestered from the male's hemolymph and that serve as an additional protein supply for the inseminated female (Friedel and Gillot, 1976). The latter function is thought to occur in species that have low-protein diets (Pardo *et al.*, 1994). The nutritive proteins are specifically endocytosed by the developing oocytes, and can be regarded as a hidden form of male parental investment.

The most interesting MAG secretions are undoubtedly those that specifically regulate female reproductive behaviour. These substances are produced in the MAG and are transferred to the female during copulation. They act like a hormone and change the female's behaviour: they inhibit remating and enhance fecundity. These substances are appropriately called remating inhibiting substances (RIS) or, more poetically, anti-aphrodisiaca (Gilbert, 1976), and fecundity enhancing substances (FES) (Gillott, 1992) respectively. RIS and FES are peptides or small proteins, which are also called sex peptides (Kubli, 1992). In crickets and in grasshoppers, a prostaglandin produced in the testis was also identified as a FES (Stanley-Samuelson and Loher, 1986).

Several RIS and FES activities have been found in many different insect orders, including Coleoptera, but we know the exact amino acid sequence from only a few peptides. As early as 1975, Baumann *et al.* isolated from *Drosophila funebris* a 27 amino acid peptide, referred to as paragonial peptide 1, that reduced female receptivity. Chen *et al.*, (1988) isolated a 36 amino acid peptide from *Drosophila melanogaster*, that combined the stimulation of fecundity and reduction of receptivity. Nearly identical peptides were isolated from sibling *Drosophila* species (Chen and Balmer, 1989). An additional peptide with FES activity was isolated from *Drosophila melanogaster* by Herndon and Wolfner (1995).

The existence of RIS and FES activities can be explained in the light of antagonistic reproductive strategies by males and females. The female's concern is to collect in her spermatheca enough sperm cells to fertilize hundreds or perhaps thousands of eggs produced in the reproductive period. Although a single mating is usually adequate to provide

the required numbers of sperm cells, it is often seen that repetitive matings take place (Thibout, 1982). One reason for this is that the female thus ensures that a process of "sperm competition" can take place: eggs are fertilized by the most competitive sperm cells, and this has a clear evolutionary benefit (Parker, 1970).

The male, however, tries to maximize its overall fertilization rate, or, in other words, promotes that his sperm cells, and not those of a rivalling male, will fertilize the eggs of his mates. It can do so in several ways. First, it can block further mating attempts by other males by mate guarding behaviour: it remains in copulatory position for several hours or even days in some species. Second, it can leave a so-called copulatory plug in the female's oviduct, which prevents additional matings until a batch of eggs is deposited that removes the plug. Third, the male can use substances like RIS and FES as a sort of "peptidergic pheromones" to prevent remating of the female (called chemical mate guarding; Miller, 1994) and to promote egg development. It seems that the last strategy increases male fitness at lowest costs; the other two strategies require relatively large investments in time or protein by the male.

Research objectives

The aim of this project is to characterize the MAC-18 antigen (referred to as *Leptinotarsa decemlineata* Male Accessory Gland Peptide, Led-MAGP) in the male accessory glands, to determine its physiological function in the context of reproductive strategies and to compare the chemical nature with the MAC-18-immunoreactive peptide in the L-NSC.

This study begins with a structural description of the relevant parts of the male reproductive organs. The sites of production and storage of Led-MAGP in the accessory glands were investigated by immunohistochemical methods at the light- and electron-microscopical level. Breeding of the beetles and assay conditions were optimized for the detection of Led-MAGP in tissue sections (Chapter 2).

Led-MAGP was purified successfully and its amino acid sequence was partially determined. Its full structure was elucidated using molecular biological methods and the gene coding for Led-MAGP and its signal peptide was identified. Surprisingly, the peptide is homologous to the N-terminus of a prion protein, the transmissible agent in Creutzfeldt-Jakob disease and bovine spongiform encephalopathy (BSE) (Chapter 3).

After the characterization of Led-MAGP, the structure was compared to the MAC-18 antigen from the L-NSC in the brain. Since a brain extract is much more complex than an accessory gland extract, the purification by HPLC of the L-NSC peptide required additional chromatographic steps. A complicating factor was the instability of the pure peptide. Purification methods were adjusted to the specific requirements of the peptide and a successful purification of some MAC-18 immunoreactive peptides was achieved. Although no full structure identification was carried out, the peptides proved to be different from Led-MAGP, in spite of their common ability to bind with the MAC-18 antibody (Chapter 4).

The continuation of the project demanded large amounts of Led-MAGP. Both the preparation of a polyclonal antiserum against Led-MAGP and the physiological experiments to determine its role in females required considerable quantities of Led-MAGP in pure form. To avoid tedious dissections of substantial numbers of MAGs, we produced Led-MAGP by employing an insect cell expression system. It is described how a baculovirus is equipped

with the Led-MAGP gene and how cultured insect cells are induced by the recombinant virus to produce bulk quantities of peptide. A polyclonal antiserum against this recombinant Led-MAGP is produced, and its specificity is determined (Chapter 5).

To provide unequivocal evidence for the transfer of Led-MAGP to the female during mating, the polyclonal antibody was used in an immunohistochemical approach to track Led-MAGP during mating on its course to the spermathecal duct of the female. (Chapter 6).

On the basis of the results obtained, an hypothesis was proposed about the function of Led-MAGP in the female. According to this hypothesis, Led-MAGP acts as a soluble receptor in the female hemolymph, that binds female hemolymph proteins in order to route them into the developing oocytes. This way, Led-MAGP stimulates oviposition and reduces receptivity. This hypothesis is compared to the concept of the *Drosophila* sex-peptide and is discussed in an evolutionary context (Chapter 7).

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2

Male accessory sex glands contain new class of exocrine peptidergic cells in *Leptinotarsa decemlineata* (Say), identified with neuropeptide-specific monoclonal antibody MAC-18

Abstract

The male Colorado potato beetle, *Leptinotarsa decemlineata* (Say) has one pair of elongate accessory glands. Using a monoclonal antibody, specific for the lateral neurosecretory cells in the brain, a small percentage of the epithelium cells is strongly immunolabelled. The antibody associates with secretory granules, which are abundant in these cells. The para-crystalline content of these granules is also identified in the lumen of the gland, which indicates that the immunoreactive cells have a secretory function. It proved to be necessary to induce accumulation of the secretary granules to allow their immunodetection, and this was accomplished by a period of starvation. Biochemical and immunological evidence suggests that the antigen is a small peptide. The cells described here belong to a new class of exocrine peptidergic cells, with possible endocrine regulatory function in female reproductive behaviour.

Introduction

For many years we are interested in hormonal regulation of reproduction in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). Early studies showed that juvenile hormone is the key factor for the maintenance of vitellogenesis, oogenesis and oviposition but it is clear that regulatory peptides are also necessary as co-factors for the endocrine control of these processes (De Wilde & de Loof 1973). This is illustrated by the fact that virgin females lay no or very low numbers of eggs, but they cannot be triggered to oviposit by injections with juvenile hormone. (Schooneveld, unpublished results). When such females are allowed to mate, oviposition starts within 2 days.

The factors which induce oviposition in *L. decemlineata* are not known. In a few other species it is becoming apparent that a complex mixture of male accessory gland (MAG) - derived substances is responsible for certain post-copulatory changes in the female's behaviour and physiology (Leopold, 1976; Chen, 1984, 1991). Examples are (1) stimulation of oviposition in e.g. *Aedes aegyptii* (Leahy and Graig (1965), *Locusta migratoria* (Lange and Louhgton, 1985), *Melanoplus sanguinipes* (Friedel and Gillott 1976), and *Drosophila funebris* (Baumann, 1974); (2) stimulation of oviduct contractions in *Locusta migratoria* (Paemen *et al.* 1991); (3) decreasing in female receptivity in Drosophila funebris (Baumann *et al.*, 1975) and (4) regulation of sperm migration in the oviduct in Rhodnius prolixus (Davey, 1958).

The nature of these male MAG components with regulatory functions in the female has been determined in a few cases. In *Drosophila suzukii* (Ohashi *et al., 1991) and Culex tarsalis* (Young and Downe, 1987), the female specific regulatory substance proved to be a small peptide. In *Drosophila melanogaster* and in *Drosophila sechellia*, a 36 amino-acid peptide has been isolated and sequenced which was responsible for the stimulation of oviposition as well as for the reduction of receptivity after mating (Chen *et al.*, 1988, Chen & Balmer, 1989).

The production site of the Drosophila melanogaster peptide may be the main cells of the MAGs. These cells were found to express a gene which probably encodes a precursor for secreted peptides (DiBenedetto *et al.*, 1990). The function of this presumed peptide is unknown. There are no further reports on the precise location of the production sites of female specific regulatory peptides.

This paper provides evidence of specific cells in the MAG's epithelium being the source of secretory peptides in L. decemlineata. We have studied the morphology, histology and ultrastructure of the MAGs and demonstrated the existence of exocrine peptidergic cell by applying immunohistochemical and immuno-dot techniques. We used the monoclonal antibody MAC-18 for this study, which is one of a panel of monoclonal antibodies which recognizes the secretory substance of the peptidergic lateral neurosecretory cells in the brain of this beetle (Schooneveld *et al.*, 1989).

Materials and methods

Animals

Colorado potato beetles of both sexes were kept together under long-day conditions (14 hrs photoperiod) and fed for at least 7 days with fresh potato foliage. This feeding period was, for certain experiments, followed by a starvation period of 7 days to enhance product accumulation in secretory cells, since a previous study showed that the MAC-18 antigen in the lateral neurosecretory cells accumulated during starvation (Schooneveld *et al.*, 1989). The entire reproductive organs of unanesthetized males were rapidly dissected under ice-cold saline (Khan et al., 1982). Care was taken not to touch the MAGs with forceps.

Immunohistochemistry

The production of monoclonal antibody MAC-18 has been described by Schooneveld and Smid (1990). Mass production was carried out *in vitro* in immunoglobulin free medium and the antibody (subclass IgM) was partly purified by precipitation with 8% polyethylene glycol according to Klein (1985).

For light microscopy, entire male reproductive organs were immersed for 10 min in ice-cold GPA (25% glutaraldehyde: saturated picric acid in water 1:3, with 2% acetic acid), followed by 10 min microwave irradiation under water cooling as described before (Smid *et al.* 1990). After 20 min of microwave stimulated washing with water-cooling, specimens were dehydrated and vacuum embedded in Paraplast-Plus. Serial sections were mounted on poly-l-lysine (Sigma) coated slides. Sections were incubated for one night at 4°C in MAC-18 diluted 1:4000 in PBS with 10 % normal swine serum, followed by a 2 h incubation at room temperature with rabbit-anti-mouse peroxidase conjugate (Dakopatts, Denmark) diluted 1:100. Labelling was visualized with N,N'-diaminobenzidin (Sigma) and sections were counterstained with Mayer's haematoxylin. Control sections received the same treatment except that the incubation with MAC-18 was replaced by an incubation in PBS.

For electron microscopy, entire male reproductive organs were fixed for 2 h in 2% glutaraldehyde in 0.1M cacodylate buffer pH 7.3 at 4°C. Postfixation in osmium-tetroxide was omitted, since it reduced the immunolabelling efficiency. Accessory glands were separated and embedded in epon. Sections of 80 nm were collected on formvar coated nickel grids. The section surface was pretreated with 4% sodium meta-periodate for 1 h prior

to immunolabelling, according to Bendayan and Zollinger (1983). Grids were incubated one night at 4°C on 20 µl drops of MAC-18 diluted 1:5000 in 1% BSA in PBS. Labelling was visualized by a 2-h incubation at room temperature with rabbit-anti mouse, prepared in the laboratory, diluted 1:50, followed by a 2 h incubation with protein A-gold complex (15 nm) prepared in the laboratory according to Slot and Geuze (1985). Sections were contrasted with 2% uranyl acetate.

Immuno-dot assay

To determine wether the MAC-18 antigen in the MAG was a small peptide, we tested its solubility in a methanol-water-acetic acid (90:9:1) mixture. Thirty pairs of MAGs were separated from the prostate and glass-homogenized in 1 ml of this extraction medium. The homogenate was centrifuged at 10,000 g, and the supernatant (the low molecular weight fraction) as well as the pellet (the high molecular weight fraction) were lyophilized, redissolved in 50 µl PBS buffer and cleared by centrifugation. For the immuno-dot assay, we used glutaraldehyde-activated nitrocellulose to increase the binding capacity for the MAC-18 antigen. The nitrocellulose strips were incubated in 1% BSA, washed in distilled water and subsequently incubated in 2% aqueous glutaraldehyde. Aliquots of one µl of both samples were spotted on dried strips of activated nitrocellulose. After drying, the nitrocellulose strips were blocked with 1% BSA. Immunodetection with MAC-18 was performed as described for paraffin sections.

Results

Gross anatomy of the male reproductive organs.

The male reproductive organs are represented schematically in Fig. 1. *L. decemlineata* has one pair of elongate MAGs, which fuse with the vasa deferentia and form the lateral ejaculatory ducts. Both lateral ejaculatory ducts fuse and form the anterior ("common") ejaculatory duct. The narrow distal extension of this duct, the posterior ejaculatory duct, ends in the aedeagus.

Histology of the MAGs

The elongate MAGs consist of a single layer of epithelial cells. The basal sides of the columnar cells rest on a basal lamella which surrounds the whole gland. The apical sides of these cells border the lumen of the gland. The basal lamella is surrounded by a thin irregularly shaped muscular layer. The gland is extensively innervated by axonal processes from the abdominal ganglion. These processes remain outside of the basal lamella.

Immunohistochemical staining of the glandular cells in the MAGs.

After incubation of MAG paraffin sections of starved males with MAC-18, about 100 cells per gland appear immunoreactive (Fig. 2). The cells are columnar shaped and randomly distributed over the gland. No such cells are labelled in the MAGs of normally fed animals.

Ultrastructural studies on starved beetles indicated that the MAC-18 positive cells are packed with granules, ranging from 300-500 nm in diameter (Fig. 3). The granules are densely labelled with immunogold particles as a consequence of MAC-18 binding. These granules have a typical para-crystalline content. The MAC-18 positive cells can be clearly

distinguished from the other cells in the MAG, because of their large amounts of secretory granules, the secretory content and the exclusive granule immuno-labelling. The gland appears to be in an inactive state, as illustrated by the small amounts of endoplasmic reticulum and tiny mitochondria. No active exocytosis of granules can be observed. The presence of large bud-like extrusions (Fig. 5) nevertheless indicates that the cells use the apocrine secretion mechanism. The typical para-crystalline deposits resulting from the dissociation of the granules, are also present in the lumen of the gland (Fig. 4). They remain clustered because of the high viscosity of the lumen's content.

In normally fed males, MAC-18 positive cells cannot be detected at the ultrastructural level, because all cells of the MAG have a similar ultrastructure, while electron-dense granules do not accumulate intracellularly. The crystalline deposits are nevertheless present in the lumen of the MAG like in starved males, and they are also immunoreactive.

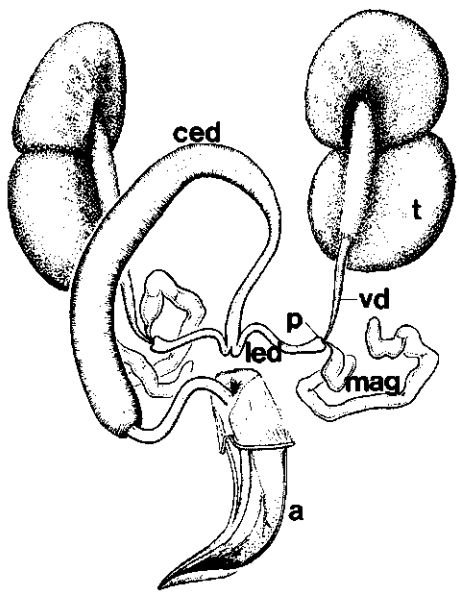

Fig. 1. Schematic drawing of the male genital tract, as situated in the abdomen. t, testis; sv, seminal vesicle; vd, vas deferens; mag, male accessory gland; pr, prostate; led, lateral ejaculatory duct; ced, common ejaculatory duct; ped, posterior ejaculatory duct; a: aedeagus. Nomenclature after Suzuki (1988).

Characterization of the immunoreactive secretory material by immuno-dot assay

To make a rough estimation of the molecular size of the immunoreactive product, MAGs of normally fed males were homogenized in a methanol-water-acetic acid mixture that allowed all poly-peptides and proteins of a mass of approximately 10,000 dalton or more to precipitate. Samples from supernatant and precipitate were spotted on nitrocellulose strips and stained with MAC-18. Only the sample from supernatant was identified positively (not shown), indicating that the molecular weight of the antigen is under 10,000 dalton.

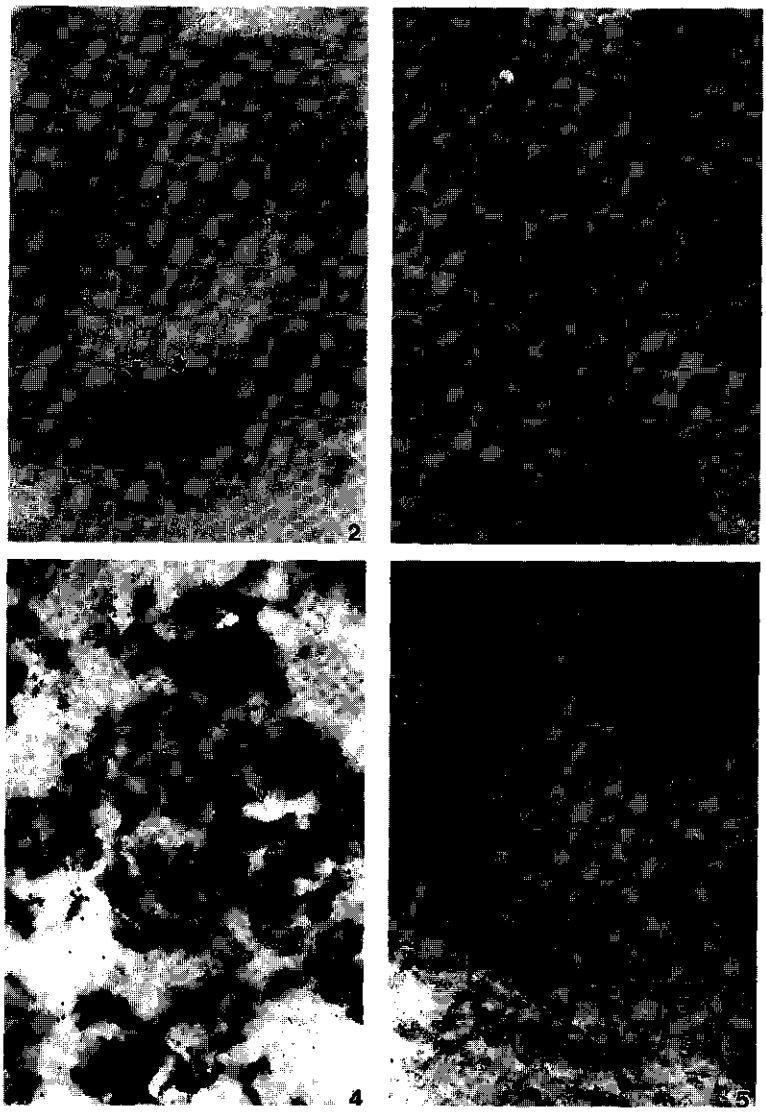

Fig. 2. LM cross-section of MAG of a beetle, starved for 7 days and immunolabelled with MAC-18. Note that the epithelium cells are columnar. Arrows indicate MAC-18 positive cells. (x 554).
Fig. 3. EM section of MAC-18 labelled MAG cell in starved male. Arrows indicate 300-500 nm secretory granules with para-crystalline content. The colloidal gold particles are associated with these granules. N, nucleus. (x 16,000).
Fig. 4. EM section of extracellular para-crystalline deposits in the lumen of an MAG of a starved beetle. Note the presence of 15 nm colloidal gold particles on these structures, showing labelling with MAC-18. (x 26720)
Fig. 5. Apical side of MAC-18 negative MAG cell in a starved beetle. A large bud-like extrusion (large arrows) has been formed towards the lumen, which indicates apocrine secretion of cell products. The adjacent cell features a similar extrusion (asterix). Small arrows indicate the lateral cell membranes. L, lumen. (x 7760)

Discussion

This study shows that a small fraction of the cells of the MAGs of male *L. decemlineata* is characterized by their stainability with monoclonal antibody MAC-18. Their detection depends on the physiological condition of the animals. Normally fed males have no immunolabelled MAG cells; only after a starvation period of 7 or more days, approximately 100 epithelial cells are immunolabelled. Although the conditions for product accumulation have not further been optimized, it appears that the fraction of MAG cells that contain the immunoreactive product is very small indeed. It should be borne in mind that the monoclonal antibody MAC-18 has originally been raised against the peptidergic content of the lateral neurosecretory cells in the brain of *L. decemlineata.* This is the first report of an immunolabelling of MAG cells with an antibody which is specific for peptidergic neuroendocrine cells.

The ultrastructure of the MAC-18 positive MAG cells is characterized by large amounts of granules with para-crystalline content, which range from 300 to 500 nm in size. The immunolabelling is associated with these granules. A detailed observation on the MAC-18 cells is not possible since they are only detectable in starved males, when they are in an inactive state. Secretion of cell product does not take place under these circumstances. Nevertheless, the presence of the MAC-18 antigen in para-crystalline content of the intracellular granules as well as in the lumen of MAGs of starved beetles is indicative of an exocrine release of the content of secretory granules. The presence of MAC-18 positive para-crystalline deposits in the lumen of MAGs of normally fed beetles shows that the antigen is also synthesized and released under this physiological condition.

Although our results show that the MAC-18 positive MAG cells use an exocrine secretory mechanism, we also considered the possibility that these cells might have an endocrine function in the male beetle, comparable to e.g. the paraneurons in the midgut of *Periplaneta americana* (Endo *et al.*, 1982). In *Rhodnius prolixus*, it was indeed recently shown that the MAGs secrete a polypeptide into the hemolymph (Sevala and Davey, 1991). Nevertheless, in no instance did we observe any indication that MAC-18 positive cells MAG cells secrete their products via their lateral or basal membranes. An endocrine function of these cells in the male is therefore unlikely.

The results of the immuno dot-assay indicate that the MAC-18 antigen in the MAG cells is a small molecule, probably a peptide, since it does not precipitate in the selective extraction medium used. We therefore consider the MAC-18 positive exocrine MAG cells to be peptidergic. The fact that the monoclonal antibody MAC-18 also recognizes the content of the secretory granules of the lateral neurosecretory cells in the brain implicates that the cell products at both locations share the same epitope. Whether the products in the MAG and in the peptidergic neurons have more chemical similarities remains to be investigated.

The phenomenon that the products of gland cells cannot be detected unless starvation conditions induce their accumulation is also observed in the endocrine lateral neurosecretory cells in the brain of *L. decemlineata* (Schooneveld *et al.*, 1989), but in a less dramatic way. At the ultrastructural level it namely appears that small amounts of granules remain present under non-accumulating conditions. This can be explained by the different secretory mechanisms used by these two cell types. The exocrine MAG cells store their products in the lumen of the MAG, while the endocrine lateral neurosecretory cells store their products in their cytoplasm and axon terminals. Consequently, the exocrine cells can secrete their

products immediately after synthesis, while the endocrine cells must keep their products in stock.

There are no previous reports on the presence of exocrine peptidergic cells in male MAGs, although there is evidence for several insect species that mating-induced stimulation of oviposition is caused by a regulatory peptide derived from the male MAGs. The absence of secretory granules in the cytoplasm of the peptidergic exocrine cells might have prevented the detection of similar cell types in other insect species. This possibility is illustrated by the fact that the ultrastructural study of the MAGs of male L. decemlineata by De Loof and Lagasse (1972), did not reveal granule-containing cells. These authors used normally fed beetles.

As a consequence, the exocrine peptidergic MAG cells in *L. decemlineata* are different from all other cell-types which have been described in ultrastructural studies on MAGs in other species. Nevertheless, the ultrastructure of the male MAGs of *Drosophila melanogaster* shows some similarities, as described by Federer and Chen (1982). There is one pair of elongate MAGs containing two cell types. The cells of one type, comprising about 5% of the total cell population in the MAG, are randomly scattered over the whole gland. They contain giant secretory granules with fibril-like content, which are released into the lumen. The nature of this secretory granule content is still unknown (Chen, 1991), and the question remains which cells are responsible for the production of the regulatory peptide which was isolated from the gland by Chen *et al.* (1988).

It is assumed that the Drosophila oviposition stimulating peptide, after being released into the oviduct, migrates through the oviduct wall into the hemolymph to act on the endocrine centres of the female (Chen, 1991; Aigaki *et al.*, 1991). Such an endocrine regulatory pathway is also possible for the L. decemlineata MAG peptide, bearing in mind that mating induces oviposition in *L. decemlineata.* It is necessary to isolate the MAG peptide to determine its function and a purification program is currently being carried out at the laboratory.

The exocrine peptidergic cells which are demonstrated here belong to a new class of peptidergic cells. We will next address the intriguing possibility of exocrine glandular cells producing a peptide with endocrine functions in the female.

Acknowledgements

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3

A peptide from the male accessory gland in *Leptinotarsa decemlineata*: Purification, characterization and molecular cloning.

Abstract

Our interest in the male accessory glands (MAGs) of *Leptinotarsa decemlineata* was raised recently by our finding that certain cells produce a secretory substance that is recognized by one of our monoclonal antibodies (MAC-18), developed for the immunohistochemical demonstration of peptidergic neurons in the brain. We undertook to isolate this substance, presumably a peptide, to find out more about its role in the post-mating physiology of the recipient of this peptide, the mated female.

This paper describes the purification and chemical characterization of the immuno-reactive peptide from 100 pairs of male accessory glands. The peptide was purified by two subsequent reversed-phase-HPLC runs, and fractions were analyzed on Western blots that were immuno-stained by MAC-18. This indicated the presence of an 8 kDa peptide in the MAG. Partial analysis of the N-terminal amino acids by automated Edman degradation revealed a sequence of 40 amino acid residues. To obtain the full amino acid sequence of this peptide, the technique of reverse transcriptase PCR (3'RACE) was used. A PCR product of 350 bp was obtained, which encoded the 3'-end of the mRNA. After cloning and sequencing, this product contained most of the genetic information of the MAG peptide. The PCR product was also used as a probe for screening a cDNA library constructed from mRNA extracted from MAGs. The nucleotide sequence coding for the signal peptide was elucidated by 5'RACE. The cDNA and 5'RACE clones were analyzed and sequenced. The sequence of the cDNA clone contained an insert of 411 bp, which agreed well with the mRNA size measured by Northern blotting.

Translation of the DNA sequences confirmed the data from partial amino acid sequence analyses and also predicted the remainder of the amino acid sequence. The entire peptide, designated Led-MAGP, consists of 74 residues; its mass was calculated and confirmed by mass spectrometry at 7971 Da. The peptide contains seven imperfect hexa-repeats, and this hexa-repeat sequence shows remarkable similarity to the hexa-repeat section of the chicken prion protein. The physiological function of the peptide has yet to be determined, but the hexa-repeat motif has recently been identified as the signal that induces internalization of the prion protein by coated-pit mediated endocytosis. Possible implications for the control of reproductive activities in *L. decemlineata* are discussed.

Introduction.

In many insect species, males manipulate their mates, thus enforcing both a reduced receptivity and accelerated oviposition. These effects are achieved chemically by transfer of certain products of the male accessory glands (MAGs) with the ejaculate (for reviews, see Chen, 1984; Raina *et al.*, 1994). In moths, loss of female receptivity is accompanied by a loss of sex-pheromone release, and this phenomenon was recently found to be induced by a pheromonostatic peptide from the MAGs of *Helicoverpa zea* (Kingan *et al.*, 1995). In *Drosophila*, the oviposition stimulating substance has been identified as a peptide (sex-peptide, SP) (Chen *et al.*, 1988; Chen and Balmer, 1989; Ohashi *et al.*, 1991; Schmidt *et al.*, 1993). RNA analysis revealed that the MAGs are the site of synthesis of SP (Chen *et al.* 1988), although the exact localization of the glandular cells that produce SP is still uninvestigated.

We adopted an immunocytochemical approach to search for cells producing peptides in the MAGs of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). A small fraction of the epithelial cells in the MAGs of *L. decemlineata* proved immuno-reactive to one

of our monoclonal antibodies, encoded MAC-18 (Smid and Schooneveld, 1992). This observation is of interest, because MAC-18 was originally raised against the lateral neurosecretory cells in the brain, that innervate the corpora allata (Schooneveld *et al.*, 1989). An electron microscopical study revealed that the immuno-reactivity in the MAGs is localized in secretory granules. These granules in the epithelial MAG cells contain a characteristic fibril-like material, and large aggregations of these fibrils were observed in the lumen of the MAGs. We postulated that the MAC-18 immuno-reactive cells are exocrine peptidergic cells, which produce a peptide that is transferred to the female during copulation (Smid and Schooneveld, 1992). The immuno-reactive substance appears to be a peptide of approximately 8 kDa (Smid and Schooneveld, 1994).

This paper describes the successful purification of the immuno-reactive peptide from dissected MAGs. Its primary structure was obtained by a combination of N-terminal sequencing of the purified peptide and molecular biology, in particular 3' and 5' RACE techniques. A cDNA library from the MAG was successfully screened to obtain the entire nucleotide sequence of the primary transcript. Some of the chemical peculiarities of our peptide are shared with some other peptides and proteins in other animals and it is inferred that these peptides may have analogous functions.

Materials and methods

Animals

Adult Colorado potato beetles of both sexes were kept together under long-day conditions (18 h photophase). MAGs were dissected in ice-cold saline solution (Khan *et al.*, 1982). We used an electronically controlled cooling device, mounted in the base of the dissection microscope, which was capable of maintaining a dissection basin with saline at 4°C. For the isolation of peptide, 14-day-old males were used, while mRNA was isolated from 7-day-old beetles.

Peptide purification.

MAGs were collected in a mixture of 80% methanol, 18% water and 2% acetic acid, with 0.1% methyl thioethanol and homogenized by sonification at room temperature. After centrifugation at 10,000 ***g*** for 2 mins, the supernatant was reduced in a Speed Vac concentrator (Savant), and lyophilized. The lyophilisate was extracted with 500 µl heptane, and centrifuged. The dried pellet was redissolved in distilled water by sonification. The sample was then clarified by centrifugation at 10,000 ***g*** for 2 mins, boiled for 5 mins and again centrifuged at 10,000 ***g*** for 10 mins. The supernatant obtained by this procedure will be referred to as the crude homogenate.

Electrophoresis was performed on a water-cooled vertical unit (Midget, Pharmacia), according to West *et al.* (1984), with some modifications. We used 20% SDS polyacrylamide gels with 0.2% bis-acryl amide, thickness 1 mm, and a 5% stacking gel. Samples, dissolved in water, were diluted 1:1 with a sample buffer of double ion strength, containing SDS and mercapthoethanol, and boiled for 2 mins. Samples of 20 µl were then applied in each slot and electrophoresed at 25mA per gel for approximately 100 mins.

Western-blotting was performed with a mini-blot device (Bio-Rad) on cellulose nitrate membranes, pore size 0.025 µm (Schleicher and Schuell, ph70) as described by LeGendre

et al. (1993), in 0.01M CAPS buffer (Sigma) with 20% methanol at pH 11.0 for 1 hr., and at 100 mA. After blotting, membranes were washed 3 times for 5 mins in phosphate buffered saline (PBS), air dried and fixed by glutaraldehyde vapour at 60°C for 1 hr.

Prior to immuno-labelling, membranes were again washed 3 times for 5 mins in PBS, and blocked for 30 mins in 1% bovine serum albumin (BSA, Sigma) in PBS with 0.25% Triton X-100. Immuno-labelling was performed by incubation of membranes with MAC-18 diluted 1:2000 in PBS with 0.5% BSA and 0.25% Triton X-100 for 1 hr at room temperature. Detection of labelling was through the use of an incubation with rabbit-anti-mouse immunoglobulins, conjugated to peroxidase (Dakopatts, Denmark), diluted 1:200 in the same buffer for 1 hr. Labelling was visualized with a 0.05% solution of 3, 3' diamino-benzidine (Sigma) as chromogen in PBS with 0.01% H_2O_2 as the peroxidase substrate. Control incubation regimes involved the omission of the first antibody; no immuno-reactive bands showed up under these conditions.

Reversed-phase high pressure liquid chromatography (RP-HPLC) separation was carried out with a Machery & Nagel C_4 column, particle size 5 µm, pore size 300 Å, dimensions 4.0 x 250mm. A linear water/acetonitrile (Merck) gradient was used, with 0.1% trifluoro acetic acid as the ion-pairing agent in both eluents. The flow-rate was 1 ml/min for all experiments. The column was loaded with crude homogenates, containing a maximum of 50 pairs of accessory glands. Either one ml fractions or single peaks were collected, concentrated with a Speed Vac concentrator (Savant), lyophilized, and redissolved in distilled water. Samples, representing 5% of each fraction, were analyzed by electrophoresis, Western-blotting and immuno-detection by MAC-18 as described above.

Analysis of the amino acid sequence by automated Edman degradation was carried out by Dr. R. van der Schors, Free University of Amsterdam, The Netherlands, with 100 pmol of peptide using an automated pulse-liquid phase peptide sequencer (Applied Biosystems, Inc., model 473).

Determination of molecular weight of the HPLC-purified MAG peptide was performed by electro-spray ionization mass spectrometry. Lyophilized HPLC fractions were redissolved in 7 mM trifluoroacetic acid in 60% acetonitrile. The mass determinations were carried out by Dr. R. van der Schors, using a Fisons BioQ triple-quadrupole mass spectrometer equipped with an electro-spray atmospheric pressure ionization source. The mobile phase was 50% acetonitrile, the flow rate was 5 µl/min.

RNA isolation

Messenger-RNA was isolated from accessory glands using a quick-prep micro mRNA isolation kit (Pharmacia), according to the manufacturer's instructions. The dissected glands were immediately immersed and homogenized by sonification in the extraction medium, containing guanidinium thiocyanate (provided in the kit) to minimize degradation of RNA by RNAse. Purified mRNA was converted to cDNA using a first strand cDNA synthesis kit (Pharmacia), primed with d(T)18 according to the manufacturer's instructions.

Total RNA was isolated from diverse tissues using TRIzol™ reagent (Life Technologies, Inc.) according to the instructions of the manufacturer.

Rapid amplification of 3'- and 5' cDNA ends (3'RACE and 5'RACE).

For PCR amplification of cDNA coding for the MAG peptide, d(T)18 was used as the downstream primer, in combination with a degenerated upstream primer, deduced by retro-translation of the amino acid sequence near the N-terminus of the MAG-peptide.

PCR was carried out according to the instructions provided with the first-strand cDNA synthesis kit. This kit included the PCR buffer, downstream primer d(T)18 and dNTP mix. The reaction mixture contained 320 pmol degenerated upstream primer, custom synthesized by Pharmacia Biotech (The Netherlands); 21 pmol downstream primer and 2.5 U *Taq* DNA polymerase (GIBCO BRL, Life Technologies Inc.) in a total volume of 50 µl. The thermal reaction was carried out in an Omnigene TR3 thermal cycler (Hybaid, UK) following the recommendations of Compton (1990): first 3 cycles of denaturation at 94°C for 30 s; annealing at 37°C for 30 s; and extension at 72°C for 60 s, with a ramp of 5 s/°C between annealing and extension temperature; followed by 30 cycles of denaturation at 94°C for 30 s; annealing at 50°C for 30 s; and extension at 72°C for 60 s.

After characterization of the PCR products by agarose gel electrophoresis, the band containing the amplified product was removed and purified with the Gene-Clean II kit (Bio 101 inc., La Jolla, California). This DNA was used as a probe to screen cDNA libraries, and for cloning with the pT7Blue T-vector kit (Novagen, Madison, Wisconsin). Plasmid DNA was purified with the Quiagen plasmid Mini Kit (Quiagen Inc., Chatsworth, California).

For 5'RACE, we used mRNA from 10 pairs of accessory glands per reaction. Single stranded cDNA was prepared as described above, using the d(T)18 primer or a gene specific primer. We used the 5'RACE protocol as described by Frohman (1990). The PCR product was cloned with the pT7Blue T-vector kit as described above.

Production and screening of a cDNA library

A cDNA library was constructed from mRNA, isolated from 100 pairs of accessory glands, in Lambda-ZAP using the ZAP cDNA syntheses kit from Statagene (CA, U.S.A). The library was probed with the 350 bp PCR product, and positive plaques were further processed according to the instructions of Stratagene.

Nucleotide sequencing

Sequencing was carried out on plasmid DNA according to the thermal cycle DNA sequencing protocol with DNA polymerase CircumVent (New England Biolabs), using α-^{33}P-dATP and template specific or universal primers in the sequencing reactions. Alternatively, sequencing was performed by automatic sequencing on a 373A-stretch sequencer (Applied Biosystems) using the Prism Ready Reaction Dyedeoxy terminator Cycle Sequencing kit (Applied Biosystems).

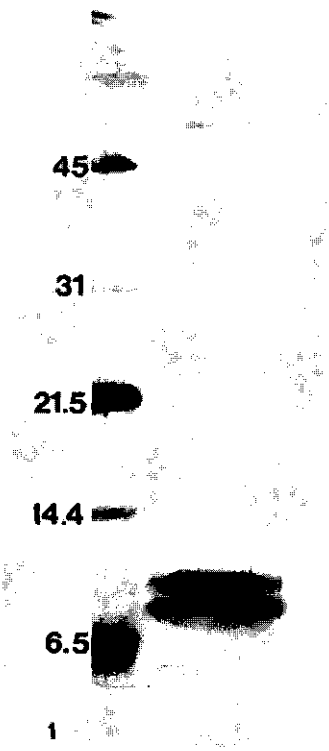
Nucleic acid analysis and blotting

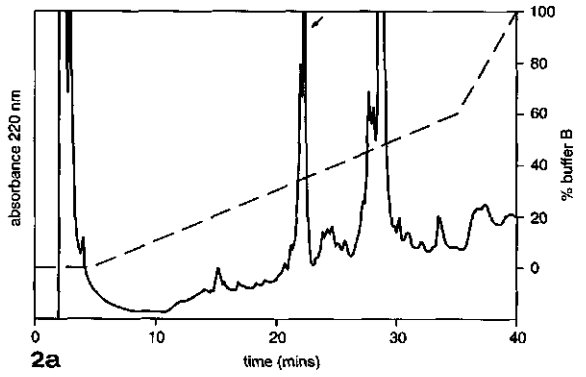
RNA analysis was performed by application of total RNA on positively charged nylon membranes (Boehringer, Mannheim) using a Bio-Dot microfiltration apparatus (BioRad, CA U.S.A.). Northern blotting of mRNA samples was performed on a denaturing formaldehyde gel electrophoresis system (Sambrook, 1989), followed by blotting on a positively charged nylon membrane (Boehringer, Mannheim). Hybridization was performed by standard procedures with α^{32}P-labelled probe.

Data bank sequence comparison

The full-length amino acid sequence of the MAG peptide as well as the nucleic acid sequence encoding it, was subjected to data bank sequence analysis. We screened the EMBL/genbank library using Fasta and TFasta, and the Swiss/PIR protein data banks using FASTA routines from Genetics Computer Group (GCG), Madison, Wi. Calculation of the molecular weight, as well as Chou-Fasman (Chou and Fasman, 1978) and Garneau/Robson (Garneau *et al.* 1978) algorithms to predict secondary structure was also done with GCG software package.

Results





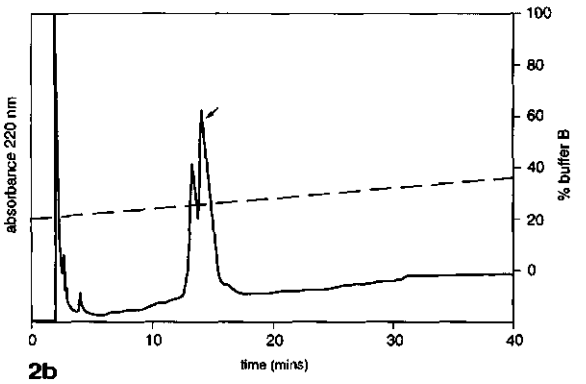


Fig. 1. Western blot after SDS-electrophoresis of crude MAG homogenate, immuno-labelled with MAC-18 (right lane). The MAG peptide appears as doublet of 8 and 9 kDa. Left lane: molecular weight markers.
Fig. 2a. Chromatogram of the first RP-HPLC run. The peak eluting after 21 mins. (arrow) was found to be immuno-reactive to MAC-18. Eluent A, water/0.1% TFA; eluent B. acetonitrile/0.1% TFA.
Fig. 2b. The peak containing MAC-18 immuno-reactivity from the first run rechromatographed on the same column, with a different elution profile. The second peak (arrow) contained MAC-18 immuno-reactivity.

Purification and N-terminal amino acid-sequencing of the MAG peptide.

Western blots of SDS-PAGE separated peptide fractions in a crude MAG homogenate revealed two sharp immuno-positive bands of 8 and 9 kDa respectively (doublet) after immuno-labelling with MAC-18 (Fig. 1). The immuno-reactive peptides will be referred to as MAG peptides throughout this paper. During the first RP-HPLC fractionation of a crude MAG homogenate, the MAG peptides came off the column with a retention time of 21 minutes (Fig. 2a). The immuno-reactivity on the Western blot appeared again as a doublet of 8 and 9 kDa. The peak containing immuno-reactivity was rechromatographed on the same column with a different elution profile; two peaks appeared that were collected separately (Fig. 2b). The 8 and 9 kDa doublet appeared to be localized in the second peak. As the two MAG peptides were apparently rather similar, we made no further attempts to separate them and subjected them together to N-terminal amino acid sequence analysis. This revealed the sequence of 40 amino acids (Fig. 3). There is one heterogeneity at position 7, where both valine and methionine may occur. We speculate that we are dealing with related peptides: a [Val^7]-MAG peptide and a [Met^7]-MAG peptide. A full structure identification by molecular techniques was carried out initially only for the [Met^7]-MAG peptide.

```
          i  c  g  a     c
1         ccggatcaacagatgcatccaggatactcggtacatcctggattttcggcgcat
      S D P  D  Q  Q  M  H  P  G  Y  S  V  H  P  G  F  S  A  H   20
                     V
55        cctggattttcgattcatcctggattcataacacatcctggattttctgcacat
          P  G  F  S  I  H  P  G  F  I  T  H  P  G  F  S  A  H   38

109       ccagggttctcgtcgcatcctggatactcagttcactctggaaacagggttcaa
          P  G  F  S  S  H  P  G  Y  S  V  H  S  G  N  R  V  Q   56

163       tctggagaccatgctcaccctgattacacgacttatcatggactctcccgaaaa
          S  G  D  H  A  H  P  D  Y  T  T  Y  H  G  L  S  R  K   74

217       tgaaagcattatgaagactaggtatatattgtcaacgcctggtccgtatctgaa
           *

271       aattgtaaattgagaataatatttgggtaactagtaattatacattcccagtgt

325       ttaaaaaaaaaaaaaaaaaa  344
```

Fig. 3. Nucleotide sequence (lower case) and amino acid sequence (capitals) of [Met^7]-MAG peptide, as revealed by 3'RACE. The degenerated primer at the 5'end is underlined, and degenerations are given above. The third nucleotide of the primer in the triplet encoding proline at position three is replaced by inosine. Met^7 can be substituted by Val. The amino acid residues revealed by N-terminal amino acid sequence analysis are in bold type, whereas the remaining amino acids are predicted from the nucleotide sequence. The polyadenylation signal is also in bold type.

The nucleotide sequence coding for the [Met^7]-MAG peptide obtained by 3' and 5'RACE

The mRNA extracted from the MAGs was used for the production of cDNA via reverse transcriptase. This single stranded cDNA was used as a template for the specific amplification of DNA between a degenerated peptide specific primer and the poly (d)T18

primer. The degenerated nucleotide primer was constructed by retrotranslation of a short sequence of amino acids as identified by previous chemical identification of part of the peptide. We selected a 20-mer degenerated nucleotide primer coding for amino acids 3-9 of the [Met^7]-MAG peptide, as shown in Fig. 3 (underlined nucleotides).

The PCR-amplified product had a size of 350 bp, large enough to code for the entire peptide. It was next cloned and sequenced, and indeed contained the complete genetic information for the peptide, including the part that was obtained by amino acid sequencing (Fig. 3), with the exception, obviously, of the first and second amino acids.

The 5' end of the mRNA was determined by using the 5'RACE technique. This revealed a nucleotide sequence that encoded a 17 amino acid hydrophobic signal peptide, starting with methionine (Fig. 4).

```
1          gtatatttcaagatgttaagattggtgctgctcatatctgtcacagtgaca      51
1                    M  L  R  L  V  L  L  I  S  V  T  V  T          13

52  gcaacatactcgtcggatcctgatcaacagatgcatccaggatactcggtgcatcctgga    111
14   A  T  Y  S  S  D  P  D  Q  Q  M  H  P  G  Y  S  V  H  P  G     33

112 ttttcggcgcatcctggattttcgattcatcctggattcataacacatcctggattttct    171
34   F  S  A  H  P  G  F  S  I  H  P  G  F  I  T  H  P  G  F  S     53

172 gcacatccagggttctcgtcgcatcctggatactcggttcactctggaaacagggttcaa    231
54   A  H  P  G  F  S  S  H  P  G  Y  S  V  H  S  G  N  R  V  Q     73

232 tctggagaccatgctcaccctgattacacgaattatcatagatcctccctaaaatgaaagc    291
74   S  G  D  H  A  H  P  D  Y  T  N  Y  H  R  S  S  L  K  *       91

292 attattgaatactaggtatatactgtcaacgcctggtccgtatgtgaaaattgtaaattg    351

352 agaataatatttgggtaactagtaattatacattctcagtgttaaaaaaaaaaaaaaaaaa    411
```

Fig. 4. Nucleotide sequence and deduced amino acid sequence of the cDNA clone of the MAG peptide. The nucleotide sequence encoding the signal peptide is confirmed by 5'RACE. The signal peptide is underlined. Nucleotides and amino acids that conflict with the PCR clone are in bold type.

Screening of the cDNA library

To confirm the nucleotide sequence obtained by 3'RACE and 5'RACE, we screened a cDNA library of accessory glands with the 350 bp PCR product from the 3'RACE experiment. The isolated clones were sequenced with universal primers and template-specific primers. One full length clone was isolated together with two shorter, but identical clones. The entire nucleotide sequence is given in Fig. 4. One additional clone appeared to be a dimeric artificial clone with a poly(A) tail at both 5' and 3' ends (data not shown). When the cDNA sequence is compared with the PCR product sequence, a few mismatches become apparent, some of which result in different amino acids after translation. At amino acid number seven, the artificial clone encoded a Val, instead of a Met. This latter heterogeneity was also detected by the N-terminal amino acid sequencing, as mentioned earlier.

Mass spectrometry.

To confirm the size of the predicted peptide, we measured its molecular weight by mass spectrometry and compared this value with the calculated value. Since we found a few mismatches between the different clones that were sequenced, probably due to allelic mutations, we calculated the masses of all possible isoforms. At position 7, a Met-Val substitution was detected by N-terminal amino acid sequencing. The PCR clone was obtained using a primer encoding [Met^7], thus this clone predicted only the [Met^7] isoform. The cDNA clones predict both Met and Val at position 7. Near the C-terminus, the cDNA clones differ from the PCR clone in a set of four amino acids (Fig. 4). Thus, there are at least four different isoforms of the MAG peptide, designated α[Met^7]- or [Val^7]-MAG peptide, with C-terminus predicted from the PCR clone, and ß[Met^7]- or [Val^7]-MAG peptide, with C-terminus predicted from the cDNA clones.

We determined the molecular weight of the authentic MAG peptide by mass-spectrometry. The peptide was purified from a homogenate of 25 MAGs by HPLC exactly under the same conditions as before. The purified peptide had a MW of 7971 Da. This value is consistent with the calculated mass of the α[Val^7]-MAG peptide, i.e. 7969.5 Da. We conclude that the peptide is not post-translationally modified. No value consistent with α or ß[Met^7]-, or ß[Val^7]-MAG peptide was obtained. Since the existence of only the α[Val^7]-MAG peptide is confirmed by mass spectrometry, we assume that this unmodified sequence represents the MAG peptide from *L. decemlineata*, designated Led-MAGP, according to the insect peptide nomenclature proposed by Raina and Gäde (1988).

		S	D	P	D	Q	Q	M	H	8
repeat	1			**P**	**G**	**Y**	**S**	**V**	**H**	14
	2			**P**	**G**	**F**	**S**	**A**	**H**	20
	3			**P**	**G**	**F**	**S**	**I**	**H**	26
	4			**P**	**G**	**F**	**I**	**T**	**H**	32
	5			**P**	**G**	**F**	**S**	**A**	**H**	38
	6			**P**	**G**	**F**	**S**	**S**	**H**	44
	7			**P**	**G**	**Y**	**S**	**V**	**H**	50
				S	G	N	R	V	Q	56
				S	G	D	H	A	H	62
				P	D	Y	T	T	Y	68
				H	G	L	S	R	K	74

Fig. 5. Amino acid sequence of the MAG peptide, here presented with aligned hexa-repeats. The 7 repeats are in bold type. Underlined amino acids are those not in the repeats but are situated in positions consistent with those in the repeats.

Northern blotting and tissue-specific expression of MAG peptide synthesis

Northern blotting of mRNA from accessory glands followed by hybridization with the 350 bp PCR product revealed a product of about 450 bp (data not shown). This value is consistent with the 411 bp sequence resulting from the cDNA clones.

We screened other tissues for the possible expression of the Led-MAGP gene. Samples containing 2 and 0.2 µg total RNA from the following tissues from animals of

different ages were analyzed: accessory glands of 5 and 28 days; brain tissues of virgin females of 42 days, mated females of 6 and 28 days, and mated females of 14 days that were starved for one week; ovaria from mated females of 7 days, from virgin females of 6 and 42 days. Hybridization was found exclusively with total RNA from accessory glands, from animals of 5 and 28 days, and in equal quantities.

Molecular characteristics of the peptide.

The amino acid sequence of Led-MAGPis highly repetitive. There are seven imperfect hexa-repeats, which becomes apparent if the amino acid sequences are arranged in a fashion to emphasize the cyclical nature of those repetitive structures (Fig. 5). The first repeat starts at the proline residue at position 9, which is followed by six subsequent repeats, most of them containing the sequence PGFS*X*H (were *X* is any amino acid). In the first and last repeat, phenylalanine is replaced by tyrosine, both being aromatic amino acids. In repeat four the uncharged polar residue serine is replaced by the hydrophobic isoleucine. The fifth amino acid of each repeat is more variable. At first sight, the peptide sequences at positions 1-7, and 50-74 are not composed of hexa-repeats, nevertheless, some residues show up at positions corresponding with those in the hexa-repeats (Fig. 5, underlined residues).

We made secondary structure predictions using the standard algorithms of Chou and Fasman (1978) and Garnier *et al.* (1978). Both algorithms predict a series of beta-turns.

A screening of protein data banks with Led-MAGP revealed some similarity with the N-terminal hexa-repeat section of the chicken prion protein, a substance inducing acetylcholine receptor-activity in brain and spinal cord tissues (Harris *et al.*, 1991) This protein of 30 kDa contains a stretch consisting of 8 hexa-repeats. The percentage of identical amino acids in the hexa-repeat sections of Led-MAGP and the prion protein is 34.9 %, the similarity is 62.8 % (Fig. 6).

```
MAG-pep S D P D Q Q V H P G Y S V H P G  16
            I           : I I I     : I I
Ch-PrP  R - P S Y P R Q P G Y P H N P G  57

MAG-pep F S A H P G F S I H P G F I T H  32
        :     : I I :     : I I :     :
Ch-PrP  Y P H N P G Y P H N P G Y P H N  73

MAG-pep P G F S A H P G F S S H - R      43
        I I :     : I I :     :
Ch-Prp  P G Y P Q N P G Y P H N - R      84
```

Fig. 6. Comparison of the MAG peptide with the prion protein from chicken [Ch-PrP, Harris et al. (1991)].

Discussion

This study on the nature and function of the MAG peptide in *L. decemlineata* was started by working out a strategy for the recovery of this peptide from dissected MAGs. The strategy presented here proved successful and contains an unusual approach, namely the use of a monoclonal antibody for detection of the MAG peptide on Western blots. This antibody, referred to as MAC-18, was selected previously for its potential to immuno-stain the peptide content of certain cells of the MAG (Smid and Schooneveld, 1992). The immuno-reactive 8 kDa peptide was purified by HPLC and its N-terminal was characterized by automated Edman degradation. These structural data enabled us to identify the gene coding for this peptide. The complete structure of the MAG peptide could next be reconstructed and the peptide is designated Led-MAGP. A search for specific expression of the MAG peptide in other tissues revealed that the peptide is expressed only in the MAGs.

The point to emphasize here is that we succeeded in identifying an insect peptide before knowing its physiological function. We did so by making use of antibody MAC-18, prepared with the specific purpose of recovering hitherto unknown neuropeptides from *L. decemlineata* (Schooneveld *et al.*, 1989). The need for the development of a bio-assay, often an insurmountable obstacle, is by-passed in this way. Our strategy opens new ways to identify peptides not likely to be identified by the classical procedures; the structural information hereby obtained, provides clues that may lead to understanding their physiological function (see below). The term coined by Lafont (1991) to denote this strategy is "reverse endocrinology"; the usual procedure is to identify a peptide after its hormonal function has been defined and a bio-assay has been developed.

Led-MAGP contains 7 imperfect hexa-repeats, as depicted in Fig. 5. It is not homologous to any known MAG peptide from other insect species, including the sex peptide from *Drosophila* (Kubli, 1992), and the pheromonostatic peptide from the MAGs of *Helicoverpa zea* (Kingan *et al.*, 1995), although repetitive sequences in MAG proteins (with unknown function) have been described earlier for *Tenebrio molitor* (Paesen *et al.*, 1992). There is, however, an interesting homology with the N-terminus of the chicken prion protein (ch-prp) as described by Harris *et al.* (1991) (Fig 6). This homology becomes striking if we consider that (1) the F-Y substitutions in 5 repeats (Fig. 6) are conservative; (2) the amino acid histidine in each prp hexa-repeat, essential for the prp's high copper affinity (Hornshaw *et al.*, 1995), is also present in each MAG peptide repeat; (3) the secondary structure predictions made by standard algorithms predict a series of beta-turns for the hexa-repeats of both origins (Bazan *et al.*, 1987); and (4) our ultrastructural study of the MAG (Smid and Schooneveld, 1992) revealed that Led-MAGP forms fibril-like crystals in the lumen of the MAG, and similar crystals have been observed in prp preparations (Chesebro, 1990).

This homology in peptide structure is interesting from a functional point of view, since the function of the prp hexa-repeat section has recently been determined as the signal that mediates the coated pit-mediated endocytosis of prp, in mammals as well as in chicken (Shyng *et al.*, 1995). The possible function of Led-MAGP with regard to trans-membrane transport of protein is discussed below. Prion proteins in general are currently attracting interest because they are considered to be the transmissible agents in certain fatal

degenerative neurodiseases in vertebrates, such as Scrapie, Creutzfeldt-Jakob disease, and Kuru (Chesebro, 1990).

Led-MAGP is supposed to be transferred from the male to the female during mating (Smid and Schooneveld, 1992) and indeed, we recently demonstrated that this peptide is deposited in the spermathecal duct (chapter 6). Its fate after the transfer is not certain, but it is probably transferred into the haemocoel through the duct wall, since its presence in the spermathecal duct can be demonstrated only briefly after mating.

Experiments to follow Led-MAGP after its release into the haemocoel, and thereby to define the possible site of action, are in progress. It was found that the peptide also exists in multimeric forms, and that it binds haemolymph proteins like vitellogenin (general discussion). The specificity and possible function of this affinity is currently under investigation. Aggregation of MAG peptides with unidentified proteins has been described by Young and Downe (1987) for a MAG peptide that inhibits female receptivity in *Culex tarsalis*, and by Miller *et al.*, (1994) for a MAG peptide or protein that turns off mating and activates oviposition in the onion fly *Delia antiqua*. The structure of these biologically active proteinaceous substances is still unresolved.

The ability of the Led-MAGP to associate with haemolymph proteins in the female may be essential to its function after release into the female. Analogous to the function of the ch-prp hexa-repeats, a signal that induces the coated pit-mediated endocytosis of prp, Led-MAGP might stimulate endocytosis of certain hemolymph proteins to which it binds. An important class of proteins that might thus be "tagged" with Led-MAGP includes the yolk protein, e.g. vitellogenin. This mechanism would accelerate the uptake of yolk protein, resulting in accelerated oocyte growth and thereby increased oviposition rates. The possibility that Led-MAGP enhances oocyte growth by increased uptake of tagged yolk proteins is currently being examined.

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4

Isolation and partial characterization of MAC-18 immuno-reactive peptides from the lateral neurosecretory cells of the Colorado potato beetle: candidate corpus allatum-activity-regulating neuropeptides.

Abstract.

Our monoclonal antibody MAC-18 recognizes the secretory granule content of the lateral neurosecretory cells in the brain and the axon terminals in the CA of *Leptinotarsa decemlineata*. The recognized antigens are considered to be candidate CA-activity regulating neuropeptides. MAC-18 also recognizes a peptide from the male accessory glands of this beetle. This peptide has been purified and its primary sequence, as well as the gene encoding it have been established (Smid *et al.*, 1997). The isolated peptide, designated MAG-peptide, shows similarity to the chicken prion protein (Harris *et al.*, 1991). Its function remains to be established. This study aims to identify the antigenic moiety in the brains and to compare it with the MAG-peptide. The antibody was used to monitor the presence of the immuno-reactive substances during an isolation program. The antigens were purified by reversed phase HPLC followed by preparative SDS-PAGE, while the presence of the antigens was monitored by Western analysis with the antibody MAC-18. Four different neuropeptides were identified by this procedure, with molecular masses between 12 and 17 kDa, as well as higher order forms of 25 and 40 kDa. Three peptides were obtained pure enough to analyze their *N*-terminal amino acid sequence by automated Edman degradation of Western blotted bands obtained after SDS-PAGE. A signal was obtained from two of these peptides, one 12 kDa peptide and a 14 kDa peptide. The identified neuropeptides were found to be different from the MAG-peptide in their molecular mass, their *N*-terminal amino acid sequences and in their chromatographic properties. The obtained sequences were different from any of the other known insect neuropeptides. Aspects on the value of the used isolation procedure are discussed.

Introduction

The regulation of reproduction and diapause in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) is mediated by changes in the juvenile hormone titre in tissues and hemolymph (De Kort, 1990). Juvenile hormone (JH) is produced by the corpora allata (CA), and variations in the activity of these glands are considered to be among the mechanisms by which the JH titre can be regulated. The CA are innervated by axons arising from the lateral and medial neurosecretory cells in the protocerebrum (Schooneveld, 1970, 1974a,b; Schooneveld *et al.*, 1989). Khan *et al.*, (1983) demonstrated the inhibitory action of these peptidergic centres on CA activity. We wish to identify the neuropeptides that serve as the messengers in this innervation and determine their function in CA regulation.

Some of the peptides that regulate CA activity in other insect species have been isolated and identified. Peptides with inhibiting action on the CA, called allatostatins, were isolated from *Manduca sexta* (Kramer *et al.*, 1991); *Diploptera punctata* (e.g. Woodhead *et al.*, 1989; Pratt *et al.*, 1991) and *Periplaneta americana* (Weaver *et al.*, 1994). An allatotropin, a peptide that stimulates CA activity, has been isolated from *M. sexta* (Kataoka *et al.*, 1989). Purification of these peptides was performed employing an in vitro assay for monitoring effects on the activity of the CA. Unfortunately, a suitable bio assay on CA activity could not be developed for *L. decemlineata*, considering the large individual

fluctuations in CA activity and low intrinsic rate of JH synthesis (de Kort, unpublished results; Khan *et al.*, 1983).

We have switched to an alternative strategy towards the purification of neuropeptides that regulate CA activity. Instead of using a bio-assay, we used immunological assays to monitor the presence of putative peptides. We previously described the production of a library of 18 monoclonal antibodies (designated "MAC's, Monoclonal Anti Colorado beetle") against unidentified brain peptides (Schooneveld *et al.*, 1989). Mice were immunized by injections of crude brain homogenates and hybridomas obtained from these immunized animals were selected for their ability to recognize any of the known peptidergic cells in brain sections of the Colorado potato beetle. Three of these monoclonal antibodies, MAC's 8, 9 and 18 specifically recognize the L-type neurosecretory cells (L-NSC), located in the protocerebral lobes, and their axonal processes in histological sections. Moreover, electron microscopic studies revealed that these MAC's recognize the contents of secretory granules, both in the cell body and in the axon terminals in the CA. They obviously have affinity for compounds that are candidate peptides with CA-regulatory functions. These observations prompted us to consider the possibility of using these antibodies for the isolation of the peptides from brain extracts. They could be used, for instance, for monitoring the peptides through successive steps in HPLC separation procedures, including their final isolation.

We developed this strategy of using antibodies for the isolation of peptides while investigating the identity of a peptide present in certain glandular cells in the male accessory glands (MAG) that were identified by immuno-histochemical methods with one of the our L-NSC-specific antibodies, MAC-18 (Smid and Schooneveld, 1992). The presence of MAC-18 immuno-reactivity in these glands was intriguing, since it is known from other insect species that MAGs produce peptides that are added to the ejaculate during mating, and enter the female hemolymph to exhibit a certain hormonal function. Such peptides are called sex-peptides and they stimulate oviposition and induce female monogamy in some *Drosophila* species (Chen, 1991). This physiological effect may well be accomplished through regulation of CA activity, thus modulating the JH level (Kelly *et al.*, 1994). The question arose whether the antigens recognized by MAC-18 in both L-NSC and MAG were identical in structure and function.

The MAG peptide has now been purified and the sequence of the amino acids as well as the gene encoding it has been established, although its function is still unknown (Smid *et al.*, 1997). This MAG-peptide from the *L. decemlineata*, proved to be different from the *Drosophila* sex peptide and, as a matter of fact, from any other known insect neuropeptide. However, its structure showed similarity to the N-terminal hexa-repeats sequence of the prion protein (prp) from chicken (Harris *et al.*, 1991). Prion proteins are raising interest since they are the transmissible agents in certain fatal degenerative brain diseases in cattle and man (Scrapie, bovine spongiformic encephalopathies (BSE) and Creutzfeldt-Jacob disease). Their normal function in the central and peripheral nervous system has yet to be determined. Whereas our MAG-peptide has a molecular mass of 8 kDa, the ch-prp measures 30 kDa.

We now wish to isolate and characterize the MAC-18 antigen in the brains of *L. decemlineata*, to find out whether this antigen is related to the MAG-peptide. In the next phase, its effects on the CA-activity will be assessed. This paper describes the purification of what indeed proved to be a peptide from homogenates of dissected brains and retrocerebral complexes. Earlier attempts to purify this peptide by "classical" methods,

employing successive HPLC steps were unsuccessful, because of its instability under the chromatographic conditions chosen. We now combined HPLC with electrophoresis and Western blotting. Three different immuno-reactive peptides were thus identified, and the *N*-terminal amino acid sequence could be determined from two of them.

Materials and methods.

Animals

Sexually mature Colorado potato beetles, *Leptinotarsa decemlineata* (Say), of a laboratory culture were kept under long-day conditions (18 h photophase). After 7 days of optimal treatment, animals were starved for 7 more days, this regime results in the accumulation of the MAC-18 immuno-reactive substance in the L-NSC (Schooneveld *et al*, 1989). Brains were dissected under ice-cold saline solution. Furthermore, MAGs of 14-day-old normally fed beetles were dissected under ice-cold saline solution.

Peptide purification by HPLC, SDS-PAGE and Western blotting.

Batches of 100 dissected brains were collected in 500 µl of a mixture of 80% methanol, 18% water, 2% acetic acid and 0.1% methyl thioethanol and were homogenized by sonification at room temperature. After centrifugation at 10,000 g the pellet was re-extracted in the same extraction mixture, centrifuged and the resulting supernatants were combined, reduced in volume in a Speed-Vac concentrator (Savant), and lyophilized. The lyophilisate was extracted with 500 µl heptane, and centrifuged. The dried pellet was redissolved in distilled water by sonification, boiled for 5 mins and centrifuged at 10,000 rpm for 5 mins. The clear supernatant obtained by this procedure will be referred to as crude homogenate.

Crude homogenates were combined to a total amount of 1000 brains and divided into three aliquots, each in 1 ml water, loaded on a Techogel C_4 500 Å column (HPLC technology, UK) and eluted by a linear gradient of 0 to 60 % acetonitrile over 60 mins, with 0,1% TFA as counter-ion. Fractions were collected every min and combined with fractions from the two identical HPLC runs. Samples representing 50 brain equivalents were taken from each fraction and analyzed by immuno-detection with MAC-18 of Western-blots on Immobilon-P membranes (Millipore) from SDS-PAGE, as described earlier (Smid *et al.*, 1997). For final purification, aliquots representing 500 brain equivalents of fractions containing immuno-reactive peptides were subsequently loaded on four lanes of a 10-20% TRIS tricine SDS gel (Novex, U.S.A.), according to the instructions of the manufacturer. Gels were electro-blotted as described, and flanks of the four lanes were immuno-labelled with MAC-18, while the inner lanes were stained by Coomassie Brilliant Blue, and briefly destained in 90% methanol, 2% acetic acid.

An homogenate of 10 MAGs was prepared as described above, and subjected to HPLC analysis under identical conditions as described for the brain homogenates, to compare the retention time of the resolved MAC-18 immuno-reactive peptides with the MAG-peptide. The identity of the MAG-peptide peak was checked by Western analysis with MAC-18 as described above. The obtained retention time for the MAG-peptide was 41 min.

Analysis of N-terminal amino acid sequence.

The peptide bands that were immuno-reactive with MAC-18 were cut out of the membrane and subjected to N-terminal amino acid sequencing. The purity of the bands was checked by total protein staining with coommassie; only those bands that were well-separated from neighbouring bands were analyzed further. Analysis of the amino acid sequence by automated Edman degradation was carried out by Dr. R. van der Schors, Free University of Amsterdam, The Netherlands, with an automated pulse-liquid phase peptide sequencer (Applied Biosystems, Inc., model 473).

Data bank sequence comparison

The obtained *N*-terminal amino acid sequences were subjected to data bank sequence analysis. We screened the Swiss/PIR protein data banks using FASTA routines from Genetics Computer Group (GCG), Madison, Wisconsin, USA.

Results

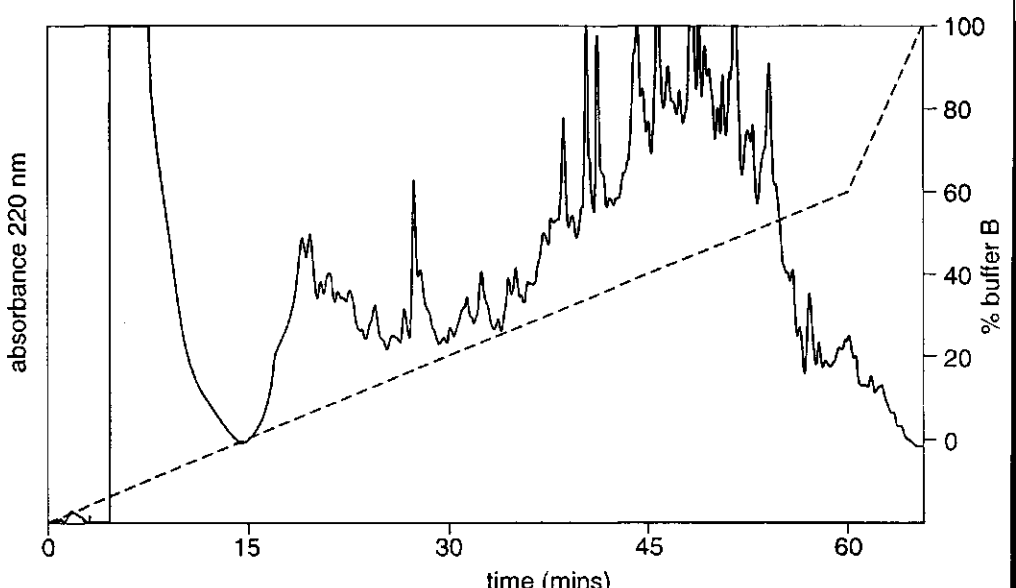

Fig. 1. Chromatographic profile of an HPLC run with 330 brain equivalents with a linear acetonitrile gradient (dashed line). Fractions 15-65 were Western-analyzed for MAC-18 immuno-reactivity (Fig. 2).

A chromatographic profile of an HPLC-run from an aliquot comprising 330 brain equivalents is depicted in Fig. 1. Two identical runs were carried out, which yielded identical extinction profiles. Fractions from each run were taken every min, starting from 15 min, and

combined. To assess the occurrence of MAC-18-positive substances, samples representing 50 brain-equivalents were analyzed by SDS-PAGE, electro-blotting and immuno-staining with MAC-18. Several of the fractions exhibited weakly to strongly immuno-reactive bands (fig. 2), only the fractions with strong bands were taken for further analysis. The following MAC-18 immuno-reactive peptides attracted our interest:

(1) a 17 kDa peptide in fraction 47,
(2) a 12 kDa peptide in fraction 50,
(3) a 25 and a 40 kDa peptide in fractions 53-55,
(4) a doublet peptide of 12-14 kDa in fraction 55.

We anticipated that the 25 and 40 kDa peptides in fractions 53-55 might result from an aggregation of the 12-14 kDa peptide doublet. To test this possibility, the samples of fraction 54 were boiled in SDS sample buffer for extended periods, i.e. 10 min, prior to loading the samples on the gel. Indeed, most high-molecular weight peptide was degraded to its 12-kDa monomer as a result from this treatment (results not shown).

Fraction 47, containing the 17 kDa peptide, appeared to contain several peptides with comparable molecular size, as revealed by total protein staining. This fraction was not analyzed further.

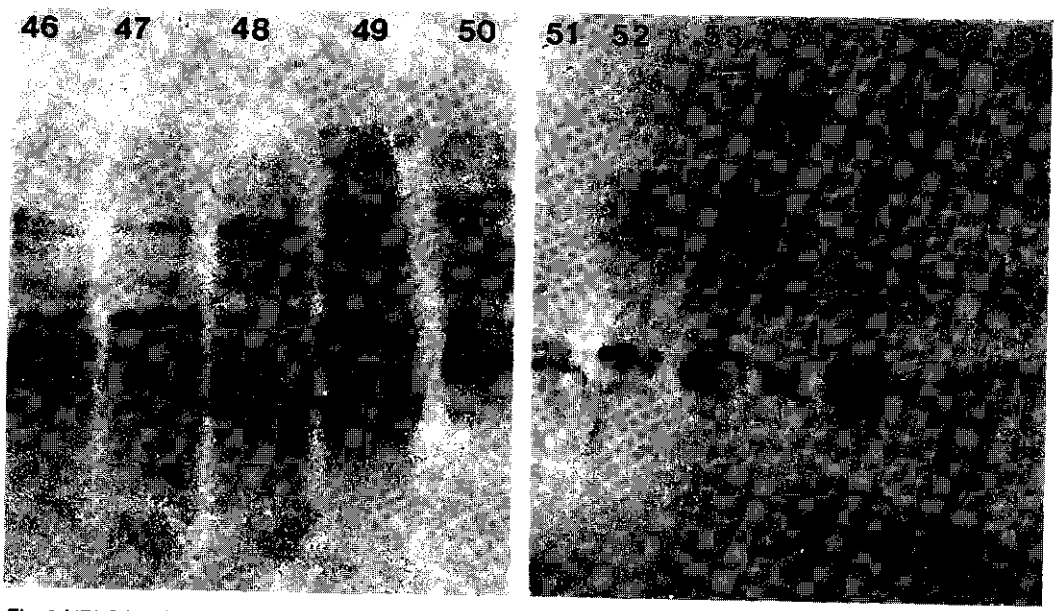

Fig. 2. HPLC fractions were further separated by SDS-PAGE, Western blotting and immuno-detection with MAC-18. Intense immuno-recognition was found in fractions 48, 51 and 54-56 (arrows).

The peptides selected for analysis of the *N*-terminal amino acid sequence were the 12 kDa peptide in fraction 50 and the 12 and 14 kDa peptides in fraction 55. The analysis was performed on coomassie-stained bands that were cut out of the Western blot from lanes that were run parallel to MAC-18 stained lanes. The 14 kDa peptide was possibly blocked at the *N*-terminus, since no signal was obtained. The 12 kDa peptide from fraction 51 yielded the sequence XXALKXDAYNLXGF after 14 cycles of Edman degradation (X=unidentified amino acid). The 12 kDa peptide from fraction 56 yielded the sequence DAPEXXQEAQ after 10 cycles of Edman degradation. Both
sequences did not show homology to any known peptide sequence, as revealed by a protein databank search.

The obtained signal was lower than expected from the intensity of the coomassie staining of the bands. We considered the possibility that this was due to peptide wash-off from the Immobilon-P membranes under the conditions used for Edman degradation. This problem, as described by Legendre *et al.* (1993) is due to the relatively weak binding force of this membrane for small peptides. Attempts to increase the efficiency by treatment of Immobilon-P membranes with polybrene after transfer of the peptides as suggested by Legendre *et al.* (1993), failed due to unidentified technical factors.

Discussion

The study presents the purification of MAC-18 immuno-reactive neuropeptides from *L. decemlineata.* Peptides in the molecular mass range of 12 to 40 kDa were detected, and these values are different from the molecular mass of the MAG-peptide, which is 8 kDa (Smid *et al.*, 1997). Furthermore, the retention time of MAG-peptide deviates from the retention times of the peptides from the brains. MAG-peptide has under identical chromatographic circumstances a retention time of 41 mins, while all immuno-reactive peptides from the brain were eluted between 47 and 55 mins. Finally, the brain peptides have higher molecular weights, than the MAG-peptide, and the obtained sequences of the N-terminus of two peptides are very different from MAG-peptide. Therefore, it can be concluded that the MAG-peptide is not present in the brains. The shared immuno-recognition of MAG-peptide and the brain peptides by MAC-18 nevertheless suggests some degree of identity; the possibility of a structural relation between the peptides of both origins awaits full structure identification of the brain peptides.

The molecular weights of the MAC-18 immuno-reactive brain peptides that are described in this study are 12, 14, 17, 25, and 40 kDa, where the latter two may result from aggregation of the 12 and 14 kDa peptides. Although peptides smaller than 2 kDa are not resolved by our technique, these results suggest that the antigens that are localized immuno-histochemically by MAC-18 in the L-NSC are much larger than the allatostatin and -tropins that at this moment have been isolated from insects, which are all below 2 kDa. We screened the L-NSC from *L. decemlineata* immuno-histochemically for the presence of these low-molecular weight allatostatins with diverse specific antisera. None of these sera yielded positive reactions on the L-NSC (Schooneveld, unpublished), which suggests that the putative allatostatin from this beetle is different from those that are characterized in other species. The results of Granger and Janzen (1987) with *Manduca sexta*, show that larger peptides of 6.8 and 13 kDa are involved in the control of CA activity, and our MAC-18 immuno-reactive peptides may be members of this category.

The isolation procedure described here is new for insect neuropeptides since it is based on a combination of HPLC, preparative electrophoresis and immunological detection, whereas the classical method employs successive HPLC steps and detection with a bio-assay. Our method is relatively simple and straightforward, since fewer purification steps are required compared to the classical method, which avoids peptide losses. This is reflected in the small amount of brain extract required to obtain sequence information; sequences were obtained from peptides isolated from "only" 500 brains. Now that commercial ready-made electrophoresis gels are available that are specially adapted to small peptides with a molecular weight of 2 kDa or higher, we anticipate that the micro-isolation of such peptides from insect tissues becomes routine. On the other hand, the resulting purified peptide is presented as a band which has been cut out of a Western blot; and shorter sequences are obtained from this material compared with HPLC purified peptides. Possibly, this problem is a result of the limited binding force of small peptides to the Immobilon-P membranes, but our efforts to overcome this problem by coating the membranes with polybrene (Legendre and Matsudaira, 1989) were unsuccessful. Alternatively, peptides can be electro-eluted from the gel instead of Western blotting, and direct sequencing after this purification step is also possible (Kurth and Stoffel, 1990).

An additional advantage of our purification method is that the nearly purified peptide is maintained in solution by SDS in the final purification step. Peptide losses by lyophilisation, or binding at surfaces of vials or pipet tips, or during additional chromatographic steps are thereby avoided. The latter cause of peptide loss may be a result of the non physiological conditions that are necessary for reversed phase (RP) HPLC with water/acetonitrile gradients, the method which is used by most researchers for the final purification steps. This method results in a very effective concentration and desalting of peptides, and especially larger peptides may be unstable under these conditions. In the case of our earlier attempts to isolate the MAC-18 immuno-reactive peptide from an homogenate of 2000 brains, we experienced that the peptide disappeared during the second HPLC-purification step. We suggest that the peptide forms aggregates as a consequence of the combination of desalting, concentrating and the increase in acetonitrile concentration. Aggregation of pure peptide during RP-HPLC analysis was noticed for the *L. decemlineata* MAG-peptide (Smid, unpublished results), and in general, acetonitrile is known to induce ß-sheet formation and denaturation in peptides (Riesner *et al.*, 1996). The fact that thus far mostly small neuropeptides have been isolated from insects may be a consequence of the common use of RP-HPLC for micro-isolation of insect neuropeptides.

We intend to continue the characterization of the MAC-18 immuno-reactive peptides that are described here by employing molecular methods, analogous to the strategy that proved to be successful for isolation of the 8 kDa MAG-peptide (Smid *et al*, 1997). We will amplify the mRNA encoding these peptides by reversed transcriptase PCR, using primers which are designed by retro-translation of amino acid sequences.

Acknowledgements

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5

Expression of a male accessory gland peptide of *Leptinotarsa decemlineata* in insect cells infected with a recombinant baculovirus.

Abstract

The male accessory glands (MAGs) of *Leptinotarsa decemlineata* produce an 8 kDa peptide, designated Led-MAGP, that is recognized by monoclonal antibody MAC-18. The site of synthesis, amino acid sequence and the gene encoding this peptide have been documented (Smid and Schooneveld, 1992; Smid *et al.*, 1997). The primary structure is homologous to the *N*-terminal hexa-repeat section of the chicken prion protein (Harris *et al.*, 1991). The biological function of the Led-MAGP has yet to be determined. For further research, large amounts of Led-MAGP is required, both for the production of a more specific antiserum, as well as for application in bio-assays. This paper describes the expression of Led-MAGP in insect cells infected with recombinant baculovirus, and the production of a polyclonal antibody against this recombinant peptide. The peptide was expressed under the control of the polyhedrin promotor. The resulting product was HPLC-purified, and analysis on Western blots immuno-labelled with MAC-18 confirmed that the correct peptide was produced. Purified recombinant peptide was also analyzed by Edman degradation and mass spectrometry; this indicated that it was *N*-terminally blocked and that the methionine residue at position 7 was oxidized. Large scale production resulted in the formation of aggregations of Led-MAGP, nevertheless a substantial proportion remained in a soluble state and could be harvested.

A polyclonal antiserum encoded #87 was produced against recombinant Led-MAGP and its specificity was tested on Western blots of authentic peptide and on LM an EM sections of MAGs. All labelling results were equal to those obtained after MAC-18 labelling. However, antiserum #87 proved to be superior compared to MAC-18, since it recognizes the MAG peptide in normally fed, sexually active males, whereas MAC-18 labelling can only be accomplished after 7 days of starvation of the males. Therefore, the new antiserum #87 enables us to study the transfer dynamics of the Led-MAGP in histological sections.

Introduction

Many male insects produce hormone-like substances that influence female behaviour after mating. These substances are produced in the male accessory glands (MAGs) and are transferred to the female during mating via the ejaculate (Chen, 1984). In a few species the peptidergic nature of such substances has been established and they are referred to as sex-peptides (Kubli, 1992). In the case of *Drosophila melanogaster*, the sex-peptide proved to be a 36 amino acid peptide, that stimulates oviposition and suppresses female receptivity (Chen *et al.*, 1988). At least part of these effects are achieved because sex-peptide activates juvenile hormone biosynthesis in the female's corpus allatum (Moshitzky *et al.*, 1996); it has allatotropic activity. In addition to this sex-peptide the MAGs of *D. melanogaster* produce several additional products (Wolfner, 1997), among them a peptide that stimulates egg-laying, and that contains a region that is similar to the egg-laying hormone from *Aplysia californica* (Herndorn and Wolfner, 1995).

We previously described the immuno-localization of a peptide in the epithelial cells and lumen of the MAGs of the Colorado potato beetle, *Leptinotarsa decemlineata* (Smid and Schooneveld, 1992). The monoclonal antibody MAC-18 which was used for this study was raised earlier against putative neuropeptides of this beetle (Schooneveld *et al.*, 1989), and specifically recognizes the peptide content of the lateral neurosecretory cells, which innervate the corpus allatum, to regulate the activity of this gland by the release of

allatotropic and or -static peptides (Khan *et al.*, 1983). From these data, we suspected that the immuno-reactive MAG peptide might be a hormone, with allatotropic activity, like sex-peptide from *Drosophila*. The amino acid sequence as well as the nucleotide sequence of the DNA encoding this peptide has now been elucidated (Smid *et al.*, 1997), but the physiological function remains to be ascertained.

The Led-MAGP is 74 amino acids long (8 kDa) and does not show any resemblance to other known insect peptides, including the *Drosophila* sex-peptide. It contains a continuous stretch of 7 imperfect hexa-repeats. These hexa-repeats are homologous to the 8 hexa-repeats that are present at the *N*-terminus of the chicken prion protein (ch-prp) (Harris *et al.*, 1991). Although the physiological function of the prion proteins is unknown, recent evidence shows that the 8 hexa-repeats serve as a signal that is recognized by the clathrin coated-pits (Shyng *et al.* 1995), which triggers the uptake of the prp molecule in the endocytotic pathway. This novel mechanism which is responsible for prp uptake is demonstrated in mouse as well as in chicken, suggesting that this type of uptake is a universal mechanism. Since a similar hexa-repeat sequence is found in the Led-MAGP of the *L. decemlineata*, the question arises whether this novel endocytosis mechanism also operates in insects.

We now wish to determine the function of the *L. decemlineata* Led-MAGP. Our strategy is to trace the peptide during and after mating and to localize its target organ by using immuno-histochemical techniques. The monoclonal antibody MAC-18 which is used so far for monitoring the Led-MAGP in immuno-histochemical assays is unsatisfactorily for this purpose since it detects the Led-MAGP in MAGs from starved, sexually inactive males (Smid and Schooneveld, 1992). However, Led-MAGP is also present in normally fed animals, as determined by biochemical procedures, such as Western blotting (Smid *et al.*, 1997). Starvation apparently induces conformational alterations, necessary for immuno-histochemical labelling by MAC-18. For immuno-histochemical detection of Led-MAGP in normally fed males, a new antibody is required that is capable to detect the Led-MAGP in sexually active males. Such requirements are more likely to be met by using an antiserum than a monoclonal antibody: an antiserum (or "polyclonal" antibody) recognizes several epitopes on the antigen, while a monoclonal can recognize only one epitope. Thus, a conformational alteration of Led-MAGP is less likely to block binding of an antiserum than of a monoclonal antibody.

It was anticipated that we would need large amounts of Led-MAGP, both for raising the antiserum as well as for subsequent bio-assays. Rather than extracting the required amounts of Led-MAGP from dissected MAGs, the baculo-virus-insect cell expression system (Summers and Smith, 1987) was exploited to produce the Led-MAGP. Insect cells would provide the best environment to produce the Led-MAGP in its physiologically active conformation. In this paper a recombinant Led-MAGP was successfully expressed in insect cells using a baculovirus recombinant, and purified. A antiserum against recombinant Led-MAGP was produced with superior specificity compared to that of MAC-18, by comparison of immuno-labelling of Led-MAGP on Western blots, as well as on LM and EM sections of MAG tissue.

Materials and methods

Insects

Adult Colorado potato beetles of both sexes were kept under long-day conditions (18 h photophase). MAGs were dissected from 14-day-old beetles in ice-cold saline solution as described before (Smid *et al.*, 1997). For certain applications (see below) animals were starved for 7 days prior to dissection.

Viruses and cells.

Sf21(K) Cells, derived from the armyworm *Spodoptera frugiperda*, were grown at 27°C in Hinks medium supplemented with 10% fetal calf serum as described (Vlak and Odink, 1979). The *Autographa californica* multiple-nucleocapsid nucleopolyhedrovirus strain AcMNPV/PAK6 (Kitts and Possee, 1993) was used as parental virus. Propagation and titration of viruses on Sf21 cells was done as described by Summers and Smith (1987).

Construction of the recombinant baculovirus

DNA recombination methods were essentially as described by Sambrook *et al.* (1989). When this experiment was initiated, only the nucleotide sequence encoding amino acids 3-74 was known (designated α-[met^7] MAGP, Smid *et al.*, 1997), whereas the *N*-terminal amino acids were known by Edman degradation. The plasmid containing this sequence was used as a template for PCR in order to obtain the entire open reading frame (ORF) of the Led-MAGP[1-74], flanked by BamH1 sites and start and stop codons. It should be emphasized that the gene fragment obtained by this procedure does not encode a signal sequence, and therefore the expected result is that the recombinant peptide remains within the cytoplasm of the infected cells.

One forward (5'-CG**GGATCC**ATGtctgatCCGGATCAACAGATGCATC-3') and one reverse primer (5'-CG**GGATCC**TCATTTTCGGGAGAGTCC-'3) were designed (BamH1 sites printed in bold, start and stop codons underlined). The first two triplets after the start codon (lower case) were deduced by retro-translation of the Led-MAGP amino acid sequence. PCR amplification was performed as described (Smid *et al.*, 1997). Standard procedures were used for the isolation of the DNA product. The BamH1 restriction fragment was ligated downstream of the polyhedrin promoter in the AcMNPV transfer vector pAcDZ1 (Zuidema *et al.*, 1990). This vector contains an *E. coli* LacZ reporter gene for easy identification of the recombinant virus. The ligated product (PacWD1) was cloned into *E. coli* DH5α cells (Gibco-BRL) according to the manufacturer's instructions. The orientation of the fragment was checked by restriction enzyme analysis using an asymmetrical Nsi1 site. The Led-MAGP ORF was checked by nucleotide sequencing, using the CircumVent^{tm} Thermal Cycle Dideoxy DNA Sequencing Kit (New England BioLabs), according to the instructions of the manufacturer. The obtained nucleotide sequence of the insert was found to be identical to the original cDNA and the used primers.

One µg purified PacWD1 DNA and 2 µg AcMNPV/PAK6 DNA were cotransfected into Sf21 cells as described by Kitts and Possee (1993). Transfected cells were assayed for the production of ß-galactosidase and cells producing recombinant virus were isolated by plaque purification. Plaques expressing ß-galactosidase activity were analyzed by PCR using forward and reverse primers as described above. In addition, positive plaques were dot-

blotted on Hybond-N filter (Amersham) and probed with ^{32}P-labelled Pacdz1-WD1 DNA using standard procedures, to confirm the insertion of the Led-MAGP ORF.

Production and purification of Led-MAGP from baculovirus infected Sf21 cells.

Sf21 cells were grown either in monolayers for the initial small-scale experiments or in spinner culture bottles, to a density of 2.10^6/ml and were infected at a multiplicity of infection of 20. Cells were harvested after 48-72 hrs, centrifuged and washed twice in phosphate buffered saline (PBS, Oxoid, Dulbecco 'A'). The pellet was homogenized by sonification in extraction buffer containing 80% methanol, 2% acetic acid and 0.1% 2-(methylthio)ethanol (Sigma). Further purification procedures, including heptane extraction, boiling in water, HPLC purification, SDS-PAGE, Western blotting and detection of Led-MAGP by monoclonal antibody MAC-18 were performed as described before (Smid *et al.*, 1997). The reversed phase HPLC column used, was a Techogel C_4 with a particle size of 5 µm and a pore size of 500Å (HPLC Technology, UK). No more than 5.10^7 homogenated cells were applied on the column per run. Identical peaks from different runs were combined when necessary.

Analysis of the N-terminal amino acid sequence and mass spectrometry.

Analysis of the amino acid sequence from HPLC purified recombinant peptide by automated Edman degradation was carried out by Dr. R. van der Schors, Free University of Amsterdam, The Netherlands, using an automated pulse-liquid phase peptide sequencer (Applied Biosystems, Inc., model 473).

Determination of molecular weight of the HPLC-purified peptide was performed by electro-spray ionization mass spectrometry. Lyophilized HPLC fractions were redissolved in 7 mM trifluoroacetic acid in 60% acetonitrile. The mass determinations were carried out by Dr. R. van der Schors, Free University of Amsterdam, The Netherlands, using a Fisons BioQ triple-quadrupole mass spectrometer equipped with an electro-spray atmospheric pressure ionization source. The mobile phase was 50% acetonitrile, the flow rate was 5 µl/min.

Production of a polyclonal antibody.

HPLC-purified Led-MAGP, resulting from the extraction of approximately $1.5.10^8$ infected cells, was used for the immunization of two female HsdPoc:Dutch rabbits. The HPLC peak with retention time of 32.6 was reduced to 10% of the original volume by evaporation in a speed vac concentrator (Savant). The volume was adjusted to 500 µl with PBS, mixed 1:1 with Freund's complete adjuvant and injected into two rabbits subcutaneously. Two identical amounts of Led-MAGP were administrated in Freund's incomplete adjuvant at four week intervals. Two weeks after the second booster injection, 20 ml blood was collected from each rabbit and designated antiserum #32 and #87 respectively.

Western blotting and immuno-histochemistry.

The specificity of serum #87 for Led-MAGP was checked by (1) Immuno-staining of Western blots of Led-MAGP purified from accessory glands by HPLC as described (Smid *et al.*, 1997), (2) Immuno-histochemical staining of paraffin sections that were prepared from MAGs taken from males that were starved for 7 days, (such males are no longer sexually

active), or from normally fed, sexually active males; tissue was fixed in Bouin Hollande Sublimé (BHS, Vieillemaringe *et al.*, 1984), as described before (Smid and Schooneveld, 1992), (3) Immunogold labelling of epon sections that were prepared from glutaraldehyde-fixed MAGs as described before (Smid and Schooneveld, 1992). For all experiments mentioned above, the antibody serum #87 was used at a dilution of 1:2000. Controls were performed under identical conditions, using pre-immune serum, diluted 1:500, instead of antiserum #87. No immuno-labelling was achieved this way.

Results

Isolation and analysis of recombinant Led-MAGP.

Recombinant peptide was purified using identical procedures as described previously for the isolation of authentic Led-MAGP from MAGs of *L. decemlineata (Smid* et al., 1997), except for that we used other but similar HPLC equipment. The retention time of authentic peptide on this set up was determined at 29.7 mins (Fig. 1a). When recombinant peptide from a methanolic extract of 10^7 infected Sf21 cells was analyzed on HPLC, the chromatograph contained a distinct peak of retention time 29.9 min (Fig. 1b). This peak was subjected to Western analysis using MAC-18 and indeed contained an 8 kDa. immuno-reactive peptide (not shown). The peptide band appeared as a doublet, exactly as we described for the authentic Led-MAGP (Smid *et al.*, 1997). A control homogenate from Sf21 cells infected with wild type AcNPV/PAK6 virus did not contain this "29.9" peak (not shown).

To further check the identity of the recombinant peptide, the *N*-terminal amino acid sequence was analyzed by automated Edman degradation. However, no signal was obtained, and it was concluded that most likely the N-terminal serine was blocked by acetylation. The sample was then analyzed for molecular weight determination by mass spectrometry. This yielded a value of 8060 Da for the recombinant peptide, while the calculated mass of α-[met^7]Led-MAGP is 8001. This discrepancy can be explained assuming that the Led-MAGP molecule is modified at two positions by: first, acetylation of the *N*-terminal serine, resulting in a blocked *N*-terminus (see above), and second, oxidation of a unique methionine residue at position 7. These modifications would result in addition of 43 and 16 Da respectively to the mass of Led-MAGP, which yields a total calculated mass of 8060 Da, a value matching the actually measured mass of 8060 Da. This approximation does not allow for other modifications. Since the authentic peptide was also not post-translationally modified (Smid *et al*, 1997), we assume that we have synthesized a recombinant peptide which is identical to the authentic peptide, except for a blocked *N*-terminus and an oxidized methionine at position 7.

Large scale production of Led-MAGP

Spinner culture bottles with 200 ml medium were used for large scale production of Led-MAGP. Conditions were similar to the above described previous Led-MAGP production and purification on analytical scale, except that the HPLC was slightly modified by insertion of an additional gradient mixer and changing the injection loop from 100 µl to 1 ml. Calibration of this new set-up showed that the retention time of authentic Led-MAGP was

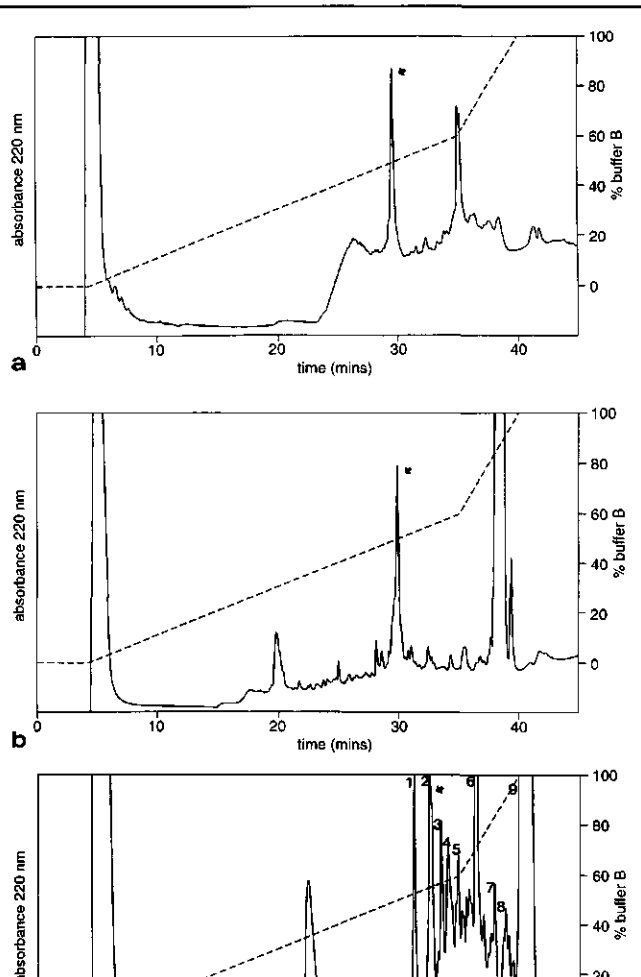

Fig. 1a. Chromatogram of reversed phase HPLC of an homogenate of 25 pairs of MAGs. Eluent A: water/0.14% TFA; B: acetonitrile/0.1% TFA. The 29.7 peak, representing the authentic Led-MAGP is indicated (arrow).
Fig. 1b. Chromatogram of reversed phase HPLC of an homogenate of 1.10^7 infected cells, same conditions as (1c). The 29.9 peak representing the recombinant Led-MAGP is indicated (arrow).
Fig. 1c. Chromatogram of reversed phase HPLC of an homogenate of 7.10^7 infected cells, conditions as described under (1a), with a slightly modified HPLC set-up, as described under materials and methods. The retention time of authentic Led-MAGP was 32.6 mins under these conditions. The numbered peaks were assayed on Western blots (Fig. 2); Peak No. 2 (arrow) is the 32.6 peak.

now 32.6 min (not shown). When methanolic extracts of infected cells were analyzed by HPLC, an entirely different chromatogram was obtained (Fig. 1c, compare to Fig. 1b). Several additional peaks were observed. Western analysis of all peaks with retention time between 30-40 min using MAC-18, revealed that the peak with retention time of 32.6 contained the majority of the 8 kDa Led-MAGP, although several other peaks also contained the immuno-reactive 8 kDa peptide (Fig. 2). Furthermore, immuno-reactive peptides smaller than 8 kDa were detected, and in some cases even different immuno-reactive proteins with higher molecular weights were detected. Our interpretation of this variability is that under the conditions of large scale production, aggregates containing the Led-MAGP as well as break-down products of Led-MAGP were formed. Such aggregates and break-down products will behave differently on the reversed phase column, and may dissociate (partially) under the denaturing conditions used for SDS-PAGE, resulting in the appearance of 8 kDa peptide on Western blot analysis of different peaks. The reproducibility between chromatograms obtained from different cultures was poor, with regard to peaks representing aggregations and break down products from Led-MAGP. Nevertheless the "32.6" peak was consistently present. Due to the formation of aggregates, a determination of Led-MAGP yield was not possible.

Production of antiserum, staining properties and specificity tests.

Antisera #32 and #87 were tested by incubation of Western blots from HPLC-purified authentic Led-MAGP. Both antisera, diluted 1:2000, detected the 8 kDa Led-MAGP, but antiserum #87 gave a significantly stronger reaction than antiserum #32 (not shown). The former antiserum was used for all further experiments.

To further compare the specificity of antiserum #87 to monoclonal antibody MAC-18, paraffin sections of MAGs of *L. decemlineata* were incubated. Initially, MAGs were dissected from males that were starved for 7 days, as described by Smid and Schooneveld, (1992). Both antibodies gave similar results, revealing a small percentage of epithelial cells, as shown in Fig. 3. However, when MAGs were dissected from normally fed males, MAC-18 staining was negative as expected, but antiserum #87 revealed a strong labelling of epithelial cells; and a much higher percentage of the epithelium was immuno-labelled compared to the MAGs of starved males (Fig. 4).

No such qualitative differences were found at the ultrastructural level. As described earlier for MAC-18 staining (Smid and Schooneveld, 1992) the immunogold label is associated with secretory vesicles in the cytoplasm containing characteristic fibril-like condensations. The same fibril-like condensations are immuno-labelled in the gland's lumen. Antiserum #87 shows exactly the same staining pattern (Fig. 5). Immuno-labelling was obtained only on MAG's from animals that were starved for 7 days. MAG's from normally fed animals were negative with MAC-18 as well as antiserum #87. The tissue processing requirements of tissue necessary for EM, apparently did not allow for the same immuno-recognition that was achieved after tissue processing for LM.

The immuno-labelling of Led-MAGP in MAG cells from normally fed beetles could thus not be achieved on EM sections. We sought the reason for this discrepancy with our LM results in the differences in tissue fixation. The fixative used for LM, BHS, is a mixture

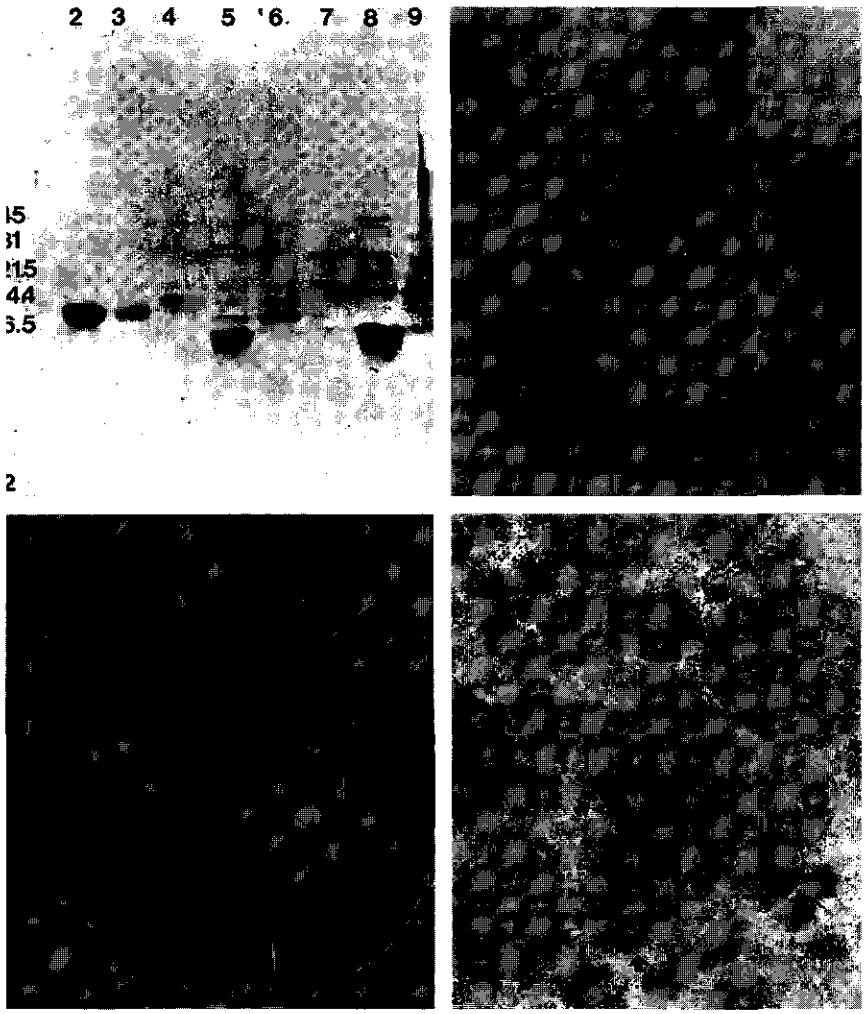

Fig. 2. Western blot of the numbered HPLC peaks in Fig. 1c., separated by SDS-PAGE and immuno-labelled by MAC-18. Molecular weights are indicated at the left. Corresponding peak numbers are indicated above each lane. Note the 8 kDa Led-MAGP band in peak 2.

Fig. 3. Light microscopical section of MAGs from males that were starved for 7 days and immuno-labelled by MAC-18. Immuno-reactive cells are indicated by arrows. Bar = 30 μm.

Fig. 4. Light microscopical section of MAGs from normally fed males, immuno-labelled by #87. Immuno-reactive cells are indicated by arrows. Note the high numbers of immuno-reactive cells, when compared to sections from MAGs of starved males (Fig. 3) Bar = 30 μm.

Fig. 5. Electron microscopical section of MAGs from males that were starved for 7 days, immuno-labelled by #87. Immuno-labelling is visualized by 10nm gold particles. Immuno-reactive granules are indicated by arrows. Note that the granules contain fibril-like crystals, which are characteristic for Led-MAGP. Bar = 500 nm.

containing 12% formaldehyde, picric acid, mercury chloride and copper sulphate (Vieillemaringe *et al.*, 1984). The fixative for EM contained 2% glutaraldehyde, neutrally buffered with cacodylate. Indeed, LM sections of MAG tissue from normally fed animals revealed immuno-reactivity only after fixation in BHS, and not in neutrally buffered glutaraldehyde. We found that the combination of a low pH and the presence of copper sulphate in the fixative was necessary to obtain immuno-reactivity (not shown). Addition of these components to our EM fixative resulted, unfortunately, in unacceptable morphology.

Discussion

This paper presents the successful production of a recombinant *L. decemlineata* Led-MAGP, and the production of an antiserum against this recombinant Led-MAGP. The data obtained from the biochemical characterization of recombinant Led-MAGP provides evidence that it originates from the expression of the inserted Led-MAGP gene: (1) Its HPLC retention time is identical to the retention time of the authentic peptide, and the corresponding HPLC peak is not present in control homogenate insect cells infected with wildtype baculovirus; (2) It is recognized by MAC-18; (3) Its molecular mass, as determined from Western blots is 8 kDa, like the authentic peptide and (4) An antiserum raised against the recombinant peptide recognizes the authentic peptide on Western blots as well as on LM and EM MAG sections.

However, the molecular mass of the recombinant peptide as determined by mass spectrometry, proved to be 60 Da higher than expected when the recombinant peptide would be unmodified, like the authentic peptide. This small difference cannot be the result from differences in the amino acid sequence, since the nucleotide sequence of the inserted Led-MAGP ORF was checked before co-transfection, but can be accounted for if we assume the occurrence of two post-translational modifications. One modification is the acetylation of the *N*-terminal serine, the other is the oxidization of the unique methionine residue at position 7, as argued before. The absence of any signal from automated Edman degradation of the *N*-terminus also suggests acetylation of the *N*-terminal serine, resulting in a blocked *N*-terminus. The reason why the recombinant peptide is modified while the authentic peptide is unclear. It may originate as a result of the expression without signal sequence of the former peptide, while the latter peptide is expressed with a 17 amino acid hydrophobic signal sequence (Smid *et al.*, 1997). This signal sequence routes the peptide through the secretory pathway into the lumen, while the recombinant peptide, which lacks such an "export ticket", remains within the cell and may undergo different modifications. The baculovirus expression system is well capable to perform diverse *N*-terminal modifications (Lanford, 1988), however, further experiments are necessary to proof the cause of the small difference in size between authentic and recombinant Led-MAGP.

Large scale production of recombinant-peptide results in the formation of a surprisingly complex mixture of compounds with immunologically similar epitopes. Western blots from SDS-PAGE-separated HPLC fractions showed that MAC-18 recognized more than one 8 kDa peptide. Different HPLC fractions proved to contain immuno-reactive materials. Our interpretation is that this multiplicity of bands is not a result of poor antibody specificity, but rather reflects the property of Led-MAGP to polymerize under the conditions of in vitro synthesis and subsequent purification steps. The capability of the baculovirus expression

system to produce oligomerized peptides has been described before (Lanford, 1988). We like to recall the presence of 7 hexa-repeats in this molecule, that are homologous to the hexa-repeats in the chicken prion protein (ch-prp, Harris *et al.*, 1991). Such proline and glycine-rich repeating sequences indicate that the molecule is coiled in solution (Bazan *et al.*, 1987), and is likely to cause self-aggregation. Indeed, preliminary results show that authentic Led-MAGP exists in multiple oligomeric forms and that only a minor part is monomeric (Smid, unpublished). This oligomerization may have important consequences for the physiological activity of the Led-MAGP. Therefore, it is necessary to determine the form in which the Led-MAGP is eventually released into the female's hemocoel: as a monomer, as a oligomer or complexed to other proteins.

The purified recombinant peptide proved highly immunogenic in rabbits. The specificity of the antiserum #87 against recombinant Led-MAGP is confirmed by tests on Western blots and LM as well as EM sections. Under the conditions which were used for immuno-labelling with MAC-18 (i.e., using starved males), antiserum #87 yields identical results. However, antiserum #87 also detects Led-MAGP in LM sections from normally fed males, as expected from the broader specificity of a polyclonal antiserum compared with a monoclonal antibody. The most probable explanation for this observation is that starvation induces a conformational change in Led-MAGP. While antiserum #87 recognizes Led-MAGP in either conformation, MAC-18 can recognize Led-MAGP only in the conformation that is present in starved males. At the ultrastructural level, the MAC-18 or antiserum #87 label is associated with fibril-like crystals, and these crystals were not found in the MAG cells of normally fed animals. We conclude that starvation induces the Led-MAGP to polymerize into higher order structures such as fibril-like crystals, and only in this form led-MAGP is recognized by MAC-18.

Unfortunately, the antiserum #87 did not allow for the immuno-localization of the MAG peptide in normally fed males at the EM level. We found that the reason for this was the different fixative used for ultrastructural preservation. This fixative was buffered at a neutral pH and did not contain copper sulphate, while the fixative for LM was acidic and did contain copper sulphate, a combination which was found to be a prerequisite to obtain immuno-labelling. The apparent role of copper sulphate is interesting in the light of the homology with the ch-prp hexa-repeats. These repeats are known to have high copper affinity, each repeat being able to bind one copper molecule (Hornshaw *et al.*, 1995). This copper binding indeed altered secondary structure of synthetic hexa-repeats, and the same may occur with our Led-MAGP. Whether the binding of copper to the Led-MAGP occurs in vivo, remains to be investigated.

Since only normally fed males are sexually active, the ability of antiserum #87 to recognize the Led-MAGP in its normal, unpolymerized form is an important improvement over the use of MAC-18 activity. The new polyclonal antiserum #87 will be used for our future research instead of MAC-18. Sections of male as well as female reproductive tissues taken from animals that are fixed while *in copula*, will be immuno-labelled with antiserum #87 to study the transfer dynamics of Led-MAGP. Then, female target tissues will be analyzed with antiserum #87 to study the possible rate of oligomerization or complexation to other proteins, while recombinant Led-MAGP may be used for in vitro and in vivo bio-assays to study its biological function.

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6

Transfer of a male accessory gland peptide to the female during mating in *Leptinotarsa decemlineata.*

Abstract

The male accessory glands of the Colorado potato beetle, *Leptinotarsa decemlineata*, produce an 8 kDa. peptide (Led-MAGP), whose primary structure and gene have been described. To investigate the possible function of this peptide in the female, we traced the passage of Led-MAGP through the male and female reproductive tracts during and after the mating process. We used immuno-histochemical procedures to localize Led-MAGP employing a specific polyclonal antiserum, that was raised against a recombinant Led-MAGP. Mating couples of Colorado potato beetles were interrupted after various time intervals after the initiation of mating, and fixed by perfusion to immobilize the transferred male accessory gland secretions and sperm cells. Serial sections of reproductive tracts were prepared and immuno-labelled.

These time-lapse studies indicated that the contents of the male accessory glands are transferred during the first few minutes of mating, and are deposited in the female spermathecal duct. Sperm cells are transferred later, 4-6 minutes after the beginning of mating. Led-MAGP is found in two forms, namely a granular, doughnut-shaped form and a homogeneously dispersed form. After deposition in the spermathecal duct, the immuno-reactive product dissolved within 4-6 minutes. It is argued that the Led-MAGP crosses the duct wall and enters the hemolymph and that its function must therefore be sought outside the female reproductive tract.

Introduction

The male accessory glands of *Leptinotarsa decemlineata* produce a variety of secretory products (Peferoen and de Loof, 1984), the functions of which are largely unknown. In *Drosophila*, peptide pheromones are among these male accessory gland products, that are transferred to the female during copulation. They enter the female's hemolymph and modify behaviour and physiology in such a way that the female refuses further matings and accelerates oogenesis and oviposition (Chen *et al.*, 1988). A single peptide, called the sex-peptide evokes both effects in *Drosophila* (see Kubli, 1996, for review).

We are interested in the function of the male accessory glands of *L. decemlineata*, and adopted an immuno-histochemical approach to detect peptides that are immunologically related to neuropeptides. We identified a population of secretory cells in these glands, immuno-localized with a monoclonal antibody encoded MAC-18 (Smid and Schooneveld, 1992). This antibody was previously prepared for the detection of the lateral neurosecretory cells, which are involved in the regulation of corpus allatum activity (Schooneveld *et al.*, 1989).

The antigen was subsequently purified from male accessory gland homogenates and its primary amino acid sequence and the nucleotide sequence encoding it determined (Smid *et al.*, 1997). It proved to be an 8 kDa. peptide, designated Led-MAGP (*Leptinotarsa decemlineata* Male Accessory Gland Peptide), containing a sequence of 7 hexa-repeats which are homologous to the N-terminal hexa-repeats of the chicken prion protein (Harris *et al.*, 1991). Prion proteins in general are presently of great interest because of their role in certain fatal degenerative neurodiseases in vertebrates, such as Scrapie, BSE, Creutzfeldt-Jacob disease, and Kuru (Chesebro, 1990). The physiological function of the

prion protein is still unknown, and the same holds true for Led-MAGP. It should be emphasized that no bio-assay for Led-MAGP is available at this time. The presence of Led-MAGP in the lumen of the male accessory glands suggests that it is transferred to the female along with the ejaculate during copulation. Knowledge as to precisely when and where in the female Led-MAGP is released during copulation, and what happens to it thereafter, may reveal its function, or alternatively rule out several candidate functions. Led-MAGP and sperm cells both pass through the ejaculatory duct on their transfer to the female during insemination, but they are derived from different compartments of the male reproductive system, namely the male accessory glands and the testes respectively. It is of interest to know whether the transfer of Led-MAGP occurs separately or mixed with the sperm cells. In case of separate transfer, the question would arise whether the peptide is deposited at the same location as the sperm cells. Both timing and place of deposition have therefore been studied in detail. We created mating situations, in such a way that the start and termination of a mating could be monitored precisely. By sacrificing mating couples at specific moments during mating and processing male and female reproductive tracts for immuno-histochemical observation, we were able to study the transfer of substances in detail.

Materials and methods

Animals

Colorado potato beetles, were obtained from a laboratory colony kept under long-day conditions (18 h photophase). All mating experiments were carried out using 10-day-old, sexually mature beetles. Males were kept together with females, but were kept apart for the two days preceding all mating experiments. Certain aspects of the mating behaviour of this beetle have been studied by Thibout (1982). His observations were of help in designing the following experiments.

Mating experiments.

Series of mating experiments were performed with virgin females. One or two males were put together with one female in a thoroughly cleaned glass jar with cover. Time was measured after observation of intromission of the aedaegus into the female uterus ($t = 0$). Copulating pairs were left in the glass jars until t = 0, 1, 2, 4, 6, 8, 10, 15, or 30 minutes, respectively (mating for this species takes about 10 mins). Males were routinely removed from the jar after the retraction of the aedeagus to prevent remating. At the end of a time interval mating was interrupted and both male and female were immediately fixated by whole-body perfusion. For this purpose, animals were decapitated and injected with 1 ml of Bouin Hollande sublimé (BHS, Vieillemaringe *et al*, 1984). This fixative was used because one of its components, copper sulphate, was found previously to be required for adequate immuno-location of Led-MAGP in male accessory glands (Smid *et al.*, in prep.). The perfused beetles were then immersed in BHS solution for 30 mins or longer. Reproductive tissues were dissected under saline solution and post-fixed in BHS overnight, washed in several changes of 70% ethanol overnight, dehydrated and embedded in Paraplast-Plus. Serial sections of 7 µm were mounted on poly-l-lysine (Sigma) coated slides.

Immuno-histochemistry.

The production and specificity of the antiserum used for this study, designated antiserum #87, is described elsewhere (Smid *et al.*, in prep). The antiserum is raised against a recombinant Led-MAGP, and recognizes authentic Led-MAGP in the glandular cells as well as in the lumen of the male accessory glands with high specificity.

Paraffin sections were deparaffinized and incubated overnight at room temperature in antiserum #87, diluted 1:2000 in 10% normal swine serum (Dakopatts, Denmark) in PBS. Secondary antibody labelling was performed by 2 hrs incubation at room temperature with swine anti-rabbit peroxidase conjugate (Dakopatts, Denmark), diluted 1:200. Labelling was visualized with N', N'-diaminobenzidin (Sigma) and sections were counterstained with Mayer's haematoxylin. Control sections received the same treatment except that the incubation in antiserum #87 was substituted by an incubation in PBS. This yielded no signal in the tissues examined.

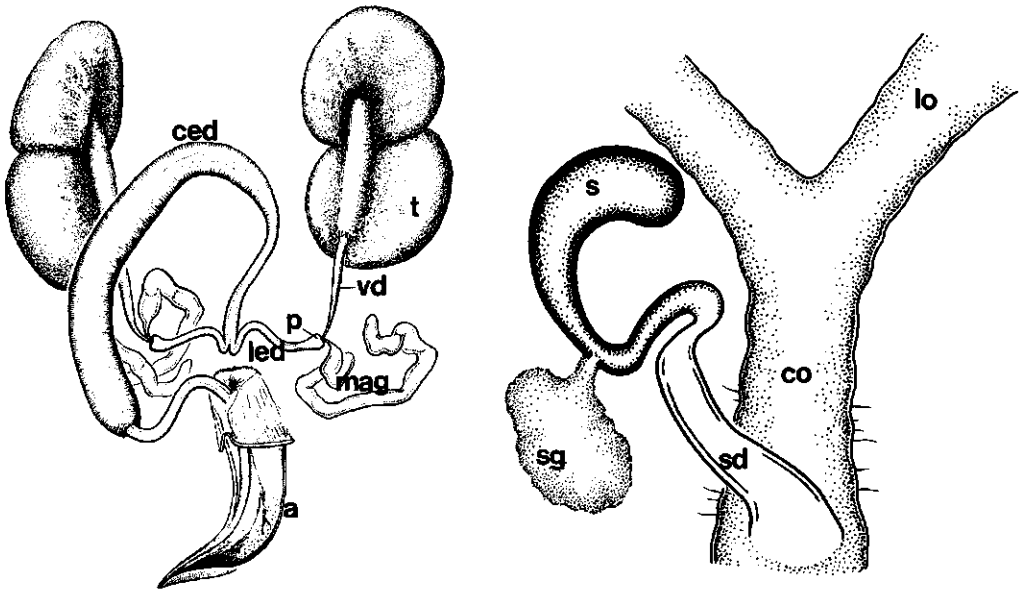

Fig 1. Schematic drawing of (a) the male reproductive tissues and (b) the female reproductive tissues. The size of the spermathecal duct and spermatheca is not drawn to scale, for clarity. t, testis; vd, vas deferens; mag, male accessory gland; p, prostate; led, lateral ejaculatory duct; ced, common ejaculatory duct; a, aedeagus; co, common oviduct; lo, lateral oviduct; sd, *spermathecal* duct; *s, spermatheca; sg, spermathecal gland.*

Results

Behavioral aspects of mating.

For a proper interpretation of the results, we needed detailed information on externally visible aspects of mating behaviour, such as intromission and withdrawal of the aedeagus. Monitoring the timing of events was essential but very time-consuming in the absence of

arrangements to favour male mating drive as well as female receptivity. Use of only virgin females and males that had been kept apart from females for two days proved most effective. Nevertheless, the onset of mating under the conditions in our laboratory remained unpredictable. Females sometimes refused motivated males, but became receptive after we replaced that male with another individual. The female apparently discriminates between individual males, and probably uses this capacity to select certain males for mating. On the other hand, males that were not motivated to mate, often changed this attitude when a second male was added to the glass jar.

Mating typically lasts approximately 10 min. In many cases, a couple remated immediately afterwards, sometimes even without the male leaving the female in between. We therefore routinely removed males after withdrawal of their aedeagus, to prevent a second mating. In certain experiments, we allowed second matings to study differences in the passage and deposition of the different male products.

Transport of substances in the male reproductive tract during mating.

A schematic drawing of the male and female reproductive tracts is given in Fig. 1. Paraffin sections of male accessory glands from males that were kept together with females reveal the presence of immuno-reactive epithelial cells (Fig. 2). When male beetles were kept without females for two days, secretory products accumulated in the male accessory glands, as the glands appear swollen compared with the glands from normally mated males. Furthermore, paraffin sections stained with antiserum #87 reveal a smaller proportion of immuno-reactive cells and an accumulation of immuno-reactive material in the lumen (Fig. 3). Such a degree of accumulation was not found in mated males. Whereas the epithelial cells appear columnar in the normally mated males (Fig. 2), they appear flattened after two days without matings (Fig. 3), due to the swelling of the male accessory glands.

The peptide in the lumen shows different degrees of condensation: it may appear finely dispersed in certain individuals while others contain doughnut-shaped granules and even large clusters of such granules (Fig. 3).

At the beginning of mating, the lateral and common ejaculatory ducts are empty. The vas deferens is packed with sperm cells. After 1 min, immuno-reactive material has reached the lateral and common ejaculatory duct as well as the endophallus (Fig. 4). Sperm cells are still not observed outside the vas deferens at this time, and this situation lasts until 4-6 mins. Thereafter, sperm cells are transmitted through the ejaculatory duct, whereas the immuno-reactive material is not. This situation lasts until the end of the copulation.

The amounts of immuno-reactive material and sperm cells released during one mating did not visibly affect the content of the male accessory gland or vas deferens, respectively. Thus, only a small proportion of stored materials is used for one mating. This is in agreement with our observation that males can remate immediately.

Transport of substances in the female reproductive tract during and after mating.

Mating experiments were routinely done with virgin females, to avoid confusion with sperm cells or immuno-reactivity remaining in the female tracts from previous matings. At the beginning of mating, the spermathecal duct and spermatheca are devoid of any immuno-reactive material or sperm cells. After 1 or 2 mins, immuno-reactive material appears in the

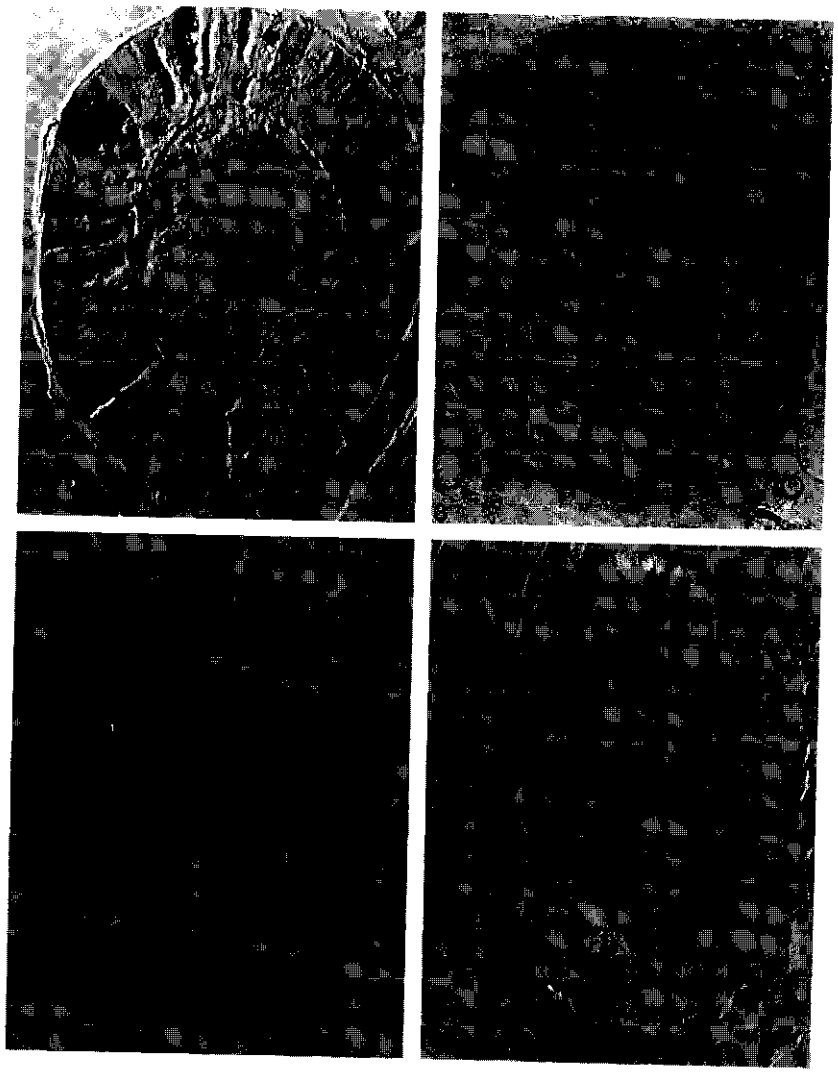

Fig. 2. Cross section of a male accessory gland, from a normally mated male, immunolabeled with antiserum #87. Arrows indicate immuno-reactive cells. Bar = 30 μm.

Fig. 3. Cross section of a male accessory gland, from a male that has been kept apart from females for two days, immuno-labelled with antiserum #87. Immuno-reactive cells, are absent in this section, small arrows indicate immuno-reactive granules in the lumen. Bar = 100 μm.

Fig. 4. Lateral ejaculatory duct of a male, 2 mins after the initiation of mating, with immuno-labelled with antiserum #87. Note immuno-reactive material coming from the male accessory glands, with homogenous as well as granular appearance. Bar = 30 μm.

Fig. 5. Spermathecal duct from a female, 2 mins after initiation of mating, immuno-labelled with antiserum #87. Note the doughnut-shaped immuno-reactive granules (arrows), coming from the male accessory glands. Bar = 30 μm.

spermathecal duct, usually adhering against the duct wall. This immuno-reactivity has the same variable appearance as described for the male, i.e. homogenous staining or doughnut-shaped granules (Fig. 5). The immuno-reactive material was never found in the spermatheca, in the spermathecal gland, or in the oviduct. The immuno-reactivity disappeared within 4-6 mins, before the transfer of sperm. Whether this disappearance is caused by proteolytic processing of the peptide and subsequent loss of immuno-reactivity, or, alternatively, by passage of the peptide through the spermathecal duct wall is not clear. The low levels of immuno-reactivity in the spermathecal duct did not allow for observation of the latter.

Four to 6 mins after the beginning of mating, the spermathecal duct is filled with sperm cells. The sperm cells migrate towards the spermatheca, and after 15-30 mins the spermathecal duct is essentially empty.

When males were allowed to re-inseminate a female immediately after a first mating, we found that such remating again began with the transfer of male accessory gland secretions, followed by transfer of sperm. As a consequence, Led-MAGP is released in this case into the mass of sperm cells that is still present in the spermathecal duct from the first mating (not shown).

Discussion

Importance of the fixation protocol.

This paper describes the transfer of male accessory gland secretions, in particular Led-MAGP, and of sperm during mating of *L. decemlineata*. Our approach was to follow the transfer of Led-MAGP with sensitive immuno-histochemical methods, rather than biochemical methods. Perfusion fixation of whole bodies caused a very rapid immobilization of substances in transfer and prevented leakage of substances from the reproductive tracts during dissection. Such a leakage is likely to occur. Meikle *et al.*, (1990) described the transfer of the enzyme esterase-6 from the male accessory glands of *Drosophila melanogaster*. This enzyme is transferred to the female in the first minute of mating, and is then found almost immediately in the hemolymph.

Phasic transfer of Led-MAGP and sperm during mating.

The transfer of Led-MAGP precedes the transfer of sperm cells. Apparently, the prostate can regulate the passage of the contents from the vas deferens and male accessory gland separately. This sequence of transfer is also found in *D. melanogaster* (Fowler, 1973). Both substances are deposited at the same place, but since there is a difference in timing, the substances are not necessarily brought in contact. For example, in *Drosophila*, esterase-6 passes through the spermathecal duct wall and enters the hemolymph before the sperm cells arrive (Meikle *et al.*, 1990). Among the male accessory gland secretions of *D. melanogaster* is a factor that cause the incapacitation of sperm present from previous matings in the seminal vesicle (Harshman and Prout, 1994). Since this male accessory gland factor is unable to discriminate between self and non-self sperm (Gilchrist and Partridge, 1995), it is essential for a male that this factor is inactivated before his sperm cells are transferred. A third peptide component of the male accessory gland secretion in *D. melanogaster* is a serine protease inhibitor, with unknown specific function,

which neither enters the hemolymph nor is inactivated, but persists in the reproductive tract for several hours and becomes mixed with the sperm cells (Coleman *et al.*, 1995). Thus, in the case of *Drosophila*, there are male accessory gland substances that, in order to exert their specific function, (1) should not become mixed with own sperm in active form (sperm incapacitation); (2) should become mixed with the sperm cells (serine esterase); (3) should enter the hemolymph immediately at the beginning of mating (esterase-6). The observed transfer sequence in the order of male accessory gland secretions vs sperm cells is the only possible sequence that allows for the combination of functions of the male accessory gland secretions in *D. melanogaster.* In conclusion, phasic transfer of male accessory gland substances and sperm is essential for a proper functioning of male accessory gland substances in *D. melanogaster*, and the same may be true in *L. decemlineata.*

Possible role of Led-MAGP.

In *L. decemlineata*, the spermathecal duct (sd, Fig. 1b), permits penetration by the long slender endophallus, which telescopes out of the aedeagus during copulation (structure is hidden in the aedeagus, (a) in Fig. 1a). Led-MAGP is deposited in the spermathecal duct a few minutes before the sperm cells arrive at the same place. Led-MAGP is nevertheless not mixed with sperm cells, as immuno-reactivity is no longer observed at the time of sperm arrival. While speculating on the possible function of the peptide, we first considered the possibility that a process of enzymatic breakdown of peptide results in the rapid decline in immuno-reactivity. In *D. melanogaster*, an example of such a process has been described for a male accessory gland protein that is involved in the elevation of egg laying. This protein, encoded Acp26Aa (Herndorn and Wolfner, 1995) is deposited within the female duct as a propeptide, and is enzymatically processed before it passes through the duct wall (Park and Wolfner, 1995). In the case of Led-MAGP, such limited enzymatic processing may also occur, but is unlikely to cause complete abolition of immuno-recognition. One should bear in mind that we developed and used a polyclonal antiserum which was raised against the entire Led-MAGP, and which would probably recognize at least part of the fragments that result from even extensive enzymatic digestion. In conclusion, a rapid enzymatic breakdown of Led-MAGP in the spermathecal duct is unlikely to be the cause of the observed decline in immuno-staining.

An alternative explanation for the rapid decline in Led-MAGP immuno-reactivity in the spermathecal duct is a fast transfer through the duct wall, in order to enter the hemolymph. As stated above, *D. melanogaster* esterase-6 is an example of a substance that is transferred to the hemolymph within minutes (Meikle, 1990). Proof for such a transfer mechanism in *L. decemlineata* awaits either the demonstration of Led-MAGP in female hemolymph, or immuno-electron microscopical analysis of the spermathecal duct wall. The latter option is at present not possible due to the specific fixation requirements necessary for antiserum #87 (Smid *et al.*, in prep.) which are inadequate for electron-microscopical observation. The former option, isolation of Led-MAGP from female hemolymph, is also proving to be a major challenge. Biochemical analysis of recombinant Led-MAGP (Smid *et al.*, in prep) and preliminary results on in vitro binding experiments (Smid, unpublished), indicate that Led-MAGP has a tendency to bind to itself to form multimers, or to other proteins to form large complexes or aggregates. This tendency may mask Led-MAGP in

female hemolymph and prevent its detection. The demonstration of Led-MAGP in hemolymph therefore, awaits methodological improvements.

In an earlier report (Smid *et al.*, 1997), an hypothesis was proposed with respect to the possible function of Led-MAGP. By analogy with the function of the homologous hexa-repeats in the chicken prion protein (Shyng *et al.*, 1995), which constitute a signal that induces coated pit-mediated endocytosis of the prion protein, Led-MAGP might stimulate endocytosis of certain hemolymph proteins to which it binds. A candidate process that could be affected this way in the female is the uptake of yolk proteins by the developing oocytes. Such a mechanism would result in the elevation of oocyte growth and hence in stimulation of oviposition. From the present study, it is concluded that Led-MAGP is indeed transferred to the female during mating, and, most likely, rapidly enters the female's hemolymph. Future experiments will test the hypothesis regarding the function of Led-MAGP.

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7

Chemical mate guarding and oviposition stimulation in insects: a model mechanism alternative to the *Drosophila* Sex-Peptide paradigm.

Abstract

Males of many insect species transfer substances within the ejaculate that enhance fecundity and inhibit remating of the post-mated female. This paper intends to bridge in this context between the research fields of reproductive physiology and evolutionary ecology, with special emphasis on sex-peptide from *Drosophila melanogaster* and Led-MAGP from *Leptinotarsa decemlineata*. It is argued that sex-peptide must be regarded as a signal from male to female, and that a mere transfer of such a signal to the female cannot induce inhibition of remating and enhancement of fecundity. The presumed activity of Led-MAGP on the other hand, is directly on the uptake of yolk-proteins, to enhance fecundity. Furthermore, it has a toxic side-effect, which increases the cost of remating for a female, and this may result in remating inhibition. The male accessory glands of *Drosophila* also produce a toxic compound, and it is suggested that this compound may have Led-MAGP-like activity. In this context, the action of sex-peptide is rather as a short term warning signal for the toxic compound.

Males of many insect species add substances to the ejaculate, that render the post-mated female sexually unreceptive and stimulate oocyte growth and oviposition. The substances are hence called remating inhibiting substances (RIS) and fecundity enhancing substances (FES), respectively (Gillot, 1988). They pass the wall of the female's receptive tract and enter the hemolymph, where they exert their effect (Wolfner, 1997). RIS and FES are produced in the male accessory glands.

As stated by Eberhard and Cordero (1995), physiological studies on RIS and FES have been carried out in a near vacuum of modern evolutionary theory. This paper intends to bring these two fields into contact. I will here compare the biological activity of two accessory gland products in an evolutionary context: the sex-peptide from *Drosophila (Chen* et al., 1988), and a peptide from the Colorado potato beetle, designated Led-MAGP (Smid *et al.*, 1997).

Female promiscuity, the problem of paternal confidence and mate guarding.

From Darwin's treatise *The descent of man and selection in relation to sex*, (1871), it becomes clear that, as cited from Smith (1984), "*Darwin believed females to be generally monogamic. This view (though a popular and comforting one among human males) is wrong for most species. A variety of circumstances compel females of most species to (at least occasionally) mate with several males during a single reproductive cycle, such that sperm from two or more ejaculates may contest the fertilization of relatively few ova.*" Although a single mating usually suffices in order to collect sperm for fertilization, mating with more than one male offers several advantages to the female compared to monogamy: (1) She gains offspring with increased genetic variability (Knowlton and Greenwall, 1984; Thornhill and Alcock, 1983); (2) She is able to select for males that produce competitive sperm, in the sense that the most viable sperm will win the 'contest' for the fertilization of her ova (a process called sperm competition; Parker, 1970); and (3) to collect younger sperm (which

gives rise to less developmental artifacts of the offspring; Baker and Bellis, 1993); (4) Remating increases her chances to select the most competitive mates, competitive in the sense of the ability of a mate to achieve access to a female, despite the presence of barriers that are raised by rivalling males.

Besides the above mentioned advantages associated with remating, there are at the same time costs involved with remating. Each remating will cost additional energy and time, and exposes the female to predation. The post-mating receptivity of a female will depend on a balance between cost and gain of an additional mating. An urge of the female to remate, renders a male with the problem of uncertain paternity: after copulation he has no guarantee that his mate will not remate, and that his sperm will fertilize her ova. Hence several male behavioral adaptations have evolved among species to increase paternal confidence. Guarding a female to prevent her from mating with rivalling males (mate guarding) is the most common adaptation. In conclusion, the actual female remating rate depends on her receptivity and on the presence and efficacy of a male in preventing remating.

"Chemical mate guarding" in insects as an adaptation to long-term sperm storage of the female.

In insects, the problem of paternal confidence is particularly complicated, due to the presence of a special organ in the female, the spermatheca, in which sperm can be stored for a prolonged time (Miller *et al.*, 1994). Eggs are fertilized just prior to oviposition by release of sperm cells from the spermatheca, which means that there may be a considerable period of time between insemination and egg fertilization. Furthermore, a female can collect sperm from several males in the spermatheca, thereby intensifying the process of sperm competition. Thus, a male must guard its mate until eggs are deposited in order to guarantee paternity. During mate guarding, however, a male cannot feed or mate another female, hence mate guarding is a costly reproductive strategy. Several insect species have developed more sophisticated mechanisms than physical mate guarding by adding the above mentioned RIS and FES to the ejaculate. RIS are generally interpreted as a form of 'chemical mate guarding' (Miller *et al.*, 1994): such substances turn the female into an unreceptive state, even in the absence of the male. FES, in addition, accelerate egg maturation, and thereby increase the success of a mating in terms of the male's offspring numbers.

Chemistry of chemical mate guarding: the sex-peptide paradigm of Drosophila.

RIS and FES activities have been demonstrated in several insect orders, but only few RIS and FES have been chemically characterized (see Miller *et al.*, 1994) The most complete picture comes from *Drosophila melanogaster* (Kubli, 1996; Wolfner, 1997). In this species, RIS and FES activity is combined in one peptide, called 'sex-peptide' (Chen *et al.*, 1988; Kubli, 1996). Injection of virgin fruit flies with sex-peptide turns off mating and stimulates oviposition, but the effects last only one day, compared with up to 11 days after a normal mating (under controlled laboratory conditions; in nature the unreceptivity after mating of the female is more variable; Wolfner, 1997). Apparently, additional factors are required for a long-term RIS and FES effect.

Sex-peptide probably has the female's corpora allata among its targets, as it increases corpus allatum activity in vitro (Moshitzky, 1996). Thus, sex-peptide may act like a hormone in the female. However, it represents a pheromone as well (Kubli, 1996); it acts as a signal between conspecific individuals. This bivalent interpretation may be confusing: as a pheromone (signal), its activity requirers the co-operation of both sexes (see below); while as a hormone, it may over-rule the female's hormonal regulatory system. Such 'aggressive' hormones may have evolved by mimicry of female messenger molecules (Eberhard, 1997). However, one should bear in mind that the activity of hormones in general is subjected to complex processes of down-regulation. If the activity of a male-derived hormone has any disadvantage for the female, it will soon become down-regulated: females will be selected for their ability to neutralize the unwanted male messenger molecule. The ability to neutralize will develop quickly, since the mechanism for such neutralisation already exists. Thus, transfer of a hormonal RIS or FES can only become an evolutionary stable strategy, if its action does not represent a disadvantage for the female; it requires the female's co-operation. I will therefore interpret sex-peptide as a pheromone, i.e. as a signal, throughout this paper.

Chemical data from species other than Drosophila.

RIS and FES from other species are less completely characterized, but in most investigated species so far they are peptides. Interestingly, some reports on efforts to isolate such peptides from *Musca domestica, Delia antiqua,* and *Culex tarsalis* (summarized in Miller *et al.*, 1994) mention the strong tendency of these peptides to bind or aggregate with other proteins, or possibly to themselves. These properties are a severe pitfall during isolation, hence full characterization of the peptides is still not accomplished. The biological activity of these 'sticky peptide' preparations after injection is identical to the effects of a normal mating: in the case of *D. antiqua* a single injection with this peptide renders the female unreceptive for her entire 6 wk lifetime. In this respect, the 'sticky' peptides are very different from the *Drosophila* sex-peptide, which activity lasts for only one day.

A prion-like 'sticky' peptide from the male accessory glands of Leptinotarsa decemlineata.

I recently described the structure of a peptide from the male accessory glands of the Colorado potato beetle, *Leptinotarsa decemlineata* (Smid *et al.*, 1997). This peptide, designated Led-MAGP (*Leptinotarsa decemlineata* Male Accessory Gland Peptide) was isolated by employing a monoclonal antibody for the detection instead of a bio-assay. Led-MAGP may be of the same category as the sticky peptides described above: it binds with itself and with other proteins. It could nevertheless be characterized, since we used an antibody for detection, instead of a bio-assay. This method allows denaturing conditions during part of the purification procedure. The biological function of Led-MAGP is still uncertain, due to its complex physio-chemical properties, however, a hypothetical function has been proposed (Smid *et al.*, 1997), and will be described below.

Analysis of its amino acid sequence showed that the peptide, with a MW of 8 kDa., contains 7 imperfect hexa-repeats at the N-terminus. Comparison of the amino acid sequence with peptide databanks, showed that the hexa-repeat section is similar to the hexa-repeats at the N-terminus of the chicken prion protein (Harris, 1991). Prion proteins are raising considerable interest at present, since they are considered to be the primary

cause of certain degenerative neurodiseases, including Creutzfeldt-Jakob disease in humans and Scrapie and bovine spongiform encephalopathies (BSE) in cattle. Prion proteins are notorious for their tendency to form very stable aggregates and this feature is characteristically involved in the diseases they cause. The hexa-repeats are thought to be responsible for the formation of prion-aggregates (Goldfarb *et al.*, 1991; Krasemann *et al.*, 1995).

The normal, non-pathogenic function of prions has attracted much less attention than the pathological aspects, and is still obscure. However, recent progress has been made concerning it's N-terminus with the hexa-repeats. It was found that this repeat sequence serves as a signal that triggers the uptake of the prion protein in the coated pit-mediated endocytosis route of nerve cells (Shyng *et al.*, 1995). This so-called internalization signal function of the N-terminal repeats has been demonstrated for mammalian and avian prion proteins. Now that a similar sequence has been found in Led-MAGP, the question is raised if this sequence functions as an internalization signal in insects as well. The possibility and consequences of this idea, together with the tendency of Led-MAGP to bind to other proteins, have been discussed in an earlier report (Smid *et al.*, 1997), in which a hypothesis is proposed on the function of Led-MAGP. This hypothesis will be described here in an evolutionary context, and compared to the role of the *Drosophila* sex-peptide.

A model mechanism for Led-MAGP.

The following role of Led-MAGP seems likely, but is still hypothetical. After transfer of Led-MAGP to the female spermathecal duct (Smid, 1998) it enters the hemolymph and binds readily to hemolymph proteins. The bound hemolymph proteins become 'tagged' this way, with a signal for endocytosis. In the adult female, the most abundant protein to become tagged is the yolk protein vitellogenin, while the process of endocytosis is most active at the surface of developing oocytes. As a result, the uptake of vitellogenin will be induced. Normally, the uptake route for vitellogenin in oocytes is by receptor-mediated endocytosis (Valle, 1993). Led-MAGP can be regarded as a soluble receptor that acts in parallel to the normal route, thereby accelerating oocyte growth. This model mechanism for Led-MAGP is essentially different from the *Drosophila* sex-peptide: the latter is assumed to function as a hormone, acting on the corpus allatum. Led-MAGP, however, actively participates in the uptake of vitellogenin.

This functional difference has important consequences for the interpretation of RIS and FES in an evolutionary context, as shown in the following section. For proper interpretation, I will define here the terms sex-peptide and Led-MAGP according to their respective mechanism, but independently from the species from which they were isolated. Thus, sex-peptide refers to a RIS and/or FES that acts as a hormone, as described for *Drosophila*, while Led-MAGP refers to a RIS and/or FES that acts on the uptake mechanism of yolk proteins, as proposed for the Colorado potato beetle.

Interpretation of RIS and FES as signals from male to female, and Zahavi's handicap principle.

In the case of sex-peptide, the RIS and FES is considered to be a signal (a pheromone) and this implies that both sexes co-operate in this matter. The signal tells the female that sperm is transferred, and the female responds by refusing further mating and

by increasing the rate of oocyte growth. Since the effect of sex-peptide is dose dependant, the female may use RIS/FES to evaluate the quantity and quality of the ejaculate (where quality must be interpreted in its broadest meaning).

I will argue that, for two reasons, this interpretation cannot directly be applied to sex-peptide. Cordero (1995) evaluated the interpretation of RIS and FES as signals from male to female, according to the handicap principle (Zahavi, 1975; Grafen, 1990, Godfray, 1995). According to this principle, a signal should represent a substantial cost (handicap) for the sender, relative to the cost of the ejaculate that it is signalling about, in order to be reliable for the receiver, and evoke a response that is reliable for the producer. In other words, the signal is reliable for the female, if it is costly to produce for the male, and more costly for those males that produce only small size or low quality ejaculates. If the signal would not be costly, the male has the possibility to cheat, that is, to signal for a better ejaculate than is actually transferred. The conclusion for the interpretation of sex-peptide as a signal, is that the cost to produce sex-peptide (a male accessory gland contains only 3 pmole sex-peptide; Balmer, 1988) is by no means related to the cost of the ejaculate and, as a consequence, males can easily cheat about the quality of their ejaculate by producing more sex-peptide. Sex-peptide can therefore not be used by the female as a signal indicating ejaculate quality or quantity.

Negative effects of RIS on female fitness.

A second argument against a signal function of sex-peptide is the fact that the action of a signal requires co-operation of both sexes. Such co-operation implies that RIS and FES activity must be advantageous not only to the male but also to the female, otherwise the female can choose not to respond to the signal. I will argue that, at the contrary, both RIS and FES constitute a conflict between the sexes.

First, consider remating inhibition. As stated above, there are costs involved in mating, such as time investment and exposure to predation (Thornhill and Alcock, 1983), and it is often concluded that refusing additional matings until sperm stored in the spermatheca is exhausted, is the optimal strategy for a female. However, as stated above, multiple mating also offers important benefits to a female. As a consequence, a female faces a trade-off between costs and gains of each additional mating. This trade-off determines the post-mating receptivity of a female: normally she will enter a refractory period after a mating that is optimal with regard to this trade-off. However, the optimal refractory period from the male's point of view is obviously longer (until all his sperm has been used) than from the female's point of view, and this results in a conflict between the sexes.

From the above, it can be concluded that transfer of a signal like sex-peptide as such cannot increase the unreceptive period beyond the optimum that follows from the female's balance between cost and gain. Hence, the term 'chemical mate guarding' (Miller *et al.*, 1994) cannot be used for sex-peptide, since the mere transfer of a signal cannot substitute for physical prevention of remating: a female will not respond to a signal that reduces her fitness. An increase of the duration of the unreceptive period can only be accomplished, if the male somehow increases the cost for an additional mating. Otherwise the male must prevent remating physically by mate guarding.

Negative effects of FES on female fitness.

Depending on the specific selection environment of a species, the female can balance energy allocation between reproduction and maintenance. A high investment in reproduction requires a high metabolic rate, which is negatively correlated with the female's life-span (Giesel, 1989; Riha and Luckinbill, 1996). In other words, the female faces the trade-off between high reproductive rate and high mortality, and the optimal balance depends on the specific environment of a species. From the male's point of view, the long-term life-span of his mate is relatively unimportant compared to the immediate reproductive rate (after insemination with his sperm), hence elevation of female reproductive rate by the action of FES from the male gives rise to a conflict between male and female: the male prefers that higher numbers of eggs are produced than the female. Again, transfer of a signal like sex-peptide cannot increase the reproductive rate beyond the optimal balance as determined by the female, since this would have a negative effect on the female fitness. It is not advantageous for the female to respond to the signal.

Induction of enhanced vitellogenesis and reduced receptivity by Led-MAGP

The proposed role of Led-MAGP as described above is to influence directly the uptake of hemolymph proteins: unlike sex-peptide it is not a signal. An important aspect of its functioning is that it seems to bind to hemolymph proteins rather unspecifically; it does not only bind to vitellogenin, which is by far the most abundant protein in a reproducing female, but also with other, somatic hemolymph proteins (Smid, unpublished). Thus, as long as the vitellogenin concentration in hemolymph is high, most Led-MAGP molecules will bind with vitellogenin. However, if the female does not respond to the increased uptake of vitellogenin by an increased production of vitellogenin, binding of Led-MAGP to other hemolymph proteins will predominate. This results in unwanted uptake of these proteins into the developing oocytes, and a decrease in the concentration of somatic proteins in hemolymph. Therefore, Led-MAGP will trigger the female to increase the production of vitellogenin, and thereby, increase metabolic rate.

The consequences of the action of Led-MAGP are several. The uptake of yolk proteins in the developing oocytes will be accelerated, thereby resulting in increased egg production. As a result, the production of vitellogenin will increase, which means that metabolic rate must be maintained at a higher level. The costs for this high metabolic rate for the female, as described above, is a decreased life-span. Furthermore, the binding of Led-MAGP to somatic hemolymph proteins, and their subsequent sequestration into the oocytes adds additional costs for the female, and it is inferred that high amounts of Led-MAGP will have a toxic effect; each additional mating will result in an increase of Led-MAGP-titre in the female's hemolymph. The female can therefore not remate until the Led-MAGP level becomes low, otherwise mating will lead to a toxic concentration of Led-MAGP. In other words, by adding Led-MAGP to the ejaculate, the male increases the cost for the female to remate. This cost is essentially a side effect of the stimulation of oocyte growth, but it will nevertheless result in remating inhibition. The female faces a trade-off between costs and gains of a remating, and the increase of cost will increase the refractory period. In summary, the effects of Led-MAGP are stimulation of oviposition, decreased life-span and extended female refractory period.

The Led-MAGP hypothesis explains how sex-peptide can act as a signal.

In this paper I have argued that ejaculate substances that enhance fecundity and refractory period, constitute a conflict between the sexes, and, for that reason, RIS and FES cannot be considered as a signal. Nevertheless, in the case of *Drosophila*, it has unequivocally been demonstrated, that a RIS and FES is combined in sex-peptide, a substance that acts as a pheromone and a hormone, and thus as a signal. The question arises, why the female responds to sex-peptide, since the response implicates that the action of sex-peptide must somehow represent an advantage to the female.

A possible explanation for this contradiction can be found in some studies of the cost of mating in *Drosophila*. It is known, that mating in this insect reduces life-span (Fowler and Partridge, 1989), and that this reduction is caused by an unidentified 'toxic' substance from the male accessory glands (Chapman *et al.*, 1995). The presence of a toxic compound in the accessory glands may seem puzzling, but can be explained if it has a Led-MAGP-like activity, the toxicity being a side-effect.

Interestingly, a *Drosophila* mutant strain (dunce) which is known to remate twice as often compared with wild type flies, suffers from increased mortality due to this frequent remating. Dunce flies remate frequently because of their inability to respond to sex-peptide (Chapman *et al.*, 1996). From this, it becomes clear that the action of sex-peptide reduces the cost of mating for the female, the cost that is caused by the toxic compound in the accessory glands. It prevents remating and thereby accumulation of the toxic compound in the female hemolymph. In this context, the immediate effect of sex-peptide is advantageous for the female (as it is, obviously, for the male). Down-regulation of the hormonal activity of sex-peptide has therefore no selective advantage for the female, and will not evolve.

Concluding remarks.

In conclusion the signal function of sex-peptide can be understood if it acts in combination with the toxic compound fro the male accessory glands, (possibly with Led-MAGP activity). This requires a different interpretation of sex-peptide. The message of sex-peptide to the female is not that sperm is collected, so that it makes sense to go into a refractory period and accelerate vitellogenesis. Instead, the message is a warning for the transfer of the toxic male accessory gland compound. Thus, chemical mate-guarding is not induced by sex-peptide, but rather by the toxic compound. It would be interesting to identify this toxic compound, as well as the 'sticky' accessory gland peptides from *Delia, Culex* and *Musca*, to see whether they are related compounds. Toxic effects of substances from the male accessory glands have been reported also for *Acanthoscelides obtectus* (Das *et al.*, 1979).

In the review of *Drosophila* male accessory gland products by Wolfner (1997), the transfer of these products to the female is poetically called a 'token of love'. However, I would suggest that 'love' is not a suitable term in this context; the transfer of toxic compounds might better be designated as a 'token of selfishness'.

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8

General discussion

Introduction

Neurohormone-like peptides in male accessory glands.

This thesis is a study on the structure and function of a peptide produced in the male accessory glands of *Leptinotarsa decemlineata*. The peptide raised our interest because it was recognized immunologically by one of our monoclonal antibodies, encoded MAC-18, that was produced to specifically localize a putative peptide hormone in the lateral neurosecretory cells (L-NSC) in the brain. Axons from these cells innervate the corpora allata, which are hormone glands that produce the insect growth regulator juvenile hormone. The L-NSC are thought to regulate the activity of the corpora allata by release of peptide hormones through their axons. It was considered to be highly relevant that MAC-18 recognizes not only these putative neuropeptides from the L-NSC, but in addition also some of the glandular cells in the male accessory gland, as will be outlined below.

The function of the male accessory glands has not yet been elucidated in *L. decemlineata*. In several other species, it has been shown that these glands produce substances that are added to the semen during copulation and affect the female's post-mating physiology and behaviour: the female becomes temporarily unreceptive for males and oviposition is accelerated. In *Drosophila melanogaster*, one peptide from the male accessory glands has been shown to evoke both reduced receptivity and accelerated oviposition after injection. This peptide is named sex-peptide and its full structure has been elucidated (Chen *et al.*, 1988; Kubli, 1996). At the time of the beginning of this study, it was suggested that (one of the) targets of sex peptide in the *Drosophila* female might be the corpora allata: by stimulation of the production of juvenile hormone, the female might increase the level of egg production (Kelly *et al.*, 1994). This idea was strengthened by the isolation of a peptide from the male accessory glands of *Locusta migratoria* (Paemen *et al.*, 1991) that is homologous to the allatotropic neuropeptide which stimulates the activity of the corpus allatum in the brains of *Manduca sexta* (Kataoka, 1989). Indeed, the allatotropic activity of sex peptide has recently been demonstrated in *Drosophila* (Moshitzky *et al.*, 1996). Thus, the idea arises that male accessory gland peptides, such as sex peptide from *Drosophila*, may be similar in function as well as in structure to allatotropic peptides from the brain.

Objectives of this thesis.

If the above described idea is applied to the bi-localization of MAC-18 immuno-reactivity in the L-NSC and the accessory glands, it becomes clear that both antigens might be similar not only in structure but also in function. The peptide in the neuroendocrine system would then have an allatotropic function; that in the male accessory glands a regulatory function in the female that was inseminated. The hypothesis raised and tested in this study was that the male accessory gland of *L. decemlineata* is producing a peptide

with an allatotropic activity in the recipient female, that has a structural resemblance to the peptide in the L-NSC. The aim of this study was, to isolate the MAC-18 antigens from the male accessory glands as well as from the L-NSC, in order to compare their structure and function.

Chemical analysis of the MAC-18 antigens

Comparison of the MAC-18 antigens from male accessory glands and L-NSC.

The MAC-18 antigen from the male accessory glands has been successfully purified and characterized. Its amino acid sequence and the nucleotide sequence of the gene encoding it is described in chapter 3. The isolated peptide was designated Led-MAGP: *Leptinotarsa decemlineata* Male Accessory Gland Peptide. The antigen from the L-NSC was only partially characterized, as described in chapter 4. It became clear that the antigens from the male accessory glands and the L-NSC are not identical. The antigens have a different molecular weight, chromatographic properties, and amino acid sequences at their N-terminus. Furthermore, mRNA encoding Led-MAGP was found exclusively in the male accessory glands, not in the brains. An additional argument against the occurrence of Led-MAGP in the L-NSC, was obtained after the production of recombinant Led-MAGP by a recombinant baculovirus. A polyclonal antibody raised against this recombinant Led-MAGP recognizes Led-MAGP in the male accessory glands (chapter 5) but fails to detect the antigen in the L-NSC (Smid, unpublished). The original observation of the presence of an immunologically detectable substance in the male accessory glands must therefore be attributed to a cross-reaction of the MAC-18 antibody.

Homology of Led-MAGP with the prion protein from chicken.

The amino acid sequence of Led-MAGP does not resemble any of the insect peptide hormones known today. Its structure resembles the N-terminus of the chicken prion protein (ch-prp; Harris *et al.*, 1991). The homology is most pronounced in a series of hexa-repeats in the N-terminus of ch-prp; Led-MAGP is much smaller than ch-prp, and its C-terminus is different.

For clarity, I will summarize some aspects of the nature and possible function of prions here because understanding of how prions work may help to understand how Led-MAGP works in the Colorado potato beetle. Prion proteins are presently in the centre of interest since they are involved in some fatal neurodegenerative brain diseases in man (Creutzfeldt-Jakob disease (CJD)) and cattle (scrapie, and bovine spongiform encephalopathies (BSE)). These diseases are caused by prp isoforms which differ from harmless prp, normally occurring in nervous tissues, only in their tertiary structure. The pathological aspects of prp have been the subject of a large body of scientific papers, especially after the recent commotion about a possible relation between the occurrence of BSE and CJD in the UK. It should be pointed out, however, that prions have also a non-pathogenic function, as they are normally occurring in healthy animals as well. The actual function of prp's is still uncertain.

The N-terminus of prp, containing the sequence with the hexa-repeats that is homologous with Led-MAGP, may represent the most interesting part of prp. Recently, this sequence was found to function as a signal that induces endocytosis of prp (Shyng *et al.*

1995). Furthermore, this sequence is also supposed to favour the formation of multimers and aggregates of prp which are characteristic of the pathogenic isoform (Goldfarb *et al.*, 1991; Krasemann *et al.*, 1995). These features of prp are shared by Led-MAGP. Led-MAGP forms aggregates (chapter 5) and small multimers (Smid, unpublished, see below), probably due to the presence of the hexa-repeats. In the model explained in chapter 3 and chapter 7, the hexa-repeat sequence functions as a signal inducing endocytosis of yolk proteins in oocytes of insects.

In conclusion, the hypothesis that the MAC-18 antigen in the L-NSC and the MAGs are identical must be rejected. The MAC-18 antigen in the male accessory glands (Led-MAGP) is different from the MAC-18 antigen in the brain.

The function of Led-MAGP.

Two strategies were considered to establish the function of Led-MAGP. The first is to study the fate of Led-MAGP after it is transferred to the female during copulation, using immunohistochemical methods; the target organ could then be localized (chapter 5, 6). The second is to develop an in vivo bio-assay to quantify the stimulation of oviposition in virgin females, by injection either of pure Led-MAGP, or of crude MAG homogenates.

Localization of the target organ of Led-MAGP.

Although monoclonal antibody MAC-18 was very efficient in the detection of Led-MAGP in the MAGs under certain conditions, it proved to be unable to detect the antigen in the form as it is transferred to the female. It was necessary therefore to produce an antibody with enhanced qualities compared with MAC-18. It was anticipated that large amounts of Led-MAGP were needed both for antibody production, and for future in vivo-testing of the activity of Led-MAGP. To avoid the tedious work of peptide purification of Led-MAGP from dissected male accessory glands, the recombinant baculovirus technique was used for the production of large amounts of recombinant Led-MAGP (chapter 5). Sufficient amounts were obtained for the generation of an antiserum with improved specificity. This antiserum, encoded #87, was successfully used to monitor the route of Led-MAGP during mating up to the female spermathecal duct, and as the peptide rapidly disappears from this duct, it was concluded that Led-MAGP is transferred to the hemolymph within minutes after its release by the male (Chapter 6). Immuno-histochemical techniques were employed once more to screen the female body cavity after mating for the presence of immuno-reactive peptide. Although some positive staining was obtained in fat-body and ovaries, the same staining was also observed in virgins (Smid, unpublished). Apparently, there is some cross reaction with substances that are endogenous to the female. Localization of possible target organs for Led-MAGP was not possible by this way.

It was next attempted to isolate Led-MAGP from hemolymph of mated females, basically following the procedures developed for isolation of MAGP from male accessory glands, that is by HPLC and detection with antiserum #87 on Western blots of SDS-PAGE gels. Immuno-reactive peptides of various sizes with various chromatographic properties were indeed present in hemolymph, but once more in mated as well as in virgin females. Some peptides, however, were found exclusively in hemolymph of mated females, but none of these peptides matched the size or chromatographic motility of Led-MAGP. It was concluded from these results that several immuno-reactive products other than Led-MAGP

are present in the female. This cross-reactivity in combination with the relatively small amount of LED-MAGP that is transferred by the male has so far prevented the detection of Led-MAGP in the female body cavity. The analytical methods should be refined to monitor the trans-body route of Led-MAGP and to detect the final site of destination.

Bio assay on binding of Led-MAGP with hemolymph proteins.

To study the possible interactions of Led-MAGP with hemolymph proteins, in vitro binding experiments were initiated. Led-MAGP, obtained from methanolic extracts of accessory glands was incubated with hemolymph and the resulting protein-peptide complexes were analyzed with native PAGE and subsequent detection on Western blots with antiserum #87. The results show that, after methanol extraction, Led-MAGP is originally present in the form of small multimers, and that these multimers bind with as yet unidentified hemolymph proteins (Smid, unpublished). This binding occurs with a variety of female as well as with male hemolymph proteins. It may be significant that Led-MAGP forms large soluble complexes under these conditions with the yolk protein vitellogenin in the female hemolymph. The observation that Led-MAGP multimers form complexes with hemolymph proteins, is implemented in a model of the function of Led-MAGP (chapter 7).

A model of Led-MAGP activity.

The model is based on two observations: (1) the homology of Led-MAGP with the chicken prion protein, and (2) the observed binding with hemolymph proteins. Led-MAGP, after its deposition into the female spermathecal duct (chapter 6) crosses the duct wall, enters the hemolymph and binds to hemolymph proteins. Led-MAGP may then, analogous to the homologous N-terminus of the prion protein, act as a signal that induces the endocytosis of the peptide-protein complex in the developing oocytes. The transfer of Led-MAGP to the female during mating would thus cause an enhanced passage of vitellogenin across the oocyte membrane, and thereby stimulate oocyte growth and, eventually, stimulate oviposition. The female cannot compensate for this increased level of vitellogenin uptake by lowering vitellogenin production, due to the fact that Led-MAGP will also bind other (somatic) hemolymph proteins, and the effect of a surplus of Led-MAGP is that it will bind to other hemolymph proteins that will become relatively abundant. Thus, a lowered vitellogenin concentration in the hemolymph will result in the undesirable uptake of somatic proteins. As a female faces a trade-off between protein allocation to reproduction versus somatic processes, the conclusion is that this balance is shifted more towards reproduction by the action of Led-MAGP. This shift is obviously beneficial to the male as his fitness is increased, but not to the female: the large investment in reproduction goes along with lower investment in maintenance, resulting in a shorter lifespan. This means that Led-MAGP is essentially "toxic" to the female, and she has to prevent that a high amount of this "toxic" compound accumulates in the hemolymph. She does so by becoming unreceptive after a mating until the Led-MAGP concentration has become low. Thus, the effect of Led-MAGP is not only a stimulation of oocyte growth, but, due to the toxic effect, also a decrease in receptivity.

It has been attempted to validate this model by injection experiments with Led-MAGP. For that reason, homogenates of accessory glands were injected into females of controlled physiological conditions such as food, age and temperature. Most importantly, the females used for this assay were not virgins like the *Drosophila* females that were used for the bio-

assay to detect sex peptide. For Led-MAGP, females were mated 7 days before the experiment; the Led-MAGP model predicts that Led-MAGP induces an acceleration of oocyte growth rather than a switch from virgin to mated physiology. Under these conditions, however, no increase in oviposition levels could be induced by injection of a male accessory gland extract, nor even by a normal mating.

Our inability to stimulate the oviposition levels of a mated female either naturally by allowing the female to remate, or artificially by injection with a homogenate of male accessory glands, can be explained when the effects of long-term inbreeding of the laboratory strain are considered. Such strain effects (i.e. effects due to long term inbreeding of insects reared under the unnatural laboratory conditions) may well be the cause of the inability to stimulate oviposition levels with male accessory gland extracts, as was shown to be the case in a strain of *Musca domestica* (Miller *et al.*, 1994). The *L. decemlineata* strain that was used for this study has been maintained for over 35 years, a period of time during which certain strain effects are likely to have developed. This strain consists of animals with an extremely high level of reproduction. This results from the lack of natural constraints as are met in the field and from the breeding method used which strongly selected for females with high reproductive activity. Egg clusters have been collected daily to hatch separate from the adult beetles, and the majority of selected eggs originate from large obviously visible egg clusters, produced by females that have the genetic outfit to produce such large clusters. The lack of most natural constrains is the cause that females can afford to allocate most of their energy in reproduction instead of maintenance. Thus, there is a strong selection for females that produce large amounts of eggs (75 per day over several weeks), resulting after 35 years in a population that consists entirely of females with a very high inherent reproductive level. The effect of Led-MAGP will be relatively small in such females compared with females that have a more average (natural) reproductive level. As a consequence, one might expect that if the balance between reproduction and maintenance is shifted artificially towards maintenance, e.g. by restricted food supply, the sensitivity for Led-MAGP will increase. Such a food-dependent effect has been reported for *Aedes aegyptii* (Klowden and Chambers, 1991). Future studies on the effects of Led-MAGP on oviposition levels of the Colorado potato beetle are perhaps better performed under restricted food supply, or, alternatively, with animals that were recently collected from field populations.

Conclusion: The strategy of reverse endocrinology.

The strategy followed in this study to determine the role of Led-MAGP is an unusual one in the sense that this peptide was isolated and characterized before its function was known. This strategy is denoted "reverse endocrinology" by Lafont (1991), the usual (classical) strategy being the isolation of a substance because of its established and/or assayable biological activity. The present study clearly demonstrates the specific advantages and disadvantages of both strategies. The classical approach requires that the biological activity of the substance to be purified is maintained during the isolation protocol, and that a convenient bioassay is available. Both requirements are unlikely to be met in the case of Led-MAGP. The purification protocol includes several steps that will interfere with the biological activity of the peptide. Further, the relative insensitivity of the laboratory strain of *L. decemlineata* to stimulate oviposition of mated females by a remating, makes it

impossible to assess the activity of the peptide. Thus, it is unlikely that the classical approach would have yielded the information that was obtained by the reverse endocrinology strategy in the case of Led-MAGP. The data presented here allowed for the definition of an assayable hypothesis as to the function of Led-MAGP, so that future experiments may unravel the function of this intriguing peptide.

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Summary

This thesis describes a study of the male accessory glands of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). These glands add various substances to the ejaculate. On mating, the ejaculate is transferred to the female, together with the substances from the male accessory glands. The function of these substances is unknown in the case of the Colorado potato beetle. From research on other insect species, we know that some of these substances stimulate the female to oviposit at a higher rate, and to refuse further matings for a certain period. These two effects are known to be evoked by the action of one peptide hormone in the case of *Drosophila melanogaster*, and this peptide is called sex-peptide. At least part of its activity is thought to be accomplished by stimulation of the corpus allatum (allatotropic) activity, as indicated by in vitro experiments. The activity of the corpora allata is normally under control of some neurons in the brain, the lateral neurosecretory cells, which innervate these glands. These neurons use as yet unidentified peptide hormones as messenger substance, and it is possible that these peptides are similar in structure and activity to sex-peptide.

Our immunohistochemical studies on the Colorado potato beetle give indirect support for the possible dual control of corpus allatum activity. We revealed that some glandular cells in the male accessory glands are labelled by a monoclonal antibody that was raised against the peptides in the lateral neurosecretory cells. The question arises whether the antigen in the accessory glands is indeed identical to the antigen in the lateral neurosecretory cells. In that case, both antigens are involved in stimulation of the corpus allatum activity. The aim of the present study is to compare the antigens in the accessory glands and the lateral neurosecretory cells, and to study the function of the former in more detail (chapter 1).

The antigen in each of the two accessory glands is present in a specific set of approximately 100 dispersed glandular cells. The immuno-reactive cells contain granules with crystalline contents, and these crystals have a rod-like appearance. Such rods are also immuno-labelled in the lumen of the gland (chapter 2).

The antigen in the accessory glands is a peptide of 8 kDa., designated Led-MAGP (*Leptinotarsa decemlineata* male accessory gland peptide). Part of the amino acid sequence has been determined. Using this structural information the gene encoding this peptide has been identified and thereby the structure of the entire peptide. The peptide is expressed exclusively in the accessory glands, not in the brains. The peptide does not resemble any known peptide hormone, but it shows a considerable degree of similarity to the N-terminus of the chicken prion protein. Prion proteins are at present in the centre of interest since they are involved in certain fatal neurodegenerative diseases in man (Creutzfeldt-Jakob disease) and cattle (scrapie, bovine spongiformic encephalopathies, better known by its acronym BSE) (chapter 3).

Four antigens from the lateral neurosecretory cells have been characterized to determine their relatedness to Led-MAGP. However, the peptides isolated all differ in size and chromatographic properties from Led-MAGP. Our initial interpretation that the antigens

from the lateral neurosecretory cells might be identical to Led-MAGP, had therefore to be rejected (chapter 4).

A recombinant baculovirus is constructed, equipped with the gene encoding Led-MAGP. This way large amounts of this peptide are produced in order to study its function. The recombinant Led-MAGP is produced by infection of insect cells cultures with the recombinant baculovirus. Large scale production is hampered by the formation of large aggregates of Led-MAGP. Nevertheless, sufficient peptide has been harvested to produce a new antibody against Led-MAGP. This antibody recognized the authentic peptide with superior specificity, compared with the monoclonal antibody used previously (chapter 5).

Microscopical analysis with the new antiserum reveals the fate of Led-MAGP during copulation. Male and female reproductive tracts were taken from mating couples for immunohistochemical analysis with the new antiserum. The route of the Led-MAGP could be analyzed in detail. Led-MAGP is transferred from the male accessory glands to the spermathecal duct in the female. Led-MAGP most probably diffuses to the hemolymph within minutes after its deposition (chapter 6).

A hypothesis is put forward as to the physiological function of Led-MAGP. This hypothesis is based on the homology of Led-MAGP with the chicken prion protein, and on the observation that it binds hemolymph protein. The N-terminus of the chicken prion protein namely contains 8 hexa-repeats, whereas Led-MAGP has and 7 hexa-repeats that are largely homologous. Although the biological function of the prion protein itself is unknown, the section with the 8 hexarepeats serves as a signal that induces the uptake of the ch-prp in the endocytosis route. By analogy, Led-MAGP could, by binding to hemolymph proteins, induce the uptake of these proteins by the developing oocytes. This way led-MAGP stimulates the growth of the oocytes on the expense of female hemolymph proteins. In other words, the balance of protein use between maintenance and reproduction, is shifted more towards reproduction. This mechanism would at the same time explain the reduction of female receptivity for mating, as remating will lead to the acquisition of too much Led-MAGP, and thus to overstimulation of reproduction at the expense of other body functions (chapter 7).

A test of the hypothesis that is proposed in chapter 7 awaits methodological improvements. Detection of Led-MAGP in the mated female, by using the specific antiserum is hampered due to cross-reactivity of several female-derived proteins. The tendency of Led-MAGP to aggregate further complicates a functional analysis. Furthermore, the available inbred laboratory strain of the Colorado potato beetle is probably un suitable for a bioassay on stimulation of oviposition (chapter 8).

Samenvatting

Dit proefschrift beschrijft een onderzoek aan de mannelijke accessorische geslachtsklieren van de Coloradokever, *Leptinotarsa decemlineata* (Say). Deze klieren voegen verscheidene stoffen toe aan het ejaculaat. Tijdens de paring wordt het ejaculaat, met deze stoffen uit de mannelijke accessorische geslachtsklieren, overgebracht naar het wijfje. De functie van deze stoffen is onbekend voor de Coloradokever. Uit onderzoek aan andere soorten weten we echter dat sommige van deze stoffen gedurende een bepaalde tijd het wijfje stimuleren tot een verhoogde ovipositie en de bereidwilligheid voor een volgende paring remmen. Het is bekend dat in *Drosophila melanogaster* deze twee effecten worden veroorzaakt door de activiteit van één peptide hormoon, het sex-peptide. Uit in vitro experimenten blijkt dat dit peptide tenminste voor een deel functioneert d.m.v stimulatie van de activiteit van het corpus allatum. De activiteit van deze klier wordt normaal gereguleerd door enkele neuronen uit de hersenen, de laterale neurosecretorische cellen (L-NSC), welke de klieren innerveren. Deze neuronen maken gebruik van tot nu toe onbekende peptide hormonen als boodschapper stof, en mogelijk zijn deze peptiden vergelijkbaar in structuur en functie met het sex-peptide.

Ons immunohistochemisch onderzoek aan de Coloradokever geeft indirecte aanwijzingen voor een dergelijke dualistische regulatie van corpus allatum activiteit. We vonden dat sommige kliercellen in de mannelijke accessorische geslachtsklieren worden herkend door een monoklonaal antilichaam dat was opgewekt tegen de peptiden uit de L-NSC. De vraag was of dit antigen in de accessorische geslachtsklieren inderdaad identiek is aan het antigen in de L-NSC. In dat geval zouden beide antigenen betrokken zijn bij de stimulering van corpus allatum activiteit. Het doel van dit onderzoek was de antigenen in de accessorische geslachtsklieren te vergelijken met die in de L-NSC, en het meer gedetailleerd bestuderen van de functie van het antigen uit de accessorische geslachtsklieren (*hoofdstuk* 1).

Het antigen in elk van de twee accessorische geslachtsklieren is aanwezig in een subpopulatie van ongeveer 100 verspreid liggende kliercellen. De immunoreactieve kliercellen bevatten korrels met een kristallijne inhoud; deze kristallen hebben een staafvormig uiterlijk. Dergelijke staafjes zijn ook immunoreactief in het lumen van de klier (hoofdstuk 2).

Het antigen in de accessorische geslachtsklieren is een peptide met een molgewicht van 8 kDa. en wordt aangeduid als Led-MAGP (*Leptinotarsa decemlineata* male accessory gland peptide). Een gedeelte van de aminozuurvolgorde is bepaald. Met gebruikmaking van deze structurele informatie is het gen dat codeert voor dit peptide geïdentificeerd, en daarmee de structuur van het gehele peptide. Het peptide wordt uitsluitend geproduceerd in de accessorische geslachtsklieren, niet in de hersenen. Het lijkt niet op enig ander peptide hormoon, maar vertoont een aanzienlijke gelijkenis met de N-terminus van het prion eiwit van de kip. Prion eiwitten staan op dit moment midden in de belangstelling, omdat ze betrokken zijn bij bepaalde dodelijke neurodegeneratieve ziekten bij de mens (ziekte van Creutzfeldt-Jakob) en in vee (Scrapie, bovine spongiform encephalopathy, beter bekend onder de afkorting BSE) (hoofdstuk 3).

Vier antigenen uit de L-NSC zijn gekarakteriseerd om hun gelijkenis met het Led-MAGP te onderzoeken. Echter, de geïsoleerde peptiden zijn alle verschillend van Led-MAGP in grootte en chromatografische eigenschappen. Onze eerste interpretatie dat de antigenen van de L-NSC identiek zouden kunnen zijn aan het Led-MAGP, moest daarom worden verworpen (hoofdstuk 4).

Er werd een recombinant baculovirus geconstrueerd, dat is uitgerust met het gen voor Led-MAGP. Op deze manier zijn grote hoeveelheden peptide geproduceerd ten behoeve van een functionele analyse. Het recombinantpeptide is geproduceerd door infectie van gekweekte insectecellen met het recombinante baculovirus. De productie op grote schaal werd bemoeilijkt door aggregaat vorming van Led-MAGP. Niettemin werd voldoende peptide geoogst voor de productie van een nieuw antiserum tegen Led-MAGP. Dit antiserum herkent het Led-MAGP met hogere specificiteit in vergelijking met het eerder gebruikte monoklonale antilichaam (hoofdstuk 5).

Het lot van Led-MAGP tijdens de paring werd opgehelderd m.b.v. microscopisch onderzoek. Het mannelijke en vrouwelijke voortplantingskanaal van parende kevers werd immunohistologisch geanalyseerd met het nieuwe antiserum. De route van Led-MAGP kon gedetailleerd worden onderzocht. Led-MAGP wordt vanuit de accessorische geslachtsklieren overgebracht naar het spermatecale kanaal van het wijfje. Led-MAGP diffundeert waarschijnlijk binnen enkele minuten na afgifte naar de hemolymf (hoofdstuk 6).

Er is een hypothese opgesteld ten aanzien van de fysiologische functie van Led-MAGP. Deze hypothese is gebaseerd op de homologie van Led-MAGP met het prion eiwit, en op de waarneming dat het bindt aan eiwitten uit de hemolymf. De N-terminus van het prion eiwit bevat namelijk 8 herhalingen van 6 aminozuren (hexa-repeats), terwijl het Led-MAGP 7 vrijwel homologe hexa-repeats bevat. Hoewel de biologische functie van het prion eiwit als zodanig niet bekend is, functioneert het deel met de hexa-repeats als een signaal dat de opname van het prion eiwit in de endocytose route induceert. Analoog hieraan zou het Led-MAGP, na binding aan hemolymf eiwitten, de opname van deze eiwitten kunnen induceren door de ontwikkelende oocyten. Op die manier stimuleert Led-MAGP de groei van oöcyten ten koste van de hemolymf eiwitten van het wijfje. Met andere woorden, het evenwicht tussen eiwitgebruik t.b.v. somatische- en voortplantingsprocessen wordt verschoven in de richting van voortplanting. Dit mechanisme verklaart tegelijkertijd de verlaging in de paringsbereidheid van het wijfje, aangezien een nieuwe paring zal leiden tot opname van te veel Led-MAGP, en dus tot overstimulatie van reproductie, ten koste van somatische processen (hoofdstuk 7).

Het testen van de hypothese die is opgesteld in hoofdstuk 7 vereist enkele verbeteringen in de methodiek. Het aantonen van Led-MAGP in de hemolymf van het gepaarde wijfje m.b.v het specifieke antiserum wordt bemoeilijkt door kruisreactiviteit met verscheidene eiwitten uit het wijfje. De neiging van Led-MAGP tot aggregatie compliceert het onderzoek naar de functie nog verder. Bovendien is de beschikbare inteeltstam van de Coloradokever niet geschikt voor een bio-toets voor de eilegstimulatie (hoofdstuk 8).

Nawoord

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Bedankt!

Hans Smid
Wadenoijen, 5 december 1997

Curriculum Vitae

In 1982 voltooide ik, Hans Marius Smid (12 april 1964, 's-Gravenhage), mijn VWO opleiding. In datzelfde jaar begon ik de HLO opleiding aan het toenmalige Van Leeuwenhoek Instituut te Delft. Stage werd gelopen bij de vakgroep Entomologie van de Landbouwuniversiteit, met als onderwerpen de anatomie van het deutocerebrum in de hersenen van de coloradokever en de immunolocalisatie van sensorische neuronen in de antennen van de coloradokever. In 1986 studeerde ik af in de richting zoölogie, en kreeg een aanstelling als analist bij bovengenoemde vakgroep. Vanaf 1986 tot 1997 heb ik onderzoek gedaan aan de endocrinologie van de coloradokever. Het onderzoek dat is beschreven in dit proefschrift is uitgevoerd in de periode 1991 t/m 1996. Vanaf 1997 doe ik fysiologisch onderzoek aan het waarnemen van geurstoffen door sluipwespen.