

Immunohistochemistry of the products of male accessory glands in several hemimetabolous insects and the control of their secretion in *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae)

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Reproduction, imaginal diapause, accessory glands, sexual maturation, *Pyrrhocoris apterus*

Abstract. Three antibodies against secretions of the male accessory glands of *Tenebrio molitor* react with specific regions of the male reproductive system in a damselfly, cockroach, cricket and the bug *Pyrrhocoris apterus*. Immunoreactivity was used to assess maturation of the system in the reproducing and diapausing *P. apterus*. In bugs reared continuously at 16 h photophase and 25°C (LD regimen), antigens detected with the PL 6.3 and PL 15.2 antibodies accumulate in the accessory glands and vas deferens (in case of PL 6.3 also in the testes) gradually during the last larval instar. The PL 3.4 antibody begins to react three days after adult emergence (1 day before the males become ready to mate), and the reaction is confined to the accessory glands. Insects reared, since the 3rd larval instar, under the diapause-inducing SD conditions (10 h photophase at 25°C and 14 h scotophase at 15°C), do not exhibit any immunoreactivity throughout the last larval instar or during the first week of adult life, and only a weak reaction to PL 15.2 is detected in the older males. Immunoreactivity to PL 6.3 and PL 15.2 can be induced in the last instar SD larvae by brains implants from the LD larvae or two injections of 1 µg makisterone A.

INTRODUCTION

Male accessory glands (MAG) of insects secrete a variety of products that provide building materials for spermatophore, elevate oviposition, render females refractory to subsequent matings, and accomplish other functions (Chen, 1984). Several components of MAG were recognized and characterized to various extents in the mealworm beetle, *Tenebrio molitor*, which produces these components in morphologically distinct parts of the glands (Table 1). Shinbo et al. (1989) reported that monoclonal antibodies to products of *Tenebrio* MAG also react with precisely localized antigens in MAG of the lepidopterans *Bombyx mori* and *Antheraea yamamai*, indicating that MAG secretions produced by various insects may contain homologous proteins. This stimulated us to test four antibodies that react with products of *Tenebrio* MAG on the reproductive system of several hemimetabolous insects. Since positive reactions occurred, we then used the antibodies to examine functional differentiation of the male reproductive system during metamorphosis of the linden bug, *Pyrrhocoris apterus*.

Information on the biology of *P. apterus* was recently reviewed by Socha (1993). Females that develop under long-day photoperiods emerge with previtellogenic ovarian follicles, promptly initiate vitellogenesis, and deposit the first batch of eggs in about one week after emergence (26°C). In females that develop under short-day photoperiods, the development of eggs is arrested at the onset of vitellogenesis. Vitellogenesis ensues only when the bugs are either transferred to a long-day photoperiod or exposed for a specific time to

natural winter conditions that terminate their diapause; such insects become insensitive to daylength and begin to reproduce as soon as the temperature becomes favourable (Hodek, 1996).

Non-diapausing males emerge with functional sperm in the spermiducts and begin mating 5–6 days after emergence. In diapausing males that were grown under short-day photoperiod (10 h light at 25.5°C and 14 h darkness at 20.5°C) since the 3rd larval instar, the sperm descends into the spermiducts only on the ninth day after imaginal ecdysis (Zachardová et al., 1989). These diapausing males do not mate and take at least 10 days to do so after diapause termination and subsequent exposure to reproduction-promoting conditions (Hodková et al., 1991). It is possible that mating is delayed, both after imaginal emergence and after diapause termination, because the accessory glands need additional time to mature. Therefore, we compared functional differentiation of MAG under reproduction-promoting and diapause-promoting conditions.

Sexual maturation and reproduction of insects are controlled by brain neurohormones, juvenile hormones, and ecdysteroids (Raabe, 1986). We determined that functional differentiation of MAG in *P. apterus* is humorally stimulated by the brain-suboesophageal ganglion complex and makisterone A. Makisterone A was previously identified as the main ecdysteroid of *P. apterus* (Zachardová et al., 1989).

MATERIAL AND METHODS

Experimental insects and rearing conditions

Damselflies, *Coenagrion puella* (L.) (Odonata: Agrionidae), and linden bugs, *Pyrrhocoris apterus* (L.) (Heteroptera: Pyrrhocoridae), were collected outdoors in the vicinity of České Budějovice, Czech Republic, whereas cockroaches, *Nauphoeta cinerea* (Olivier) (Blattaria: Blaberidae), and crickets, *Gryllus bimaculatus* de Geer (Ensifera: Gryllidae), were obtained from the laboratory cultures. Some of the collected *P. apterus* adults were immediately sacrificed for histological examinations and others were used to establish a laboratory culture under a long-day (LD) photoperiod comprising a 16 h photophase and 8 h scotophase, which were both maintained at 25°C. All insects reared under these conditions promptly reproduced; this is consistent with the fact that a 16 h photophase is longer than the critical diapause-inducing daylength of 15.75 h (Saunders, 1983). The ability of males to mate was tested by combining 2–6 males of known age (0–4 days after emergence) with 1–3 adult females (4 days old). Females of this age are just about to initiate mating and lay the first batch of eggs in another 3–5 days (Socha, 1993).

Bugs were reared in 1 liter jars that were supplied with corrugated paper as a walking substrate. Drinking water and linden seeds were provided ad libidum. Freshly ecdysed 3rd instar larvae of the third laboratory generation were segregated into groups of 10–20 individuals and placed in Petri dishes (9 cm in diameter). Some of these dishes were maintained under the LD regimen, and other were transferred to diapause-inducing short day (SD) conditions comprising a 10 h photophase at 25°C and 14 h scotophase at 15°C. Hodek (1971a) showed that exposure of adult bugs to low temperatures (+2 to +6°C) for just 2–4 days causes a reproduction block in 60–80% females, even if they are reared in a long-day photoperiod. We used a scotophase temperature that is just below the developmental (Pouvreau, 1963; Honěk & Kouček, 1990) and diapause-terminating (Hodek & Hodková, 1986) temperature thresholds. Since it has been demonstrated in other insects that thermoperiods with a cryophase below the threshold temperature are efficient in inducing diapause (Beck, 1982), we assume that bugs reared under our SD conditions entered a deep diapause, comparable to that evoked by natural field conditions.

Immunohistochemistry

The entire reproductive system of water-anaesthetized males was dissected under saline and compared with previous descriptions of reproductive morphology of the damselflies (St. Quentin & Beier, 1968), cockroaches (Feliubadaló et al., 1996), crickets (Kaulenas et al., 1975), and of the *P. apterus* (Merle,

1969). Changes of the reproductive system during metamorphosis were examined in *P. apterus*. To this end, some insects were sacrificed each day between the end of the penultimate larval instar and the first day after imaginal emergence. A representative specimen of the reproductive system was drawn to scale with camera lucida.

Dissected tissues were fixed with Bouin's solution. Standard techniques were employed to dehydrate, embed in paraplast, and section specimens at 7 µm, and to rehydrate sections. Immunohistochemistry was completed in accordance with the protocol supplied with the Amersham commercial kit: sections were pre-treated with 10% normal goat serum for 30 min at room temperature and subsequently incubated with primary antibodies overnight at 4°C; with secondary Goat-anti-Mouse antibody, conjugated to biotin, for 1 h at room temperature; and with biotin-streptavidin-horse radish peroxidase complex for an additional 1 h at room temperature. A positive reaction was revealed with diaminobenzidine (Sigma) staining.

We used four monoclonal antibodies that recognize specific secretions of male accessory glands of the adult mealworm, *Tenebrio molitor*. These antibodies were kindly provided by G.M. Happ of the University of Vermont, and their nomenclature and specificity are summarized in Table 1. We used them in a 1 : 500 dilution with phosphate buffered saline containing 0.1% Triton X-100.

TABLE 1. Distribution of antigens recognized by tested antibodies in the accessory sex glands of *Tenebrio molitor* males.

Antibody	Reactive cells	Antigens	Reference
PL 3.4	Type 3 cells in BAG	27.6 & 29.4 kD kD	Grimnes & Happ, 1986
PL 6.3	Type 7 & 5 cells in BAG	9.6 & 5 kD	Grimnes et al., 1986a
PL 15.2	TAG secretion and endocuticle	80–90 kD	Grimnes et al., 1986b
PL 21.1	Type 4 cells in BAG	23 kD	Shinbo et al., 1987

BAG – bean-shaped accessory glands; TAG – tubular accessory glands.

Hormone applications

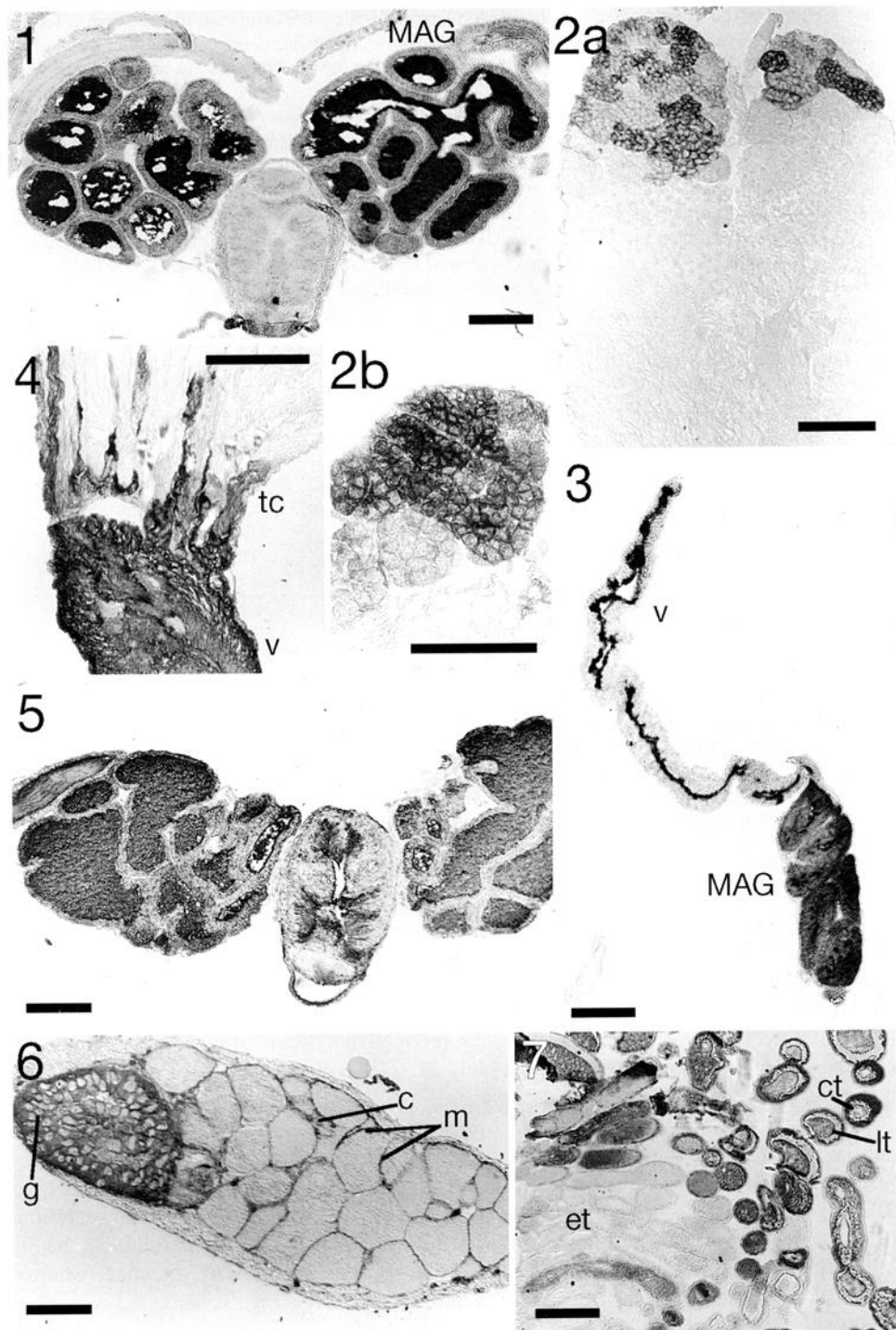
Two treatments were performed to disclose possible involvement of neurohormones and makisterone A in the development of accessory glands. A likely source of neurohormones, the brain-suboesophageal ganglion complex of LD larvae (5th day of the last instar), was implanted into the 2nd day last instar SD larvae. The implant was inserted into the body cavity of a water-anaesthetized larva through an incision inflicted on the ventral side of abdomen with a pair of scissors. Makisterone A of plant origin was dissolved in 8% ethanol and the solution injected into SD larvae on the 2nd day and again on the 5th day of the last instar.

RESULTS

Reactivity of tested antibodies

Antibodies were first tested on the sections of the male accessory glands of *T. molitor*. All bound to specific MAG regions, consistently with the reports listed in Table 1. An identical procedure was then used with the reproductive systems of our experimental species. No reaction was detected with antibody PL 21.1, whereas the three remaining antibodies recognized specific cells or secretions.

In reproductive male *P. apterus*, reaction with PL 3.4 was confined to MAG and was particularly strong in the gland lacunae, where the secretion accumulates (Fig. 1). PL 6.3 reacted with differentiating spermatocysts in the most apical region of testes follicles (Fig. 2), with secretions in spermiduct (vas deferens) lumen, and with MAG cells and secretions (Fig. 3). Antigen that are recognized by PL 15.2 were detected in the spermiducts, beginning with the outlets of testicular follicles (Fig. 4), and in the MAG lacunae, whereas MAG secretory cells were nearly devoid of immunoreactivity (Fig. 5).



In male *C. puella* damselflies, which lack MAG, antibodies reacted in the testes. Antibodies PL 3.4 and PL 6.3 only recognized cells that are sparsely scattered between the follicles, whereas PL 15.2 also reacted with the extracellular matrix that separates the follicles, and reacted vigorously with a gland-like region at the base of testes (Fig. 6). Reactivity of PL 15.2 with the interfollicular matrix also occurred in the cricket *G. bimaculatus*. In this species, PL 15.2 reacted with some of the numerous tubular MAG; however, other MAG tubuli were devoid of immunoreactivity (Fig. 7). No clear immunoreactivity to PL 3.4 and PL 6.3 antibodies was detected in the cricket. During preliminary testing of PL 3.4 and PL 15.2 (PL 6.3 was not included) on the cockroach *N. cinerea*, PL 3.4 reacted with the interfollicular matrix, and both this antibody and PL 15.2 react in some of the numerous tubular MAG (data not shown).

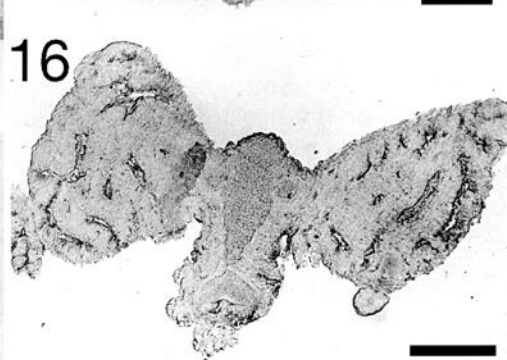
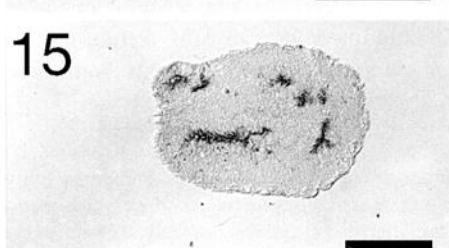
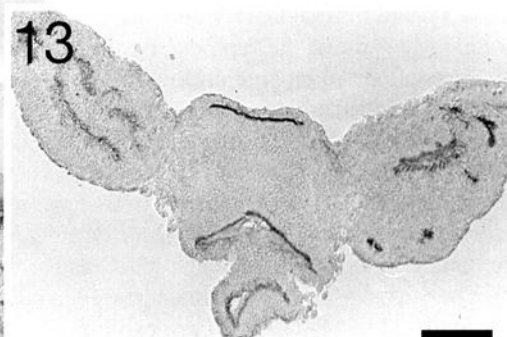
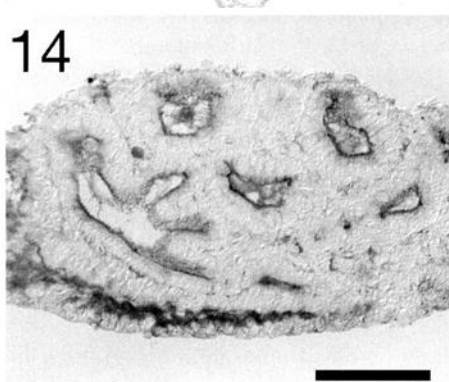
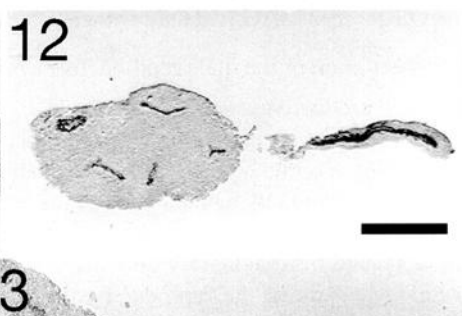
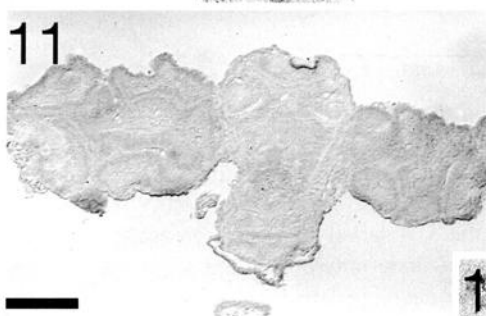
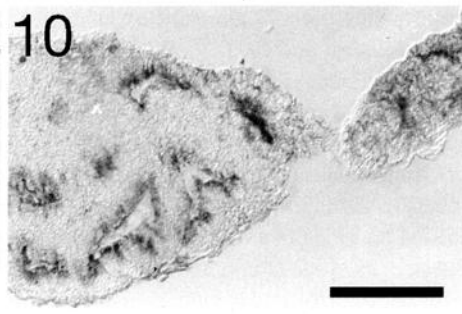
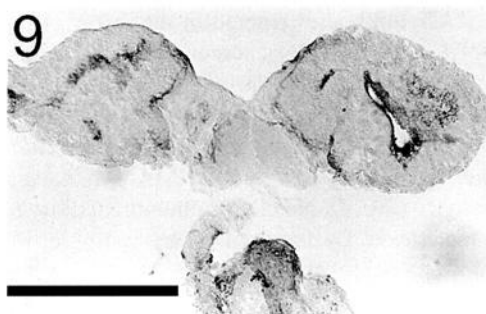
Differentiation of the male reproductive system in bugs grown under LD regimen

The reproductive accessory glands of *P. apterus* males are rudimentary until the beginning of the last larval instar. The whole reproductive system rapidly develops during this instar (Fig. 8). Freshly ecdysed last instar larvae contain clearly defined testes and rudimentary spermiducts with anlagen of the accessory glands and the ejaculatory duct. Testes enlarge more or less gradually throughout the last larval instar, whereas MAG, spermiducts, and the ejaculatory duct begin to differentiate only during the second half of the instar. All parts of the reproductive system are developed in newly emerged adults but MAG continue to enlarge during the first 4 days of imaginal life. On day 4 they shrink somewhat and thereafter maintain this acquired size. Since the shrinkage also occurs in males that are separated from females, it cannot be due to a discharge of MAG contents during copulation.

Antigens that are detectable by use of the tested antibodies occurred gradually in specific parts of the reproductive system. Antibodies PL 6.3 and PL 15.2 reacted with the secretory cells of spermiducts and MAG as early as freshly ecdysed last instar larvae (Fig. 9). The intensity of the reaction increased in course of the instar (Table 2), but no secretion accumulated in MAG lumen prior to imaginal ecdysis (Fig. 10). Immunoreactivity to PL 6.3 was detected in the testes only after ecdysis, and immunoreactivity to PL 3.4 in MAG first appeared in three day old adults (Table 3).

Our mating test revealed that males begin mating on day 4 after adult emergence, but half of the females that mated with these males laid unfertilized eggs (egg laying occurred when the females were 8–9 days old). Other 4 day old males proved fully fertile; neither the number nor the hatchability of deposited eggs were significantly different from those laid by females paired with older males (Table 4).

Figs 1–7. 1–5: Reaction of tested antibodies in reproducing males of *Pyrrhocoris apterus* (bars = 100 μ m). 1 – reaction to PL 3.4 in the lacunae (l) of MAG (d = ductus ejaculatorius). 2a, b – immunoreactivity to PL 6.3 in the apical section of testicular follicles (tf); note that staining is restrained to early spermato-cysts. 3 – material reacting with PL 6.3 in the lumen of vas deferens (v) and in both the cells (somewhat weaker reaction) and secretion of MAG. 4 – reaction to PL 15.2 in testes calyx (tc) and spermiducts (vas deferens, v). 5 – reaction to PL 15.2 in the lacunae (l) of MAG. 6 – immunoreactivity to PL 15.2 in the matrix (m) separating testes follicles, in cells (c) sparsely distributed in the matrix, and in the “glandular” part (g) of testes of the damselfly *Caenagrion puella* (bar = 100 μ m). 7 – sections through numerous MAG tubes of the cricket *Gryllus bimaculatus* stained for the PL 15.2 antigen: some tubes (et) are devoid of the antigen, others contain antigen either in the cells (ct), the lumen (lt), and/or in both the cells and the lumen (bar = 100 μ m).



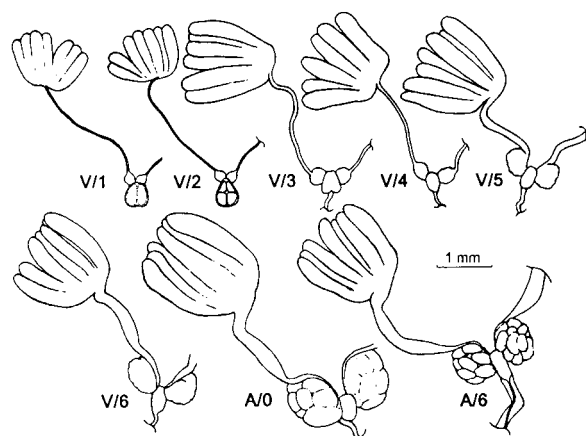


Fig. 8. Schematic drawing of male reproductive system of *P. apterus* on consecutive days of the last larval instar (V/1–V/6) and in newly emerged (A/0) and 6 days old adults (A/6) of *P. apterus*: a – MAG; d – ductus ejaculatorius; t – testes; v – vas deferens.

TABLE 2. Development of immunoreactivity in course of the last larval instar (day 7 = newly emerged adult) in *P. apterus* reared under LD conditions.

Day	Antibody PL 6.3		Antibody PL 15.2	
	Accessory glands	Vas deferens	Accessory glands	Vas deferens
0	(+)	(+)	+	–
1	+	+	+	–
2	+	+	+	–
3	++	+	++	+
4	++	++	++	++
5	++	++	++	++
6	++	++	+++	++
7	++	++	+++	+++

Immunoreaction intensity: +++ maximal; ++ moderate; + slight; (+) just detectable; – absent. Antibodies PL 3.4 and PL 21.1 never reacted in the larvae.

TABLE 3. Development of immunoreactivity to PL 3.4 in MAG of adult *P. apterus* males reared under LD conditions.

Day after emergence	1	2	3	4	6	8	11	15
Reactivity	–	–	+	+++	+++	+++	++	++

Immunoreaction intensity: +++ maximal; ++ moderate; + minimal; – absent. Reaction with the PL 6.3 and PL 15.2 was maximal in all adults; the PL 21.1 antibody never reacted.

Figs 9–16. 9–10: Development of immunoreactivity in the male reproductive system of *P. apterus* last instar larvae grown under LD photoperiod (bar = 100 μ m). 9 – small amount of the PL 6.3 antigen in the cells lining the lacunae (l) of rudimentary MAG at the beginning of the instar. 10 – reaction to PL 15.2 in vas deferens (v) and MAG at the end of the instar (day 6). 11 – lack of immunoreactivity to PL 6.3 in a diapausing male 2 days after imaginal emergence (bar = 100 μ m). MAG and ductus ejaculatorius (d) are shown. 12–16: Induction of antigens in the diapause-destined larvae (bar = 100 μ m). 12, 13 – reaction to PL 6.3 in vas deferens (v) and MAG in day 6 last instar larvae implanted with active brain-suboesophageal ganglion on day 2. 14 – reaction to PL 15.2 in such a larva. 15 – occurrence of PL 6.3 antigen in the MAG of a 12 day old last instar larva injected with 1 μ g makisterone A on days 2 and 5. 16 – PL 15.2 antigen (arrows) in MAG of such a larva.

TABLE 4. Fertility of *P. apterus* males reared under LD conditions.

Age of mating males	Number of test groups	Eggs laid per female	Egg hatchability (%)
4 (a)	3	42 ± 16	3 ± 4
4 (b)	2	62 ± 17	66 ± 28
5	7	52 ± 21	42 ± 19
6–7	5	69 ± 19	49 ± 17

Each test group consisted on two females 4 days after adult emergence and four males 0–4 days after emergence. The age of males at mating and the size and hatchability of the first egg batch (deposited when the females were 8–9 days old) were recorded; males mating at the age of 4 days were either nearly infertile (a) or exhibited normal fertility (b).

TABLE 5. Immunoreactivity in *P. apterus* males, 7 days (reproducing adults) or 11 days (diapausing adults) after emergence.

Antibody	Testes	Accessory glands	Vas deferens
Reproducing adults			
PL 3.4	–	+++	–
PL 6.3	+++	+++	+++
PL 15.2	–	+++	+++
Diapausing adults			
PL 3.4	–	–	–
PL 6.3	–	–	–
PL 15.2	–	(+)	(+)

Immunoreaction intensity: +++ maximal; ++ moderate; + minimal; (+) just detectable; – absent.

TABLE 6. Induction of antigens in male accessory glands of pre-diapausing last instar larvae of *P. apterus* by implant of brain-suboesophageal ganglion or by injection of makisterone A.

Day	Brain-suboesophageal ganglion implant			Makisterone A injection		
	PL 3.4	PL 6.3	PL 15.2	PL 3.4	PL 6.3	PL 15.2
3	–	–	+	not tested		
6–8	–	–	+	–	+	++
9–10	–	+	++	–	+	+++
12	–	+	++	–	+	++

Implants of brain-suboesophageal ganglion from day 5 last instar LD larvae were inserted into day 2 last instar SD larvae. Other SD larvae received injections of 1 µg makisterone A on days 2 and 5 of the last instar. Only PL 15.2 reacted in the non-treated SD males that were at least one week after adult emergence (data not shown). Immunoreaction intensity: +++ maximal; ++ moderate; + minimal; – absent.

Induction of antigens in the reproductive system of diapausing males

Morphological development of the reproductive system of last larval instar males that were grown under the SD regimen was not distinguishable from that described above, except that development was protracted. Under LD conditions with a constant 25°C temperature, the final larval instar lasted 6.3 ± 0.5 days (average ± standard deviation, $n = 20$) in females and 6.2 ± 0.4 days in males, whereas, under SD conditions with scotophase at 15°C, the instar lasted 15.8 ± 2.6 days and 18.1 ± 1.0 days, respectively. The body size of

females was not significantly affected by the rearing regimen; under LD conditions newly emerged females weighed 39.5 ± 1.5 mg and under SD conditions they weighed 40.6 ± 4.1 mg. In contrast, body weight (of newly emerged LD males $38.2 \text{ mg} \pm 1.1$ mg) was significantly ($p = 0.0009$) lower than the weight established in SD males (40.6 ± 2.8 mg).

In spite of apparently normal morphogenesis, the reproductive system of bugs grown under SD conditions and examined at imaginal ecdysis was devoid of antigens detectable with tested antibodies (Fig. 11). A weak reactivity to PL 15.2, but to no other antibody (Table 5), appeared as late as 11 days after emergence, at which time our experiments were terminated.

Antigens recognized by PL 6.3 and PL 15.2 antibodies appeared when the diapause-destined larvae were either implanted with the brain-suboesophageal ganglion complex taken from the LD larvae, or injected with 1 μ g makisterone A (Table 6). Immunoreactivity to PL 15.2 occurred one day after the brain implantation, i.e. on the third day of the last larval instar, and immunoreactivity to PL 6.3 occurred on the sixth day of the instar (Figs 12–14). The effect of makisterone A was first examined on day 8, i.e. 48 h after the second injection, and reactions with both PL 6.3 and PL 15.2 were positive at that time (Figs 15 and 16). Occurrence of antigen(s) recognized by the PL 3.4 antibody was not induced in last instar larvae subjected to either treatment (adults were not examined).

DISCUSSION

Evolutionary conservation of the secretions of male accessory glands

Our results demonstrate that males of remote insect taxa contain antigens in their reproductive systems that are recognized by three monoclonal antibodies raised against specific products of the male accessory glands of *Tenebrio molitor*. In our study, none of these antibodies reacted in any organ other than the male reproductive system. We conclude that secretions of insect reproductive system include highly conserved components. Most of the secretions are produced in MAG but some components occur also in the testes and spermiducts. The reaction in testes suggests that similar proteins are important, possibly because they provide nutrition, for both the rapidly growing spermatocytes in testes and the mature sperm which is mixed with the products of MAG. A special region at the base of damselfly testes, which does not contain spermatocytes and structurally resembles a gland, probably fulfills the usual roles of MAG, which are lacking in Odonata.

There is no data on the functions of MAG secretions in *P. apterus* and the time sequence in which they appear provides the only cue of their use. Early occurrence of PL 6.3 and PL 15.2 antigens may indicate their potential importance for sperm maintenance and nutrition, while the late appearance of PL 3.4 suggests its involvement in either sperm activation or spermatophore formation. Copious amounts of all three antigens in MAG lacunae of mating males imply that these antigens constitute major components of the ejaculate. Since mated and unmated *P. apterus* females lay similar numbers of eggs (Sláma, 1971), secretions of MAG that are transferred during copulation do not appear to stimulate egg development.

Reproductive diapause may begin in the larval stage

Reproductive diapause is manifested in adults, but is often induced in larvae. Larvae of *P. apterus* that are reared under a short-day photoperiod since the beginning of the last

instar emerge as diapausing adults (Hodek, 1971b). An earlier exposure to SD conditions is even more efficient for diapause induction (Saunders, 1983). Our study extends earlier observation (Zachardová et al., 1989) that larvae reared since the 3rd instar under a short-day photoperiod combined with a thermoperiod are altered in development by the last instar; the duration of the instar is extended, body weight is elevated, and none of the studied secretions of the male reproductive system are produced. An extension of the instar is understandable because SD larvae were maintained diurnally for 14 h at the subthreshold temperature of 15°C. They could develop daily for only 10 h, when the temperature was maintained at 25°C. Therefore, their development should be 2.4 times longer than that of LD bugs, which were continuously at 25°C. Deriving from the last instar length of 6.2 days (males) and 6.3 days (females) of the LD larvae, the expected instar length of the SD bugs should be 14.9 and 15.1 days, respectively. In the case of SD females, the actual last instar length of 15.8 days is close to this calculated one, however the actual last larval instar length of SD males is 18.1 days. This is a significant prolongation that provides time for additional body growth. In contrast to the female larvae that attain similar body weights under both LD and SD conditions, the 40.6 mg body weight of newly emerged SD male adults is significantly higher than the 38.2 mg weight of LD males. Apparently, males destined to enter reproductive diapause apparently accumulate more reserves than the non-diapausing ones. The body weight of diapausing bugs continues to increase after imaginal ecdysis (Zachardová et al., 1989).

We regard the delay of imaginal ecdysis and the additional body growth as pre-diapause syndromes that are manifested in the larvae. A third syndrome, which is most important in respect to reproduction, is the lack of secretions in the testes, spermiducts and MAG. Although morphogenesis of the reproductive system seems to proceed normally (Fig. 8) and spermiogenesis is merely delayed (Zachardová et al., 1989), the reproductive system remains in a non-functional state in respect to the secretion of specific products.

The hormonal control of imaginal diapause in *P. apterus* males

Morphogenesis of the reproductive system during metamorphosis, which is driven by ecdysteroids (Dorn et al., 1986, 1993; Joshi & Sehna, 1989), occurs in both LD and SD larvae in a manner that is consistent with the observation that makisterone A titre changes in the second half of the last larval instar are alike in both LD and SD insects (Zachardová et al., 1989). In insect species without imaginal diapause, for example in the mealworm beetle (Grimnes & Happ, 1987), functional differentiation of MAG seems to be linked to their ecdysteroid-dependent morphogenesis. In *P. apterus* SD larvae, however, MAG begin secreting their specific products (antigens PL 6.3 and PL 15.2) only when early last instar larvae are injected with makisterone A or implanted with the brain-suboesophageal ganglion complex from the LD larvae. We interpret these results as evidence that a specific level of makisterone A at the beginning of the last larval instar triggers secretion of a neurohumoral factor that promotes sexual maturation. MAG of SD larvae do not mature because the endogenous makisterone A level is not elevated at a proper time. This can be circumvented by providing exogenous makisterone A or by implanting larvae with an activated source of the neurohumoral factor. Thus, we believe that pre-diapause syndromes in last instar larvae of *P. apterus* are controlled by ecdysteroids and neurohormone(s).

Reproduction blockage and manifestation of other diapause syndromes in adult *P. apterus* are controlled by several hormones (Sláma, 1964) whose roles apparently vary

depending on the intensity of diapause. Zachardová et al. (1989) claimed that young diapausing adults contain high levels of makisterone A that may be responsible for inhibition of vitellogenesis. Suppression of vitellogenesis by makisterone A was demonstrated in the non-diapausing bug *Oncopeltus fasciatus* (Aldrich et al., 1981; Dorn et al., 1994), but more data are needed to reconcile the role of ecdysteroids in reproductive diapause.

A well known cause of reproductive failure in many diapausing adults is the lack of juvenile hormone (JH). Exogenous hormone or its analogues stimulate reproduction, but often only in adults that have passed a critical age (Sehnal, 1985). For example, Burov et al. (1982) demonstrated that the sunn bug, *Eurygaster integriceps*, which has an obligatory imaginal diapause lasting from June/July until March/April of the next year, becomes fully responsive to JH analogues only after completing previtellogenesis (about 20 days after emergence) and/or spermiogenesis (60 days). Thereafter, maintenance of diapause is apparently due to the lack of JH because administration of its analogues readily stimulates normal reproduction, whereas natural completion of diapause requires additional time. It is known that the function of MAG is controlled by JH (Gillott & Gaines, 1992), thus, the lack of JH in diapausing insects apparently keeps these glands non-functional. This was shown in *E. integriceps*, in which MAG are already sensitive to JH in the young adults, long before the gonads are ready to respond (Burov et al., 1982).

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