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Sexual activity in macropterous and brachypterous males of a flightless bug, *Pyrrhocoris apterus* (Heteroptera)

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Abstract. The long-winged (macropterous) and short-winged (brachypterous) adult males of *Pyrrhocoris apterus* (L.) from temperate (Czech Republic) and Mediterranean (Israel) populations were analysed for the sexual activity and the functional activity of their accessory glands. The sexual activity of the males reared either under long-day (18L : 6D) or short-day (12L : 12D) conditions was determined by their capability to mate with 5-day-old reproductive females of the brachypterous morph and to fertilize the eggs. The functional activity of accessory glands was characterized by the presence of a specific immuno-marker. Sexual activity of fasting macropterous males from both temperate and Mediterranean populations was almost as high as that observed in the reproductive brachypterous ones. These findings were also confirmed by an immunotest. Contrary to the temperate macropterous males, the feeding arrest in temperate macropterous females was coupled with a non-diapause inhibition of reproduction in spite of long days. A similar kind of difference was observed also in the Mediterranean macropterous bugs reared under short-day conditions. The results showed the sexual difference in reproductive activity of the macropterous morph in *P. apterus*.

INTRODUCTION

The firebug, *Pyrrhocoris apterus* (Linnaeus, 1758) is a convenient experimental model for biological research (see review Socha, 1993). Short-winged (brachypterous) and long-winged (macropterous) specimens may be produced (Seidenstücker, 1953; Tischler, 1959; Honěk, 1976) and two generations develop per year in the Czech Republic in warmer years and places (Socha & Šula, 1992). The wing dimorphism and diapause of this flightless heteropteran species are genetically determined but influenced by environmental variables, mainly by photoperiod and temperature (Socha, 1993). Under constant short-day conditions (photophase < 16 h), all bugs from temperate population become brachypters and enter diapause (Hodek, 1968; Saunders, 1983), while under longer days the bugs do not diapause and a fraction of population becomes macropterous (Honěk, 1976). There is a latitudinal gradient in the wing-form response of *P. apterus* to photoperiod with the critical daylength 12.5 and 16.5 h of light in the Mediterranean (Israel) and temperate (Czech Republic) population, respectively (Socha, 2001). The critical daylength for determination of diapause in this bug was also 4–5 h shorter in Mediterranean population than in the temperate one (Socha & Šula, 1996; Socha, unpubl.). Geographical differences in photoperiodic response regulating wing length and diapause, an occurrence of wing morph-specific types of reproductive arrest in females (Socha & Šula, 1996) and higher adipokinetic and locomotory activities in macropters than in brachypters (Kodrík & Socha, 1999; Socha & Kodrík, 1999; Socha & Zemek, 2001) indicate that macropterous morph might play a specific role in the life strategy of this heteropteran, most probably in its dispersal.

The adult life of *P. apterus* males may last from two months to one year, depending mostly on their sexual activity. In males, sexual behaviour was studied only in the specimens of brachypterous morph (Žďárek, 1967, 1968, 1970; Zachardová et al., 1989; Hodková et al., 1991; Hodková, 1994). Non-diapausing males start to mate 3 to 5 days after the imaginal ecdysis; in males the sexual activity does not fluctuate throughout the imaginal life. Whereas in females the overall physiology is deeply affected by diapause (Sláma, 1964a), in males the absence of mating behaviour is probably the most significant character of a diapausing specimen. The diapause in males was presumed not to be easily characterized using other physiological criteria (Sláma, 1964b). However, further studies revealed that diapause of brachypterous males can be recognized also by means of other specific markers, e.g. by the accumulation of hexameric storage protein in the haemolymph (Šula et al., 1995; Socha & Šula, 1996) and the absence of particular proteins in their accessory glands (AG) (Šauman & Sehnal, 1997).

Recent research showed that, contrary to brachypters, the macropterous females from temperate population may enter two different types of reproductive arrest, i.e. that of non-diapause type under a long-day photoperiod, and hibernal diapause induced by a short-day photophase (Honěk, 1985; Socha & Šula, 1996; Šula et al., 1998). Thus, the macropterous specimens of the temperate population of *P. apterus* can distinguish between two daylengths thresholds, inducing either hibernal diapause or reproductive arrest of non-diapause type. While the hibernal diapause in brachypterous bugs of both sexes is characterized by a very high level of the hexameric proteins in haemolymph, its content in haemolymph of macropterous males and females is at most as low as in the

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reproductive adults. In both the long-day temperate and short-day Mediterranean macropterous females the reproductive arrest of non-diapause type is characterized by prolonged pre-oviposition period (Socha & Šula, 1996). Two possible interpretations of the extended pre-oviposition period and occurrence of non-diapause reproductive arrest in macropterous females were proposed. It might either represent a relict of a summer diapause (aestivation) or be associated with macroptery and relate to the migratory activity of the macropterous morph rather than to photoperiodically induced diapause (Socha & Šula, 1996). When the second interpretation is valid, then the macropterism-associated reproductive arrest can represent a relict of "oogenesis-flight" syndrome (Johnson, 1969). In spite of the above findings, data concerning the reproductive activity in the macropterous males of *P. apterus* are missing.

Since the wing dimorphism has been discussed with allocation of reproduction and dispersal in flying insects and most results so far are restricted to females of these species (Zera & Denno, 1997), the aim of the present study was to describe the relationship between wing morphs and reproduction in males of non-flying species. The relationship between wing morphs and reproduction in insects with a nonfunctional wing dimorphism (i.e. with non-flying macropters) is of great interest. The present paper was focused therefore on the comparison of the sexual activity of macropterous males with that of the brachypterous ones and finding whether the reproductive arrest of non-diapause type occurs also in the macropterous males from temperate (Czech Republic) and Mediterranean populations (Israel).

MATERIAL AND METHODS

Experimental animals

The laboratory stock cultures of *P. apterus* were reared on linden seeds and water at temperature 26 ± 1°C. The temperate (T) cultures producing specimens of brachypterous and macropterous morphs were established from the bugs collected at České Budějovice (Czech Republic, 49°N) and bred under a constant photoperiod of 18 h light-6 h dark (Socha et al., 1997; 1998). Water and linden seeds were replenished twice a week. The long-day conditions are known to prevent diapause and allow continuous breeding. The animals of T culture destined for diapause were kept from eggs until adult stage under a constant short-day (12L : 12D) photoperiod. The wild-type diapausing males of brachypterous morph were collected from the field (České Budějovice) in the second half of October. The standard Mediterranean culture (M), originating from Ma'agal Michael (Israel, 33°N) population, was reared under a constant photoperiod of 16L : 8D. The highest proportion of macropters in M culture was induced under a photoperiod of 12L : 12D (at 26 ± 1°C) (Socha & Šula, 1996; Socha, 2000). To obtain sufficient number of macropterous males for our experiments we transferred the eggs laid by females from the M culture in a photoperiod of 16L : 8D to a photoperiod of 12L : 12D and allowed them to develop in these conditions at 26 ± 1°C until the adult stage.

Freshly ecdysed adult males of a particular wing morph removed from T and M cultures were transferred into small glass jars (0.25 l) in groups of 10–15 specimens and kept under the same photoperiodic regimen, in which they developed. When they were 10 days old, they were individually used for experiments.

The experimental groups of adult males were marked as follows: temperate population - reproductive brachypters (T-br-R), diapausing brachypters from the laboratory (T-br-D_L) and the field (T-br-D_N), and macropters (T-ma); Mediterranean population - brachypters (M-br) and macropters (M-ma).

Sample preparation and electrophoresis

Electrophoretic analysis of the accessory gland (AG) proteins was performed using 1, 5, and 10 days old adult males; ten specimens were analysed in each experimental group. The AG were dissected out from the body and individually homogenised in 15 µl of the sample buffer (0.06 M Tris, 0.5% dithiothreitol, 8.54 M urea and 2% SDS; pH adjusted to 6.8 by HCl). Simultaneously the condition of the midguts was inspected. After homogenization in an Eppendorf micro tube at 2,000 rpm and centrifugation at 10,000 g for 10 min the supernatants were used. Aliquots of 0.4 AG were taken for the polyacrylamide gel electrophoresis under denaturing conditions (in SDS) according to Laemmli (1970) on 5–20% gradient slab gels 0.7 mm thick. Molecular weight markers were purchased from Pharmacia LKB. The gels with separated proteins were used for immunoblotting.

Immunoblotting methods

AG proteins separated by PAGE were electroblotted onto nitrocellulose membrane (0.45 µm pore, Schleicher & Schuell) according to Towbin et al. (1979) using a wet blotter (Hoeffer Scientific Instruments). Five per cent Skim Milk dissolved in phosphate-buffered saline containing 0.5% Tween 20 (PBS-Tween) was used to saturate nonspecific binding sites. Nitrocellulose sheets were then overlaid with the first monoclonal antibody solution (dilution 1:50,000 in PBS-Tween) and incubated for 1 h at room temperature.

This monoclonal antibody PL - 15.2 was kindly provided by G.M. Happ (University of Vermont). It was originally raised against a secretory 80–90 kDa antigen from tubular AG of the adult mealworm *T. molitor* (Grimness & Happ, 1986) and its nomenclature, specificity and immunohistochemical application to *P. apterus* was described by Šauman & Sehnal (1997). The antibody gives a positive reaction with an AG protein from the reproductive adult males of *P. apterus*.

Antigen-antibody complexes were visualized by means of another reaction with second antibody SwAM/Px (swine anti-mouse immunoglobulin labelled with horseradish peroxidase, Sevac Praha) diluted 1:1000 in PBS-Tween and kept for 1 h at room temperature. The substrate for peroxidase, 3,3-diaminobenzidine tetrahydrochloride, was dissolved in 0.1 M Tris-HCl buffer, pH 7.0, and the reaction continued in the presence of hydrogen peroxide for 1–2 min.

Mating and fertility tests

The mating tests were carried out in Petri dishes (6 cm in diameter). Usually a set of about 8 dishes was observed simultaneously. Thirty-two to forty-four males of each experimental group (T-br-R; T-br-D_L; T-ma; M-br; M-ma), 10 days old, were tested for their mating activity. They were individually tested in isolated couples in each dish (the isolated-couple test) with 5-day-old reproductive virgin females of brachypterous morph; females of this age had very high attractiveness and receptivity (cf. Žďárek, 1967). The test consisted of one 30 min observation period. In each experimental group, the number of males with successful courtship completed by the connection (copulation) was recorded.

Fertility of 10-day-old macropterous males was determined based on their capability to fertilize the eggs laid by reproduc-

tive females of the brachypterous morph. The fertility test was also carried out in 6 cm Petri dishes. Ten macropterous and reproductive brachypterous males were tested in isolated couples. Ten days later the males were removed and each female kept individually to lay three consecutive egg batches; fertilization of eggs was subsequently evaluated.

Figure preparation and statistical analysis

The pictures of representative blots for 1, 5 and 10 days old males of five experimental groups were digitalized by a gel documentation system (White/Ultraviolet transilluminator, UVP) and resulting images in TIFF format were subjected to densitometry. The programme "Quantiscan" (Microbial Systems Ltd. 1991, distributed by BIOSOFT) was used for quantitative evaluation of immunopositive bands. The integrated areas across the corresponding peaks were compared and expressed as relative numbers.

The graphs were made by means of the software GraphPad Prism, version 3.0 (San Diego, CA, USA). Statistical evaluation of results was performed using t-test (hatchability of eggs) or the contingency tables followed by Fisher's exact test (mating activity) or Chi-square test (food content in midguts).

RESULTS

Immunoblotting

On the first day of adult life the immunoreaction in all experimental groups of males was almost negligible (Fig. 1), while conspicuous reactions of similar intensities were shown in the reproductively active brachypterous and macropterous males from both temperate and Mediterranean populations on days 5 and 10. On the other hand, immunoblotting of AG proteins after SDS-PAGE revealed almost no reaction of the antibody in the homogenates of laboratory and field-collected diapausing males of the temperate population. The quantitative estimate of the intensities of immunoreactions showed (a) the rise of intensity in all experimental groups (except of field-collected diapausing males), and (b) this increase (between day 1 and 5) was very steep in males of all groups except laboratory and field-collected diapausing brachypters (Fig. 2). This resulted in either steadily low levels of the antigen in diapausing brachypterous males, or 5 to 6 times higher levels in other groups at days 5 and 10.

Mating and fertility

Analysis of mating behaviour showed a distinct difference in mating activity between diapausing brachypterous males and males of other experimental groups (Fig. 3). The lowest percentage (2.5%) of mating males was observed in the diapausing laboratory brachypterous group. On the other hand, the percentage of mating in the macropterous males from the temperate (89.5%) and Mediterranean (82.5%) populations was only slightly lower than that in the temperate and Mediterranean brachypterous males (97.7% and 93.8% , respectively).

Fertility tests of long-day reared macropterous and reproductive brachypterous males mated with 5-day-old reproductive adult females of the brachypterous morph showed almost no differences in fertilization of three successive egg batches laid by these females. Hatchability of eggs was as high as 80.0 ± 22.2% and 85.9 ± 11.0% in case of macropterous and brachypterous males, respectively. The difference was statistically non-significant ($P = 0.537$).

Dissection of midguts revealed high amounts of food in the reproductive brachypterous males, much lower quantities in the diapausing laboratory brachypters and no food in the macropterous males and field-collected diapausing males of the brachypterous morph (Table 1). All

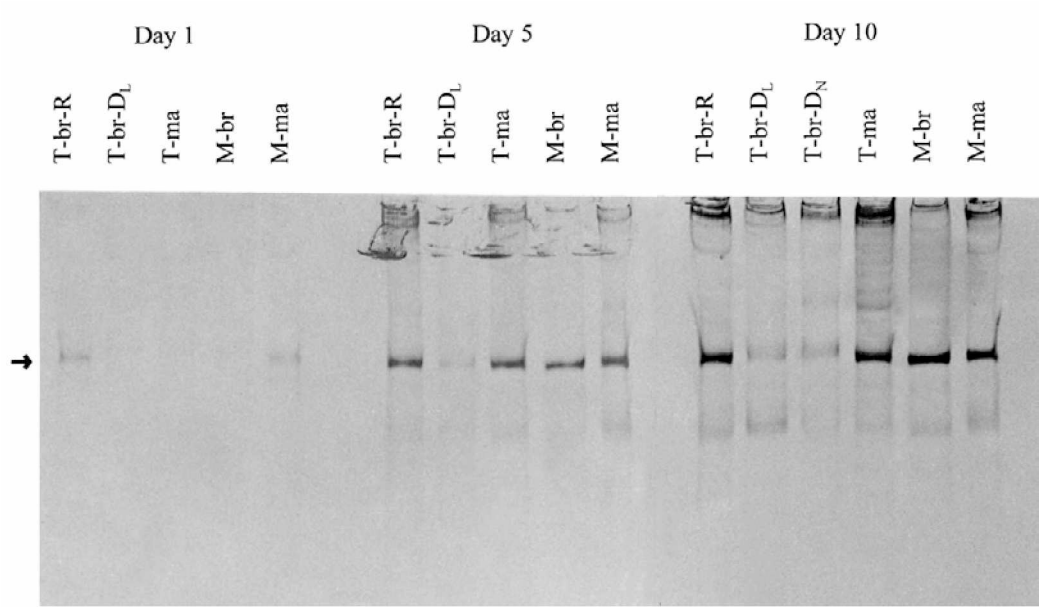

Fig. 1. Immunoblotting of AG proteins in 1, 5 and 10 days old males after SDS-PAGE: 1 - T-br-R; 2 - T-br-D_L; 3 - T-br-D_N; 4 - T-ma; 5 - M-br; 6 - M-ma. The arrow indicates the position of a detected antigen. For abbreviations see Material and Methods.

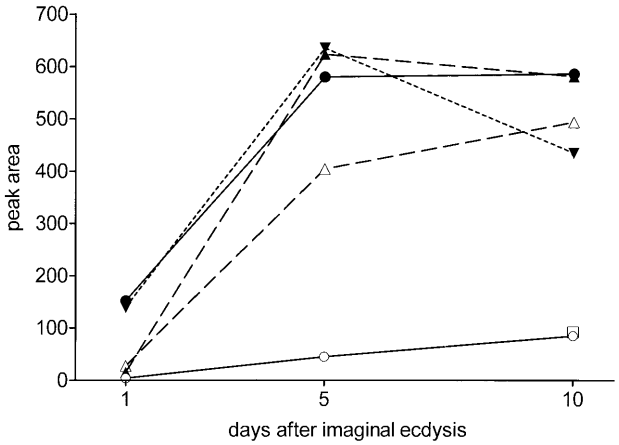

Fig. 2. The typical picture of changes in quantity of the antigen in AG detected by PL 15.2 antibody in 1, 5 and 10 days old males of all experimental groups. The quantity is expressed as the area of the peak after densitometry evaluation of the immunoblot (Fig. 1). Symbols used: ● T-br-R, ○ T-br-D_L, □ T-br-D_N, △ M-br, ▲ T-ma, ▼ M-ma. For abbreviations see Material and Methods.

the results showed that mating activity and fertility of macropterous males of both populations were not inhibited by the fact that they refused feeding.

DISCUSSION

It is known that a hexameric storage protein (M_r ~ 480,000) accumulates in a high quantity in the haemolymph of diapausing adults, while its amount in non-diapausing reproductive bugs is negligible (Šula et al., 1995). We found an absence or very low concentration of this protein also in the haemolymph of the macropterous females of both temperate and Mediterranean populations during their reproductive arrest (Socha & Šula, 1996).

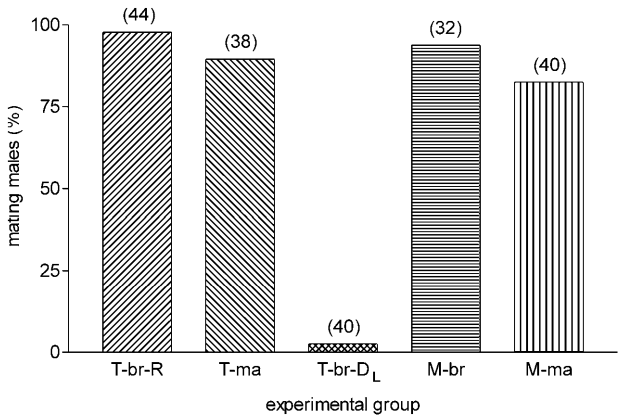

Fig. 3. Mating activity of 10-day-old adult males of five different experimental groups individually kept in single couples with 5-day-old reproductive adult females of brachypterous morph. Numbers of males in experimental groups are shown in parentheses above the bars. The Fisher's exact test revealed that reproductive and diapausing brachypterous males were significantly different ($P < 0.0001$), while there were no significant differences between reproductive brachypterous and macropterous males both in Mediterranean ($P = 0.282$) and temperate ($P = 0.569$) populations. For abbreviations see Material and Methods.

TABLE 1. The presence of food in midguts of adult males of *P. apterus*.

Experimental group	Rearing photoperiod	Number of males analysed	Midgut with food % (n)	Midgut half- or near-empty % (n)	Midgut without food % (n)
T-br-R	18L:6D	17	94.1 (16)	0 (0)	5.9 (1)
T-br-D_L	12L:12D	13	0 (0)	92.3 (12)	7.7 (1)
T-br-D_N	12L:12D	13	0 (0)	0 (0)	100.0 (13)
T-ma	18L:6D	15	0 (0)	13.3 (2)	86.7 (13)
M-br	12L:12D	10	100.0 (10)	0 (0)	0 (0)
M-ma	12L:12D	13	0 (0)	0 (0)	100.0 (13)

The differences between experimental groups as to the amounts of food in midguts were statistically significant (χ^2 = 136.6, df 10, $P < 0.0001$).

Similarly, very low levels of the hexameric protein were found in both temperate and Mediterranean males, however, their reproductive status was not determined. In the present paper we found no profound differences between the mating activity of 10-day-old macropterous and reproductive brachypterous males reared under long-day conditions. Finding that macropterous males are sexually active was supported by the immunoreactivity test showing the normal function and differentiation of their AG (in contrast to diapausing brachypterous males) and by the capability of these males to fertilize the eggs laid by reproductive brachypterous females. All these results clearly show that there is no reproductive arrest of non-diapause type in macropterous males as that reported earlier for the macropterous females (Socha & Šula, 1996). While the pre-oviposition period in macropterous females with reproductive arrest of non-diapause type is considerably longer (19 and 38 days in T-ma and M-ma females, respectively) than in normal reproductive brachypterous females (~ 7 days) (Socha & Šula, 1996), mating activity of 10-day-old macropterous males is already almost as high as in the reproductive brachypterous males of the same age (present results). However, it cannot be excluded that macropterous males may develop mating activities a little later than the reproductive brachypterous ones, though the results of Western blotting five day after emergence were similar between these wing morphs. Anyway, our results revealed sex-dependent differences in reproductive status of the macropterous morph of *P. apterus*: while in the females the reproductive arrest of non-diapause type is characterized by the long pre-oviposition period (Socha & Šula, 1996), no such prolongation of the pre-reproductive period occurs in the males.

Considering the values of photoperiodic regulation of the mating activity of brachypterous bugs (slower induction, faster activation), there is also a higher tendency of *P. apterus* males to remain or become active when compared with females (Hodková et al., 1991). In the brachypterous morph of this bug, the higher tendency to reproductive activity in males than in females was recorded particularly in early autumn (Hodek, 1971). Moreover, it is known that males and females of some insects can differ not only in the tendency to enter diapause, but also in diapause development, the intensity of diapause and in post-diapause development (Danks,

1987). In some species, females are more likely to enter diapause than males, as in the tick *Dermacentor reticulatus* (Szymánski & Černý, 1981), the butterfly *Batus philenor* (Sims & Shapiro, 1983) and the mosquito *Aedes geniculatus* (Sims & Munsterman, 1983). In most annually eusocial insects, only the females have a diapause (Danks, 1987). In *Coccinella septempunctata* the activity of the male accessory glands is independent of diapause and females can be fertilized by males kept under diapause conditions (Hodek & Honěk, 1996).

We found that in contrast to sexual difference in the reproductive activity of the macropterous morph of *P. apterus*, both the females (Socha et al., 1998) and males (this paper) of this wing morph spontaneously cease feeding after a short period of a food intake. Since the corpora allata (CA) of non-diapause starving females of the brachypterous morph of *P. apterus,* reared under a long-day photoperiod, are inhibited via nervous connections from the brain (Hodková, 1982), a similar mechanism of temporary nervous inhibition of CA resulting in the reproductive arrest of non-diapause type can be presumed also in fasting macropterous females. On the contrary, the probable inhibition of CA in fasting macropterous males is not associated with inhibition of reproduction. The present study shows that it does not interfere with the post-imaginal development and function of AG, mating activity and the capability of males to fertilize eggs.

The present results are in accordance with the finding that the sexual behaviour of brachypterous males exposed to artificial starvation is not inhibited (Žďárek, 1968). According to this author, the sexual instinct does not disappear during starvation; its intensity only decreases after a longer period of starvation in brachypterous males. An appearance of the mating behaviour coincides with the time of release of spermatozoids and activation of AG in males and with the beginning of vitellogenesis in females. Žďárek's and our findings show that lasting feeding is not essential for the appearance of the mating instinct in both the macropterous and reproductive brachypterous males. On the contrary, unfed females of the brachypterous morph (Žďárek, 1968) and fasting macropterous females (Socha & Šula, 1996) were found to be sexually inactive. Separation of feeding and mating behaviour in macropterous males of *P. apterus* might play a significant role in the dispersal and spreading of the genotype for macroptery into another area, despite the lack of food. This presumption is in a good accordance with findings that lack of food and/or mates induces or increases a seasonal flight activity in some heteropterans, e.g. *Neacoryphus bicrucis* and *Oncopeltus fasciatus* (Tauber et al., 1986).

In summary, we showed that the method based on the immunoreactivity of AG might be a useful approach that allows us to distinguish the reproductive males from the diapausing ones. The data obtained from immunoblotting, mating and fertility tests showed that sexual activity of the macropterous males from both the temperate and Mediterranean populations is almost as high as that found for reproductively active males of the brachypterous morph. Sexual behaviour of macropterous males from both temperate and Mediterranean populations is normal even if they do not feed. *P. apterus* thus belongs to the species characterized by the sexual difference in the reproductive activity of the macropterous morph.

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