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ORIGINAL ARTICLE

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Spermatophore characteristics in bushcrickets vary with parasitism and remating interval

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Abstract Male bushcrickets provide females with a nuptial gift, a spermatophore, which is transferred to females at mating. The spermatophore consists of a gelatinous mass, the spermatophylax, and the sperm-containing ampulla. Male spermatophore size is positively correlated with insemination rate and female refractory period and therefore with male reproductive success. In this study, we examined spermatophylax weight, ampulla weight and sperm number in males of *Poecilimon mariannae* parasitized by the acoustically orienting fly *Therobia leonidei.* We show that in parasitized males, spermatophylax weight decreases with the level of parasitism. In line with the hypothesis that parasitism is a cost to reproduction, we found that spermatophylax weight was reduced at remating. In contrast, the replenishment of the spermatophylax in unparasitized males was complete after 2 days and was increased no further after 3 days. Both sperm number and ampulla weight showed an increase over time since last mating and sperm production was estimated at a constant rate of 500,000 per day in all individuals, regardless of parasitism. The allocation of investment in components of the spermatophore varies greatly with parasitism and remating. Both factors had rather independent effects on spermatophore constitution, revealing functional constraints acting on spermatophore characteristics in bushcrickets, which are important for understanding the selection pressures working on its components.

Key words Spermatophylax · Sperm competition · Host-parasitoid relationship · Ormiini · Orthoptera

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Introduction

Males of a variety of insect orders provide their mates with a nutrient gift during or prior to copulation (Thornhill and Alcock 1983; Zeh and Smith 1985). Gifts can include prey items, products of male metabolism, adapted parts of male's body or, in sexually cannibalistic species like spiders and mantids, the male itself (see Vahed 1998 for a review). The evolution of courtship feeding has been extensively studied and evidence supports both a role of sexual selection for increased male fertilization success and natural selection for increased male parental investment (Simmons and Parker 1989).

Courtship feeding is widespread in the Orthoptera, and is believed to have evolved several times independently (Gwynne 1997). Male bushcrickets (Tettigonioidea) feed their mates with products of the accessory gland, the spermatophylax, which is attached to the sperm-containing ampulla and transferred to the female during copulation (Boldyrev 1915; Gwynne 1997). The ejaculate of bushcrickets comprises sperm and seminal fluid as well as accessory secretions associated with the complex spermatophore. The spermatophylax and ampulla constitute a single spermatophore, which, in some species may attain 30% of the male's body mass (reviewed in Wedell 1993a, 1994a; Vahed and Gilbert 1996). After copulation, the female feeds on the spermatophylax while sperm are transferred to her spermatheca. In most bushcrickets, once the spermatophylax is eaten the female consumes the ampulla. The spermatophylax is thus considered to have evolved as a sperm protection device, representing a barrier to ampulla removal by the female before sperm have been transferred (Sakaluk 1984; Reinhold and Heller 1993; Wedell 1993a, 1993b). Hence, the optimal spermatophylax size should be just large enough to ensure complete transfer of the ejaculate and any reduction in spermatophylax size should result in impaired insemination (Simmons 1995a).

The number of sperm transferred is also believed to be under strong selection (Parker 1970; Simmons and Siva-Jothy 1998), given that bushcrickets remate regularly and sperm from a number of males compete for fer-

tilization in a female's reproductive tract (Wedell 1991; Achmann et al. 1992; Gwynne and Snedden 1995).

In this paper, we focus on the costs associated with spermatophylax and sperm production in males. One cost of signalling is parasitism by acoustically orienting tachinid flies of the tribe Ormiini (Zuk and Kolluru 1998). *Therobia leonidei* is the only representative of ormiine flies in Europe (Leonide 1969) and has been found to attack the singing sex of three different families of bushcrickets. In populations of the Greek phaneropterine bushcricket *Poecilimon mariannae*, males were found to be parasitized in large proportions, up to 65% at the end of their season, whereas the mute females were never found to be infected (Lakes-Harlan and Heller 1992; Lehmann and Heller 1998). After locating a male bushcricket, the female fly deposits planidium larvae on his abdomen which burrow inside the host. The larvae produce a breathing funnel on the host's abdomen, on average 4 days later, visible as a brown dot (Lehmann and Heller 1998). Infected host males inevitably die following parasitoid emergence, on average 11 days after infection (Lehmann and Heller 1997; Lehmann 1998). We expect that parasitism will reduce a male's investment capability into spermatophore production unless males are able to compensate the costs related to infection.

We hypothesis that: (1) parasitized males might reduce both spermatophylax weight and sperm number in concert as predicted by optimal allocation theory, or (2) that active suppression of host reproduction may occur as a result of direct parasitoid influence (see Adamo 1997 for a review). Reproduction is inevitably coupled with costs for the host, making its suppression an advantage for parasitoid survival and growth (Beckage 1985; Hurd 1990, 1993; Hurd and Webb 1997). Alternatively, (3) the infection might influence the different parts of the spermatophore independently. An alteration in spermatophore allocation may be caused by functional constraints between host interest and the physiological costs induced by bearing the fly larvae. To test the different hypotheses, we investigated the effects of parasitism in this species with respect to the allocation of investment into the different parts of the spermatophore (spermatophylax, ampulla weight and sperm number).

Methods

The phaneropterine bushcricket *P. mariannae* is a medium-sized species (mean body length around 2 cm) confined to areas of central Greece and inhabiting roadside verges and grassy patches (Willemse and Heller 1992). Animals were collected from a grassy ridge near Vrissiá (22°19' E, 39°15° N, Nomos Lárissa). Individuals of both sexes were collected as penultimate-instar nymphs and reared until adulthood in stock cages. They were provided with unlimited access to fresh green leaves and flowers of local plant species. Males used in the experiment molted to adulthood within a 3-day period, leading to little variation in male age. Adult animals were removed from stock cages and housed individually in 200-ml plastic containers. During the experiment, males were released into the field (Kanália 21°59' E, 38°53' N, Nomos Fthiótida) within a period from 20 June to 10 July and checked every night for parasitism. The fly larvae produce a breathing funnel in the host abdomen, which is visible as a brown dot 4 days after infection (Lehmann and Heller 1998). After visible infection, males survive up to 7 days (Lehmann 1998; Lehmann and Heller 1997). We scored the level of parasitism by counting the number of breathing funnels in every male.

Individuals four days after intestation after the breathing funnel had newly formed were used for determining the effect of levels of parasitism on spermatophore production by placing them in mating trials on the same night.

Treatments

To measure the influence of the number of parasitoid larvae on spermatophore size, four groups of males were used: one control group contained unparasitized males (n=21), and three experimental groups consisted of infected males with one (n=18), two (n=14) or three (n=16) larvae. These treatment groups reflected the distribution of parasitism found in samples of males collected in the field, where 50% had a single parasitoid larvae and a further 35% were double or triple infected (Lehmann 1998).

In the field experiment, females were removed from the study area to prevent males from mating. We demarcated the experimental area with wooden rods and surrounded it with a gauze fence 80 cm high. Prior to the experiment, all individuals were removed from the surrounding area over a period of 7 nights and during the experiment, we checked the surrounding area for invading individuals. No females were found within the enclosure and so we presumed no mating had taken place. Unparasitized males were housed separately until they were randomly chosen for the experiment. Mating history did not differ between parasitized and unparasitized males. The effect of the remating interval was studied by dividing males of each of four groups into two random subsets. The half of the males were remated after 2 days while the second half were mated after 3 days.

The experimental design produced 12 datasets: four for the males with different number of larvae by three treatments: mating on day 0, 2 and 3. The univariate statistical analysis was done by repeated-measures two-factor ANOVA with interaction. Multivariate analysis was done by two-factor repeated measures MANOVA (Wilkinson 1998).

Matings

Males were mated with randomly chosen virgin females from the stock cages. To obtain matings, individual pairs were assigned to 500-ml plastic boxes and observed until copulation. Spermatophores were removed from the female immediately following copulation. The whole spermatophore was weighed fresh on a Mettler PM 460 scale (accuracy ±1 mg). Afterwards, both spermatophylax and ampullae were dissected and the mass of each component determined. Weighings were carried out "blind" by the second experimenter. Total handling time for weighing was 5 min. Water loss due to desiccation was checked by repeated weighing of the whole spermatophore; no significant loss during a 5-min weighing window was found.

Sperm number

Sperm-containing ampullae were stored in 1.5-ml Kryovials (Nalgene) in liquid nitrogen. They remained frozen until their transfer to the laboratory where the ampullae were placed into 1-ml isotonic water and ruptured mechanically with a pair of tweezers and a scalpel. The liquid was homogenized by drawing through a syringe and afterwards diluted in a saline solution (Lehmann and Festing 1998). The high number of samples (n=138) made it desirable to use an automated counting procedure. Sperm counts were made with a particle counter (Coulter Z1). This process was developed to detect particles of a given volume and count them in an electrical conducting solution (Festing 1996). The user can adjust the size of the particle to a desired range. For this study, single

sperm of *P. mariannae* were measured under a microscope and the mean size (sperm head 25 μm in length and 2.5 μm width, n=20) used as the measurement limit. After homogenization of the whole ampulla, some remains from the ampullae walls caused problems for the counts (Lehmann and Festing 1998) and for this reason all counts were done after 5 min during which tissue was separated from sperm by gravity. For each probe, four counts were made and the mean used in further analysis (see Simmons 1986). All counts were corrected for the dilution factor.

Counting under the microscope as a control was done on random samples using a haemocytometer (improved Neubauer) and this method gave similar results (correlation Pearson's R^2=0.83, n=35, P<0.01).

Results

The weight of the whole spermatophore in unparasitized *P. mariannae* was on average 133.19±22.75 (SD) mg (n=21) which constituted 22.79±4.10% of male body weight. The transfer of the spermatophore followed immediately after genital contact in this species and without any previous time in copula. The transfer of the whole spermatophore lasted nearly 2 min (mean 118.67±16.75 s, n=30). This time was divided into three phases. First, the male transferred the ampulla to the female (36.70±7.70 s). Second, the male drew near the female and pressed the ampulla to the ventral side of her abdomen (26.40±11.00 s) and finally, the male spent the rest of the time (55.57±15.09 s) passing the spermatophylax to the female. The pair separated after the whole spermatophore had been transferred. There was no influence of parasitism or day of mating on copulation times (ANOVA: level of parasitism, df=1,24, F=0.01, P=0.92 NS; day of mating, df=2,24, F=2.86, P=0.07 NS).

Spermatophylax size

The spermatophylax in *P. mariannae* weighed on average 109.04±20.55 mg in unparasitized males at their initial mating, which accounts for 18.55±3.63% of male body weight (n=21). The spermatophylax was the largest part of the spermatophore and accounted for 81% of the whole donation.

Spermatophylax size decreased with remating (two-factor repeated-measures ANOVA within subjects: df=1,61, F=60.6, P<0.001) and this reduction was enhanced by parasitism (df=3,61, F=5.3, P<0.01). Unparasitized males showed similar spermatophylax weight at the initial mating as well as after a remating interval of 2 and 3 days. In parasitized males, there was a great change between spermatophylax size at remating compared to the initial mating (Fig. 1); nevertheless, there was no significant interaction between the change in spermatophylax weight – comparing initial mating and remating – and the duration of the remating interval (df=1,61, F=1.7, P<0.20 NS).

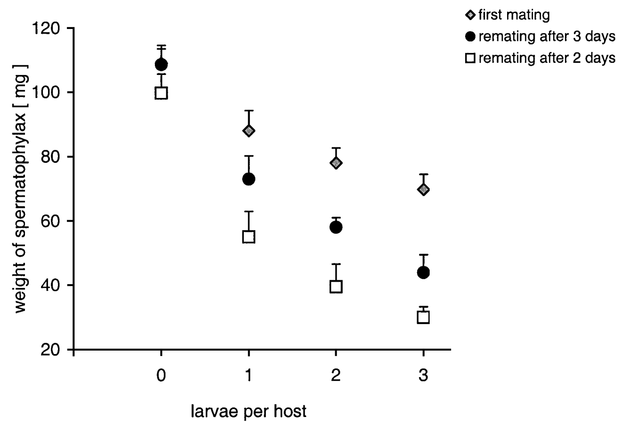


Fig. 1 Spermatophylax weight (mean±SE) in unparasitized and parasitized *Poecilimon mariannae* males at the initial mating (*diamonds*), and after a remating interval of 2 (*squares*) or 3 (*circles*) days in comparison to the level of parasitism. Number of tested individuals (given in the order: initial mating, remating after 3 and after 2 days) for unparasitized males: 21, 11, 10; males with one maggot per host: 18, 11, 7; two maggots: 14, 7, 7, and three maggots: 16, 7, 9. Unparasitized males had similar-sized spermatophylaces in the initial mating and after remating. Parasitized individuals transferred smaller spermatophylaces in all matings compared to unparasitized males; additionally, spermatophylax size decreased at remating compared to the initial mating and this reduction was enhanced by the degree of parasitism (two-factor repeated-measures ANOVA; see text for details)

Ampulla size

Ampulla size was drastically reduced at remating compared to the initial mating (two-factor repeated-measures ANOVA within subjects: df=1,58, F=149.9, P<0.001), but there was no significant interaction between the change in ampulla size with the duration of the remating interval (df=1,58, F=3.0, P=0.09 NS). Change in ampulla weight from the initial mating to remating was not influenced by parasitism (df=1,58, F=0.6, P=0.61 NS); absolute ampulla size was reduced in parasitized males (two-factor repeated-measures ANOVA between subjects: df=3,58, F=6.4, P<0.001). Parasitized males produced smaller ampullae than unparasitized males, but the number of fly larvae did not further influence ampulla size (Fig. 2). The same was true for the duration of the remating interval: the day of remating had no significant influence on the change across rematings, but the absolute ampulla weight was in all classes of males greater after 3 days than after 2 days (df=1,58, F=6.3, P<0.05). The interaction between parasitism and remating was not significant (df=3,58, F=0.7, P=0.58 NS), indicating that the two factors are independent.

Sperm number

An obvious reduction in the number of sperm was only found in threefold-parasitized males (Fig. 3). These males transferred fewer sperm especially during the initial mating than males of the other three groups, giving a significant reduction in sperm number with the level of

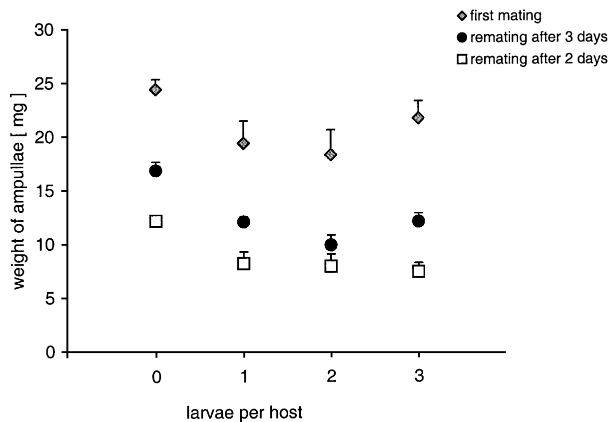


Fig. 2 Ampulla weight (mean±SE) in parasitized and unparasitized *P. mariannae* males on the initial mating (*diamonds*), and after a remating interval of 2 (*squares*) or 3 (*circles*) days in comparison to the level of parasitism. Number of tested individuals (given in the order: initial mating, remating after 3 and after 2 days) for unparasitized males: 21, 11, 10; males with one maggot per host: 18, 11, 7; two maggots: 14, 7, 7, and three maggots: 16, 7, 9. Parasitized males produced smaller ampullae than unparasitized males, but the number of fly larvae did not decrease it further. Ampulla size was reduced at remating compared to the initial mating and was greater after 3 days than after 2 days (two-factor repeated-measures ANOVA; see text for details).

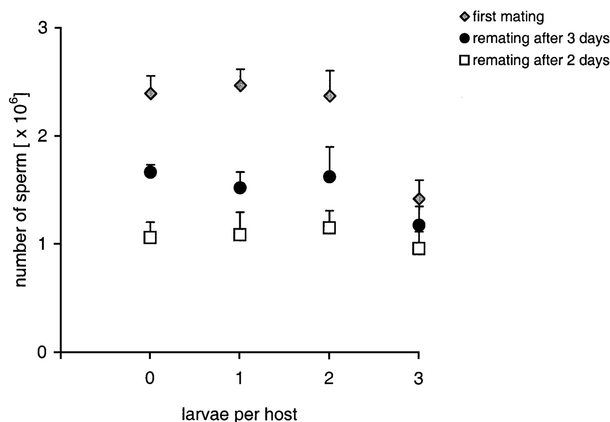
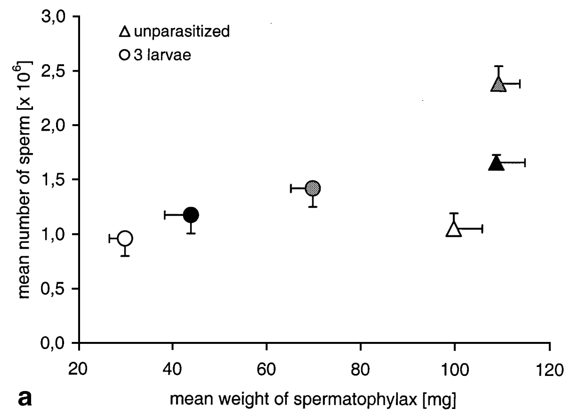


Fig. 3 Sperm number (mean±SE) in parasitized and unparasitized *P. mariannae* males on the initial mating (*diamonds*), and after a remating interval of 2 (*squares*) or 3 (*circles*) days in comparison to the level of parasitism. Number of tested individuals (given in the order: initial mating, remating after 3 and after 2 days) for unparasitized males: 21, 11, 10; males with one maggot per host: 18, 11, 7; two maggots: 14, 7, 7, and three maggots: 16, 7, 9. The number of sperm was not significantly different between unparasitized and parasitized males infected with one or two fly larvae. In all three groups, the number of sperm was reduced after remating. Parasitized males bearing three larvae transferred fewer sperm at their initial mating, but not at remating (two-factor repeated-measures ANOVA; see text for details)



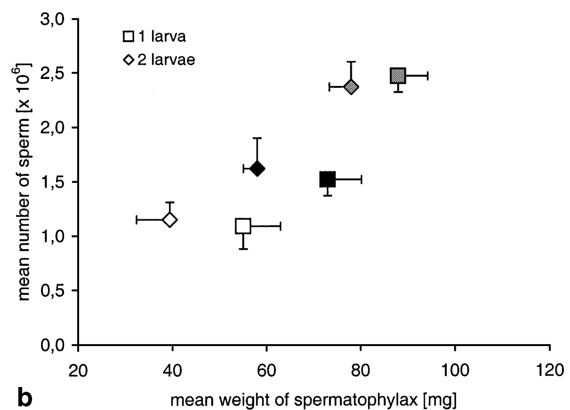


Fig. 4a,b Sperm number (mean±SE) plotted against spermatophylax weight (mean±SE) for the 12 groups of *P. mariannae* males at the initial mating (*grey symbols*), and after a remating interval of 2 (*open symbols*) or 3 (*black symbols*) days. **a** Unparasitized males (*triangles*) in comparison to parasitized individuals bearing three (*circles*) parasitoid larvae. **b** Parasitized individuals bearing one (*squares*) or two (*diamonds*) parasitoid larvae. The allocation into spermatophylax weight compared to number of sperm varied with remating interval and parasitism (repeated-measures MANOVA; see text for details)

parasitism (two-factor repeated-measures ANOVA: level of parasitism, df=3,61, F=9.1, P<0.001).

Two-factor repeated-measures ANOVA indicated a significant change in sperm number with remating (within subjects: remating, df=1,61, F=73.0, P<0.001). The pattern of change across rematings also differed with the duration of the remating interval (interaction: df=1,61, F=4.9, P<0.05): the largest sperm number was transferred at the initial mating while the smallest amount was transferred at the remating interval of 2 days. After a remating interval of 2 days, the amount of sperm transferred was 1.06±0.08 million (n=33), after 3 days, 1.52±0.08 million (n=36; data pooled from all males). The production rate could be calculated as half a million sperm cells per day for parasitized and unparasitized males. There was also a significant interaction between the change in sperm number and the level of parasitism (interaction: df=3,61, F=3.5, P<0.05). In fact, threefold-infected males had only slightly lower sperm numbers after remating than at the initial mating, whereas in all other males, a great change with the duration of the remating interval was obvious.

Allocation

Univariate statistics confirmed that spermatophylax weight, ampulla weight and sperm number increased

with remating interval. Analysis of the relative investment into spermatophore compartments showed an increase in both ampulla weight and sperm number, suggesting that these components covary. We found a positive and significant relationship between ampulla weight and sperm number (y=0.00008+56765.1x; n=134, R=0.57, P<0.001). The residuals of the regression between ampulla weight and sperm number revealed a significant influence of both the day of mating and the level of parasitism on relative sperm number in the ampullae. Unparasitized males had relatively more sperm, corrected for the size of ampulla than parasitized males (two-factor ANOVA: level of parasitism, df=3,122, F=5.9, P<0.001). Even so, sperm number in the ampullae was relatively higher at the initial mating than at rematings after 2 and 3 days (day of mating, df=2,122, F=4.5, P<0.05). There was also a positive interaction between both factors (df=6,122, F=2.3, P<0.05).

We then focused on the relative investment into sperm number compared with spermatophylax mass. Multivariate analysis of variance showed that the allocation into spermatophylax mass compared to sperm number varied with remating interval (Wilks' λ=0.697, df=4,58, F=6.310, P<0.001) and parasitism (λ=0.218, df=12,153, F=9.975, P<0.001). There was no remating×parasitism interaction (λ=0.808, df=12,153, F=1.076, P=0.384 NS), suggesting that both factors had rather independent effects. This led to a shift in the relative investment into either sperm number or the spermatophylax size (Fig. 4).

Discussion

Male bushcrickets generally require time to recuperate before they are ready to mate again. Furthermore, species transferring larger spermatophores appear to need a longer period than species providing smaller ones (Wedell 1994b). The spermatophore mass of 23% body weight in *P. mariannae* is at the top end of the range found in bushcrickets (Wedell 1994a; Vahed and Gilbert 1996) and this may explain why males are not willing to remate the day after mating (G. Lehmann, personal observation). Our results show that spermatophore size increases with male remating interval as found in other bushcrickets (Simmons 1995b; Reinhold and von Helversen 1997). In *Requena verticalis*, mating history of high-diet males had no significant influence on spermatophore size (Gwynne 1990), while males of the wartbiter *Decticus verrucivorus* showed reduced spermatophore size after their initial mating but size remained constant over subsequent matings (Wedell 1993b). Males in both studies (Gwynne 1990; Wedell 1993b) were allowed to adjust their remating interval freely and therefore may not reduce spermatophore size, but instead invest in an adjusted spermatophore size by prolonging their remating interval. The bushcricket spermatophore is attached externally to the female and so reduced spermatophore size should lead to a reduced ampulla attachment period because female feeding on the spermatophylax is size dependent. As a consequence, reduced spermatophylax size reduces the number of sperm transferred to female's spermatheca (see Gwynne 1997 for a summary).

In our study, all unparasitized males were able to replenish their spermatophylax after 2 days; their spermatophylax was not significantly smaller than at the initial mating. This may be explained by the fact that spermatophylax size is critically coupled with sperm transfer. A male benefits if he transfers a spermatophylax large enough to protect its ejaculate. Further enhancement in spermatophylax size in turn would reduce a male's capability for future matings (Simmons 1995b).

This is consistent with the result that spermatophylax size is not further enhanced in unparasitized males after a remating interval of 3 days. Parasitized males, however, showed a reduction in spermatophylax mass compared to their initial mating and there was a negative correlation between spermatophylax size and the number of parasitoid larvae per host. This indicates that an infection with the parasitoid fly reduces drastically a male's ability to replace the spermatophylax. We conclude that this reduction in spermatophylax size is due to the extra costs of bearing a parasitoid inside a male's body. No evidence of tissue damage was observed in dissected parasitized males, as was also the case in the reproductive organs of a gryllid infected by a closely related ormiine fly (Adamo et al. 1995a). Fly larvae dissected from males immediately after forming their breathing funnel (a mean of 4 days after infection) were only 1 mm in length, while they reached close to 10 mm and around 10% of a male's body weight 7 days after the appearance of the breathing funnel (Lehmann 1998). Such rapid growth was reported for *T. leonidei* by Leonide (1969) and is also found for the ormiine *Ormia ochracea* in North American gryllid species (Adamo et al. 1995b). The use of host nutrition for maintaining growth undoubtedly explains the reduction in spermatophlyax size found in parasitized males.

Our results could be explained by a preference of the female flies for laying larvae on the males that sing the most, and if these long-duration singers tend to invest more energy into singing they might have smaller spermatophylaces. We believe that this is unlikely for two reasons:

(1) Unparasitized control males and animals released into the field were chosen randomly from the same stock. Therefore it is very unlikely that all unparasitized males represent a "large-spermatophylax male type".
(2) Probably a stronger argument against prior differences might be seen in the fact that song verses produced on a given night did not differ between parasitized males of *P. mariannae* compared to healthy males in an early stage of parasitism (Lehmann and Heller 1997; Lehmann 1998). The verse number was only reduced from the eighth day after infection onwards.

Sperm number and ampulla weight were both reduced in rematings, but contrary to the spermatophylax, both compartments showed an increase over time not only in parasitized but in all males. Assuming a linear increase over time, sperm number can be calculated as being replaced at a rate of half a million sperm cells per day in all males. Sperm production is very likely to be restricted by the number of germ cells in the testis. This result is consistent with the nearly linear increase in sperm number with time since last mating in the Australian bushcricket *R. verticalis* (Simmons 1995b). The change in ampulla size and sperm number at remating may be simply a numerical increase over time. This build-up is reduced with parasitism but the reduction was small compared to the influence of remating. Some questions might arise from the fact that sperm number in males with one or two parasitoids is not influenced by parasitism. Is sperm production not under a male's *direct* control? Alternatively sperm production could be simply physiological cheap? A third possibility is that a male invests actively as much as he can into sperm production and reduces the investment into the spermatophylax production? These three alternatives need further investigation.

Allocation

Comparative studies revealed high correlations between number of sperm and size of spermatophylax among species (Wedell 1993a, 1994b; Vahed and Gilbert 1996). The authors concluded that the main function of the spermatophylax is to ensure sperm transfer. In our study, this correlation is reduced by the different effects of parasitism and remating on the size of the spermatophore (see Fig. 4). Unparasitized males transfer in every mating the same amount of spermatophylax whereas parasitized males transferred much smaller spermatophylaces. In all males, sperm number was mainly influenced by remating and to a much smaller degree by parasitism. An obvious reduction in the number of sperm was only found in threefold-parasitized males at their initial mating.

In two bushcricket species, such a decoupling between spermatophore compartments was shown to be due to male response to variance in female quality (Simmons et al. 1993; Simmons and Kvarnemo 1997) and explained as an adaptive variation (Simmons 1995b). In our experiments, all matings were obtained using virgin females, reducing variance in female quality, which is therefore unlikely to explain the alteration in spermatophore characteristics.

Our results do not support an optimal investment of males into the different parts of the spermatophore. As outlined previously, the amount of sperm transferred is directly coupled to spermatophylax size in all studied bushcrickets (see Gwynne 1997 for a review). Under such a scenario, a correlation between both compartments would be an advantage for males. This is obviously not the case in parasitized males, which transfer the same number of sperm but a spermatophylax reduced in size compared to unparasitized males (cf. Fig. 4).

Alternatively, the parasitoid might influence a male's investment into the spermatophore actively (see Adamo 1997 for review). Reproductive events are inevitably coupled with costs for the host, making their suppression an advantage for parasitoid survival and growth (Beckage 1985; Hurd 1990, 1993; Hurd and Webb 1997).

The fly larvae of *T. leonidei* do not completely suppress host reproduction: all parasitized males in our study were able to transfer a spermatophore. If the parasitoid suppresses the replacement of the spermatophylax only partly, this might explain its reduction in size in parasitized males. This is not achieved by active destruction of the accessory glands, because no evidence for direct damage of tissue was found in dissected males.

Despite the direct influence of the parasitoid, a non-adaptive alteration in spermatophore allocation may be caused by functional constraint between host interest and the physiological costs induced by bearing fly larvae. The direction of this allocation shift may shed some light on the selection forces related to spermatophylax production in bushcrickets and its evolutionary implications. We favor a functional explanation with respect to our results: physiological costs due to parasitism reduce a male's investment capacity into the spermatophylax. There is correlational evidence that sperm number is restricted by testis size and related to cell division speed, which seems to be unaffected by parasitoid infection. We therefore argue that the selection pressure is different for these two compartments.

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