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Immune Challenge and Pre- and Post-copulatory Female Choice in the Cricket *Teleogryllus commodus*

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Abstract Life history theory predicts a trade off between the expression of male sexual traits and the immune system. To test for this trade off, male crickets *Teleogryllus commodus* were injected with bacterial lipopolysaccharides (LPS) to induce an immune response and their subsequent pre- and post-copulatory sexual attractiveness to virgin and non-virgin females was assessed. Pre-copulatory attractiveness was quantified based on the time taken for males to court and mate with a female. Post-copulatory attractiveness was measured as the time that elapsed between mating and a female interrupting sperm transfer by removing the externally attached spermatophore. We found no difference in pre- or post-copulatory attractiveness between LPS and control males. In contrast, virgin females retained spermatophores for almost twice as long as non-virgins, presumably to enhance fertilization and begin egg-laying. Finally, we note that although LPS is a widely used immune elicitor in insects, its effect might be transitory. After 24 h there was no detectable elevation in haemolymph antibacterial activity of LPS injected crickets compared to that of controls.

Keywords Immunocompetence · LPS · male attractiveness · trade off · pre-copulatory choice · cryptic female choice

Introduction

Throughout its lifetime, an individual will accumulate resources that it can then allocate to the production and maintenance of traits that will enhance its fitness.

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Trade offs in how resources are allocated to different traits inevitably arise because the allocation of resources to one trait will deplete the resources available for another trait (Rowe and Houle 1996). One particular case study of trade offs between different life history traits is the trade off between male reproductive traits and the immune system. Both male sexual traits (e.g. sexual signaling, courtship), and maintaining and using the immune system are generally costly in terms of resources such as energy and nutrition. Consequently, a trade off between the two traits might be expected if resources are limited (Sheldon and Verhulst 1996). Trade offs between male sexual signaling and immune function are of particular interest because they are pivotal in explaining how male sexual traits might function as reliable signals of male quality to females (i.e. the immunocompetence handicap hypothesis Folstad and Karter 1992, e.g. Rantala et al. 2000; Ryder and Siva-Jothy 2000; Rantala et al. 2002; Rantala and Kortet 2003; Simmons et al. 2004; Simmons and Roberts 2005).

A standard empirical test for a trade off between immune function and sexual characters is to experimentally up regulate either sexual signaling or immune system activity and examine the consequence for investment in the other trait (Schmid-Hempel 2005). For example, exposing males to a greater number of females increases male reproductive effort and/or sexual signaling. Increased reproductive effort results in weaker expression of several components of the immune system in the crickets *Allonemobius socius* (Fedorka et al. 2004) and *Gryllodes sigillatus* (Gershman et al. 2010; Kerr et al. 2010), mealworm beetles *Tenebrio molitor* (Rolff and Siva-Jothy 2002), fruit flies *Drosophila melanogaster* (Mckean and Nunney 2001) and spiders *Hygrolycosa rubrofasciata* (Ahtiainen et al. 2005). Similarly, injection of male *T. molitor* with juvenile hormone enhances the attractiveness of their pheromones, but suppresses two components of immune function (Rantala et al. 2003a). Some studies experimentally infect males with parasites to test whether mounting an immune response is costly and therefore reduces sexual trait expression. For example, nematode infection reduces spermatophylax (nuptial gift) size in *G. sigillatus* (Luong and Kaya 2005), tapeworm infection reduces the attractiveness of male pheromones in *T. molitor* (Worden et al. 2000) and male crickets, *Teleogryllus oceanicus*, infected with a parasitoid fly show reductions in various aspects of reproductive effort (Kolluru et al. 2002).

Rather than using live parasites to induce an immune response, another approach is to use non-living immune elicitors that activate the immune system without direct pathogenic effects. One widely used immune elicitor is lipopolysaccharide (LPS), a surface molecule of gram negative bacteria. Several studies that inject individuals with LPS report that it negatively affects life history and sexual traits (e.g. Moret and Schmid-Hempel 2000; Ahmed et al. 2002; Jacot et al. 2004, 2005b; Reaney and Knell 2010). For example, in the field cricket *Gryllus campestris*, injection of nymphs with LPS causes costly upregulation of the adult immune system (Jacot et al. 2005a), and alters the morphology of the male forewing thereby negatively affecting the production of sexual advertisement calls (Jacot et al. 2005b). Injecting adult males with LPS lowers their call rate, which reduces sexual attractiveness (Jacot et al. 2004). Similarly, injecting males with LPS leads to the production of smaller spermatophores in *G. sigillatus* thereby reducing insemination success (Kerr et al. 2010), and reduced the time spent calling by sagebrush

crickets *Cyphoderris strepitans*, which lowers a male's mating rate (Leman et al. 2009).

Here we test for a trade off between sexual attractiveness and immune function in the Australian black field cricket *Teleogryllus commodus*. Most studies that test for the predicted trade-off focus on male traits that are used to attract females to mate (i.e. those that are under pre-copulatory sexual selection). Fewer studies have investigated traits that are under post-copulatory sexual selection due to cryptic female choice. We investigated the effect of immune challenge on both pre-copulatory and post-copulatory attractiveness. Rather than using indirect measures of attractiveness (e.g. call rate which is correlated with mating success; Brooks et al. 2005; Bentsen et al. 2006) we directly determined how an immune challenge affected the propensity of females to mate with a male (female choice), and then how much sperm the female will allow the male to transfer after mating (cryptic female choice).

Male *Teleogryllus commodus* produce a long-distance advertisement call to attract females (Loher and Rence 1978; Evans 1988), and switch to a courtship call when the female is nearby (Campbell and Shipp 1979). A sexually receptive female will mount the male, who then transfers an externally attached spermatophore. After she dismounts, the male guards her to prevent her from removing the spermatophore until the sperm inside have migrated to her spermatheca (Bussière et al. 2006). Once guarding ends most females remove the empty spermatophore (Loher and Rence 1978; Loher 1981). Male reproductive success therefore depends on a male's ability to: (a) attract females; (b) successfully court those that approach; and (c) ensure sperm are transferred after mating. In this study, we focused on the effect of an immune challenge on short-range, pre-copulatory attractiveness (i.e. male courtship success) and post-copulatory attractiveness (ability to induce spermatophore retention). In previous work we have shown that a male's latency to mating when placed with a female (i.e. the time taken for the female to mount the male) is significantly repeatable across females and predicts actual mating success (Shackleton et al. 2005). We therefore use latency as our measure of male pre-copulatory attractiveness. To measure post-copulatory male attractiveness we recorded how long a female retains a spermatophore after mating when the male is prevented from guarding so that the female controls sperm transfer. Spermatophore retention time is linearly related to the total number of sperm transferred to the female (Hall et al. 2010).

Immune system activation has been shown to affect pre-copulatory sexual advertisement in several crickets (Jacot et al. 2004, 2005b; Tregenza et al. 2006). For example, in the congeneric *T. oceanicus*, an immune challenge with a nylon filament reduced the propensity of males to court females and altered their courtship call (Tregenza et al. 2006). The courtship call of *T. commodus* is under post-copulatory sexual selection because call structural components affect the length of time that a female retains a spermatophore after mating (Hall et al. 2008). We therefore predict that an immune-challenged male *T. commodus* will be less attractive to females and have a longer latency to mating. Previous studies have shown that males with a shorter latency to mating have a greater spermatophore retention time (Bussière et al. 2006; Hall et al. 2010). Consequently, we predict that females will more rapidly remove spermatophores of immune-challenged males.

Materials and Methods

Study Population

We used crickets from stock populations established from >200 wild caught crickets collected in Canberra, A.C.T., Australia. Each generation was bred from >100 pairs and housed in 6-8 large communal tanks maintained at 26–28 °C on a 12:12 photoperiod with ad libitum dry cat food (KiteKat Krunch, Uncle Ben's, Raglan, Australia) and water. Immature females were separated from males as soon as their ovipositor became visible to ensure virginity. Exposure of juvenile crickets to acoustic sexual signals during rearing can affect investment into immune function as adults (e.g. Bailey et al. 2011). All crickets used in the study were reared in the same constant temperature room and were therefore exposed to similar levels of acoustic background noise from calling stock males.

Male Attractiveness and Immune System Activation

Male attractiveness was assessed in mating trials. To measure male pre-copulatory attractiveness we noted (a) the time from the introduction of a female until the male started to produce the courtship call ('time to call'); and (b) the time from the onset of courtship calling until the female mounted the male ('time to mount'). Previous studies in *T. commodus* have recorded 'latency to mate' as the sum of these two measures (Shackleton et al. 2005; Bussière et al. 2006). Here we also report the combined measure of 'latency to mate', but we recorded 'time to call' and 'time to mount' to test whether an immune challenge affects one, neither or both components of 'latency to mate'. 'Spermatophore retention time' was measured as the time from when a male was experimentally removed immediately after spermatophore transfer was completed, until the female removed the spermatophore (see Bussière et al. 2006).

It is worth noting that our four measures of male "attractiveness" reflect different male and female processes. Time to call is a measure of a male's readiness to begin courtship and transfer a spermatophore. Time to mount is a measure of a female's willingness to mate with a courting male. Therefore time to call can be considered a male driven process, whereas time to mount is under female control and measures male attractiveness in the strict sense of active female choice. Consequently, latency to mate is a combination of both male and female medicated processes. Spermatophore retention time is under female control and therefore can be considered as a measure of male attractiveness in the strict sense.

We injected males with LPS from *Serratia marcescens*, a bacterium that is potentially lethal to crickets (Adamo et al. 2001) to induce an immune response. We conducted two separate behavioural experiments that were performed several months apart. In both experiments, males were either injected with LPS or a control saline and then pre- and post-copulatory attractiveness was assessed in mating trials. We predicted that males injected with LPS would take longer to begin courtship and to induce a female to mate (i.e. longer time to call and time to mount). Because of the link between latency to mate and spermatophore retention time (see above, Bussière et al. 2006), we then predicted that LPS males would have their spermatophores

removed sooner than control males. In the first experiment, we used virgin females. There is, however, the potential for virgins to be less choosy than already mated females (Kokko and Mappes 2005 e.g. Bateman et al. 2001). In the second experiment we therefore used both virgin and non-virgin females. In this second experiment, we predicted that non-virgins would be more discriminating than virgins in the mating trials. For example, it is possible that any discrimination against LPS males might only be observed with non-virgin females. Finally, in a third experiment we assessed the effect of LPS injections on one component of the immune system: lysozyme-like activity of the haemolymph. Lysozyme is an antibacterial enzyme that aids in defence by hydrolysing bacterial cell walls. The standard assay for lysozyme-like activity is to measure the rate at which haemolymph clears a bacterial suspension (methods for *T. commodus* in Drayton and Jennions 2011). We predict that injection with LPS will elevate lysozyme-like activity in the haemolymph.

Experiment 1: Male Immune Challenge and Mate Choice by Virgin Females

Males were tested in 11 blocks (n=8–10 males/block). Each morning, half the males in a block were injected between the abdominal sternites using a 10 μl syringe (SGE Analytical Science) with 10 μl of LPS solution [5 mg of LPS derived from *Serratia marcescens* (Sigma-Aldrich, L 6136)/1 ml Grace's Insect Medium (Sigma-Aldrich, G8142)]. This is equivalent to 100 μg LPS/g of cricket (mean adult mass≈500 mg). The other 'control' males were injected with 10 μl of Grace's Insect Medium. Males were then housed individually in plastic tubs (9x9x5cm) with food and water provided ad libitum. Males resumed normal activities (e.g. feeding and grooming) after injection. Males used for the mating trials in Experiment 1 were virgins.

Male attractiveness was assessed in mating trials conducted 24 h later. Each male was placed on a piece of clean filter paper under an inverted plastic tub (9×9×5 cm). The trial started with the introduction of a virgin female. We recorded 'time to call' and 'time to mount'. After a spermatophore was transferred and the female dismounted, the male was removed by lifting the container and gently coaxing him out with a pencil. There was minimal disturbance of the female. The time the female took to remove the spermatophore was then recorded. If a male did not call within 60 min he was discarded. Only one female failed to remove the spermatophore within 2 h of its transfer and was excluded from the analyses of spermatophore retention time. All trials were performed under red light. Males and females were weighed before each trial.

Experiment 2: Does Mate Choice Differ Between Virgin and Already Mated Females?

Males were tested in nine blocks (n=10–60 males/block). For each block, half the males were injected with 10 μl of LPS solution [5 mg of LPS/1 ml phosphate-buffered saline (PBS- 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 L distilled water, pH 7.4)], and the other half with 10 μl of PBS. One block of males was injected per day. After injection, males were housed individually in small plastic tubs (9×9×5 cm) with food and water provided ad libitum. Again, males resumed normal activities after injection. Males were injected in the morning and mated to a

stock female in the afternoon to ensure that the spermatophores used in the mating trials the following day were produced after the injection. This ensured that females could potentially gain information about a male's immune status from the spermatophore, as well as pre-copulatory cues such as courtship calls. Following the afternoon mating the male was returned to his individual tub. Consequently, males used for the mating trials in Experiment 2 were non-virgins.

The following morning (22 h after injection), male attractiveness was assessed in two mating trials, which proceeded as in Experiment 1. In the first trial, half the males were introduced to a virgin and the other half to a previously mated female. The latter females were former virgin stock females that had been mated to another stock male the previous day (to ensure sperm transfer we placed each female in a narrow tube for an hour after mating to prevent her from prematurely removing the spermatophore). After the first trial, experimental males were allowed 150 min to produce another spermatophore and regain sexual readiness (Loher and Rence 1978). In the second trial they were introduced to a female of the opposite mating status to that in their first trial. Males and females were weighed before each trial. Again, if a female did not remove a spermatophore within two hours of transfer, she was excluded from the analyses of spermatophore retention time (n=11). Inclusion of these females did not change the main results.

If a male did not call or transfer a spermatophore after 2 h in their first trial they were returned to their own tub. These males were then reintroduced to the same female at the start of the second trial. If these males still did not call or transfer a spermatophore, they were placed with a new female the next day. If they still did not mate they were discarded. For each male we noted the time between injection and the commencement of trials that provided data.

Experiment 3: LPS and Lysozyme-Like Activity

We tested for an effect of LPS injections on immune function by measuring lysozyme-like activity in the haemolymph. We also compared male and female lysozyme activity. Virgin crickets were randomly assigned to one of three treatments: LPS, PBS or control. LPS individuals were injected with 10 μl of LPS solution (5 mg of LPS/1 ml PBS) (n=44 males, 52 females). PBS individuals received a 10 μl injection of PBS (n=42 males, 53 females). Controls were handled like LPS and PBS individuals but received no injection (n=30 males, 51 females). Lysozyme activity was assessed after 24 h (see Drayton and Jennions 2011).

To collect haemolymph, we made a small puncture under the pronotum and collected 2 μl of haemolymph with a Gilson pipette. The haemolymph was then added to 8 μl of PBS, and frozen to induce cell lysis. Where possible, two samples were taken per cricket to assess the repeatability of our measurements. Later, samples were thawed and we added 90 μl of a *Micrococcus lysodeikticus* solution (3 mg/ml PBS). The samples were then loaded into a microplate spectrophotometer (PowerWave 340, Bio-Tek Instruments Inc.) and we measured lysozyme activity at 490 nm and 30 °C as the rate of change in optical density (OD) from 10 to 30 min and from 0 to 80 min. These two measures were highly correlated (r_s=0.985, n=231, P<0.001). For subsequent analyses, we used the latter measure. Several control samples (PBS and *Micrococcus lysodeikticus* solution without haemolymph) were loaded onto each

plate to control for any daily fluctuations in the assays. The rate of change in optical density for each haemolymph sample was calculated as the sample slope minus the mean control slope. Compared to the cricket samples, very little change in optical density was observed in the control samples. We are therefore confident that our assays captured the antibacterial activity of the cricket haemolymph. All crickets were weighed prior to haemolymph extraction.

Statistics

To test for an effect of immune treatment on time to call, time to mount, latency to mate and spermatophore retention time, we ran four separate linear mixed models estimated using REML in S-PLUS 7.0. Where necessary, variables were transformed to ensure that residuals were normally distributed and homoscedastic. We used a model simplification approach, initially fitting a full model with all two-way interactions, and then removing fixed terms until the final model only contained significant terms (Crawley 2002) (random terms were always retained). For Experiment 1 block was the only random factor, for Experiment 2 both block and male identity were random factors (as each male was measured twice). For both experiments, treatment (LPS or control) was a fixed factor. For Experiment 1, male and female mass and age were fixed effect covariates. In Experiment 2, female mating status (virgin or non virgin) was a second fixed factor, and male and female mass, female age, and time since injection were fixed effect covariates. We only included female age in the models for Experiment 2 as male and female age were correlated (r_s=0.83, n=266, P<0.001). Using male age produced almost identical results. To allow the reader to assess the effect size for terms excluded from the final model, we present the significance value associated with the parameter estimate for each term when included in the final model.

To test for a relationship between pre-copulatory attractiveness and spermatophore retention time (Bussière et al. 2006) we ran separate models as described above but with spermatophore retention time as the dependent variable and either time to call, time to mount or latency to mate as a predictor.

The repeatability of our measure of lysozyme activity in the haemolymph was assessed using a one-way ANOVA with cricket identity as a factor. It was highly repeatable (see Results). We therefore used mean lysozyme activity of each cricket as our dependent variable and ran a general linear model with treatment (LPS, PBS, Control) and sex as fixed factors, and cricket weight as a fixed covariate. All crickets used in Experiment 3 were the same age.

Unless otherwise stated summary statistics are presented as mean ± SE, tests are two-tailed and α=0.05.

Results

Experiment 1: Mate Choice by Virgin Females

We injected 53 males with LPS and 54 males with control saline. Of these 3 were found dead 24 h after injection (1 LPS male, 2 control males) and 14 LPS and 11 control males did not call giving a final sample size of 38 LPS and 41 control males.

Summary statistics are shown in Table 1. An immune challenge with LPS had no significant effect on any of the four measures of male attractiveness. Heavier males took significantly longer to mate, but there were no other significant effects (Table 1).

The relationship between spermatophore retention and time to call differed between treatments ($F_{1,48}$=7.027, P=0.011). Time to call and spermatophore retention time were, as predicted, negatively related for LPS males ($F_{1,16}$=3.049, P=0.100), but not significantly so. Unexpectedly, however, control males who took longer to call had their spermatophores retained for longer ($F_{1,22}$=5.165, P=0.033), but this relationship was sensitive to removal of a possible outlier male ($F_{1,21}$=2.210, P=0.152). Again, unexpectedly, males that took longer to be mounted had their spermatophores retained for longer ($F_{1,49}$=7.529, P=0.009; no interaction with treatment: $F_{1,47}$=0.034, P=0.855). The relationship between net latency to mate and spermatophore retention differed between treatments ($F_{1,47}$=8.135, P=0.006). Spermatophore retention time and latency to mate were unrelated for LPS males ($F_{1,15}$=1.077, P=0.316), while control males that took longer to mate had their spermatophores retained for longer ($F_{1,22}$=11.300, P=0.003).

Finally, to assess the relationship between time to call and time to mount we included time to call as a covariate in the model for time to mount. These two measures of pre-copulatory attractiveness were unrelated ($F_{1,60}$=0.150, P=0.700).

Experiment 2: Mate Choice by Virgin and Already Mated Females

We injected 105 males with LPS and 105 males with control saline. 10 LPS and 10 control males were found dead 22 h after injection, and 20 LPS and 22 control males did not mate after three trials and were discarded. The final samples were therefore 75 LPS males (11 mated to a virgin only; 4 mated to a non-virgin only) and 73 control males (11 mated to a virgin only; 4 mated to a non-virgin only). Summary statistics are presented in Table 2.

As in Experiment 1, an immune challenge with LPS had no effect on any measure of male attractiveness (Table 2). The mating status of the female did not affect latency

Table 1 The effect of mass, age and immune challenge with LPS on four measures of male attractiveness in Experiment 1

	Time to call			Time to mount			Latency to mate			Spermatophore retention time		
	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Treatment	1,66	0.032	0.859	1,60	0.158	0.692	1,59	0.800	0.375	1,51	1.791	0.187
Male mass	1,66	1.415	0.239	1,60	3.387	0.071	1,60	5.201	**0.026** *	1,51	0.029	0.866
Female mass	1,66	0.309	0.580	1,60	0.439	0.510	1,59	0.009	0.923	1,51	0.987	0.325
Male age	1,66	1.061	0.307	1,60	0.019	0.892	1,59	0.003	0.957	1,51	1.521	0.223
Female age	1,66	1.329	0.253	1,60	0.136	0.714	1,59	0.0002	0.988	1,51	0.019	0.892

P-values are from the final model (if the effect of the term was significant) or for the effect of the term when it alone was added to the final model (see text)

* *P*-values significant at the 0.05 level are in bold

Table 2 The effect of mass, age, female mating status and immune challenge with LPS on four measures of male attractiveness in Experiment 2

	Time to call			Time to mount			Latency to mate			Spermatophore retention time		
	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Treatment	1,133	0.997	0.320	1,133	0.457	0.500	1, 133	0.539	0.464	1, 137	0.433	0.512
Mating status of female	1,99	1.454	0.231	1,98	0.599	0.441	1, 98	1.738	0.190	1, 106	19.758	**<0.0001** *
Male mass	1,99	0.221	0.639	1,98	0.114	0.736	1,98	0.172	0.679	1, 105	0.336	0.564
Female mass	1,99	0.749	0.389	1,98	0.635	0.428	1,98	1.128	0.291	1,106	6.346	**0.013** *
Female age	1,99	0.007	0.935	1,98	0.409	0.524	1,98	0.032	0.859	1,105	1.204	0.275
Time since injection	1,99	1.380	0.243	1,98	0.013	0.911	1,98	1.352	0.248	1,105	0.086	0.770

P-values are from the final model (if the effect of the term was significant) or for the effect of the term when it alone was added to the final model (see text)

* *P*-values significant at the 0.05 level are in bold

to mating, time to calling or time to mounting, however, virgin females took significantly longer to remove spermatophores (27.2±2.3 versus 15.0±1.4 min n=132, 123; Table 2). There was a weak but significant trend for heavier females to take longer to remove a spermatophore (Fig. 1). No other relationships were significant (Table 2).

There was no relationship between spermatophore retention time and time to call ($F_{1,89}$=0.263, P=0.610), time to mount ($F_{1,88}$=3.205, P=0.077), or latency to mate ($F_{1,88}$=0.015, P=0.901). Likewise, the time to call was not correlated with the time to mount ($F_{1,98}$=0.004, P=0.948).

Experiment 3: LPS and Lysozyme Activity

Our measure of lysozyme activity was highly repeatable ($F_{161,162}$=6.521, P<0.001, r_I=0.734). Neither an immune challenge with LPS or the act of injection had an effect on lysozyme activity ($F_{2,228}$=0.299, P=0.741). There was no sex difference ($F_{1,\ 229}$= 1.421, P=0.234) or difference in the effect of the treatments between the sexes ($F_{2,225}$=0.051, P=0.951). Finally, there was no relationship between body mass and lysozyme activity ($F_{1,\ 229}$=0.063, P=0.802).

Discussion

We predicted that the injection of male crickets with a relatively large dose of LPS (100 μg/g of cricket, see Jacot et al. 2004, 2005b; Shoemaker and Adamo 2007 for

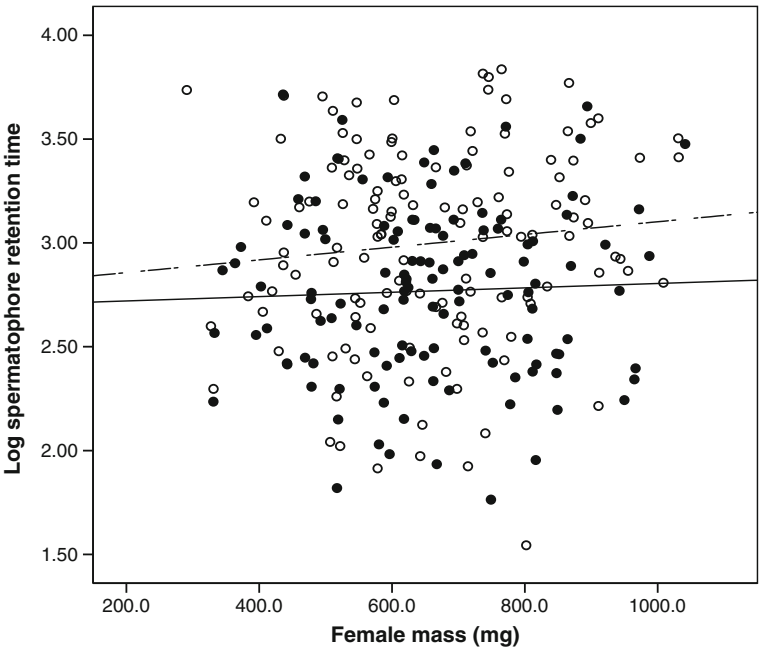

Fig. 1 The relationship between spermatophore retention time and female mass for Experiment 2. Virgin females are shown as open circles (○), with a dashed line. Non-virgin females are shown by closed circles (●) and a full line

comparative doses) would activate the male immune system and, due to a trade-off between immunity and sexual signaling, reduce male attractiveness. We predicted that LPS injected males would take longer to begin courtship and to induce females to mate, increasing the latency to mating. In addition, because *T. commodus* females have been shown to remove the spermatophores of less attractive males sooner (see Bussière et al. 2006), we further predicted that LPS injected males would suffer a reduction in spermatophore retention time. Females might also be able to detect changes in the spermatophores of LPS injected males and remove them sooner, irrespective of how long it took them to mate. However, we found no difference in the pre-copulatory attractiveness or spermatophore retention times of immune-challenged and control males.

The Trade-Off Between Sexual Signals and Immune Function

Maintaining and using the immune system has been shown to be nutritionally and energetically demanding in several insects (Feder et al. 1997; Siva-Jothy and Thompson 2002; Freitak et al. 2003; Rantala et al. 2003b; Jacot et al. 2005a). Sexual advertisement has also been shown to be condition-dependent (Wagner and Hoback 1999; Holzer et al. 2003; Scheuber et al. 2003; Hunt et al. 2004; Hedrick 2005) and to incur substantial energetic costs in crickets (Prestwich and Walker 1981; Kavanagh 1987; Hack 1998), including *T. commodus* (Kavanagh 1987; Hunt et al. 2004). Immune function and sexual advertisement should therefore trade off as they both draw from the same pool of limited resources (Sheldon and Verhulst 1996). Injection with LPS is well known to induce an immune response in many insect species (e.g. Moret and Schmid-Hempel 2000; Ahmed et al. 2002; Korner and Schmid-Hempel 2004), including crickets (Jacot et al. 2005a). Accordingly, activation of the immune system with LPS should reduce investment in courtship as resources are diverted into immunity. Many studies have found the predicted effect of immune elicitors such as LPS on reproductive effort (e.g. Jacot et al. 2004, 2005b; Fedorka and Mousseau 2007; Reaney and Knell 2010) but others, including ours, have not. Vainikka et al. (2007) found that an immune challenge with several different elicitors (including LPS) did not decrease the attractiveness of male pheromones in mealworm beetles. Shoemaker and Adamo (2007) found no changes in egg production when female crickets *Gryllus texensis* were chronically injected with 20 μg of LPS from *S. marcescens* (equivalent to 40 μg/g of cricket, mean adult mass of *G. texensis*≈ 0.5 g), and only one aspect of egg production was affected at large doses (100 μg of LPS/injection, equivalent to 200 μg/g of cricket). Finally, although injection with LPS reduced spermatophore size in the cricket *Gryllodes sigillatus*, it did not reduce calling activity (Kerr et al. 2010). It is, of course, possible that the effect of experimental immune activation on sexual signaling is generally overestimated in the literature due to a publication bias against studies that fail to demonstrate the expected outcome, even though they use the standard protocol (Jennions et al. 2012).

There are several reasons why we might not have observed a trade off between male attractiveness and immune function in *T. commodus*. First, with food and water provided ad libitum, LPS injected males might have compensated for any resources lost due to immune activation by feeding more (Vainikka et al. 2007). Immune challenged bumble bees *Bombus terrestris* only suffer a reduction in survival relative to controls when starved (Moret and Schmid-Hempel 2000), suggesting that fed bees

can compensate for any resources lost due to immune activation. Similarly, Simmons (2012) found that crickets, *Teleogryllus oceanicus,* that were immune challenged as juveniles suffered reductions in sperm viability when their access to food was restricted. However, crickets given ad libitum access to food suffered no such trade off (Simmons 2012).

Second, LPS injected males might conserve resources necessary to maintain immune activation and courtship by reducing investment in traits less closely related to fitness (e.g. less grooming) (Shoemaker and Adamo 2007). Courtship is an important determinant of male reproductive success (Bussière et al. 2006; Hall et al. 2008), so it might be protected against short-term changes in immune activation (e.g. Shoemaker and Adamo 2007). In some instances, immune challenged males may go further than simply maintaining courtship at pre-challenge levels, and instead increase levels of sexual signaling if the probability of surviving the immune challenge is perceived to be low. Such terminal investment has been demonstrated in mealworm beetles *Tenebrio molitor*, where repeatedly immune challenged males actually become more attractive to females (Kivleniece et al. 2010; Krams et al. 2011).

Third, the effect of LPS on the immune system might be sufficiently brief in duration that it has a barely detectable effect on the resource pool of signaling males. Fourth, injection with LPS causes an immune response in several insect species (e.g. Moret and Schmid-Hempel 2000; Ahmed et al. 2002; Korner and Schmid-Hempel 2004; Jacot et al. 2005a), but we cannot exclude the possibility, although it seems unlikely, that LPS failed to activate the immune system of *T. commodus*. We found no effect of LPS injections or injection per se on lysozyme-like activity in the haemolymph 24 h after treatment. If an immune response in LPS injected crickets proceeded quickly (i.e. <24 h), it is possible that we conducted our immune assays too long after injection to detect an actual elevation in lysozyme activity. Studies have shown, however, that injection with LPS can have long lasting effects on immunity [up to 24 days in crickets (Jacot et al. 2005a) and 14 days in bees (Korner and Schmid-Hempel 2004)] that can arise within two hours of injection (Korner and Schmid-Hempel 2004). Even so, variation in the rate of clearance of LPS probably varies widely among species that differ in their natural exposure to pathogens. Our results differ from Simmons (2012) who found that injection of congeneric juvenile *Teleogryllus oceanicus* with LPS caused a peak in haemolymph lysozyme activity 24–36 h after injection. Juvenile crickets might, however, allocate resources to the immune system in a different way than sexually mature adults (as used in our study). At present it therefore seems premature to conclude that LPS does not activate the immune system of *T. commodus*. Crucially, other studies that measure several aspects of immune function have found effects of experimental treatment (e.g. immune challenge, diet) on only some of the immune parameters measured (e.g. Rolff and Siva-Jothy 2002; Rantala et al. 2003a, b; Jacot et al. 2005a). Consequently, it is possible that injection with LPS induces an immune response in *T. commodus* that was not reflected in our single measure of immune function.

Female Mating Status and Mate Choice

We found that virgin females retained spermatophores for almost twice as long as already mated females. Virgin females might leave spermatophores in for longer to

ensure that at least some of their eggs can start to be fertilized, allowing them to initiate egg-laying and avoid delays in reproduction. Once fertilization is assured, however, females can then afford to be more choosy (Kokko and Mappes 2005). We might therefore expect that mated female *T. commodus* will be more willing to prematurely remove spermatophores (thereby interrupting sperm transfer) from less attractive males. Our results, however, do not support this prediction. In Experiment 2 females did not remove the spermatophores of less attractive males (e.g. males with a longer latency to mate) sooner. Furthermore, in Experiment 1 females actually retained the spermatophores of less attractive males for longer than those of attractive males. At present, our results are therefore difficult to explain. They also partly conflict with our earlier finding that females increase spermatophore retention when mating with more attractive males that have a short latency to mating (Bussière et al. 2006). There are, however, differences in study design (e.g. the number of prior matings by test females) that do not make our present and previous studies directly comparable. Furthermore, the different studies used crickets from populations (this study: Canberra, A.C.T., previous study: Smiths Lakes, N.S.W.) that differ in many call parameters and life history traits (J. Hunt, unpublished data).

In conclusion, we found no evidence to indicate a trade-off between courtship and immunity in *T. commodus*. We did, however, detect a difference in the post-copulatory behaviour of virgin and non-virgin females, indicating that any effect of an immune challenge on male attractiveness is small compared to the effect of female mating status.

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