Inbreeding, immunity and male sexual signaling in the black field cricket *Teleogryllus commodus*

Jean Mia Drayton

A thesis submitted for the degree of Doctor of Philosophy of The Australian National University

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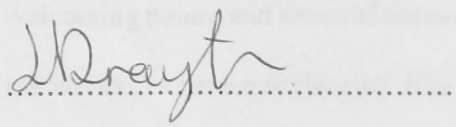
DECLARATION

The research presented in this thesis is my own original work. Several individuals made important contributions to the chapters, and this is recognized by co-authorship. Michael Jennions contributed to the ideas developed throughout this thesis and to the analysis of the data. Michael is a co-author of all chapters. Together, Kobus Boeke and I jointly injected the crickets with immune elicitors and ran all of the immune assays for Chapter 1, and he is acknowledged for this with co-authorship. Matthew Hall is a co-author of Chapter 4 and 5. Matthew assisted in calculating multivariate attractiveness scores and running the statistical analyses for these chapters. John Hunt, a co-author of Chapter 3 and 5, ran the selection analyses and assisted with the statistical analyses. Richard Milner, a co-author of Chapter 3 and 4, assisted with feeding and maintaining the crickets, and with recording male calls. The contributions of others are acknowledged in the 'Acknowledgements' section of each chapter. I am, however, the principal contributor to all aspects of the work, none of which has been submitted for a previous degree.



Jean M. Drayton

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Title page photo: Hendrik Maat.

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Wow, I'm writing the acknowledgements. That must mean I have made it!

I have come to realise that a PhD is somewhat of a team effort. It could not be attempted or completed by one person. There are many wonderful people that I need to thank who made it all possible. So here goes…

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THESIS ABSTRACT

Male sexual traits might be honest signals of male quality because they are costly to produce and/or maintain. Only high quality males can bear the costs of extravagant sexual display, creating a positive relationship between quality and trait expression. I conducted a series of experiments to test for the honesty of male sexual signals in the cricket *Teleogryllus commodus*. Males produce two acoustic sexual signals prior to mating: advertisement and courtship calls.

I first focused on the role of immunity in sexual selection. The immunocompetence handicap hypothesis (ICHH) proposes that sexual signals reliably indicate male immunocompetence because of a trade off between sexual trait expression and immunity. I first tested for the predicted trade off between male sexual attractiveness and immunity (Chapter 1). Males were injected with bacterial lipopolysaccharides (LPS) to induce an immune response and their subsequent attractiveness to females was assessed. There was no effect of LPS injections on male attractiveness.

The ICHH predicts that the expression of sexual traits should be positively correlated with immunocompetence. I tested this by measuring courtship and advertisement call structure, advertisement calling effort, and immune function (Chapter 5). I also calculated the multivariate attractiveness of calls. Immune function was not related to call structure or multivariate attractiveness. There was a weak, positive relationship between advertisement calling effort and lysozyme-like activity. Therefore a female preference for higher calling effort might select for males with higher lysozyme activity.

Finally, the assays used to measure insect immunity presumably reflect disease resistance. If disease resistance enhances fitness, we might expect a positive relationship between fitness and immune function. I tested this prediction by comparing the immune function of inbred (which should have reduced fitness) and outbred males (Chapter 2). Inbreeding did not affect lysozyme activity, but caused a significant increase in haemocyte counts. Therefore it is not always the fittest individuals that have the highest measured immune function.

I then investigated the effect of inbreeding on sexual traits. If sexual traits are reliable signals of male fitness (e.g. male condition, heterozygosity), then they should show reduced expression with inbreeding (because inbreeding reduces fitness). I first tested for inbreeding depression on advertisement calling (Chapter 3). Inbreeding reduced calling effort but had no effect on call structure. I compared the number of females that were attracted to the calls of inbred and outbred males in the field. There was no difference. I also compared the multivariate attractiveness of inbred and outbred males. Again, there was no difference. Therefore, inbreeding does not affect the attractiveness of the actual call but, because inbred males call less, they will attract fewer females.

Finally, I tested for inbreeding depression on courtship call structure and multivariate attractiveness (Chapter 4). Inbreeding resulted in a small but significant change in call structure, but did not affect multivariate attractiveness. The results suggest that the courtship call of *T. commodus* is not a reliable signal of aspects of male quality that are affected by inbreeding (which generally reduces fitness-enhancing traits).

INTRODUCTION AND OVERVIEW

Females of many species choose mating partners based on male sexually selected ornamentation and displays (review: Andersson 1994; Hettyey et al. 2010). Such preferences may be selected for in females because the traits that females use in mate choice reveal some aspect of male quality from which females can benefit (Kokko et al. 2006). Mate choice can directly benefit females if it increases, for example, their fecundity or longevity, or indirectly because males provide genes that increase offspring fitness (Hunt et al. 2004a). Sexually selected traits are thought to be honest indicators of male quality because they are costly to produce or maintain (e.g. predation, energy expenditure (review: Kotiato 2001)). Only high quality males are able to bear the costs of well developed sexually selected traits because they pay a smaller marginal cost to increase investment (Zahavi 1975; Grafen 1990 but see Getty 2006). This should lead to a positive correlation between sexual trait expression and male quality, allowing females to choose high quality mates based on sexually selected traits.

Here, I describe a series of experiments in which I test for the honesty of male sexual signalling in the black field cricket *Teleogryllus commodus*, a gryllid cricket endemic to Australia. *T. commodus* inhabits open grasslands and is largely nocturnal. Mating behaviour consists of pair formation, courtship, spermatophore transfer, and post copulatory mate guarding by males. Males produce a long-distance repetitive advertisement call to attract sexually receptive females for mating. Males call using a stridulatory apparatus consisting of a file and a scraper on the forewings. Sound is produced by rubbing the raised forewings together. Males call from simple burrows that they have dug into the

ground, grass tufts, cracks, rocks or other shelters (Loher and Rence 1978; Evans 1983; Evans 1988).

Once a female approaches a male, the male then switches from advertisement calling to produce the short-range courtship call to induce the female to mate (Campbell and Shipp 1979). A sexually receptive female will respond by climbing onto the male's back, and spermatophore transfer then takes place in which the spermatophore is transferred from the male to the female's genital opening. When mating is completed, the female dismounts and the male engages in guarding behaviour in which he stays close to the female and touches her with his antennae (Loher and Rence 1978; Loher 1981). The male acts aggressively towards a female if she tries to leave or remove the spermatophore, thereby preventing the female from prematurely interrupting sperm transfer (Loher 1981; Bussière et al. 2006). After the guarding period is over, a female will remove and usually consume the spermatophore. Females then oviposit in the burrow of the male that they have just mated, or may seek other oviposition sites such as the base of grass tufts (Evans 1983).

Recent studies have quantified female choice for advertisement and courtship calls in *T. commodus*. Female choice exerts strong positive directional sexual selection for greater advertisement calling effort and moderate levels of stabilizing and directional multivariate sexual selection on finer-scale advertisement call structure (Brooks et al. 2005; Bentsen et al. 2006). Furthermore, diet manipulations have shown that advertisement calling effort is affected by nutrition (Hunt et al. 2004b; Maklakov et al. 2008). The fine-scale structure of the courtship call is under multivariate post-copulatory sexual selection because its influences how long a female will retain a spermatophore after mating (Hall et al. 2008), which determines the number of sperm transferred (Bussière et al. 2006; Hall et al. 2010).

I first focused on the role of immunity in sexual selection and honest signalling. According to the immunocompetence handicap hypothesis, male secondary sexual traits can reliably signal resistance to parasites because the expression of sexual traits is costly due to immune suppression (Folstad and Karter 1992). Males that have genotypes that confer greater resistance to parasites, and/or have acquired sufficient resources (i.e. are in good condition) to maintain an effectively functioning immune system will minimize any costs imposed by parasites and can therefore invest more heavily in sexual trait expression. This should lead to a positive correlation between male quality and sexual attractiveness. In vertebrates, the trade off between sexual signaling and immune function is thought to be mediated by testosterone. High levels of testosterone enhance the development of sexual traits, but suppress the immune system (Folstad and Karter 1992; but see Boonekamp et al. 2008).

Although the immunocompetence handicap hypothesis was originally vertebrate based, it is becoming apparent that similar trade offs can underlie sexual signaling in invertebrates. In particular, studies with insects have shown that male sexual traits can signal immunocompetence to females (e.g. Rantala et al. 2000; Ryder and Siva-Jothy 2000; Rantala and Kortet 2003; Simmons et al. 2004). It has been suggested that the trade off between immune function and sexual signals in insects is mediated by juvenile hormone which affects resource allocation (Rolff and Siva-Jothy 2002; Rantala et al. 2003a). More generally, a trade-off between the immune system and sexual signaling is expected because both traits compete for limiting resources (e.g. energy, nutrients) (Sheldon and Verhulst 1996). Consequently, only males who are in good condition can produce extravagant condition-dependent sexual signals and maintain an effective immune system (Westneat and Birkhead 1998; Rantala et al. 2003b).

It is possible to test for the predicted phenotypic trade off between sexual traits and the immune system by experimentally up regulating either sexual signaling or immune function and examining the consequence for investment in the other trait (Schmid-Hempel 2005). I first tested for the predicted trade off between sexual attractiveness and immune function in *T. commodus* (Chapter 1). I did this by injecting males with bacterial lipopolysaccharides (LPS) to induce an immune response, and examining their subsequent pre- and post copulatory attractiveness to females. I also examined the effect of LPS injections on immune function (lysozyme-like activity in the haemolymph).

I then tested for the predicted positive relationship between the expression of male sexual traits and immune function in *T. commodus* (Chapter 5). I examined the relationship between fine scale advertisement and courtship call structure, the amount of time that a male spent advertisement calling per night (i.e. calling effort), and two measures of immune function (lysozyme-like activity and haemocyte counts). Using the results of previous selection analyses (Brooks et al. 2005; Hall et al. 2008), we could also calculate the predicted multivariate attractiveness of advertisement and courtship calls (i.e. the multivariate call trait combinations that females most strongly prefer). We then tested for any relationships between predicted multivariate attractiveness and immune function.

Many studies of sexual selection and immunity in invertebrates assume that the parameters used to assay an individual's immune system (e.g. encapsulation rate) reflect the ability of that individual to resist disease (Adamo 2004). Increased disease resistance will presumably enhance fitness, and therefore we might expect that individuals with higher fitness will have elevated measures of immunity. Indeed, several studies have found positive

correlations between fitness enhancing traits (e.g. pheromone attractiveness, mobility) and measures of immune function (e.g. Ryder and Siva-Jothy 2000; Ahtiainen et al. 2004; Rantala and Kortet 2004). In general, however, direct empirical tests of whether less fit individuals show reduced expression of specific immune components are rare. Such tests are therefore needed. I report on an experiment in which I tested for a relationship between two widely used measures of immune function and fitness using inbreeding to manipulate fitness (Chapter 2). Using inbreeding, it is possible to create classes of individuals that can be assigned either high or low fitness/quality (Bolund et al. 2010; review: Cotton et al. 2004). Inbreeding increases homozygosity across the genome and reduces many components of offspring fitness (review: Keller and Waller 2002; Armbruster and Reed 2005). Therefore, inbred individuals can be unambiguously assigned as having low fitness relative to outbred individuals. I examined the effect of inbreeding (full-sibling mating) on lysozyme-like activity and haemocyte counts in *T. commodus* (Chapter 2). If either of these measures are correlated with fitness, we expect both of these measures to decline with inbreeding.

I then turned my attention to the effect of inbreeding on sexually selected traits themselves to test if these traits are reliable signals of overall male quality in *T. commodus*. One way to test whether male sexual traits signal aspects of male quality is to create classes of individuals that can be assigned high or low phenotypic quality and to compare the expression of sexual traits between these classes. An aspect of quality that can be easily manipulated in the laboratory is condition and this is usually achieved by changing external environmental factors that affect condition such as diet (e.g. Holzer et al. 2003; Judge et al. 2008). An alternative, yet underexploited method of creating classes of individuals that can be assigned high- or low quality status is to manipulate condition via inbreeding (see

above). Inbreeding could affect sexual traits because of lower heterozygosity at loci that directly code for them. If, however, sexual traits are condition dependent (review: Cotton et al. 2004), and inbreeding reduces the general ability of individuals to acquire/assimilate the energetic and nutrient resources needed for sexual signaling (i.e. lowers condition *sensu* Rowe and Houle 1996), then we expect reductions in sexual traits with inbreeding.

Inbreeding depression in sexually selected traits might also indicate that these traits can reveal a male's genome wide heterozygosity to females. Recent models of mate choice evolution have proposed that females can benefit from mating with males with above average heterozygosity (Mays and Hill 2004; Kempenaers 2007; Fromhage et al. 2009). This is because more heterozygous males may be more likely to carry locally rare alleles that are dissimilar to those in the average female, and so mating with heterozygous males yields offspring with above average heterozygosity (i.e. heterozygosity is 'heritable'; Mitton et al. 1993; Brown 1997). Female choice for heterozygous males will therefore elevate offspring fitness whenever heterozygosity increases offspring performance. A decline in sexual trait expression with inbreeding provides direct evidence that these traits can signal genome-wide heterozygosity. More generally, inbreeding in normally outbreeding species almost always reduces fitness (Falconer and Mackay 1996). Inbreeding should reduce many components of male fitness (e.g. performance capacity, condition). If a sexual trait is a signal of one or more of these measures of male quality, and/or genome-wide heterozygosity, then inbreeding should lead to signals that are less attractive to females.

First, I examined the effect of inbreeding on advertisement calling (Chapter 3). I investigated the effect of one generation of full-sibling mating on calling effort and fine-

scale call structure. I then tested if inbreeding reduced the attractiveness of advertisement calls in a field play back experiment by monitoring how many wild females were attracted to inbred and outbred calls. Using the field data, we could conduct a selection analysis to identify the major axes of linear and nonlinear multivariate sexual selection on call structure. This allowed us to then compare the multivariate attractiveness of inbred and outbred calls along each major axis of selection. I then investigated the effect of inbreeding on the courtship call (Chapter 4). I examined the effect of one generation of full sibling mating on fine scale call structure, along with several life history traits. Using a previous selection analysis (Hall et al. 2008, see above), we could then calculate the multivariate attractiveness of each inbred and outbred courtship call and compare the attractiveness of the two call types. Finally, I briefly discuss my results within the context of honest signaling in the Concluding Remarks section.

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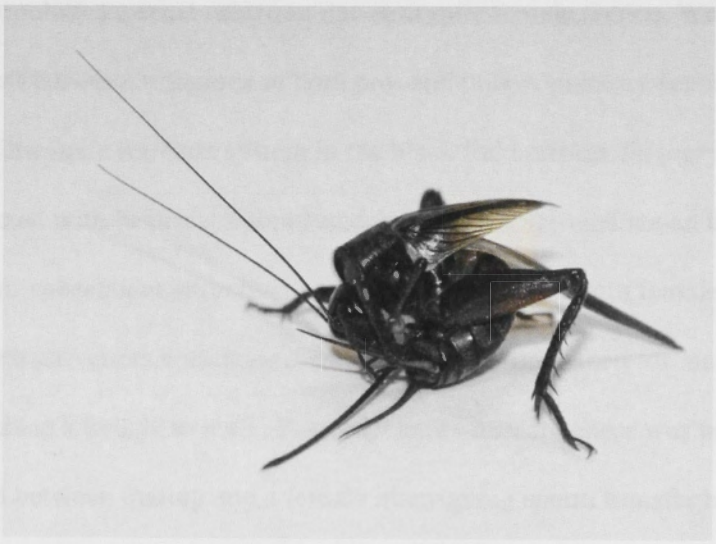
CHAPTER 1

IMMUNE CHALLENGE AND PRE- AND POST-COPULATORY FEMALE CHOICE IN THE CRICKET *TELEOGRYLLUS COMMODUS*



A female *T. commodus* removing a spermatophore. Photo: Alex McWilliam.

Jean M. Drayton [1], J.E.(Kobus) Boeke[2], Michael D. Jennions[1]

1. Evolution, Ecology & Genetics, Research School of Biology, The Australian National University, Canberra, A.C.T. 0200, Australia.

2. Centre for Ecological and Evolutionary Studies, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands.

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ABSTRACT

Several studies in insects suggest that male secondary sexual traits can signal aspects of their immunocompetence. Most studies have investigated male traits that attract females and are under pre-copulatory sexual selection. Fewer studies have investigated traits that are under post-copulatory sexual selection due to cryptic female choice. We tested for a predicted trade off between measures of both pre- and post-copulatory sexual attractiveness and the status of the male immune system in the black field cricket *Teleogryllus commodus*. Males were injected with bacterial lipopolysaccharides (LPS) to induce an immune response and their subsequent attractiveness to virgin and non-virgin females was assessed. Pre-copulatory attractiveness was quantified based on the time taken for males to initiate courtship and induce a female to mate. 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 immune challenged and control males. In contrast, we showed that virgin females retained spermatophores for almost twice as long as non-virgins. Hence any effect of an immune challenge on male post-copulatory attractiveness is small compared to the effect of female virginity on sperm transfer success. Finally, we note that although LPS is widely used to provoke an immune response in crickets and other insects, the effect might be transitory. After 24 hours there was no detectable elevation in haemolymph antibacterial activity of LPS injected crickets compared to that of control crickets.

KEY WORDS: cryptic female choice, immunocompetence, mate choice, pre-copulatory choice, sexual selection

INTRODUCTION

According to the immunocompetence handicap hypothesis, male secondary sexual traits can reliably signal resistance to parasites if the expression of sexual traits suppresses the immune system (Folstad and Karter 1992). The basic idea is that the marginal costs from immune suppression due to sexual trait expression are lower for higher quality males. This might be because these males have genotypes that confer greater resistance to parasites, and/or because they initially acquired more resources (i.e. are in better condition) and can more readily maintain an efficiently functioning immune system and fight off infections. Given the sexually selected benefits of sexual trait expression, all else being equal, this should lead to a positive correlation between male quality and sexual attractiveness. In vertebrates, the trade off between sexual signaling and immune function is thought to be mediated by testosterone. High levels of testosterone enhance the development of sexual traits, but suppress the immune system (Folstad and Karter 1992; but see meta-analyses of Roberts et al. 2004). Another possibility is that causality is reversed and immune system activation suppresses testosterone levels (see Boonekamp et al. 2008).

Although the immunocompetence handicap hypothesis was originally formulated for vertebrates, it is becoming apparent that male sexual traits can signal immunocompetence to females in insects (e.g. Rantala et al. 2000; Ryder and Siva-Jothy 2000; Rantala et al. 2002; Rantala and Kortet 2003; Simmons et al. 2004). It has been suggested that the trade off between immune function and sexual signals in insects is mediated by juvenile hormone which affects resource allocation (Rolff and Siva-Jothy 2002; Rantala et al. 2003a). More generally, a trade-off between immune system function and sexual signaling is expected because both traits compete for limiting resources such as energy and nutrients (Sheldon

and Verhulst 1996). Consequently, only males who are in good condition can produce extravagant condition-dependent sexual signals and maintain an effective immune system (Westneat and Birkhead 1998; Rantala et al. 2003b).

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), and tapeworm infection reduces the attractiveness of male pheromones in *T. molitor* (Worden et al. 2000). The use of live parasites to induce an immune response does not, however, provide a good test for a trade-off as it might simply reflect the direct effects of the parasite on sexual traits (e.g. tissue damage; see Shoemaker and Adamo 2007). This limitation can be overcome by using non-living substances that activate the immune system without actually causing disease.

One widely used immune elicitor is lipopolysaccharide (LPS), a surface molecule of gram negative bacteria that activates the insect immune system without direct pathogenic effects. 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; Jacot et al. 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 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*. We investigate 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 (cryptic female choice).

MATERIALS AND METHODS

Study species

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 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. Our measure of post-copulatory male attractiveness depends on cryptic female choice, which is measured as how long a female retains a spermatophore 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).

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. All adults used in experiments were ≥10 days post maturation to ensure they were sexually mature.

Male attractiveness and immune system activation

Immune system activation has been shown to affect pre-copulatory sexual advertisement in several crickets (see above; Jacot et al. 2004; Jacot et al. 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). Furthermore, inbreeding, which lowers fitness, affects courtship call structure (Drayton et al. 2011). Consequently, it is plausible that courtship calling, which affects both pre-and post-copulatory attractiveness, is sensitive to an immune challenge. 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). We therefore predict that females will more rapidly remove spermatophores of immune-challenged males.

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'); (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 affect 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).

We injected males with LPS from *Serratia marcescens*, a bacterium which is potentially lethal to crickets (Adamo et al. 2001) to induce an immune response. We conducted two behavioural experiments in which males were either injected with LPS or a control saline. Pre- and post-copulatory attractiveness was then assessed in mating trials. 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). In a second experiment we therefore used both virgin and 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).

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 10µl syringe (SGE Analytical Science) with 10µl of LPS solution [5mg of LPS derived from *Serratia marcescens*

(Sigma-Aldrich, L 6136)/1ml Grace's Insect Medium (Sigma-Aldrich, G8142)]. This is equivalent to 100µg LPS/g of cricket (mean adult mass ≈ 500mg). The other 'control' males were injected with 10µl of Grace's Insect Medium. Males were then housed individually in plastic tubs (9x9x5cm) with food and water.

Male attractiveness was then assessed in mating trials conducted 24 hours later. Each male was placed on a piece of clean filter paper under an inverted plastic tub (9x9x5cm). 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 hours 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 10µl of LPS solution [5mg of LPS/1 ml phosphate-buffered saline (PBS-8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in 1L distilled water, pH 7.4)], and the other half with 10µl of PBS. One block of males was injected per day. After injection, males were housed individually in small plastic tubs (9x9x5cm) with food and water. 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 precopulatory cues such as courtship calls. Following the afternoon mating the male was returned to his individual tub.

The following morning (22 hours 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 two hours 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. We noted the time between injection and the commencement of trials that provided data for each male.

Experiment 3: LPS and lysozyme-like activity

We tested for a detectable 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 (5mg of LPS/1ml 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 hours.

To collect haemolymph, we made a small puncture under the pronotum and collected 2µl 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 90µl of a *Micrococcus lysodeikticus* solution (3mg/ml PBS). The samples were then loaded into a microplate spectrophotometer (PowerWave 340, Bio-Tek Instruments Inc.) and we measured lysozyme activity at 490nm and 30°C as the rate of change in optical density (OD) from 10 to 30 minutes and from 0 to 80 minutes. 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. 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.

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 died (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 died, 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 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 minutes 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 (*Teleogryllus commodus*) with a relatively large dose of LPS (100μg/g of cricket, see Jacot et al. 2004, Jacot et al. 2005b, Shoemaker and Adamo 2007 for comparative doses) would active the male immune system and, due to a trade-of between immunity and sexual signaling, reduce male attractiveness. We therefore predicted that LPS injected males would take longer to initiate courtship and/or require a longer period of courtship 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 have shorter spermatophore retention times than control males. 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. Contrary to these predictions there was 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 up-regulating 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 depend on nutritional condition (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). More specifically, advertisement calling in *T. commodus* is condition dependent (Hunt et al. 2004) and energetically expensive (Kavanagh 1987). It is also plausible that courtship calling in *T. commodus* incurs similar energetic/nutritional costs. Immune function and sexual advertisement should therefore show a phenotypic 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 sexual signaling 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; Jacot et al. 2005b; Fedorka and Mousseau 2007; Reaney and Knell 2010) but others, including ours, have not. For example, 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. Similarly, Shoemaker and Adamo (2007) found no changes in egg production when female crickets *Gryllus texensis* were chronically injected with 20μg of LPS from *S. marcescens* (equivalent to 40μg/g of cricket, mean adult mass of *G. texensis* ≈ 0.5g), and only one aspect of egg production (egg weight) was affected at large doses (100μg of LPS/injection, equivalent to 200μg/g of cricket). 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. in press).

There are several reasons why we might not have observed a trade off between male attractiveness and immune system activation in *T. commodus*. First, with food and water provided *ad libitum*, LPS injected males might have readily compensated for any resources lost due to immune activation by feeding more (Vainikka et al. 2007). For example, immune challenged bumble bees *Bombus terrestris* only suffer a reduction in survival relative to controls when starved (Moret and Schmid-Hempel 2000). This suggests that well fed bees can readily compensate for any resource loss associated with immune activation. 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 or locomotory activity) (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). Third, the effect of LPS on the immune system might be sufficiently brief in duration, especially compared to an actual on-going parasitic infection, that it has a barely detectable effect on the availability of resources used by signaling males. Fourth, injection with LPS induces a measurable 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 can not exclude the possibility, although it seems unlikely, that LPS failed to activate the immune system of *T. commodus*. Indeed, we found no effect of LPS injections or injection *per se* on lysozyme-like activity in the haemolymph 24 hours after treatment. If an immune response in LPS injected crickets proceeded quickly (i.e. <24 hours), 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 bumble 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 parasites and pathogens. 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 or even diet) on only some of the immune parameters measured (e.g. Rolff and Siva-Jothy 2002; Rantala et al. 2003a; 2003b; 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.

Sex differences

It has been argued that differences in life history strategies might lead to males investing less into immunity than females (Rolff 2002), but this depends on several simplifying assumptions that are probably violated in many species (Kokko and Stoehr 2006). Studies investigating immunological sex differences in invertebrates have produced mixed results (e.g. Gray 1998; Adamo et al. 2001; Rolff 2001; Siva-Jothy and Thompson 2002; Fedorka et al. 2004; Zuk et al. 2004). In an earlier study, Zuk et al. (2004) found that male *T. commodus* have a stronger encapsulation response than females. We failed to detect a sex difference in lysozyme-like activity in our study. Thus, as noted earlier, conclusions can vary depending on which component of the immune system is measured (see also results for the cricket, *Gryllus bimaculatus*; Rantala and Roff 2005). In general, far more empirical data is needed before we can draw any conclusion about general trends, or reasons for, sex differences in insect immune systems.

Female mating status and mate choice

Neither virgin females nor once mated females exhibited a preference for control males over LPS injected males based on any of our four measures of male attractiveness. Virgin females did, however, retain 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 are fertilized, allowing them to start egg laying and avoid delays in reproduction. Interestingly, heavier females also took longer to remove spermatophores, which one might expect given that they require more sperm to fertilize all their eggs because of their greater fecundity. Once fertilization is assured, females can then afford to be more choosy (Kokko and Mappes 2005). We might therefore expect that mated female

T. commodus should 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 neither already mated nor virgin females removed the spermatophores of less attractive males (e.g. males that took longer to induce a female to mount them; Shackleton et al. 2005) sooner to reduce the amount of sperm transferred (Hall et al. 2010). Furthermore, in Experiment 1 females actually retained the spermatophores of less attractive males for significantly 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 time 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 and timing 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: Smith's Lake, N.S.W.) that differ in many call parameters and key life history traits (J. Hunt, unpublished data).

Conclusion

There was no detectable effect of LPS injections on male attractiveness based on either pre-copulatory female choice or post-copulatory cryptic female choice. There is therefore no evidence to indicate a trade-off between short range attractiveness and immunity in *T. commodus*. We did, however, detect a difference in the post-copulatory behaviour of virgin and non-virgin females, indicating that if there is an effect of an immune challenge on male attractiveness it is small compared to the effect of female mating status.

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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.

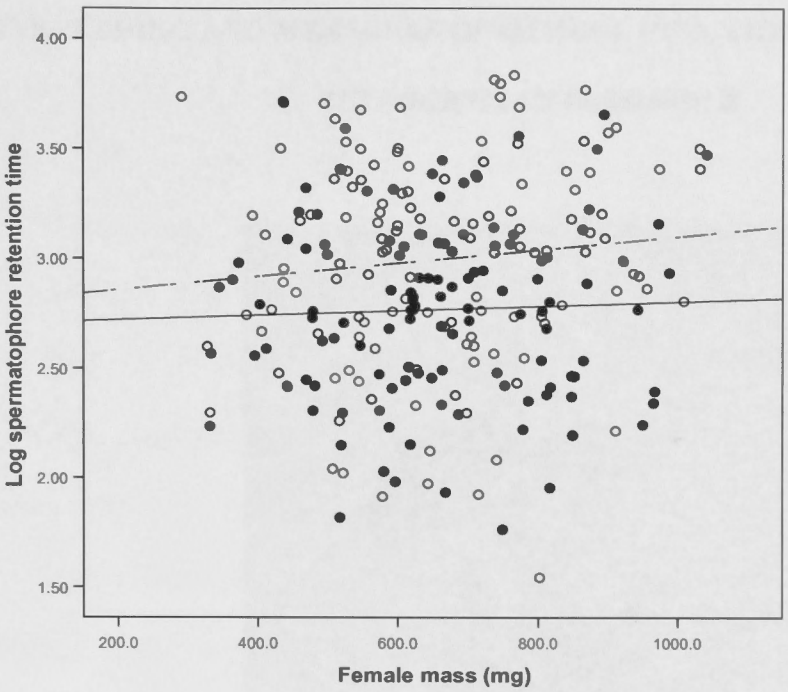
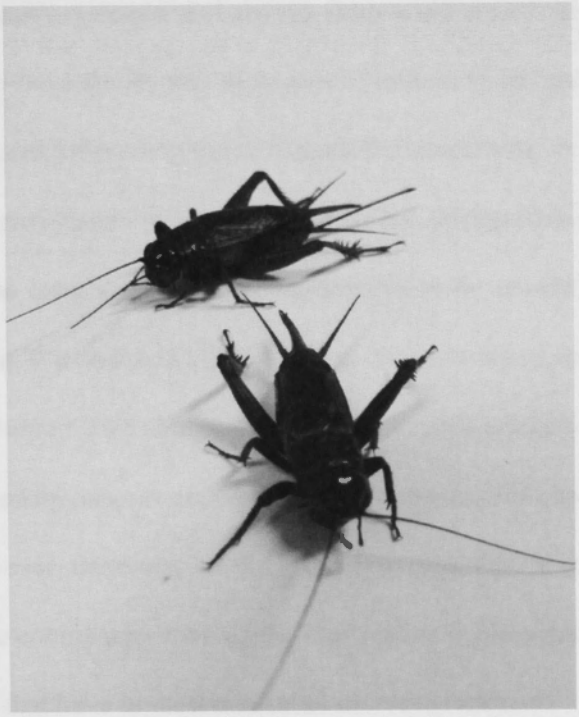


Figure 1. The relationship between spermatophore retention time and female mass for Experiment 2. Virgin females are shown as open circles (○), with a dashed line. Non-virgin females are shown by closed circles (●) and a full line.

CHAPTER 2

INBREEDING AND MEASURES OF IMMUNE FUNCTION IN THE CRICKET *TELEOGRYLLUS COMMODUS*



A male (front) and female (back) *T. commodus*. Photo: Hendrik Maat

Jean M. Drayton [1] and Michael D. Jennions[1]

1. Evolution, Ecology & Genetics, Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia.

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ABSTRACT

Studies of sexual selection and immunity in invertebrates often assay components of the immune system (e.g. encapsulation response, haemocyte counts) to estimate disease resistance. Because increased disease resistance is thought to enhance fitness in most cases, we might expect a positive relationship between fitness and measured immune function. Indeed, several studies have shown that measures of immunity are correlated with fitness enhancing traits. We used inbreeding to investigate the relationship between fitness and two commonly used assays of insect immunity in the cricket *Teleogryllus commodus*. Previous studies in *T. commodus* have shown inbreeding depression for several life history and sexually selected traits. We compared the lysozyme-like activity of the haemolymph and haemocyte counts of inbred (full-sibling mating) and outbred crickets. If these measures of immune function are positively correlated with fitness, we expect both measures to decline with inbreeding. However, there was no change in lysozyme-like activity and a significant increase in haemocyte counts with inbreeding. Our results demonstrate that it is not always the fittest individuals that have highest measured immune function.

KEY WORDS: inbreeding, immunity, *Teleogryllus commodus*, lysozyme, haemocytes, fitness, sexual selection.

INTRODUCTION

The immunocompetence handicap hypothesis states that sexual signals can reliably indicate male genetic quality (see Hunt et al. 2004) because of a trade-off between parasite resistance and sexual trait expression mediated by the effects of testosterone (Folstad and Karter 1992; for an update see Boonekamp et al. 2008). It is now apparent that comparable trade-offs could underlie sexual signaling in invertebrates. For example, several studies of insects show that immune challenge with non-pathogenic substances increases immune activity but decreases investment in sexual traits (e.g. Jacot et al. 2004; Jacot et al. 2005; but see Sadd et al. 2006). Likewise, an increase in sexual signaling or behaviour can suppress immune function (e.g. Rantala et al. 2003b). Insects are popular subjects to investigate links between immunocompetence and expression of sexual signals. Compared to vertebrates they have relatively simple immune systems that can be assayed using well established protocols; for example, by measuring the encapsulation response, lysozyme-like activity and phenoloxidase activity of the haemolymph, and by counting haemocytes (review: Lawniczak et al. 2006).

Many studies of sexual selection and immunity in invertebrates assume that the parameters used to assay an individual's immune system (e.g. encapsulation rate, lysozyme-like activity in the haemolymph) reflect the ability of that individual to resist disease (Adamo 2004a; b). In addition, it is assumed that the relationship between immune measures and disease resistance is positive (i.e. individuals with a larger measured immune function are more resistant, review Lawniczak et al. 2006). In several studies, higher values of measured immune function (e.g. encapsulation, lysozyme-like activity) are correlated with greater resistance against real pathogens (Adamo 2004a; Rantala and Roff 2007). Increased disease

resistance will presumably have a positive effect on fitness, and therefore we might expect that individuals with higher fitness will have elevated measures of immunity. In accordance with this prediction, several studies have found positive correlations between fitness enhancing traits (e.g. pheromone attractiveness, courtship display, mobility, dominance) and measured immunity (e.g. encapsulation rate, phenoloxidase activity, lysozyme-like activity, haemocyte count) (e.g. Ryder and Siva-Jothy 2000; Rantala et al. 2002; Ahtiainen et al. 2004; Rantala and Kortet 2004; Ahtiainen et al. 2006). In other cases, the relationship between immunity and fitness may be less clear (Viney et al. 2005; Lawniczak et al. 2006). Larger measured immune responses do not always indicate greater resistance against actual disease (Adamo 2004a; b). Moreover, even if disease resistance is accurately measured, the relationship between immunity and fitness may still be obscure; the most robust immune response may not confer the greatest fitness, if this immune response reduces the resources that can be allocated to reproduction. Fitness might therefore be increased by having an intermediate immune response rather then a maximal one (Viney et al. 2005).

One way to investigate the relationship between measurable immune responses (and presumably disease resistance) and fitness is to create classes of individuals that can be assigned high or low fitness and to assay the immune function of these classes. One aspect of fitness that can be easily manipulated in the laboratory is condition. This is usually achieved by changing external environmental factors that affect condition such as diet or parasite loads. Diet manipulations have shown that individuals given high quality diets have higher measurable immune responses than those given less food or poorer diets (e.g. Feder et al. 1997; Siva-Jothy and Thompson 2002; Rantala et al. 2003a; Jacot et al. 2005). An alternative, yet underexploited method of creating classes of individuals that can be assigned high or low fitness is to manipulate fitness using inbreeding (e.g. Bolund et al.

2010; review: Cotton et al. 2004). Inbreeding increases homozygosity and usually results in a decline in fitness known as inbreeding depression (review: Keller and Waller 2002; Armbruster and Reed 2005). This reduction in fitness mainly arises due to the unmasking of partially or fully recessive deleterious alleles, as well as the loss of advantageous heterozygosity (Charlesworth and Charlesworth 1999; Charlesworth and Willis 2009). Inbreeding could affect the immune system due to lower heterozygosity at loci that directly affect traits involved in immunity, or by reducing the general ability of individuals to acquire and/or assimilate the energetic and nutrient resources that are available to be allocated to the immune system (i.e. lowering condition *sensu* Rowe and Houle 1996; review: Tomkins et al. 2004). More generally, inbreeding should reduce many components of fitness such as disease resistance, performance capacity and condition, and therefore, inbred individuals can be unambiguously assigned as low fitness relative to outbred individuals.

In this study, we investigated the relationship between two widely used measures of immune function and fitness by comparing the immune function of inbred and outbred individuals. We investigated the effect of one generation of full-sibling mating on lysozyme-like activity in the haemolymph and haemocyte counts in *Teleogryllus commodus*, a gryllid cricket endemic to Australia. Lysozyme is an antibacterial enzyme that hydrolyses bacterial cell walls. Its activity is assayed by measuring the rate at which an individual's haemolymph clears a bacterial suspension (e.g. Rantala and Roff 2005). Haemocytes are the cellular components of haemolymph and a subset of these cells are involved in an immune response by transporting molecules to the site of infection, by ingesting foreign particles, or by encapsulating parasites (Korner and Schmid-Hempel 2004; Lawniczak et al. 2006).

Inbreeding reduces fitness across a wide range of taxa (Crnokrak and Roff 1999; Keller and Waller 2002; Armbruster and Reed 2005) and has strong negative effects in *T. commodus*. One generation of inbreeding (full sibling mating) in *T. commodus* causes a reduction in hatching success, nymph survival, adult lifespan, and affects sexually selected male traits such as calling effort and call structure (Drayton et al. 2007; Drayton et al. 2010). We therefore assume that inbred crickets are less fit and in poorer condition (*sensu* Rowe and Houle 1996) than their outbred counterparts. Both haemocyte counts and lysozyme-like activity have been used as a proxy for immunity and disease resistance (e.g. Rolff 2001; Ahtiainen et al. 2004; Rantala and Kortet 2004; Simmons et al. 2005; Simmons and Roberts 2005; Tregenza et al. 2006), and have been shown to correlate with fitness enhancing traits (e.g. Ryder and Siva-Jothy 2000; Ahtiainen et al. 2004; Rantala and Kortet 2004; Ahtiainen et al. 2006) in other invertebrate species. If, in *T. commodus*, either of these measures are correlated with individual condition or fitness, we expect both of these measures to decline with inbreeding. Empirical tests directly testing whether less fit individuals show reduced expression of specific immune components are rare. Such tests are therefore needed.

MATERIALS AND METHODS

Generation and rearing of inbred and outbred crickets.

Inbred individuals were created by full-sibling matings (inbreeding coefficient, $F = 0.25$) and their fitness compared with that of outbred crickets ($F \approx 0$ assuming panmixis in the field population, which is in an area surrounded by many km^2 of suitable habitat). Full-

sibling families were derived from ~70 wild caught gravid females collected in May 2006, at Smith's Lake, NSW, Australia. To start, the offspring of these wild caught females were reared to adulthood and separated before sexual maturity to ensure virginity. They were then paired with a non-sibling (i.e. different wild caught mother) to create full-sibling families. Each full-sibling family was reared communally in a 43 x 30 x 13cm plastic tub with dry cat food (KiteKat Krunch, Uncle Ben's, Raglan, Australia) and water *ad libitum*. Females were separated from their brothers as soon as their ovipositors became visible to ensure virginity.

We created 33 experimental blocks that each used two unrelated full-sibling families (i.e. not derived from the same wild caught female). In each block, brothers and sisters from both full-sibling families were mated to create two inbred genotypes. Outbred genotypes were created by reciprocal mating of a male and a female from each family (see Fig. 1). This design generated four offspring genotypes (two inbred and two outbred) per block. It was balanced for the relative maternal and paternal genetic contribution of each family to inbred and outbred offspring, which is necessary to control for variation among families in breeding values for focal traits (see Tomkins et al. 2010). After mating females were provided with moist cotton wool ("egg pads") in which to lay eggs. We checked egg pads every three days for newly hatched nymphs. Nymphs from the same mother were reared communally for 20 days in 9 x 9 x 5cm plastic tubs with food and a piece of moist cotton wool. They were then transferred to individual containers (9 x 9 x 5cm) with a vial of water plugged with cotton wool, cat food and a cardboard egg cup for shelter. Nymphs were not transferred to individual containers immediately after hatching to reduce the risk of being crushed by their water tube. In total we set up 5073 nymphs. The density during the communal stage was very similar because, on average, we set up 37.5 ± 3.3 (± SE) nymphs

of each inbred genotype and 39.4 ± 3.3 nymphs of each outbred genotype per block. Food and water was replaced every 10 days. As nymphs neared maturity we checked daily for the moult into adulthood to record their development time (i.e. days taken to reach maturity). All adults used to test the effect of inbreeding on immune function were ≥10 days post maturation to ensure that they were sexually mature. Furthermore, because crickets were reared and maintained in individual containers (apart from the brief period of communal rearing as nymphs), all crickets used in the experiment were virgins. Crickets were maintained at 26 - 28°C on a 12:12 photoperiod.

Lysozyme-like activity in the haemolymph

We used a sterile pin to make a small puncture under the pronotum from which we collected 2µl of haemolymph with a Gilson pipette. This was added to 8 µl of phosphate-buffered saline (PBS: 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in 1L distilled water, pH 7.4), and frozen to induce cell lysis. The samples were later thawed and 90µl of a *Micrococcus lysodeikticus* solution (3mg/ml PBS) added. We then loaded samples into a microplate spectrophotometer (PowerWave 340, Bio-Tek Instruments Inc.) to measure lysozyme activity at 490nm and 30°C as the rate of change in optical density from 10 to 30 minutes and from 0 to 80 minutes. These two measures were highly correlated (r_s= 0.991, P < 0.001, n = 1015) and we only present analyses using the later. Crickets were weighed before haemolymph extraction (mass and linear body size, such as pronotum width, are closely correlated, r = 0.946, P < 0.001, n = 2731, M Jennions, unpublished data). Crickets were 18-25 days post maturation (22.2 ± 0.06 days) when haemolymph was taken. There were no age differences between inbred males, inbred females, outbred males and outbred females (Kruskal-Wallis Test: χ^2_3 = 0.587, P = 0.899). Lysozyme activity measured in this

way is highly repeatable ($r_I = 0.74$, $P < 0.001$, J Drayton, J Boeke and M Jennions, unpublished data).

Haemolymph samples from all groups (inbred males, inbred females, outbred males and outbred females) were run together on the same MicroWell plates (Nunc, Roskilde, Denmark). Several control samples (containing PBS and *Micrococcus lysodeikticus* solution, but no haemolymph) were also 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 then calculated as the sample slope minus the control slope. This was then multiplied by -1 to make the value more intuitive (i.e. a greater value indicates more lysozyme-like activity in the haemolymph).

Haemocyte counts

At the same time we collected haemolymph for the lysozyme assays, an additional 2µl of haemolymph was collected and mixed with 8µl of anticoagulant (3.92g NaOH, 8.532g NaCl, 6.328g EDTA, 8.616g citric acid in 1L distilled water). This mixture was then expelled onto a Neubauer haemocytometer, and we counted the haemocytes in six of the 0.04mm^2 central squares, following a chequer-board pattern, to estimate the number of circulating haemocytes per ml of haemolymph. For 56 crickets, we counted the haemocytes in a further six squares to measure the repeatability of the count. It was repeatable ($F_{55,56} = 3.132$, $P < 0.001$, $r_I = 0.521$).

Statistics

To test for an effect of inbreeding on lysozyme-like activity and haemocyte counts we ran separate linear mixed models estimated using restricted maximum likelihood in S+ 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, including all two way interactions, and then removed fixed terms until the final model only contained significant terms (random terms were always retained) (Crawley 2002). First, we tested for an effect of inbreeding on lysozyme activity and haemocyte counts. Inbreeding was included as a fixed factor in the models. Sex and adult age (days post maturation) were included as a fixed factor and a fixed effect covariate respectively. Block and the interaction between inbreeding and block were included as random factors. This controlled for variation in the mean value of immune parameters across blocks (i.e. random intercepts), and differences in the effect of inbreeding among blocks (i.e. random slopes). We then added the two life history traits (development time and weight at haemolymph collection) to the models as fixed effect covariates to investigate any relationships between immune parameters and life history traits. To allow the reader to assess the influence of terms excluded from the final models, we present the significance value associated with the parameter estimate for each term if it is included in the final model. We tested the repeatability of our haemocyte counts using a one way ANOVA with cricket identity as the factor. Unless otherwise stated summary statistics are mean ± SE, tests are two-tailed and $\alpha = 0.05$. To allow comparison of the biological importance of different variables we calculated standardised effect sizes by converting the F-statistic for each model parameter into the common currency of Pearson's r using the Metawin 2.0 calculator (Rosenberg et al. 2000).

RESULTS

Lysozyme-like activity in the haemolymph

Summary statistics are presented in Table 1. Lysozyme-like activity was measured for 452 inbred (204 males, 248 females) and 578 outbred crickets (237 males, 341 females). The relationship between age at haemolymph collection and lysozyme-like activity differed significantly between the sexes ($F_{1,967} = 11.178$, $P = 0.001$), so we ran separate models for each sex.

Inbreeding had no effect on the lysozyme-like activity of males or females. Older females had higher lysozyme-like activity levels than younger females, but there was no effect of age for males. For males, the relationship between development time and lysozyme-like activity differed between inbred and outbred crickets ($F_{1,382} = 7.034$, $P = 0.008$), so to assess the effect of life history traits on lysozyme-like activity, we ran separate models for inbred and outbred males. Outbred males who took longer to reach sexual maturity had higher lysozyme-like activity, but there was no such relationship for inbred males. Heavier inbred males had lower lysozyme-like activity, but there was no such relationship for outbred males. The relationship between weight and lysozyme activity did not differ significantly between inbred and outbred males ($F_{1,380} = 3.468$, $P = 0.063$, although the interaction was marginally non-significant). There was no relationship between female lysozyme activity and either development time or weight (Table 1).

Haemocyte counts

Summary statistics are presented in Table 2. Haemocyte counts were made for 329 inbred (159 males, 170 females) and 369 outbred crickets (179 males, 190 females). Inbreeding significantly increased the number of circulating haemocytes, and females had significantly more haemocytes than males (Fig. 2). Crickets that took longer to mature had higher haemocyte counts. There was no detectable effect of adult age or body mass at the time of haemolymph collection on haemocyte count (Table 2).

Relationship between haemocyte count and lysozyme-like activity

When we included lysozyme activity in the final model for haemocyte counts, we found that crickets with higher lysozyme activity also had more circulating haemocytes in their haemolymph ($F_{1,503} = 215.52$, $P < 0.001$, $r = +0.5477$) (Fig. 3). This relationship did not differ between the sexes ($F_{1,502} = 0.000$, $P = 0.997$), or between inbred and outbred crickets ($F_{1,502} = 0.684$, $P = 0.409$).

DISCUSSION

Studies of vertebrates suggest that inbreeding can reduce immune defence and increase susceptibility to parasitism and disease (e.g. Townsend et al. 2009; reviewed in Keller and Waller 2002). However, studies with non-domesticated vertebrates often rely on indirect measures of inbreeding. For example, they compare individuals with varying levels of heterozygosity, or those from small and large populations, rather than performing controlled breeding experiments (but see Ilmonen et al. 2008 and references within for exceptions). Furthermore, without such experiments, it is difficult to control for the level of inbreeding in the parents of inbred individuals, thereby potentially confounding estimates

of inbreeding depression in offspring (e.g. Szulkin and Sheldon 2006). Invertebrates are more amenable to controlled breeding designs but studies investigating inbreeding depression in invertebrate immunity have reported mixed results. One generation of brother-sister mating significantly reduced the encapsulation response of female, but not male, pupae of the moth *Epirrita autumnata* (Rantala and Roff 2007), and had no effect on encapsulation ability in the bumble bee *Bombus terrestris* (Gerloff et al. 2003). Inbred and outbred termites, *Zootermopsis angusticollis* do not differ in their encapsulation response, or in survival following bacterial and fungal infections when kept individually. When kept in groups and exposed to high doses of fungal disease, however, inbred termites had reduced survival, and higher cuticular microbial loads. This suggests that inbreeding does not affect the immunity of individuals, but might affect social mechanisms of pathogen control such as allogrooming (Calleri et al. 2006). Inbred lines of the red flour beetle *Tribolium castaneum*, did not differ from the original outbred stock population in their susceptibility to tapeworm infection (Stevens et al. 1997). Crickets, *Gryllus firmus*, from inbred lines did not differ from individuals resulting from 'outbred' crosses between lines in encapsulation ability or the lysozyme-like activity of the haemolymph (Rantala and Roff 2006). In contrast, however, decorated crickets *Gryllodes sigillatus* from inbred lines had a greater encapsulation response than outbred crickets (Gershman et al. 2010). Such differences between studies may reflect differences in the genetic architecture of immune parameters between species, populations or even between the sexes. For example, in the abovementioned study of the moth *Epirrita autumnata*, the author's findings suggest that in males, genetic variation for the encapsulation response is predominantly additive. In females however, additive variation is reduced and encapsulation shows higher levels of directional dominance (Rantala and Roff 2007). Because inbreeding depression is due to the effects of directional dominance (Lynch and Walsh 1998), as expected, inbreeding

depression was found in female, but not male, encapsulation response (Rantala and Roff 2007).

Our results using the cricket *Teleogryllus commodus* do not support the prediction that inbreeding reduces two widely used measures of immunity. Instead, inbreeding was associated with a significant increase in haemocyte count, and there was no effect on lysozyme-like activity. There are several possible explanations for the higher number of circulating haemocytes in inbred crickets. First, if inbred crickets have a reduced investment in other fitness traits, they might, as a byproduct, invest more into haemocyte production than outbred crickets. For example, in a study with similar results to our own, Gershman et al. (2010) found that inbreeding increased encapsulation ability in decorated crickets, *Gryllodes sigillatus*. The authors speculated that because inbred crickets have reduced reproductive effort, they have more resources to invest into immunity. How inbreeding affects egg laying or provisioning in female *T. commodus* is unclear, but inbred males do have reduced calling behaviour (Drayton et al. 2010). Second, inbreeding might disrupt physiological mechanisms responsible for regulating haemocyte production resulting in overproduction. If so, inbred crickets risk an overly responsive immune system which could result in autoimmunity (e.g. Sadd and Siva-Jothy 2006) and/or the uneconomical use of energetic or nutritional resources diverted to immunity (e.g. Siva-Jothy and Skarstein 1998; Lochmiller and Deerenberg 2000). Given the increase in the number of circulating haemocytes with inbreeding it seems plausible that the function relating haemocyte count to fitness is non-linear, so that there is an optimal peak, where counts well below this optimum run the risk of severe pathology, and counts above carry autoimmune and resource costs (Siva-Jothy and Skarstein 1998).

Inbreeding in normally outbreeding species almost always reduces offspring fitness (Falconer and Mackay 1996), and has strong negative effects on key life history and sexually selected traits in *T. commodus* (Drayton et al. 2007; Drayton et al. 2010). We are confident that the inbred crickets used in the current study suffered comparable reductions in fitness to those reported in the previous studies of Drayton et al. (2007; 2010) for the following reasons. First, although they were a separate group of crickets, the inbred crickets used in the current study were bred (i.e. full sibling mating) and reared under almost identical conditions to those used by Drayton et al. (2007). Second, the crickets used by Drayton et al. (2010) (where substantial inbreeding depression was found in male calling behaviour) are the same individuals used in the current study to investigate the effect of inbreeding on immunity. We can therefore be confident that our inbred crickets have reduced fitness. Both haemocyte counts and lysozyme-like activity have been used as a proxy for disease resistance in other invertebrates (e.g. Rolff 2001; Ahtiainen et al. 2004; Rantala and Kortet 2004; Simmons et al. 2005; Simmons and Roberts 2005; Tregenza et al. 2006), and increased disease resistance is thought to enhance fitness in most cases (but see Viney et al. 2005). Furthermore, haemocyte count and lysozyme-like activity have been shown to correlate with fitness enhancing traits (e.g. Ryder and Siva-Jothy 2000; Ahtiainen et al. 2004; Rantala and Kortet 2004; Ahtiainen et al. 2006). If lysozyme-like activity and haemocyte counts correlate positively with fitness in *T. commodus*, we would expect both to decline with inbreeding. For any of the reasons listed above, however, we have shown that inbred individuals had a larger haemocyte count (and therefore potentially higher immunocompetence) than outbred individuals. Furthermore, there was no difference in lysozyme-like activity between inbred and outbred crickets. Our results demonstrate that it is not always the fittest individuals that have highest measured immune function.

It is worth noting that there are two general approaches to measuring immune function, either by measuring baseline levels (pre-challenge or constitutive: e.g. Simmons et al. 2005; Simmons and Roberts 2005; Tregenza et al. 2006) or by measuring levels after an immune challenge is administered (induced: e.g. Adamo 2004a; Gershman et al. 2010). Our study investigated the effect of inbreeding on constitutive immunity. While it would have been informative to also investigate the effect of inbreeding on induced immunity, we had to balance the pursuit of large sample sizes needed for a statistically powerful test of inbreeding effects (our sample sizes are far larger than those of most comparable studies), against the logistical constraints of measuring both constitutive and induced immunity. There is, however, evidence that constitutive and induced immune measures are correlated in *Teleogryllus*. Specifically, pre-challenge haemocyte counts correlate with the degree of encapsulation of a nylon filament insert in both *T. oceanicus* and *T. commodus* (Simmons et al. 2005; Simmons and Roberts 2005; Tregenza et al. 2006). The relationship between pre- and post challenge lysozyme-like activity in *T. commodus* is currently unknown. It is plausible, however, that baseline levels reflect immunocompetence as a high baseline level of lysozyme could facilitate a more rapid immune response against invading pathogens. Furthermore, in scorpionflies *Panorpa vulgaris*, baseline lysozyme-like activity is positively correlated with the phagocytic capacity of the haemolymph which is an important defence against particles such as bacteria and fungal spores (Kurtz et al. 2000). In *Gryllus texensis*, however, although the increase in lysozyme-like activity in response to an immune challenge predicted survival after bacterial infection, baseline lysozyme activity level did not (Adamo 2004a). Whether this is a general finding that will be replicated in other species is unknown.

In conclusion, in *T. commodus* two commonly used measures of immune function were not correlated with fitness if we make the reasonable assumption that inbreeding lowers fitness. One generation of inbreeding increased one measure of immune function and did not affect another. Our statistically robust results are based on very large sample sizes and involved a direct experimental manipulation of fitness. They provide empirical support for more general warnings of equating greater immunity with elevated fitness (e.g. Viney et al. 2005).

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Table 1. The effect of four variables on lysozyme-like activity in the haemolymph.

	lysozyme-like activity of males				lysozyme-like activity of females			
	d.f.	*F*	*P*	*r*	d.f.	*F*	*P*	*r*
inbreeding	1,25	2.352	0.138	+0.2932	1,27	1.457	0.238	+0.2263
age at haemolymph collection	1,383	0.015	0.902	+0.0063	1,530	30.059	**<0.001**	+0.2317
development time	Inbred: 1,173 Outbred: 1,208	Inbred: 0.188 Outbred: 13.139	Inbred: 0.665 Outbred: **<0.001**	Inbred: -0.0329 Outbred: +0.2438	1,529	0.631	0.428	+0.0345
weight at haemolymph collection	Inbred: 1,174 Outbred: 1,207	Inbred: 4.153 Outbred: 0.555	Inbred: **0.043** Outbred: 0.457	Inbred: -0.1527 Outbred: +0.0517	1,529	0.328	0.567	+0.0249

P-values are from the final model if the term was significant, or when it alone was added to the final model if non-significant (see text). *P*-values significant at the 0.05 level are in bold.

The effect size *r* is +/- to show the direction of the change in lysozyme-like activity with inbreeding (+ = increase with inbreeding), or the direction of the correlation with the three continuous traits.

Table 2. The effect of five variables on haemocyte counts.

	haemocyte counts			
	d.f.	*F*	*P*	*r*
inbreeding	1,27	5.912	**0.022**	+0.4238
sex	1,637	23.148	**<0.001**	0.1873
age at haemolymph collection	1,636	2.664	0.103	+0.0646
development time	1,637	6.692	**0.010**	+0.1020
weight at haemolymph collection	1,636	3.319	0.069	+0.0721

P-values are from the final model if the term was significant, or when it alone was added to the final model if non-significant (see text). *P*-values significant at the 0.05 level are in bold.

The effect size *r* is +/- to show the direction of the change in haemocyte counts with inbreeding (+ = increase with inbreeding), or the direction of the correlation with the three continuous traits.

Block	Matings			
	Inbred 1	Outbred 1	Outbred 2	Inbred 2
1	A♂ A♀	A♂ B♀	B♂ A♀	B♂ B♀
2	C♂ C♀	C♂ D♀	D♂ C♀	D♂ D♀
3...	E♂ E♀	E♂ F♀	F♂ E♀	F♂ F♀

Figure 1. The mating design showing Block 1 (comprising full sibling families A and B), Block 2 (comprising full sibling families C and D) and so on. There are two inbred and two outbred offspring genotypes per block.

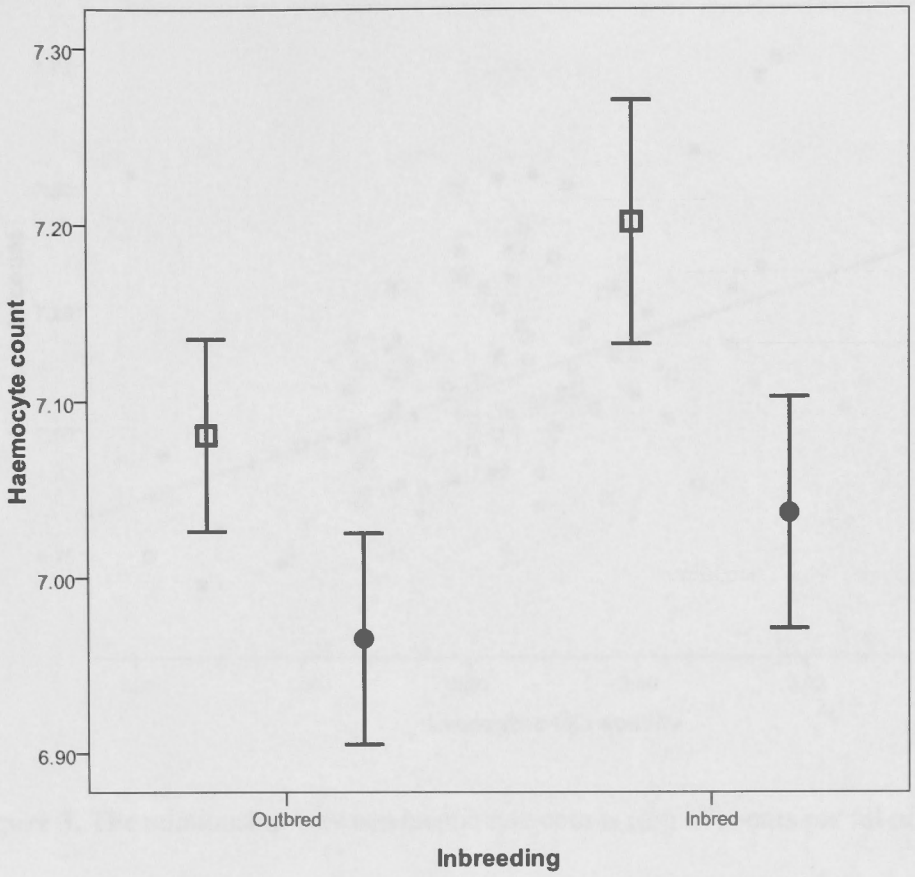

Figure 2. The haemocyte counts (log of counts per ml of haemolymph) of inbred and outbred males (●) and females (□). Error bars represent 95% confidence intervals for the means.

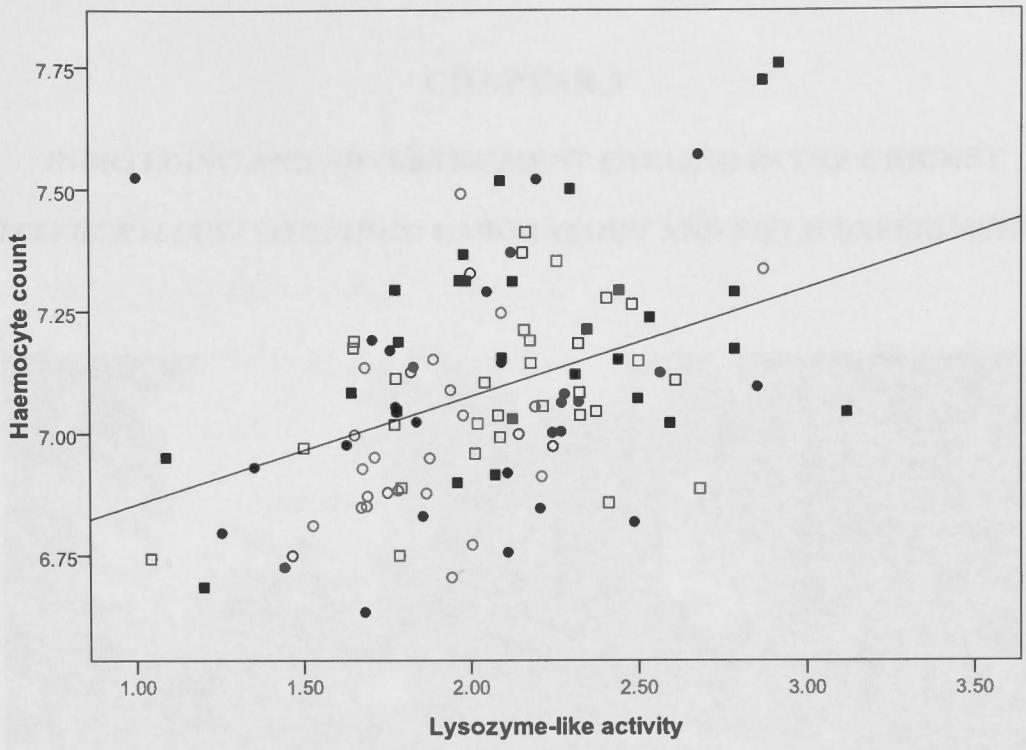

Figure 3. The relationship between haemocyte counts (log of counts per ml of haemolymph) and lysozyme-like activity (the square root of the rate of change in optical density, which has been multiplied by -1 so that a larger value corresponds to greater lysozyme activity). Values plotted are the means for inbred males (●), inbred females (■), outbred males (○) and outbred females (□) in each block. This graph is for illustrative purposes as the statistical tests used in the text take into account variation in sample sizes and across block variation.

CHAPTER 3

INBREEDING AND ADVERTISEMENT CALLING IN THE CRICKET *TELEOGRYLLUS COMMODUS*: LABORATORY AND FIELD EXPERIMENTS

The set up of the playback experiments at Smith's Lake. Photo: Kate Drayton.

Jean M. Drayton[1], Richard N.C. Milner[1], John Hunt[2] and Michael D. Jennions[1]

1. Evolution, Ecology & Genetics, Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia.

2. Centre for Ecology & Conservation, University of Exeter in Cornwall, Penryn, TR10 9EZ, UK.

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ABSTRACT

If sexually selected traits reveal a male's heterozygosity or condition to females, then such traits should exhibit declines with inbreeding. We tested this by examining the effect of inbreeding on advertisement calling in male crickets *Teleogryllus commodus*. We investigated the effect of one generation of full-sibling mating on calling effort and fine-scale call structure. Inbreeding reduced calling effort but had no effect on call structure. We then compared the attractiveness of inbred and outbred calls in the field by monitoring how many wild females were attracted to each call type. From the field data we conducted a selection analysis to identify the major axes of linear and non-linear multivariate sexual selection on call structure. A comparison of multivariate attractiveness of inbred and outbred calls along each major axis of selection revealed no difference in attractiveness. Our results suggest that inbred male calls have a fine-scale structure that is no less attractive to females than that of outbred calls. However, because inbred males call less often, and female *T. commodus* prefer males with a higher calling effort, inbred males will suffer reductions in mating success. Females who base mate choice on call rate are therefore using a signal correlated with male heterozygosity and/or condition.

KEY WORDS: male attractiveness, selection analysis, heterozygosity, calling effort, call structure, sexual selection

INTRODUCTION

For many years, theoretical models have been based on the assumption that female mate choice in non-resource based mating systems is driven by males signaling additive genetic benefits that increase offspring fitness through the inheritance of 'good genes' (Hunt et al. 2004a; Radwan 2008). More recently, however, attention has focused on non-additive genetic benefits. Specifically, it has been asked whether females show directional mating preferences for males with higher than average levels of genome wide heterozygosity (e.g. Mays and Hill 2004; Kempenaers 2007; Fromhage et al. 2009). This is because, under certain conditions, more heterozygous males are more likely to carry locally rare or dissimilar alleles to those of a female, so that mating with such males increases offspring heterozygosity (i.e. heterozygosity is heritable in the sense that the heterozygosity of offspring will resemble that of their parents; Mitton et al. 1993, but see Puurtinen et al. 2009). For example, if an individual migrates into a new population, it might bring with it locally rare alleles. If this individual then mates with a local individual, the resulting offspring with not only be relatively heterozygous, but they will also carry the rare alleles brought in by their immigrant parent. Other females then choosing these heterozygous sons as mates will, in effect, be choosing carriers of recent immigrant genes that are probably dissimilar to their own, thereby producing relatively heterozygous offspring themselves (Reid et al. 2006). This should elevate fitness because inbred organisms and/or those with lower heterozygosity tend to perform less well than outbred ones (although there is still debate about the strength of the correlation between heterozygosity and fitness; reviewed by Kempenaers 2007). Consequently, females mating with more heterozygous males should produce fitter offspring. Females can detect such males using male secondary sexual

traits if the expression of these traits is correlated with heterozygosity. For example, if male condition is positively correlated with heterozygosity, then increased heterozygosity will correlate with the expression of condition-dependent sexual traits. Females can then express a directional preference for highly ornamented and therefore relatively heterozygous males (Brown 1997; Fromhage et al. 2009; Ilmonen et al. 2009).

It is possible to test for a correlation between heterozygosity and the expression of sexually selected ornamental traits using inbreeding. Inbreeding (the mating of relatives) increases homozygosity across the genome and tends to reduce fitness (inbreeding depression). Inbreeding depression has been reported in a wide range of traits across numerous taxa (Lynch and Walsh 1998; Crnokrak and Roff 1999; DeRose and Roff 1999; Keller and Waller 2002; Armbruster and Reed 2005). Inbreeding depression is thought to arise from either or both the unmasking of recessive deleterious alleles and the loss of advantageous heterozygosity (Charlesworth and Charlesworth 1987; 1999). In addition, epistatic interactions between loci might play a role in some species (van Oosterhout et al. 2003). Consequently, inbreeding depression is largely due to the effects of dominance interactions, specifically directional dominance, and does not occur in traits with a purely additive genetic basis (Charlesworth and Charlesworth 1987; Lynch and Walsh 1998). Traits that are closely related to fitness (i.e. life history traits, including sexual signals) are predicted to show reduced additive genetic variance because they are more closely associated with fitness and are therefore under stronger selection which depletes variation (Mousseau and Roff 1987; DeRose and Roff 1999; Blows and Hoffmann 2005; Hunt et al. 2007). Such traits are also expected to show higher levels of directional dominance, and therefore inbreeding depression, because mutations affecting these traits are typically deleterious and recessive. Traits that are less closely related to fitness (i.e. morphological traits) show

substantial levels of additive genetic variance and less directional dominance, and therefore tend to exhibit lower levels of inbreeding depression (Lynch and Walsh 1998; Roff 1998; DeRose and Roff 1999).

Inbreeding depression in sexually selected traits might be due to effects of changes in heterozygosity at loci that directly code for sexual traits. Many sexually selected traits are condition dependent (reviewed by Cotton et al. 2004), however, so if inbreeding reduces the general ability of males to acquire and assimilate resources (i.e. lowers condition), then sexually selected traits are predicted to show strong inbreeding depression. Hence, sexually selected traits will signal the negative effects of reduced heterozygosity at the many loci across the genome that affect condition (Rowe and Houle 1996; Tomkins et al. 2004). These two mechanisms of inbreeding depression on sexual traits (direct effects or via inbreeding depression of condition) are not mutually exclusive.

More generally, if sexually selected traits are condition dependent, and inbreeding reduces male condition, then sexual traits should show declines with inbreeding. In this case, it may not be a male's heterozygosity *per se* that sexual traits will signal but rather his underlying condition. In such systems, females choosing males based on sexually selected traits will normally gain genetic benefits for their offspring if some of the variation in male signaling is due to additive genetic variation in condition (Tomkins et al. 2004). In this case, inbreeding can be viewed as way of experimentally reducing a male's condition if condition is subject to inbreeding depression (see Drayton et al. 2007).

Several studies report substantial declines in sexually selected traits with inbreeding. For example, inbreeding in guppies *Poecilia reticulata* impairs male reproductive and courtship

behaviour (van Oosterhout et al. 2003; Mariette et al. 2006), reduces sexual colouration (Sheridan and Pomiankowski 1997; van Oosterhout et al. 2003) and decreases actual mating success (Mariette et al. 2006). Likewise, inbreeding in the least killfish, *Heterandria formosa*, reduces the frequency of male mating attempts (gonopodial thrusting) (Ala-Honkola et al. 2009). Similarly, female mice *Mus musculus* find the courtship scent marks of inbred males less attractive than those of outbred males (Ilmonen et al. 2009), and inbred male *Drosophila* have delayed and abnormal courtship (Miller et al. 1993), altered courtship songs (Aspi 2000) and reduced competitive mating success (Sharp 1984; Miller et al. 1993). Finally, inbred male butterflies *Bicyclus anynana* suffer a reduction in mating success (Joron and Brakefield 2003). Traits that are involved in post-copulatory sexual selection (e.g. sperm quality and quantity) can also show substantial inbreeding depression (e.g. Roldan et al. 1998; Margulis and Walsh 2002; Konior et al. 2005; Gage et al. 2006; Zajitschek et al. 2009). Importantly, several of the studies that have investigated multiple traits and/or different populations have shown that the traits that females explicitly use as cues during mate choice are those that tend to show more substantial inbreeding depression (e.g. Sheridan and Pomiankowski 1997; Aspi 2000). Although these data are limited, they suggest that such traits might be good indicators of genome wide heterozygosity and/or male condition.

Here, we test the prediction that traits that are important mate choice cues will be sensitive to the effects of inbreeding in the Australian black field cricket, *Teleogryllus commodus*, using a population from Smith's Lake, Australia. Specifically we predict that if such mate choice cues reveal condition and/or relative heterozygosity they should be reduced by inbreeding. Males produce a long-distance advertisement call to attract sexually receptive females (Campbell and Shipp 1979; Evans 1988). We have already shown that female

choice exerts strong positive directional sexual selection for greater calling effort and moderate levels of stabilising and directional multivariate sexual selection on finer-scale features of advertisement calls (for details of which traits or trait combinations are favored see Brooks et al. 2005; Bentsen et al. 2006). Furthermore, through diet manipulations, calling effort has been shown to be affected by nutrition (Hunt et al. 2004b; Maklakov et al. 2008). We have previously found substantial inbreeding depression in advertisement calling in another Australian population of *T. commodus* from Canberra (Drayton et al. 2007). In the current study, we quantify the effect of one generation of brother-sister mating (inbreeding coefficient $F = 0.25$) on nightly calling effort and finer-scale advertisement call structure. Studies have shown that the effect of inbreeding on male reproductive success can be masked in the laboratory relative to natural conditions (e.g. Joron and Brakefield 2003). We therefore examined the attractiveness of inbred and outbred advertisement calls in the field by monitoring how many wild females were attracted to each call type. This did not allow us to test whether natural conditions increase the relative difference in the expression of calls by inbred and outbred males compared to those in the laboratory (because calls were recorded in the laboratory, see below), but it did allow us to ensure that female choice was measured in a more natural context (i.e. over greater distances and with confounding natural effects on signal propagation such as wind and vegetation). The field experiment allowed us to conduct a formal selection analysis (e.g. Brooks et al. 2005; Bentsen et al. 2006; Hall et al. 2008) to identify the major axes of linear and non-linear multivariate sexual selection on the finer scale call structure. We could then compare the multivariate attractiveness of inbred and outbred males along each major axis of selection under field conditions.

METHODS

Generation and rearing of inbred and outbred crickets

Inbred individuals were created by one generation of full sibling matings ($F = 0.25$) and their fitness compared with that of outbred crickets ($F = 0$). Full sibling families were derived from ~ 70 wild caught gravid females collected in May 2006, at Smith's Lake (at the Bungwahl cemetery), New South Wales, Australia. Male and female offspring from wild caught females were reared to adulthood (males and females were separated before sexual maturity to ensure virginity) and then paired at random (such that pair members did not share a wild caught mother) to create full sibling families. Each full sibling family was reared communally in separate large plastic tubs with cat food (KiteKat Krunch) and water *ad libitum*. Females were separated from their brothers before sexual maturity to ensure virginity.

The full sibling families were grouped in sets of two to generate mating blocks ($N = 33$ blocks). We ensured that the two families in a block did not share a grandmother. In each block, brothers and sisters from both full sibling families were mated to create two inbred genotypes. Outbred genotypes were created by reciprocal matings of a male and a female from each family in the block. For example, in a block consisting of full sibling families A and B, inbred progeny were generated by mating brothers and sisters from family A, and brothers and sisters from family B, while outbred progeny were generated by mating a male from family A with a female from family B, and a male from family B with a female from family A. This design generated four offspring genotypes (two inbred and two outbred) per block such that maternally and paternally derived genes from each family are, on average, equally represented in inbred and outbred males. After mating females were provided with

moist cotton wool ("egg pads") in which to lay their eggs. Egg pads were checked every three days for hatching nymphs. Upon hatching, nymphs from a given pairing were reared communally for 20 days in 9x9x5cm plastic tubs with food and a piece of moist cotton wool. They were then transferred to individual containers (9x9x5cm) with a water tube, cat food and a cardboard refuge. Nymphs were not transferred to individual containers immediately after hatching because they are small and easily crushed by a water tube. On average, 37.5 ± 3.3 (± SE) nymphs of each inbred genotype and 39.4 ± 3.3 nymphs of each outbred genotype were set up individually for each block. Food and water was replaced every 10 days. Nymphs nearing maturity were checked daily to record development time (days from hatching to adult eclosion). The males used to test the effect of inbreeding on calling were >10 days post maturation to ensure sexually maturity. All crickets were kept at 26-28°C on a 12:12 photoperiod.

Calling effort

Male nightly calling effort was measured using a custom-built electronic monitoring device similar to that of Hunt et al. (2004b) and Drayton et al. (2007). In brief, the device consists of 128 recording chambers (9x9x5cm). Each chamber has one condenser microphone mounted in the lid and housed one male. The device samples the chambers throughout the night. During sampling, the microphone of one chamber is activated and, if the sound level produced by the male in that chamber is 10 dB or more above the background noise, a 1 representing that the male is calling is recorded. Otherwise, a 0 representing no calling is recorded. The microphone of that chamber is then de-activated and the next microphone in the next chamber is activated, so that only one microphone (and therefore only one chamber) is sampled at a given time. Each recording chamber (and therefore each male)

was sampled 10 times per second. The nightly calling effort of a male was calculated in two ways. First, if a male was recorded as calling for any of the 10 sampling events/second, he was recorded as calling for that second, and nightly calling effort was defined as the total number of seconds called per night. Second, calling effort was calculated as the total number of sampling events (i.e. the total number of tenths of a second) that a male called per night. The two measures of calling effort are highly correlated ($r_s = 0.993$, $P < 0.001$, $N = 1409$), so we only present the results of analyses using seconds called/night. Calling effort was measured for 10 hours per night (19h00- 05h00) at 26-28°C. Males were 12-23 days post maturation (14.4 ± 0.02 days), and their calling effort was recorded over 2-5 nights (3.2 ± 0.01 nights). Males were weighed before they were put into a chamber.

The structure of the advertisement call

The advertisement call

Male crickets call using a stridulatory apparatus consisting of a file and scraper on the forewings. The most basic call unit is a pulse. Each pulse is produced by closing the wings once (Kavanagh 1987). In the advertisement call of *T. commodus* there are two types of sound pulses. The first are longer, more intense and grouped into chirps. The second are shorter, softer and grouped into trills. Chirps and trills are arranged into the main repeated unit of calling, a phrase, which consists of a single chirp followed by one to several trills (Bentley and Hoy 1972; Hill et al. 1972) (see Fig. 1).

Recording and analysis of the advertisement call

Males were set up in individual, acoustically isolated 9x9x5cm recording chambers with a condenser microphone mounted in the lid. We recorded calls using a digital recorder (MicroTrack 24/96, M-Audio). A power unit, containing a 9 volt battery powered the microphone. Chambers were checked though out the evening for calling males. If a male was heard calling, we recorded 1-2 minutes of his call. He was then weighed. Calls were recorded 15-36 days post maturation (23.0 ± 0.46 days). Because advertisement calls were recorded opportunistically and males do not call on demand, despite intensive effort on our part, sample sizes for advertisement call structure are lower than those for calling effort.

Calls were analyzed using Raven Pro 1.3 sound analysis software (Cornell Laboratory of Ornithology, Ithaca, NY, USA www.birds.cornell.edu/raven). We measured the following five parameters of five randomly chosen phrases from the call of each male: dominant frequency (DF), the interval between the last trill pulse of the selected phrase and the first chirp pulse of the next [inter-call duration (ICD)], the number of pulses per chirp [chirp pulse number (CPN)], the duration of the interval between the last two pulses in the chirp [chirp inter-pulse duration (CIPD)] and the number of trills in a phrase [trill number (TN)] (Fig. 1). These five call parameters were measured because they have been shown to capture variation that predicts male attractiveness to females (e.g. Brooks et al. 2005; Bentsen et al. 2006; Hunt et al. 2007).

Male attractiveness in the field

Playback experiments were conducted at Smith's Lake, Australia (32°22′S, 152°30′E) in February-March, 2009, on a grassy area surrounded by Eucalypt forest. Playbacks were conducted at night. Eight playback units were evenly spaced around the circumference of a

circular arena (20 m diameter). A playback unit consisted of a pair of speakers (Logitech LS11 stereo speakers, one speaker faced into, the other out of the arena), a digital audio player (SanDisk Sansa c200) and a small tent (for rain protection) covering the unit. The speakers were placed on a 60x60cm piece of black Coreflute that was coated with sticky insect trap coating (TangleFoot) to capture approaching females (Fig. 2). Each playback unit continuously played the call of one male each night. Speakers were powered from the mains by cables that ran around the circumference of the arena. The calls of inbred and outbred males were played from alternating units around the circumference of the arena (Fig. 2). Call playback intensity was calibrated nightly to 60dB SPL at 80 cm using a sound level meter (Digitech QM-1589) (see Bentsen et al. 2006). To ensure that there were females present during trials, at least 50 wild adult females were collected from a nearby site (<5km, Bungwahl cemetery) and released in the centre of the arena, under cardboard egg cartons, immediately before each trial. The collected females were released to boost an unknown number of wild females that were already at the field site. Consequently, the females attracted to the calls could have been released females and/or resident females. Because females were from the wild, we had no prior knowledge of their previous mating or rearing experience and were therefore unable to control for factors such as females mating status, age or condition. In the wild, however, males will encounter females that vary in such factors, and so we were recreating similar conditions under which mate choice occurs in nature. Playbacks were started between 21h00-22h00 and ended at 05h30. The number of trapped females at each unit was counted when the trial ended. The number of females caught at a unit provides a measure of attractiveness of the call played by that unit.

To create the playback recordings for each test male we selected 15 consecutive phrases from his original laboratory recording, selecting from the beginning of the first chirp pulse

of the first phrase to the end of the intercall duration (ICD) of the 15th phrase. We then repeated this selection to create a continuous call loop that was played throughout the night. Recordings were generated using Audacity 1.2.6 Free Digital Audio Editor (www.audacity.sourceforge.net) and saved as uncompressed WAV audio files.

The attractiveness of inbred and outbred calls was compared in two experiments. First, inbred and outbred males were paired by block. Second, males were tested irrespective of block of origin. Of the males whose advertisement calls we recorded, 42 could be paired by block (i.e. $N = 21$ pairs of an inbred and outbred male from the same block). For the first experiment, male pairs were randomly assigned into groups of four (i.e. 8 males per group). Each night, the eight calls of one group were played. The calls of males from the same pair were played from adjacent units. To test the 21st pair, three other pairs that had already been tested were grouped with this pair to create the same level of background calling experienced by other pairs tested.

The second experiment tested the attractiveness of males that could not be paired by block ($N = 16$ inbred and 16 outbred males). Males were randomly assigned into groups of four inbred and four outbred males. Each night, the calls of one group were tested.

We also included a silent control (a playback unit was set up and powered, but no call was played) in the second experiment. The control was placed between two units that were playing calls. Its position was randomly changed each night.

Statistics

Calling effort

Calling effort (seconds/night) was not normally distributed, so we used non-parametric tests. The calling effort of each male was measured over several nights, so we could test repeatability among males using a Kruskal-Wallis test. Calling effort was repeatable (see Results), so we calculated a mean value per male. We tested for any relationship between calling effort, and weight, development time and adult age using Spearman rank correlations (r_s).

To examine inbreeding depression in calling effort we took two approaches. First, to control for block/family effects, we conducted a meta-analysis with each block contributing one effect size. For each block we calculated the mean and standard deviation for the number of seconds of calling/night by inbred and outbred males respectively. We then calculated Hedge's g as the difference between the inbred and outbred means standardised by the pooled standard deviation. We corrected for small sample size effects using J to generate the final effect size Hedge's d. All the equations used are in Rosenberg et al. (2000) (note: some authors refer to Hedge's d as g, e.g. Cooper et al. 2009). We then conducted a standard random effects model meta-analysis in Metawin 2.0 to calculate the grand mean effect size after weighting effect sizes by the inverse of their variance. In practice this means that we calculated the mean effect size across blocks giving greater weighting to blocks where more males were monitored. Inspection of the 95% confidence interval of the mean effect size reveals whether there is a significant difference in call rate between inbred and outbred males. We also tested whether there was greater variation in effect sizes among blocks than expected by chance using the test statistic Q_T. Statistical methodology and equations are in Rosenberg et al. (2000). Second, we ran a Wilcoxon

matched-pairs test to compare the mean calling effort per block between inbred and outbred males. This test need not give the same result as the meta-analysis as it makes a different assumption (i.e. equal weighting for each block).

Structure of the advertisement call

The repeatability of each call parameter among males was assessed using a one-way ANOVA or Kruskall-Wallis test. All parameters were highly repeatable (see Results), so we calculated a mean value for CIPD, ICD, CPN, DF and TN for each male. To test for an effect of inbreeding on CIPD, ICD and CPN we ran separate linear mixed models in S+ 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, including all two-way interactions, and then removed fixed terms until the final model only contained significant terms (random terms were always retained as part of the experimental design) (Crawley 2002). For all models, block and the interaction 'block by inbreeding' were included as random factors, allowing us to control for any differences in call parameters across blocks (i.e. random intercepts) and differences in the effect of inbreeding among blocks. The initial models contained inbreeding as a fixed factor and development time, adult age (days post maturation) and male mass as fixed effect covariates. To allow the reader to assess the influence of terms excluded from the final model, we present the P - value associated with the parameter estimate for each term if it is included in the final model.

DF and TN were not normally distributed and could not be transformed. Because of lower sample sizes, we used a different non-parametric approach than the one taken for calling

effort. To test for inbreeding depression, we calculated the mean value of inbred and outbred males for each block and compared these using Mann-Whitney U tests (we do not present Wilcoxon tests, as pairing by block reduced the sample size due to missing values in blocks; Wilcoxon tests did, however, yield the same conclusions). We assessed relationships between the two call parameters and development time, age and mass using Spearman Rank correlation (r_s).

To compare traits, we calculated the standardized coefficient of inbreeding δ (Lande and Schemske 1985) which is the percentage change with inbreeding, calculated as (outbred trait value- inbred trait value)/outbred trait value. A negative value indicates that inbred individuals had a larger value for the trait, interpretation of which depends on the direction of selection on the trait. We also present δ for calling effort and the five call parameters after one generation of full sibling inbreeding in a Canberra population of *T. commodus* (Drayton et al. 2007). Furthermore for both studies we calculated Hedge's *d* (see above) to compare the effect of inbreeding between the current study and Drayton et al. (2007) (to allow the comparison we treat males as independent data points). We then calculated the difference in effect sizes between the two studies for each trait and tested whether they differed significantly using a *Z*-test.

Male attractiveness in the field

For the first experiment we tested for a difference in the number of females attracted to the calls of inbred and outbred males with a paired *t*-test (i.e. paired by block, note that a highly conservative approach of pairing by night yielded the same conclusion). For the second experiment we used an independent samples *t*-test. Finally, to maximize the sample size,

we pooled the data from both experiments and ran an independent samples *t*-test (i.e. one data point per mating type per block).

Summary statistics are presented as mean ± standard error. When two sample size values are presented the first is for inbred, and the second for outbred males.

Estimating linear and non-linear selection, and comparing multivariate attractiveness

To estimate linear, quadratic and correlational selection on the five call parameters we used the multiple regression approach of Lande and Arnold (1983). We standardized call traits using a *Z*- transformation (i.e. mean = 0, variance = 1) and converted absolute fitness (i.e. the number of females attracted to a call) to relative fitness by dividing the absolute fitness of each call by the mean across all calls (i.e. population mean). Linear and non-linear selection gradients were estimated in separate regression models. We doubled quadratic regression coefficients to obtain the correct quadratic selection gradients (Stinchcombe et al. 2008). To start, we ran separate multiple-regression models for inbred and outbred calls and then used a sequential model building approach to compare linear and non-linear selection between the call types (Draper and John 1988; Chenoweth and Blows 2005). To determine if sexual selection differed between breeding types we evaluated if the addition of linear by breeding type, quadratic by breeding type or correlational by breeding type interaction terms improved the fit of the linear and non-linear regression models, respectively (see Appendix A in Chenoweth and Blows 2005; Hall et al. 2008 for application of this approach).

There were no detectable differences between breeding types (see Results) so we re-ran the analysis on the full dataset to generate linear selection gradients (β) and the γ matrix of quadratic and correlational selection coefficients. Relative fitness was not normally distributed, so we used randomization testing (see Mitchell-Olds and Shaw 1987) to assess the significance of each β and γ. Relative fitness values were randomized across the calls and selection gradients re-calculated using the Monte Carlo option in PopTools (ver. 2.6.9, CSIRO). This process was repeated 9999 times to obtain the expected distribution for each gradient under random female choice. The *P*-value is the proportion of randomizations in which the gradient estimate was equal to or farther away from zero than the original estimate (Bisgaard and Ankenman 1996; Blows and Brooks 2003).

To detect the major axes of nonlinear sexual selection we conducted a canonical rotation of the γ matrix (Phillips and Arnold 1989). This produced a **M** matrix containing 5 eigenvectors $\mathbf{m}_{1\text{-}5}$ (linear combinations of weighted values of the original five call parameters). The strength of linear and nonlinear sexual selection on each eigenvector is given by θ and λ_i (i.e. the eigenvalue), respectively. The values of θ and λ_i are the regression coefficients when **m** scores are regressed against relative fitness in separate linear and quadratic regression models (Bisgaard and Ankenman 1996). As with terms in the γ matrix, we tested the significance of θ and λ_i using randomization tests in PopTools.

Finally, to compare the multivariate attractiveness of the calls of inbred and outbred males we compared the **m** scores between inbred and outbred males using randomization tests (with 10,000 permutations) implemented in Poptools. To compare each **m** score, we first ran an ANOVA comparing the **m** score across inbred and outbred males. The **m** scores were then randomly shuffled between treatments (i.e. inbred versus outbred) and a second

ANOVA on this shuffled data set performed. A Monte-Carlo simulation with 10,000 permutations was then performed and the number of times the F value of the ANOVA based on the real data exceeded the F value based on the shuffled data was calculated and converted to a two-tailed P -value following the protocol outlined in Manly (1997).

It might be argued that although we have treated them as such in the statistical analyses, the attractiveness scores of each male call are not independent of those of other male calls that were played on a given night (i.e. for every female that was attracted to one male call, there is one fewer choosing another male). However, the entire basis of selection analyses of mating success in large, natural populations (i.e. using the standard multiple regression methods of Lande and Arnold (1983) and subsequent elaborations), is that this non-independence is ignored. In contrast, say, two-choice mating experiments in the laboratory often explicitly take into account non-independence in the statistical analysis (e.g. use of the difference in association time between two males). Our study is an intermediate between these two extremes. We therefore also performed an extremely conservative analysis in which we simply compared the total number of females that were attracted to each call type (inbred or outbred) in a night using a paired t-test.

Visualization of the fitness surface

We used thin-plate splines (Green and Silverman 1994) to visualize the major axes of the fitness surface extracted from the canonical rotation. This non-parametric approach provides a less constrained view of the surface than the best quadratic approximation (Blows et al. 2003). We used the Tsp function in the *fields* package in R (http://www.r-

project.org) to fit a spline surface using the value of the smoothing parameter, λ, that minimized the generalized cross-validation (GCV) score. We then plotted the surface in R using both the perspective and contour map views.

RESULTS

Calling effort

We monitored the calling effort of 1409 males. The calling effort of each male was measured over several nights, so we could test the repeatability of calling effort among males. Calling effort was repeatable ($\chi^2_{1408} = 3387.79$, $P < 0.001$, $r_I = 0.550$). Both inbred and outbred males that took longer to mature had a lower calling effort (inbred males $r_s = -0.290$, $P < 0.001$, $N = 664$, outbred males $r_s = -0.235$, $P < 0.001$, $N = 745$). Both older ($r_s = 0.112$, $P = 0.002$, $N = 745$) and heavier outbred males ($r_s = 0.109$, $P = 0.003$, $N = 738$) had a higher calling effort. For inbred males, there was no significant relationship between age and calling effort ($r_s = 0.060$, $P = 0.120$, $N = 664$), or weight and calling effort ($r_s = 0.020$, $P = 0.612$, $N = 663$). The correlations are, however, not significantly different for inbred and outbred males (age: $Z = 0.97$, $P = 0.33$; weight: $Z = 1.66$, $P = 0.11$).

On average, outbred males called 0.13 standard deviation units more per night than inbred males (Hedge's $d = 0.135$; 95% CI: 0.01 - 0.240). There was therefore a significant negative effect of inbreeding on calling effort (~30% reduction, Table 1). There was no significant heterogeneity in effect size among blocks ($Q_{Total} = 29.73$, $df = 27$, $P = 0.327$). This suggests that inbreeding had a constant effect and did not differ among families. Any differences in the direction and/or magnitude of inbreeding depression among blocks is

likely to be attributed to sampling error. In the second analysis, where each block was weighted equally, inbreeding still significantly reduced calling effort (Wilcoxon's test: $Z = 2.11$, $P = 0.035$, $N = 28$ blocks).

Structure of the advertisement call

In total, we measured call parameters for 98 males. All five call parameters were repeatable among males (Table 2). Inbreeding had no effect on any call parameter (CIPD, ICD and CPN: see Table 3, DF: Mann-Whitney test: $Z = 0.307$, $P = 0.759$, $N = 19, 24$, TN: Mann-Whitney test: $Z = 0.416$, $P = 0.677$, $N = 19, 24$, Table 1).

Older males had both shorter CIPDs and ICDs, but age had no effect on the CPN. In contrast, males who took longer to mature had longer ICDs and more pulses in their chirps, but there was no effect on CIPD. The only effect of mass on calling was that heavier males had a lower CPN (Table 3). There was no interaction between inbreeding and male age, development time or mass affecting CIPD, ICD or CPN [all $P > 0.05$, except for ICD: the interaction between inbreeding and development time ($P = 0.046$), and for CPN: the interaction between inbreeding and male age ($P = 0.042$). For these two interactions, inspection of the graphs of ICD on development time, and CPN on male age showed that the regression lines for inbred and outbred males intercepted outside the range of development times/ages that were included in the experiments. We therefore removed these interactions from the respective models].

DF was not correlated with development time, age or weight. TN was not correlated with development time or weight. None of these correlations differed significantly between

inbred and outbred males (Table 4). Older inbred males had fewer trills/phrase in their calls but there was no such relationship for outbred males. The relationship between age and TN differed significantly between inbred and outbred males (Table 4).

Hedge's *d* for the effect of inbreeding on calling effort and the five call parameters for both this study and Drayton et al. (2007) are presented in Table 1. The effect sizes for inbreeding differed significantly between the Canberra (Drayton et al. 2007) and Smith's Lake populations (this study) for CIPD and ICD, with a significantly larger effect of inbreeding on both traits in the Canberra population.

Male attractiveness in the field

There was no difference in the number of females attracted to the calls of inbred and outbred males in either the first ($t_{20} = 1.083$, $P = 0.292$, inbred males = 4.1 ± 0.75, outbred males = 3.2 ± 0.71 females/night) or second experiment ($t_{30} = 0.269$, $P = 0.790$, inbred males = 2.0 ± 0.66, outbred males = 2.3 ± 0.66 females/night). The pooled data also showed no inbreeding effect ($t_{72} = 0.587$, $P = 0.559$, inbred males = 3.1 ± 0.54, outbred males = 2.7 ± 0.50 females/night). In addition, when we simply compared the total number of females attracted to each call type (inbred or outbred) per night there was no effect of inbreeding ($t_9 = 0.823$, $P = 0.432$). Therefore when calling effort was standardized between inbred and outbred males (because calls were played continuously), there was no difference in attractiveness, suggesting that inbreeding did not alter any aspects of call structure that are important to females. No females were caught on the silent control. Therefore females caught at speakers are actively attracted to the call playbacks.

Estimating linear and non-linear selection, and comparing attractiveness

Inbred and outbred calls did not differ in the strength of linear ($F_{5,68} = 0.693$, $P = 0.630$), quadratic ($F_{5,58} = 1.123$, $P = 0.357$) or correlational sexual selection ($F_{10,38} = 0.582$, $P = 0.818$), so we estimated sexual selection gradients on the combined data set. There was significant linear selection favouring lower dominant frequency (DF), shorter intercall durations (ICD) and higher chirp pulse number (CPN). The only significant non-linear selection was negative quadratic selection (stabilizing) on CIPD (Table 5).

Canonical rotation of the $\boldsymbol{\gamma}$ matrix of non-linear selection gradients revealed significant concave up (disruptive) selection along $\mathbf{m}_1$ and stabilizing selection along $\mathbf{m}_5$, indicating that sexual selection approximates a multivariate saddle (Fig. 3). There was also significant positive directional selection along $\mathbf{m}_2$ (favouring a combination of higher CPN, higher TN and a lower DF: Fig. 4a) and along $\mathbf{m}_4$ (favouring higher CPN, longer CIPD and shorter ICD: Fig. 4b) (Table 6). None of the axes differed between inbred and outbred calls: $\mathbf{m}_1$ ($P = 0.084$), $\mathbf{m}_2$ ($P = 0.107$), $\mathbf{m}_3$ ($P = 0.573$), $\mathbf{m}_4$ ($P = 0.702$) and $\mathbf{m}_5$ ($P = 0.695$), suggesting inbred and outbred males do not differ in multivariate attractiveness.

DISCUSSION

We tested the prediction that traits important in female mate choice are sensitive to the effects of inbreeding, and therefore provide a signal of a male's phenotypic condition, his level of heterozygosity or both. The advertisement call traits that we examined are known to influence female mate choice in *Teleogryllus commodus*: female choice exerts strong positive directional selection for greater calling effort and stabilising and directional

multivariate selection on the five finer-scale aspects of call structure (Brooks et al. 2005; Bentsen et al. 2006; Hunt et al. 2007).

Calling effort and inbreeding

We found that one generation of full sibling mating substantially reduced male nightly calling effort. While this could be due to the effect of inbreeding at loci that directly code for calling behaviour (indicating directional dominance for the mechanical traits underlying calling), it seems more likely that inbreeding depression in calling effort is mediated by effects on condition. Calling in crickets, including *T. commodus*, has been shown to be condition dependent (Wagner and Hoback 1999; Holzer et al. 2003; Scheuber et al. 2003; Hunt et al. 2004b; Hedrick 2005; Judge et al. 2008; Maklakov et al. 2008) and to incur substantial energetic costs (Prestwich and Walker 1981; Kavanagh 1987; Hack 1998). Given the detrimental effects of inbreeding on general fitness in this species (Drayton et al. 2007), it is plausible that inbreeding reduces the ability of males to acquire and assimilate the nutritional and energetic resources needed for high calling rates. Studies with *T. commodus* (Bentsen et al. 2006), and other crickets (Hedrick 1986; French and Cade 1989; Crnokrak and Roff 1995; Holzer et al. 2003) have shown that females prefer males with higher calling effort. Female *T. commodus* may benefit from this preference because calling effort is condition-dependent and might therefore signal male genetic quality or direct benefits (Hunt et al. 2004b). It may also be easier to locate males that call for longer periods (Bentsen et al. 2006). Given the reduction in calling effort with inbreeding, females choosing males based on call rate are using a signal that is correlated with male condition and/or heterozygosity. The relative importance of male heterozygosity or condition in generating benefits of female mate choice in *T. commodus* is uncertain, although it is

conceivable that calling effort signals both characters. Models have shown that the evolution of mate choice for heterozygosity requires a positive correlation between the heterozygosity of parents and offspring (Fromhage et al. 2009). The extent to which parents and offspring resemble each other in heterozygosity in the field population of *T. commodus* is unclear. Nevertheless, the decline in calling effort of inbred males indicates that calling effort does signal aspects of male quality.

Call structure, attractiveness and inbreeding

We found no effect of inbreeding on any of the finer-scale call parameters (ICD, CPN, CIPD, TN and DF), indicating low directional dominance variance for these traits (Roff 1998; Rantala and Roff 2007). These traits do not seem to be condition-dependent in *T. commodus* as reducing dietary protein had no effect on any of these call parameters (Hunt et al. 2004b). Nonetheless, these call parameters influence female choice (Brooks et al. 2005; Bentsen et al. 2006), even though they do not seem to reflect dietary condition or levels of inbreeding (in the $F = 0$ to 0.25 range). These finer scale call parameters might therefore have evolved to signal information other than male condition or heterozygosity to females. For example, finer scale call parameters could play a role in species recognition (Hill et al. 1972), which is supported by the significant stabilizing selection detected for finer scale call structure in this study and others (e.g. Brooks et al. 2005; Bentsen et al. 2006; Hunt et al. 2007), indicating that males who deviate in either direction from optimum trait value combinations might not be recognized by conspecific females.

Despite our study finding no effect of inbreeding on the five measured structural components of the advertisement call, it is possible that inbreeding altered unmeasured call

characteristics that are still detected by females. Consequently, we tested the attractiveness of advertisement calls in the field by measuring the number of females that approached the calls of inbred and outbred males. We tested the attractiveness of advertisement calls in the field because studies have shown that estimates of inbreeding depression on male attractiveness can be masked in the laboratory. This could arise because the detrimental effect of inbreeding on trait development or production is magnified under harsher field conditions and/or because female choice differs between the laboratory and field. For example, Joron and Brakefield (2003) demonstrated that a small reduction in mating success of male butterflies in captivity was greatly accentuated in a greenhouse environment where unconstrained flight and a fuller expression of courtship behaviour were possible. Unlike the study by Joron and Brakefield, however, male sexual traits were not expressed under field conditions in our study as inbred and outbred calls were recorded from laboratory reared males. Nonetheless, we ensured that female choice was expressed in the field. By testing the attractiveness of the calls here we created conditions that were as close as possible to those under which females would naturally express a choice for inbred and outbred males while also controlling for calling effort by using a playback design.

In our field experiment call rate did not differ between inbred and outbred calls because each call was broadcast continuously throughout the night. This allowed us to isolate the effect of any structural changes in the advertisement call. There was no difference in the number of females that were attracted to each call type, suggesting that inbreeding had no effect on call structures important to females. This was further supported by the lack of any difference in the scores for the major axes of multivariate selection between inbred and outbred males. This, however, does not mean that inbreeding will not affect male reproductive success. Recall that inbred males called significantly less (30% reduction)

than outbred males. They will therefore attract far fewer females as an earlier field study at the same site showed a very strong female preference for higher call rate (Bentsen et al. 2006). It is also noteworthy that we detected inbreeding depression in calling effort under favorable laboratory conditions of unrestricted food, no competition for calling sites and controlled temperatures. Under harsher field conditions, the reduction in calling would most likely exceed 30%. Furthermore, once a male has attracted a female, he produces a different courtship song, to induce her to mate. Inbreeding depression in the courtship song is currently under investigation in our laboratory. There is evidence that courtship songs are condition dependent (Tregenza et al. 2006, but see Wagner and Reiser 2000; Gray and Eckhardt 2001). For example, male *T. oceanicus* produce different courtship songs after suffering an immune challenge (Tregenza et al. 2006). There is also a link between courtship call features and cryptic female choice (spermatophore retention by females) in *T. commodus* (Hall et al. 2008). Any changes in the courtship song with inbreeding might further reduce the reproductive success of inbred males if they fail to successfully court females.

A comparison of studies

We have previously investigated the effect of inbreeding on advertisement calling in a Canberra (Australia) population of *T. commodus*. In accordance with the results of the current study, we found no effect of inbreeding (one generation of brother sister mating) on dominant frequency and trill number in the Canberra population. In contrast, in the Canberra population inbreeding had no significant effect on nightly calling effort, but caused highly significant changes in CIPD, ICD and CPN (Drayton et al. 2007; Table 1). The effect sizes for inbreeding however, only differed significantly between studies for

CIPD and ICD, with the effect of inbreeding being of a larger magnitude in the Canberra population for these two traits (Table 1). It would therefore seem that aspects of advertisement call structure vary in their susceptibility to inbreeding depression between the Canberra and Smith's Lake population (450 km apart). Several factors might account for these differences. One possibility is that the two populations differ in the level of directional dominance variance (and therefore susceptibility to inbreeding depression) underpinning different components of call structure because of differences in the strength of selection imposed by females (which depletes additive genetic variance) for different trait combinations between the populations. To test this prediction would require a population comparison of female choice criteria. In addition, natural selection on call traits might differ between the populations due to differences in the occurrence of acoustically orientating predators or parasitoids (e.g. Zuk et al. 1993; Rotenberry et al. 1996).

Multivariate sexual selection on call structure

While the main aim of the selection analysis was to compare the multivariate attractiveness of inbred and outbred males, the resulting measure of multivariate sexual selection deserves mention. The selection analysis revealed significant concave up (disruptive) selection along $\mathbf{m}_1$ (most heavily loaded with ICD and CPN) and stabilizing selection along $\mathbf{m}_5$ (heavily loaded with CIPD, and to a lesser degree, ICD). The resulting fitness surface is saddle shaped (Fig. 3), indicating two fitness peaks. The large loading of CIPD in $\mathbf{m}_5$ and the resulting involvement of this trait in significant stabilizing selection is in contrast to both Brooks et al. (2005) and Bentsen et al. (2006), who failed to find strong evidence for a role of CIPD in stabilizing selection in *T. commodus* from Smith's Lake. It is possible that there are temporal differences in multivariate sexual selection operating in the Smith's Lake

population. We also found evidence of directional selection on call traits. Selection favoured a lower dominant frequency (DF), shorter intercall duration (ICD), a higher number of pulses in the chirp (CPN), a higher number of trills in a phrase (TN) and longer intervals between the pulses in the chirp (CIPD). Our results showing directional selection on DF, CPN and CIPD are consistent with Bentsen et al. (2006) who investigated multivariate sexual selection on call structure and calling effort in the same field population of *T. commodus*.

Interestingly, Bentsen et al. (2006) found significant directional selection for longer ICD, whereas Brooks et al. (2005) and our study found significant directional selection for shorter ICDs. In both our study and Brooks et al. (2005), calls were played to females in a continuous loop. However, Bentsen et al. (2006) varied the number of phrases repeated in a five minute loop, meaning that there was a silent period between the end of the last phrase in the loop and the first phrase in the next loop. Playbacks therefore consisted of discrete calling bouts and inter-bout intervals of silence. Females preferred males with longer call bouts, and these were the loops that contained both longer phrases and a greater number of phrases. A longer interval between phrases (i.e. longer ICD) increases the overall bout length by lengthening each phrase. Consequently, in this case, selection for shorter inter-bout intervals (less time 'off air') overrides selection for shorter intercall durations (ICD), which increases total time on air when calling is continuous (Bentsen et al. 2006).

In conclusion, our results show that the actual call of an inbred ($F = 0.25$) male *T. commodus* is no less attractive to a female than that of an outbred male in a natural field setting. This is either because inbreeding does not affect the structure of the advertisement call, or because any structural changes are inconsequential to females. It is, of course,

possible that the effects of inbreeding on call structure will only become apparent in this population after more severe inbreeding (e.g. Zajitschek et al. 2009), or under harsher rearing conditions. We did find, however, that inbred males have a far lower call rate than outbred males. This should cause a marked reduction in the number of females mating with inbred males. This is because males who call less often are harder for females to locate and/or because female *T. commodus* actively prefer males who call more (Bentsen et al. 2006). In sum, advertisement calling (i.e. calling effort) signals a male's inbreeding status, and females can therefore discriminate against inbred males. Although the avoidance of inbred males may not be the reason why female mating preferences evolved, and the effect of mating with relatively outbred males on offspring fitness is unknown, the predicted reduction in the mating success of inbred males clearly has fitness consequences for individuals, and wider ramifications for population genetic structure.

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Table 1. The means ± SE for inbred and outbred males, δ (% change with inbreeding) and Hedge's *d* for the effect of inbreeding on calling effort and the five call parameters from this study and from Drayton et al. 2007 for comparison. *Z* and *P*- values are for the test of a significant difference in effect sizes between the two studies for each trait.

Trait	Mean ± SE (*N*), this study		δ this study	δ Drayton et al. 2007	*d*, this study (95% confidence interval)	*d*, Drayton et al. 2007 (95% confidence interval)	*Z*	*P*
	Inbred	Outbred						
Calling effort (s/night)	234.06 ± 38.93 (664)	335.32 ± 39.56 (745)	**30.2**	-5.0	0.10 (-0.01, 0.20)	-0.02 (-0.50, 0.45)	0.49	0.63
CIPD (s)	0.015 ± 0.001 (37)	0.016 ± 0.001 (61)	6.3	**-34.4**	0.14 (-0.27, 0.55)	-2.34 (-3.06, -1.62)	5.86	**<0.001**
ICD (s)	0.179 ± 0.025 (37)	0.157 ± 0.007 (61)	-14.0	**-350.8**	-0.21 (-0.62, 0.20)	-1.92 (-2.59, -1.24)	4.24	**<0.001**
CPN	6.21 ± 0.16 (37)	5.74 ± 0.13 (61)	-8.0	**-17.9**	-0.45 (-0.86, -0.04)	-1.04 (-1.64, -0.44)	1.58	0.11
DF (Hz)	4031.93 ± 24.38 (37)	4018.85 ± 11.74 (61)	-0.3	0.0	-0.11 (-0.52, 0.30)	0 (-0.57, 0.57)	0.31	0.76
TN	2.13 ± 0.19 (37)	1.96 ± 0.13 (61)	-8.6	0.4	-0.16 (-0.57, 0.25)	0.01 (-0.56, 0.58)	0.46	0.64

CIPD: chirp inter-pulse duration, ICD: inter-call duration, CPN: chirp pulse number, DF: dominant frequency, TN: trill number.

Bold values of δ indicate a significant difference between inbred and outbred males.

Bold *P*-values indicate that the effect sizes for a trait differ significantly between the two studies.

Table 2. The repeatability of call parameters among males (five calls per male, $N = 98$ males).

Call Parameter	Test Statistic	*P*-Value	r_I
CIPD	$F_{97,392} = 16.917^{*}$	<0.001	0.888
ICD	$F_{97,392} = 5.832^{*}$	<0.001	0.707
CPN	$F_{97,392} = 10.384^{*}$	<0.001	0.824
DF	$\chi^2_{97} = 464.424^{\dagger}$	<0.001	0.979
TN	$\chi^2_{97} = 393.908^{\dagger}$	<0.001	0.908

CIPD: chirp inter-pulse duration, ICD: inter-call duration, CPN: chirp pulse number, DF: dominant frequency, TN: trill number. r_I: intra-class correlation coefficient.

*One-way ANOVA, †Kruskall-Wallis test.

Table 3. The effect of inbreeding, development time, age and weight on CIPD, ICD and CPN. *P*-values are from the final model if the term was significant, or when it alone was added to the final model if non-significant (see text). *P*-values significant at the 0.05 level are in bold.

	CIPD			ICD			CPN		
	d.f.	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*
Inbreeding	1,14	0.711	0.413	1,14	0.185	0.674	1,14	1.658	0.219
Development time	1,53	2.465	0.122	1,53	5.180	**0.027**	1,53	5.658	**0.021**
Age	1,54	9.857	**0.003**	1,53	4.193	**0.046**	1,52	0.223	0.639
Weight	1,53	1.719	0.196	1,52	1.761	0.190	1,53	4.613	**0.036**

Table 4. Correlations between DF (dominant frequency) and TN (trill number), and development time, age and weight. *Z* and *P* values are for the comparison of r_s between inbred and outbred males.

	DF				TN			
	r_s inbred (*P*)	r_s outbred (*P*)	*Z*	*P*	r_s inbred (*P*)	r_s outbred (*P*)	*Z*	*P*
Development time	-0.233 (0.165)	0.029 (0.823)	1.29	0.197	0.132 (0.436)	-0.166 (0.200)	1.45	0.146
Adult age	-0.241 (0.150)	0.111 (0.393)	1.73	0.084	-0.442 **(0.006)**	-0.020 (0.880)	2.28	**0.023**
Weight	-0.304 (0.067)	0.059 (0.651)	1.81	0.071	-0.036 (0.833)	-0.112 (0.389)	0.37	0.711

All r_s $N = 37, 61$. *P*-values significant at the 0.05 level are in bold.

Table 5. The vector of standardized linear selection gradients (β) and the matrix of standardized quadratic and correlational selection gradients (γ).

		γ				
	β	CIPD	DF	ICD	TN	CPN
CIPD	0.228	**-0.234**				
DF	**-0.275**	-0.060	-0.009			
ICD	**-0.258**	-0.334	0.426	-0.045		
TN	-0.083	0.279	-0.241	-0.191	-0.023	
CPN	**0.310**	-0.349	0.009	0.493	-0.066	0.038

Gradients in bold are significant at $P < 0.05$. 10,000 permutations to test for significance.

Table 6. The matrix of eigenvectors from the canonical rotation of γ. The linear (θ) and quadratic (λ) gradients of selection along each eigenvectors are given.

						Selection	
	CIPD	DF	ICD	TN	CPN	θ	λ
$\mathbf{m_1}$	-0.354	0.379	0.588	-0.345	0.515	-0.148	**0.466**
$\mathbf{m_2}$	-0.124	-0.624	0.027	0.437	0.636	**0.297**	0.056
$\mathbf{m_3}$	0.284	0.500	0.290	0.765	0.008	-0.209	-0.087
$\mathbf{m_4}$	0.431	0.351	-0.574	-0.177	0.575	**0.343**	-0.326
$\mathbf{m_5}$	0.770	-0.307	0.489	-0.271	0.015	0.161	**-0.381**

Gradients in bold are significant at $P < 0.05$. 10,000 permutations to test for significance.

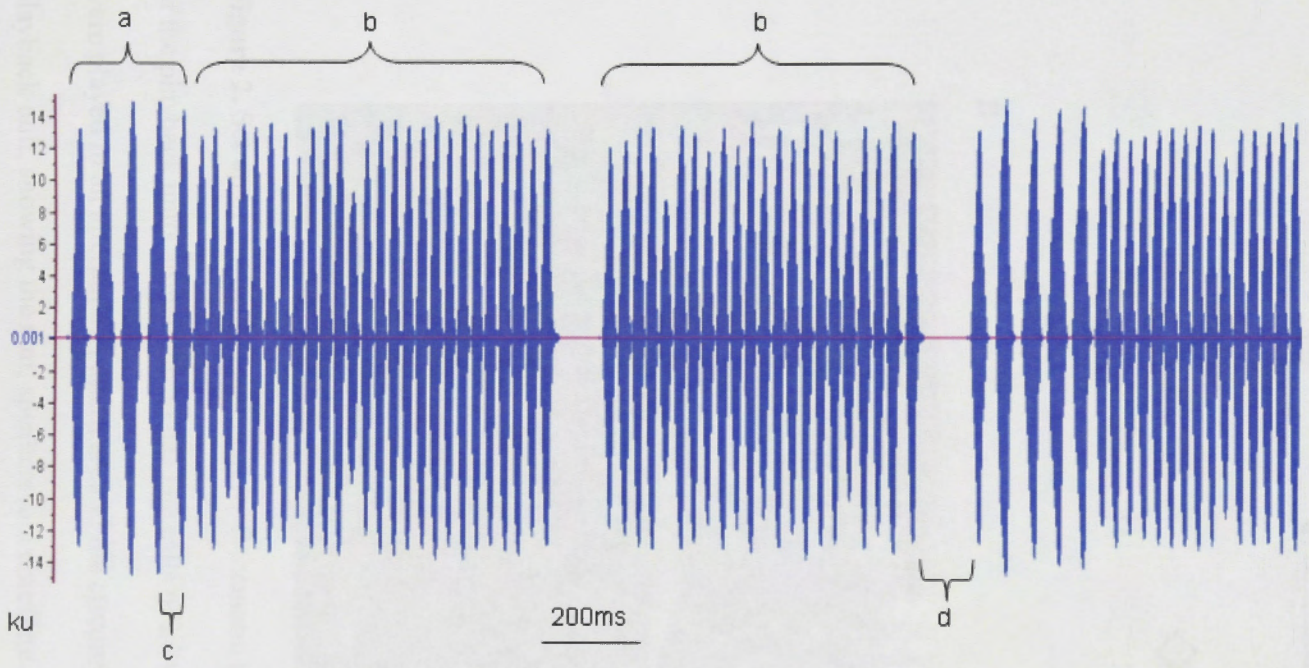
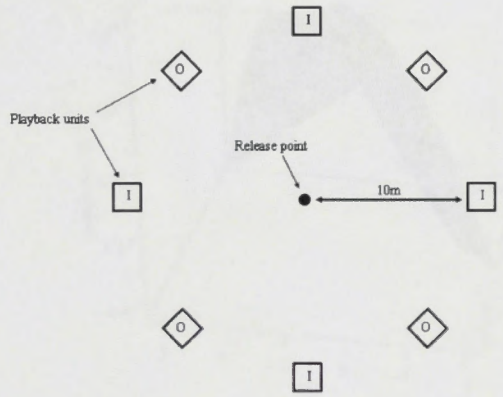


Figure 1. The advertisement call of male *T. commodus*. Louder pulses repeated at a lower rate are group into chirps (a) and softer pulses repeated at a higher rate are grouped into trills (b). Each phrase consists of a single chirp followed by a variable number of trills (the phrase in the figure has two trills). We measured the dominant frequency (DF), the number of pulses per chirp (CPN), the chirp inter-pulse duration (CIPD) (c), the inter-call duration (ICD) (d), and the number of trills in a phrase (TN). The horizontal axis is time (ms), while the vertical is amplitude (kU).

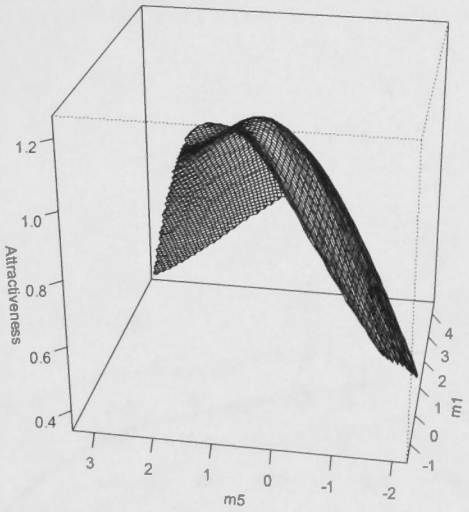
A



B

Figure 2. Set up of the field experiment (a) Schematic diagram showing the placement of the playback units around the arena. The calls of inbred (I) and outbred (O) males were played in an alternating pattern around the circumference of the arena (b) one playback unit, showing the tent, speakers and coreflute covered in Tangle-Trap. The digital audio player was housed in the plastic box for rain protection.

A



B

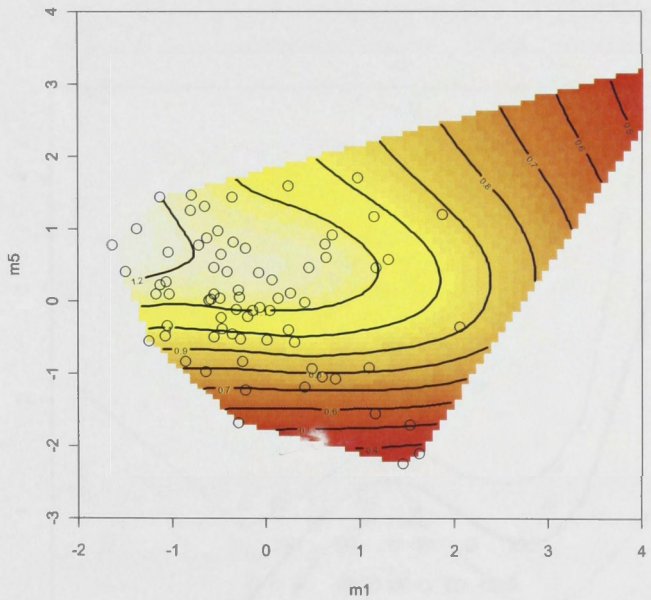
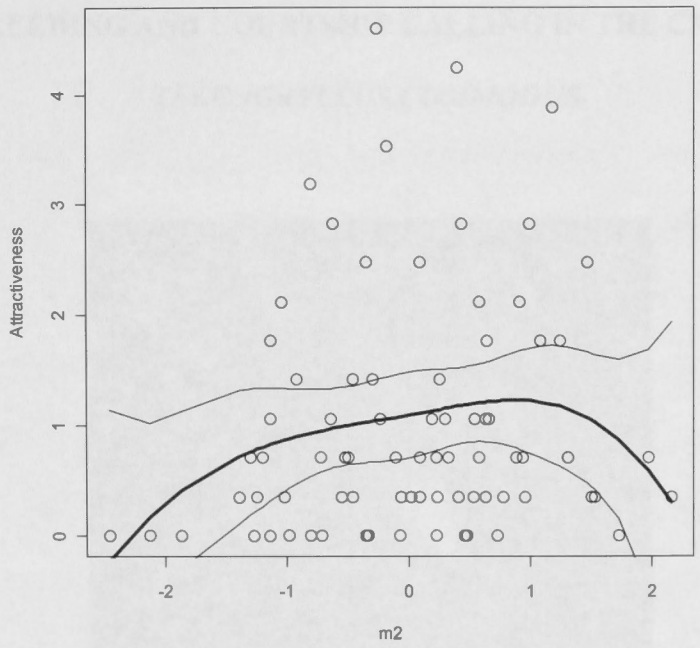


Figure 3. Thin-plate spline visualization of the fitness surface along the two major axes of non-linear selection, $\mathbf{m_1}$ and $\mathbf{m_5}$. (a) Perspective view, (b) contour view with data points overlaid. Yellow regions indicate higher fitness.

A



B

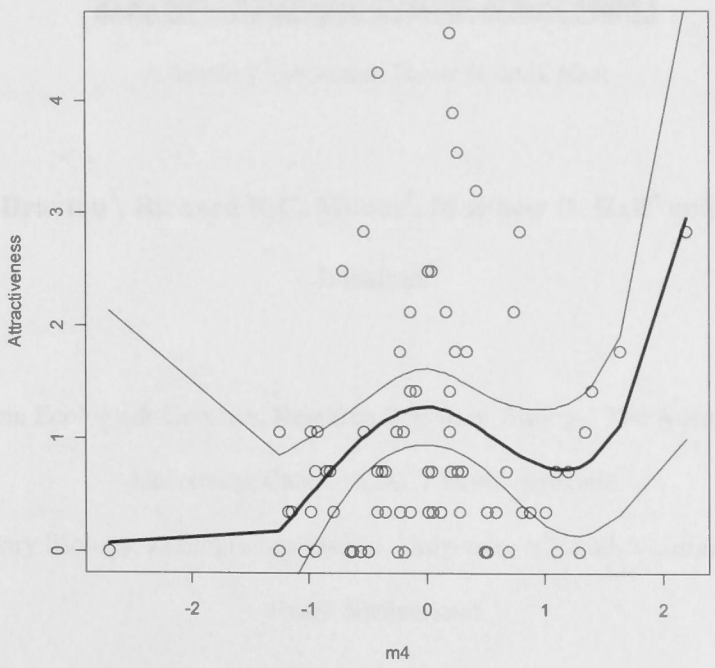


Figure 4. Univariate cubic spline visualization of sexual selection operating on (a) $\mathbf{m_2}$ and (b) $\mathbf{m_4}$ with data points overlaid. 95% confidence intervals are also provided.

CHAPTER 4

INBREEDING AND COURTSHIP CALLING IN THE CRICKET *TELEOGRYLLUS COMMODUS.*

A female *T. commodus*. Photo: Hendrik Maat

Jean M. Drayton[1], Richard N.C. Milner[1], Matthew D. Hall[2] and Michael D. Jennions[1]

1. Evolution, Ecology & Genetics, Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia.

2. Evolutionary Biology, Zoologisches Institut, University of Basel, Vesalgasse 1, CH-4051 Basel, Switzerland.

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ABSTRACT

Male field crickets produce two acoustic signals for mating: advertisement calls and courtship calls. While the importance of advertisement calling in mate attraction is well understood, the function of courtship calling is less clear. Here we tested if the courtship call of male crickets *Teleogryllus commodus* signals aspects of male quality by comparing the calls of inbred and outbred males. We examined the effect of one generation of full sibling mating on fine-scale call structure, along with several life history traits. Inbreeding reduced nymph survival but had no significant effect on weight or development time. Inbreeding resulted in a small but significant change in two of the six call parameters measured. We then tested if inbreeding affects call trait combinations that are important to females by using the results of a previous selection analysis to compare the multivariate attractiveness of the calls of inbred and outbred males. There was no difference. We conclude that the courtship call of *T. commodus* is not a reliable signal of aspects of male quality that are affected by inbreeding (which generally reduces fitness-enhancing traits). It might, however, signal components of male fitness that are not affected by changes in heterozygosity.

KEY WORDS: female choice, heterozygosity, condition dependence, selection analysis, courtship call, sexual selection

INTRODUCTION

Males of many species possess multiple sexually selected ornaments that females may use when evaluating prospective mates (Moller and Pomiankowski 1993; Candolin 2003). Field crickets are a useful model system to investigate multiple sexual signals during mate choice. Males produce two acoustic sexual signals prior to mating: an advertisement call and a courtship call. The advertisement call attracts sexually receptive females from a distance for mating (Loher and Rence 1978; Campbell and Shipp 1979; Evans 1988). Female preferences for advertisement calls have been well studied (e.g. Hedrick 1986; Stout and McGhee 1988; Wagner 1996; Wagner and Reiser 2000; Holzer et al. 2003; Brooks et al. 2005; Bentsen et al. 2006; Wagner and Basolo 2007). Several studies have shown that preferences for specific aspects of advertisement calling are likely to exist because they provide information about male traits that confer direct benefits to females (Wagner and Basolo 2007) as well as traits that are likely to confer indirect genetic benefits if they are heritable and positively correlated with net fitness, such as body condition (Scheuber et al. 2003a,b; Judge et al. 2008), dietary intake (Maklakov et al. 2008) and immune function (Jacot et al. 2004). At a higher level, it is also well established that advertisement calling signals species identity. As expected for a signal used in species recognition, there is evidence for strong stabilising selection on advertisement calls (e.g. Brooks et al. 2005; Bentsen et al. 2006). At a more proximate level, stabilising selection on advertisement calls can also be imposed by limitations of the female auditory and nervous system (Brooks et al. 2005).

In contrast, the courtship call has received less attention and explaining its evolution and maintenance is more challenging (Gray and Eckhardt 2001; Zuk et al. 2008; Rebar et al. 2009). Once a female approaches a male and the two are in physical contact, the

male switches from advertisement calling to produce the distinctive courtship call. This raises an obvious question. If the advertisement call is sufficient to bring a male and a female together to mate, why produce a second signal (Tregenza et al. 2006; Rebar et al. 2009), especially when there are costs of acoustic signals for both males (review: Zuk and Kolluru 1998; Kotiaho 2001) and females (e.g. Martin and Wagner 2010)? It appears, however, that the courtship call is important to induce female mounting behaviour (Crankshaw 1979; Burk 1983; Adamo and Hoy 1994; Libersat et al. 1994; Balakrishnan and Pollack 1996; but see Bailey et al. 2008), which is necessary for spermatophore transfer to the female. Several recent studies have demonstrated female preferences for specific courtship call components that appear to signal aspects of male fitness. For example, female variable field crickets *Gryllus lineaticeps* prefer courtship calls with a higher chirp rate, which can generate a different pattern of mate choice than that initially predicted based on preferences for advertisement calls (Wagner and Reiser 2000). Female Mediterranean field crickets *Gryllus bimaculatus* prefer courtship calls with a greater expression of components that are positively correlated with an immune response (encapsulation rate) (Rantala and Kortet 2003). Likewise, male *Teleogryllus oceanicus* produce structurally different courtship calls after an immune challenge. Furthermore, females responded more rapidly to males in response to playback of courtship calls that were experimentally manipulated to resemble those of males with higher encapsulation ability (Tregenza et al. 2006). Rebar et al. (2009) showed that the courtship calls of attractive *T. oceanicus* males differ from those of less attractive males, and that a previously unattractive male could be made more attractive by muting him and playing the courtship call of a more attractive male.

One way to test whether male sexual traits signal aspects of male quality is to create classes of individuals that can be assigned high or low phenotypic quality and to

compare the expression of sexual traits between these classes. An aspect of quality that can be easily manipulated in the laboratory is condition. This is usually achieved by changing external environmental factors that affect condition such as diet or food availability (e.g. Holzer et al. 2003; Scheuber et al. 2003a,b; Judge et al. 2008). The few studies that have investigated the condition dependence of the courtship call by manipulating diet have found no effect (*G. texensis*: Gray and Eckhardt 2001; *G. lineaticeps*: Wagner and Reiser 2000). An alternative, yet underexploited method of creating classes of individuals that can be assigned high or low quality status is to manipulate condition via inbreeding (e.g. Bolund et al. 2010; Drayton et al. 2010; review: Cotton et al. 2004). Inbreeding (the mating of relatives) increases homozygosity across the genome and usually results in a decline in fitness known as inbreeding depression (review: Keller and Waller 2002; Armbruster and Reed 2005). This reduction in fitness mainly arises due to the unmasking of partially or fully recessive deleterious alleles, as well as the loss of advantageous heterozygosity (Charlesworth and Charlesworth 1999; Charlesworth and Willis 2009). Inbreeding could affect sexual traits due to lower heterozygosity at loci that directly code for them. If, however, sexual traits are condition dependent (review: Cotton et al. 2004), and inbreeding reduces the general ability of individuals to acquire and/or assimilate the energetic and nutrient resources that are available to be allocated to sexual signalling (i.e. lowers condition *sensu* Rowe and Houle 1996; review: Tomkins et al. 2004), then we expect reductions in sexual traits with inbreeding.

Inbreeding depression in sexually selected traits may also indicate that these traits can reveal a male's genome wide heterozygosity to females. Recent models for the evolution of female mate choice have proposed that females can benefit from mating with males with above average heterozygosity (Mays and Hill 2004; Kempenaers 2007;

Fromhage et al. 2009). This is because, under certain conditions based largely on rates of immigration between meta-populations, males with greater heterozygosity are more likely to carry locally rare alleles dissimilar to those in the average female. Consequently, mating with such males yields offspring with above average heterozygosity (i.e. mean heterozygosity is heritable; Mitton et al. 1993; Brown 1997 but see Puurtinen et al. 2009). Female choice for heterozygous males will therefore elevate offspring fitness whenever heterozygosity increases offspring performance. A decline in sexual trait expression with inbreeding provides direct evidence that these traits can signal genome wide heterozygosity. More generally, inbreeding in normally outbreeding species almost always reduces fitness (Falconer and Mackay 1996). We predict that inbreeding will reduce many components of male fitness such as immune function, performance capacity and condition. If a sexual trait is a signal of one or more of these measures of male quality, and/or genome wide heterozygosity, then inbreeding should lead to signals that are less attractive to females.

In this study, we investigate the extent to which courtship call parameters signal male fitness in the black field cricket *Teleogryllus commodus*. To do this, we quantified the effect of one generation of brother-sister matings ($F = 0.25$) on courtship calls. We have previously shown that inbreeding ($F = 0.25$) reduces egg hatching success, nymphal survival and adult lifespan in *T. commodus* (Drayton et al. 2007). There is therefore good evidence for inbreeding depression in fitness. We have also shown that inbreeding reduces the advertisement call rate (Drayton et al. 2010), and alters fine-scale advertisement call structure (Drayton et al. 2007). In contrast, there is currently no data on whether or not the courtship call signals aspects of male quality. Hall et al. (2008) have shown that courtship calls of *T. commodus* are under post-copulatory sexual selection because call components affect the length of time that a female retains the

externally attached spermatophore after mating. The longer the attachment time, the greater the number of sperm transferred (Bussiere et al. 2006; Hall et al. 2010, although after approximately an hour, the number of sperm transferred levels off, see Fig. 3 in Bussiere et al. 2006). Hall et al. (2008) characterized the major axes of multivariate sexual selection on courtship call structure using spermatophore attachment times as the measure of fitness. They found significant non-linear multivariate selection on call structure and male size (Hall et al. 2008). We worked with the same study population and measured the same courtship call parameters as Hall et al. (2008). This allowed us to compare the multivariate attractiveness of inbred and outbred males along significant axes of multivariate selection. If the courtship call signals aspects of male quality that are reduced by inbreeding, such as male condition or heterozygosity, then we predict that the call will change significantly with inbreeding, and that this will lower the predicted multivariate attractiveness of inbred males. Finally, we also quantified the effect of inbreeding on three life history traits in both males and females: maturation weight, development time and nymph survival.

MATERIALS AND METHODS

Generation and rearing of inbred and outbred crickets

Inbred individuals were created by full sibling matings ($F = 0.25$) and their fitness compared with that of outbred crickets ($F = 0$). Full sibling families were derived from approximately 70 wild caught gravid females collected in May 2006, at Smith's Lake, NSW. Offspring from wild caught females were reared to adulthood (when communally rearing all crickets in the study, males and females were always separated before sexual maturity to ensure virginity) and then paired at random (a male and female in a pair did

not share the same wild caught mother) to create full sibling families. Each full sibling family was reared in a separate 43x30x13 cm plastic tub with dry cat food (KiteKat Krunch, Uncle Ben's, Raglan, Australia) and water provided *ad libitum*.

The full sibling families were grouped into pairs, hereafter known as blocks ($N = 33$ blocks). We ensured that the two families in a block were unrelated to each other. In each block, brothers and sisters from both full sibling families were mated to create two inbred genotypes. Outbred genotypes were created by reciprocal matings of a male and a female from each family in the block. For example, in a block consisting of full sibling families A and B, inbred progeny were generated by mating brothers and sisters from family A, and brothers and sisters from family B, while outbred progeny were generated by mating a male from family A with a female from family B, and a male from family B with a female from family A (Fig. 1). This design generated four offspring genotypes (two inbred and two outbred) per block. After mating females were provided with moist cotton wool ("egg pads") for egg laying. These were checked every three days for emerging nymphs. Upon emerging, nymphs from the same pairing were reared communally for 20 days in 9x9x5cm plastic tubs with food and a piece of moist cotton wool. After 20 days, each nymph was transferred to an individual container (9x9x5cm, i.e. one nymph/container) with a small tube of water plugged with cotton wool at the open end, a piece of cat food and a cardboard egg cup for shelter. Nymphs were not transferred to individual containers immediately after emerging because they were small enough to be crushed by the water tube. The density of nymphs during the communal stage was very similar because, on average, we set up 37.5 ± 3.3 (± SE) nymphs of each inbred genotype and 39.4 ± 3.3 nymphs of each outbred genotype per block. It is unlikely that nymphs competed for resources during the communal rearing because they were very small and therefore spacing between individuals was large, and

food and water was provided *ad libitum*. Once set up individually, food and water was replaced every 10 days. Dead nymphs were recorded to calculate nymph survival (the proportion of nymphs that reached the final adult moult). Nymphs nearing maturity were checked daily to record development time (days from hatching to the final adult moult). All crickets were weighed after their final adult moult (i.e. maturation weight). All the males used to test the effect of inbreeding on courtship calling were at least 10 days post maturation to ensure that they were sexually mature. Crickets were maintained at 26-28°C on a 12:12 photoperiod.

The structure of the courtship call

The courtship call

Male crickets call using a stridulatory apparatus consisting of a file and scraper on the forewings. The most basic unit of a call is the pulse. Each pulse is produced by closing the wings once (Kavanagh 1987). Pulses of the courtship call are grouped together into chirps and trills. These are arranged into repeated phrases. Each phrase consists of a single, amplitude-modulated chirp, followed by a variable number of trills that are consistent in their intensity (Hall et al. 2008) (Fig. 2).

Recording and analysis of the courtship call

To record courtship calls, we set up males in 9x9x5cm recording chambers (one male per chamber) with a condenser microphone mounted in the lid. To induce a male to call, a virgin stock female was introduced into the recording chamber. We recorded 1-2 minutes of the courtship call using a digital recorder (MicroTrack 24/96, M-Audio,

Irwindale, CA, USA). A power unit, containing a 9 volt battery, was in series between the microphone and the recorder to power the microphone. If the female mounted the male during recording, we gently shook the chamber to interrupt the mating. After each recording, the male and female were removed from the chamber and weighed. We also noted the age of the male and the ambient temperature. Male crickets *T. oceanicus* can adjust the quality of their ejaculates according to female matings status (Thomas and Simmons 2007). It is therefore possible that male *T. commodus* might adjust courtship depending on the phenotype of the female. Consequently, only virgin females were used to induce courtship and the allocation of females to inbred and outbred males was randomised.

Courtship calls were analyzed using Raven Pro 1.3 sound analysis software (Cornell Laboratory of Ornithology, Ithaca, NY, USA; www.birds.cornell.edu/raven). We measured the following six call parameters of five randomly chosen phrases from the courtship call of each male: dominant frequency (DF), the number of pulses in the chirp [chirp pulse number (CPN)], the duration of the interval between the last two pulses in the chirp [the chirp inter-pulse duration (CIPD)], the duration of the interval between the chirp and the first trill [chirp-trill interval (CTI)], the number of trills in a phrase [trill number (TN)] and the length of the first trill [trill 1 length (T1L)] (Fig. 2). Calls were filtered before analysis to remove ambient noise below 3kHz and above 6kHz.

Statistics

Structure of the courtship call

The repeatability of each call parameter among males was assessed using a one-way ANOVA or Kruskall-Wallis test. All call parameters were highly repeatable (see Results), so we calculated a mean value of CIPD, CTI, DF, T1L, TN and CPN for each male. To test for an effect of inbreeding on CIPD, CTI, T1L and CPN we ran separate linear mixed models 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 that included all two-way interactions. We then removed fixed terms, starting with interactions, until the final model only contained significant terms. Random terms were always retained as part of the experimental design (Crawley 2002). For all models, block and the interaction between inbreeding and block were included as random factors. This controlled for variation in the mean value of call parameters across blocks (i.e. random intercepts), and differences in the effect of inbreeding among blocks (i.e. random slopes). The initial models contained inbreeding as a fixed factor and development time, male age, male mass, the mass of the female used to induce courtship, and ambient temperature as fixed effect covariates. To allow the reader to assess the influence of terms excluded from the final model, we present the *P*-value associated with the parameter estimate for each term if it is individually included in the final model.

The model for CIPD showed a significant interaction between inbreeding and female mass (but none of the other covariates). We therefore ran separate models for inbred and outbred males to test for the effects of other covariates. We used Spearman Rank correlations (r_s) to assess the bivariate relationships between DF or TN and development time, male age, male and female mass and temperature, as neither call parameter could be transformed to fit parametric assumptions.

To test the effect of inbreeding on CIPD, TN and DF we ran separate meta-analyses. Each block contributed one effect size (Hedge's *d*) per call parameter based on the standardised difference in means between inbred and outbred males. We corrected for small sample size effects using *J* [see Rosenberg et al. (2000); note: some authors refer to Hedge's *d* as *g*, e.g. Cooper et al. 2009)]. We then ran a random effects model in Metawin 2.0 to calculate the mean effect size. This weights each effect size by the inverse of its variance, which gives greater weighting to blocks where the estimate of the inbreeding effect was more precise. We then examined the non-parametric, bootstrapped 95% confidence interval to determine whether the mean effect of inbreeding differed significantly from zero. This meta-analysis is equivalent to a standard parametric two-way mixed model ANOVA (block by inbreeding), but does not require that the call parameters are normally distributed.

Maturation weight, development time and nymph survival

To test for an effect of inbreeding on maturation weight, we ran a linear mixed model in S-Plus 7.0 (see above). Block and the block by inbreeding interaction were random factors and the initial model contained inbreeding and sex as fixed factors. Development time could not be transformed to conform to parametric assumptions so we calculated separate means for each sex for inbred and outbred individuals in each block and compared the means using two Wilcoxon's tests. There was no inbreeding effect (see Results). To test for any difference between the sexes we therefore pooled inbred and outbred individuals, calculated the mean development time for males and females in each block, and compared the means with a Wilcoxon's test. For the effect of inbreeding on nymph survival, we calculated the proportion of inbred and outbred

nymphs that survived to adult eclosion in each block and compared the values with a paired *t*-test.

To compare traits, we calculated the standardized coefficient of inbreeding, δ (Lande and Schemske 1985) which is the percentage change with inbreeding, calculated as (outbred trait value - inbred trait value)/outbred trait value. A negative value indicates that inbred individuals had a larger value for the trait, interpretation of which depends on the direction of selection on the trait. We also calculated the effect size, *d* (see above), for inbreeding for each trait using the grand mean and standard deviation for all inbred and outbred crickets (i.e. ignoring block). A positive value of *d* indicates that the outbred trait value is larger than the inbred trait value (conversely a negative value indicates a larger inbred trait value).

Summary statistics are presented as mean ± standard error. When two sample size values are presented the first is for inbred, and the second for outbred crickets. All tests are two-tailed and $\alpha = 0.05$.

Selection analysis and comparing multivariate attractiveness

We measured the six courtship call parameters used by Hall et al. (2008) to characterise multivariate sexual selection on the courtship call, where male fitness was measured as spermatophore retention time for mating pairs of crickets collected from the same study site. The selection analysis of Hall et al. (2008) identified the major axes of multivariate sexual selection on courtship call structure. Using the courtship call and weight data from our study, we could then calculate the value of each inbred or outbred male along each major axis of selection. This allowed us to compare the multivariate attractiveness

of the courtship calls of inbred and outbred males. Full details of the selection analysis are given in Hall et al. (2008). In brief, to characterize sexual selection on the courtship call and body size, Hall et al. (2008) used the multiple regression approach of Lande and Arnold (1983). Before analysis, they converted absolute fitness (i.e. spermatophore attachment time) to relative fitness by dividing the attachment time of each male by the sample mean and standardized all other traits to a mean of zero and standard deviation of one. Separate multiple regressions (Lande and Arnold 1983) were then used to estimate the vectors of linear selection gradients ($\boldsymbol{\beta}$) and the matrix of quadratic and correlational selection gradients ($\boldsymbol{\gamma}$), with the reported quadratic regression coefficients doubled to obtain quadratic selection gradients (Stinchcombe et al. 2008). Finally, a canonical rotation of the $\boldsymbol{\gamma}$ matrix was used to detect the major axes of nonlinear sexual selection (Phillips and Arnold 1989). This analysis produced an **M** matrix with 7 eigenvectors $\mathbf{m}_{1\text{-}7}$ (linear combinations of weighted values of the original traits) that describe the major axes of the nonlinear response surface (see Table 3 in Hall et al. 2008), with the strength of nonlinear sexual selection on $\mathbf{m}_i$ given by the eigenvalue λ_i.

To compare the multivariate attractiveness of the courtship call between inbred and outbred males we calculated a score for the two eigenvectors with the strongest nonlinear selection ($\mathbf{m}_6$ and $\mathbf{m}_7$) for each male (Table 3: female only treatment in Hall et al. 2008). Specifically, all call traits were first standardised, multiplied by the respective trait loadings of the eigenvectors and then summed in order to calculate an individual's multivariate attractiveness. We then compared **m** scores between inbred and outbred males using linear mixed models with inbreeding as a fixed factor, and block and the block by inbreeding interaction as random factors.

RESULTS

Courtship call parameters

In total we recorded and successfully analysed the calls of 451 males (199 inbred and 252 outbred). All six call parameters were repeatable (Table 1). Inbreeding had no effect on CTI, T1L or CPN (Table 2 and 3). Inspection of the confidence intervals from the meta-analyses showed that inbreeding significantly increased courtship call DF (Hedge's d = -0.198; 95% CI: -0.374 to -0.030). There was also a significant increase in the CIPD of the calls of inbred males (Hedge's d = -0.178; 95% CI: -0.333 to -0.026), but there was no effect of inbreeding on TN (Hedge's d = -0.089; 95% CI: -0.295 to 0.0740, for all meta-analyses N = 26 blocks) (see Table 2).

Several of the covariates we examined affected call parameters (Tables 3 and 4). Males with a slower development time had a significantly higher CPN, older males had significantly shorter CTIs and, at higher temperatures, the CTIs were also shorter. Heavier males had a significantly longer T1L. None of these relationships differed between inbred and outbred males (all interactions $P > 0.05$). In contrast, the relationship between female mass and CIPD differed between inbred and outbred males ($F_{1,377} = 6.981$, $P = 0.009$). For inbred males courtship calls performed for heavier females had significantly longer CIPDs but there was no effect of ambient temperature. While for outbred males, courtship calls performed for heavier females had significantly shorter CIPDs, and at higher temperatures the CIPD was also significantly shorter (Table 3).

The calls of heavier inbred and outbred males had a significantly lower DF. For outbred, but not inbred, males those with a longer development time had a call with a

significantly lower DF, and, at higher temperatures, the DF of the courtship call was also significantly higher. However, none of these correlations differ significantly between inbred and outbred males. Heavier inbred males had a significantly lower TN, while the TN of outbred males was significantly lower at higher temperatures. Finally, outbred male courtship calls directed at heavier females had a marginally non-significant increase in the number of trills ($P = 0.051$). The correlations between trill number and male mass, female mass and temperature were significantly different for inbred and outbred males (Table 4).

Maturation weight, development time and nymph survival

Inbreeding significantly reduced nymph survival ($t_{28} = 2.98$, $P = 0.006$; outbred survival: 71.9 ± 2.0%, inbred survival: 64.5 ± 2.1%, δ = 10.3%, Hedges $d = 0.642 \pm 0.269$). Despite the loss of over 30% of nymphs, we still recorded life history traits for 1584 females and 1620 males. There was a marginally non-significant trend for inbred crickets to have a lower maturation weight ($F_{1,28} = 3.773$, $P = 0.062$, inbred females: 564.7 ± 5.4 mg, $N = 732$, outbred females: 591.9 ± 5.2 mg, $N = 852$, δ = 4.6%, Hedges $d = 0.182 \pm 0.051$; inbred males: 611.9 ± 5.8 mg, $N = 764$, outbred males: 634.6 ± 5.4 mg, $N = 856$, δ = 3.6%, Hedges $d = 0.143 \pm 0.050$). Males were significantly heavier at maturation than females ($F_{1,3141} = 75.837$, $P < 0.001$).

Inbreeding had no effect on male or female development time (male: $Z = 1.37$, $P = 0.170$, $N = 29$ blocks, inbred males: 92.7 ± 0.7 days, $N = 767$, outbred males: 90.1 ± 0.7 days, $N = 867$, δ = -2.9%, Hedges $d = -0.130 \pm 0.050$; female: $Z = 0.44$, $P = 0.658$, $N = 29$ blocks, inbred females: 87.3 ± 0.8 days, $N = 736$, outbred females: 86.3 ± 0.7 days, N

= 868, δ = -1.2%, Hedges d = -0.051 ± 0.050). Males took significantly longer to mature than females (Z = 3.51, P < 0.001, N = 33 blocks) (all Wilcoxon's tests).

Selection analysis and comparing multivariate attractiveness

The position of inbred and outbred males along the two strongest vectors of selection did not differ significantly ($\mathbf{m}_6$: $F_{1,27}$ = 0.105, P = 0.749, Hedges d = -0.061 ± 0.095, $\mathbf{m}_7$: $F_{1,27}$ = 0.201, P = 0.658, Hedges d = 0.039 ± 0.095). There is therefore no evidence that inbred and outbred males differ in the multivariate attractiveness of their courtship calls and their ability to induce a female to retain their spermatophore for a longer period to increase the total amount of sperm transferred.

DISCUSSION

General evidence for inbreeding depression

One generation of full sibling mating in the cricket *Teleogryllus commodus* significantly reduced nymph survival (δ: 10.3%). There was a marginally non-significant trend for inbreeding to lower maturation weight (δ: 3.6 to 4.6%), but there was no detectable effect on development time (δ: -1.2 to -2.9%). Traits that are closely linked to fitness are expected to show higher levels of inbreeding depression than traits that are less closely related to fitness. Inbreeding depression is due to the effects of directional dominance, and does not occur in traits with a purely additive genetic basis (Charlesworth and Charlesworth 1987; Lynch and Walsh 1998). Traits that are more closely related to fitness are expected to be under stronger selection which depletes additive genetic variation (Mousseau and Roff 1987; DeRose and Roff 1999; Blows and Hoffmann

2005; Hunt et al. 2007) and because mutations affecting such traits are typically deleterious and recessive, traits closely tied with fitness are expected to show higher levels of directional dominance, and therefore inbreeding depression (see Tomkins et al. 2010). In contrast, traits that are less closely related to fitness and therefore under weaker selection show substantial levels of additive genetic variance and less directional dominance, and therefore tend to exhibit lower levels of inbreeding depression (Lynch and Walsh 1998; Roff 1998; DeRose and Roff 1999; Wright et al. 2008). In accordance with these predictions, we found strong inbreeding depression in nymphal survival, a key fitness trait. Weight may also be closely linked to fitness because female fecundity generally increases with body size in insects (Honek 1993), and male fighting success is strongly affected by size in *T. commodus* (Shackleton et al. 2005). Inbreeding depression on weight was, however, marginally non-significant. We found no effect of inbreeding on development time, a result that is consistent with a previous study of *T. commodus* (Drayton et al. 2007) and with the finding of low inbreeding depression on development time in the cricket *Gryllus firmus* (Roff 1998). This indicates little directional dominance for development time. It is not known how strongly development time affects fitness. More generally, our results agree with those of two previous studies in *T. commodus* showing widespread inbreeding depression on major fitness traits. Drayton et al. (2007) found that inbreeding (full-sibling mating, F = 0.25) led to lower egg hatching success (δ: 11.9%), reduced nymph survival (δ: 33.2%) and a shorter adult lifespan (δ: 13.4 to 31.5%). Drayton et al. (2010) found that inbreeding (F = 0.25) significantly reduced male advertisement call rate (δ: 30.2%), which is a strong predictor of male mating success (Bentsen et al. 2006). Taken together these results provide clear evidence that inbreeding reduces fitness in this species.

Courtship call structure and male attractiveness

A small but growing number of studies are now using inbreeding as an experimental method to manipulate male quality (e.g. Bolund et al. 2010; Drayton et al. 2010 and references therein). Given the well established effects of inbreeding on fitness (reviews: Keller and Waller 2002; Armbruster and Reed 2005), inbred individuals can be unambiguously assigned as low phenotypic quality relative to outbred individuals. If male sexually selected traits reflect aspects of quality that are reduced by inbreeding such as condition and/or heterozygosity then sexual traits should decline with inbreeding. Accordingly, several studies have documented strong inbreeding depression in sexually selected male traits: studies of guppies *Poecilia reticulata* (Sheridan and Pomiankowski 1997; van Oosterhout et al. 2003; Mariette et al. 2006), least killfish, *Heterandria formosa* (Ala-Honkola et al. 2009), mice *Mus musculus* (Ilmonen et al. 2009), *Drosophila* (Sharp 1984; Miller et al. 1993; Aspi 2000), butterflies *Bicyclus anynana* (Joron and Brakefield 2003) and even of *T. commodus* itself (Drayton et al. 2010) have all shown that inbreeding reduces traits known to attract and induce females to mate such as sexual colouration and displays (i.e. traits under pre-copulatory sexual selection), ultimately reducing male mating success. Traits subject to post-copulatory sexual selection (e.g. sperm quality and quantity) can also show substantial inbreeding depression (e.g. Roldan et al. 1998; Margulis and Walsh 2002; Konior et al. 2005; Gage et al. 2006; Fitzpatrick and Evans 2009; Zajitschek et al. 2009). In general, these studies suggest that a comparison of inbred and outbred males is a useful method to determine whether or not pre- or post-copulatory sexually selected traits are good indicators of male quality and/or genome wide heterozygosity.

It is plausible that the courtship call might signal male quality in *T. commodus*. Producing the courtship call in the cricket *Acheta domesticus* is twice as energetically

expensive as producing advertisement calls (Hack 1998). There is also evidence from the congeneric *T. oceanicus* that males produce structurally different courtship calls after suffering an immune challenge (Tregenza et al. 2006), suggesting that the courtship call can signal male fitness. Furthermore, courtship call structure influences post-copulatory female choice in *T. commodus* (Hall et al. 2008). We therefore predicted that if the courtship call signalled aspects of male quality that are reduced by inbreeding, such as condition, (and ultimately net fitness) there should be significant changes in the structure and attractiveness of the courtship call with inbreeding. We found that that one generation of full sibling mating increased both the dominant frequency (DF, δ: -0.8%) and the chirp inter-pulse duration (CIPD, δ: -2.4%) of the courtship call. There was no effect on any other call parameter (δ: -2.1 to 2.0%). The effect of inbreeding on DF may have been mediated, in part, by the tendency for inbred males to be smaller, because both larger inbred and outbred males had calls with a lower DF. The increased CIPD of inbred males could indicate a slight reduction in male condition with inbreeding. Each pulse of the call and the silent interval that precedes it is produced by one cycle of wing closure (the pulse) and wing opening (the interval) (collectively termed one wing stroke). The duration of the CIPD therefore corresponds to the time taken for a male to reopen his wings after producing one pulse of sound (Prestwich and Walker 1981; Kavanagh 1987; Simmons et al. 2005). The increase in the CIPD means that inbred males were taking slightly longer to reopen their wings after producing sound pulses then outbred males. Because the energetic cost of calling increases as the wing stroke rate increases (Prestwich and Walker 1981), this suggests that inbred males may have slightly less energy reserves to allocate to calling. Despite finding significant, albeit small, changes in two structural components of the call, we found no evidence that inbreeding reduces the post-copulatory multivariate attractiveness of the courtship call.

There are several scenarios that could explain the lack of substantial inbreeding depression in the courtship call and resulting multivariate attractiveness. First, the courtship call is not a reliable signal of male quality or condition in *T. commodus*. The courtship call does not appear to reflect male condition in the cricket *Gryllus texensis* as it was unaffected by dietary treatment, and was unrelated to two measures of condition (residual mass and fat reserve) (Gray and Eckhardt 2001). Likewise, female crickets *G. lineaticeps* prefer courtship calls with a higher chirp rate but this trait was not affected by nutrition (Wagner and Reiser 2000). These studies and our data suggest that, at least for three cricket species, the courtship call, despite its potential energetic cost (Hack 1998), is a poor indicator of male condition. Interestingly, work on the closely related species *T. oceanicus* suggests that the courtship call is not an obligatory requirement to induce females to accept a spermatophore. On the Hawaiian island of Kauai, a recent morphological mutation ('flatwing') has rendered males silent because they lack sound-producing wing structures. This mutation confers a selective advantage as it protects silent males against acoustically orientating parasitoid flies (Zuk et al. 2006). Females must still mate with silent males or the mutation would have been eliminated by selection. Comparison of courtship behaviour between the present population (where the flat wing mutation occurs) and the ancestral population (collected before the mutation arose; therefore, there are no flatwing males in the ancestral population) shows that in both populations approximately 55% of females mounted males in the absence of the courtship call. Consequently, females in the ancestral population were willing to mount silent males. However, the tendency for females to mount males was increased by broadcasting a courtship call when a silent male courted a female (Bailey et al. 2008). Likewise, deafened female *T. commodus* will still mount males, but only after the courting male approaches and makes antennal contact with the female (Loher and

Rence 1978). These studies suggest that in both *T. commodus* and *T. oceanicus*, while the courtship call increases the tendency of females to mount males, it is not an absolute requirement for mating. Selection on the courtship call as an indicator of male quality might therefore be weak. In this case, the courtship call may be a sexual signal that provides unreliable information to females but is maintained because it is cheap to produce (although potentially energetically costly it is only produced for a few minutes) and there is still a weak vestigial female preference for it (Moller and Pomiankowski 1993).

Second, it is possible that the courtship call might show stronger inbreeding depression in response to more severe inbreeding (i.e. $F > 0.25$, see Zajitschek et al. 2009). The available evidence suggests, however, that the courtship call of *T. commodus* does not signal male condition or genome-wide heterozygosity. Even if more severe inbreeding does change courtship call structure or attractiveness, the fact that inbreeding at the same level as the current study had a strong effect on advertisement call rate (δ: 30.2%; Drayton et al. 2010) implies that the courtship call is a far less accurate indicator of traits that are strongly correlated with fitness and are affected by inbreeding. This is further supported by the fact that the males used by Drayton et al. (2010) (where a 30.2% reduction in calling effort was found) are the same individuals used in this study to investigate inbreeding depression on courtship calling. Third, the courtship call might provide information to females about male traits that are not affected by inbreeding (i.e. traits with little directional dominance), in which case, we should not expect changes in the courtship call with inbreeding. If this is the case, then the advertisement call (which shows strong inbreeding depression, Drayton et al. 2007; 2010) and the courtship call must signal different aspects of male quality to females (e.g. Moller and Pomiankowski 1993). Consistent with this notion, females use the courtship call in post-copulatory

mate choice because it affects the timing of spermatophore removal by females (e.g. Hall et al. 2008), and therefore the number of sperm transferred (Bussiere et al. 2006; Hall et al. 2010). Fourth, it is important to note that our measure of multivariate attractiveness was of post-copulatory attractiveness (i.e. spermatophore retention time, Hall et al. 2008). It is likely that the courtship call of *T. commodus* is also under pre-copulatory sexual selection and will influence whether or not a female will mount a male (e.g. Wagner and Reiser 2000; Tregenza et al. 2006). It is possible that inbreeding will affect measures of pre-copulatory courtship call attractiveness (e.g. latency to mounting). This is an area for future study. Finally, by using the previous selection analysis to predict the multivariate attractiveness of males, we have assumed that the fitness surface described by Hall et al. (2008) is stable across time. Although we worked on the same population of *T. commodus*, under almost identical laboratory conditions, our study was done approximately two years later than Hall et al. (2008). We therefore can not rule out the possibility that we found no difference in multivariate attractiveness because the fitness surface has shifted over time.

On a more general note, it is worth noting that a female may not necessarily produce lower quality offspring after mating with an inbred male. An inbred male cannot pass on his inbred status to his offspring, because diploid sexual organisms inherit genes, not genotypes. Genes inherited from each parent combine to produce new offspring genotypes (Falconer and Mackay 1996; Szulkin and Sheldon 2006), and if an inbred male mates with an unrelated, genetically dissimilar female, then the resulting offspring should be heterozygous and have higher fitness relative to their father. In this sense, the negative effects of inbreeding will be wiped clean, at least genetically, in the offspring of an inbred male (Szulkin and Sheldon 2006). Consequently, an inbred male will not necessarily be a lower quality sire relative to an outbred male because the offspring of

an inbred male will not necessarily have reduced fitness (provided that the female is unrelated to the inbred male). In this case, there may be no reason to expect that a sexual trait will reveal the poor genetic quality, in relation to inbreeding, of an inbred male. If however, due to population structuring, a female is more likely to be related to an inbred male compared to an outbred male, she will benefit by mating with the outbred male, thereby producing outbred, heterozygous offspring (e.g. Reid et al. 2006). Females can detect such outbred males if male sexual traits are correlated to levels of genome wide heterozygosity (Kempenaers 2007; Fromhage et al. 2009). In any case, if sexually selected traits reveal information about male characteristics that could confer direct or indirect benefits to females (e.g. sperm quality, nuptial feeding, condition or heterozygosity) and if inbreeding adversely affects such characteristics, then sexual traits should show declines with inbreeding. In this sense, inbreeding can be viewed as a way of experimentally reducing fitness to then determine if such reductions in fitness are mirrored in sexual traits (see Bolund et al. 2010; Drayton et al. 2010). Whether or not the offspring of inbred males have reduced fitness is an area for future study.

In conclusion, inbreeding had only a small effect on the structure of the courtship call and did not change post-copulatory multivariate attractiveness. It is therefore unlikely that the courtship call signals aspects of male quality that are reduced by inbreeding such as condition or heterozygosity. However, the courtship call influences female post-copulatory choice in this species (Hall et al. 2008) and consequently, it is possible that the courtship call signals information to females about other male properties that are not affected by inbreeding.

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Table 1. The repeatability of call parameters among males ($N = 451$ males).

Call parameter	Test Statistic	*P*-value	r_I
chirp inter-pulse duration (CIPD)	$F_{450, 1712} = 5.309^{*}$	<0.001	0.683
chirp-trill interval (CTI)	$F_{450, 1712} = 2.974^{*}$	<0.001	0.497
trill 1 length (T1L)	$F_{450, 1712} = 5.783^{*}$	<0.001	0.705
chirp pulse number (CPN)	$F_{450, 1712} = 7.329^{*}$	<0.001	0.760
dominant frequency (DF)	$\chi^2_{450} = 1823.283^{\dagger}$	<0.001	0.840
trill number (TN)	$\chi^2_{450} = 1376.254^{\dagger}$	<0.001	0.758

*One-way ANOVA, †Kruskall-Wallis test.

Table 2. Mean ± SE for inbred and outbred males, δ (% change with inbreeding) and Hedge's *d* for the six courtship call parameters. Bold values of δ and *d* indicate a significant difference between inbred and outbred males.

	Mean ± SE			
Call parameter	Inbred ($N = 199$)	Outbred ($N = 252$)	δ	Hedges d ± SE
CIPD (ms)	29.3 ± 0.5	28.6 ± 0.4	**-2.4**	**-0.097 ± 0.095**
CTI (ms)	146 ± 5.0	143 ± 3.5	-2.1	-0.056 ± 0.095
T1L (s)	2.723 ± 0.092	2.668 ± 0.081	-2.1	-0.042 ± 0.095
CPN	9.302 ± 0.145	9.495 ± 0.115	2.0	0.100 ± 0.095
DF (Hz)	3978.55 ± 9.32	3947.48 ± 7.84	**-0.8**	**-0.243 ± 0.095**
TN	3.084 ± 0.122	3.078 ± 0.106	-0.2	-0.004 ± 0.095

Table 3.The effect of inbreeding and five covariates on four courtship call parameters: CIPD, CTI, T1L and CPN. *P*-values are from the final model if the term was significant, or when it alone was added to the final model if non-significant. *P*-values significant at the 0.05 level are in bold.

	CIPD						CTI			T1L			CPN		
	Inbred			Outbred											
	d.f.	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*
Inbreeding	-	-	-	-	-	-	1,27	0.01	0.907	1,27	0.07	0.797	1,27	2.64	0.116
Development time	1,164	1.78	0.184	1,207	1.67	0.198	1,378	3.19	0.075	1,353	1.88	0.172	1,390	6.61	**0.011**
Male age	1.164	0.36	0.551	1,207	0.55	0.460	1,379	6.81	**0.009**	1,353	0.35	0.555	1,389	0.11	0.745
Male mass	1,150	2.09	0.151	1,185	0.22	0.639	1,343	0.21	0.647	1,354	6.77	**0.010**	1,353	3.59	0.059
Female mass	1,165	4.36	**0.038**	1,208	4.51	**0.035**	1,367	0.34	0.563	1,341	0.12	0.725	1,377	0.02	0.894
Temperature	1,161	0.09	0.761	1,208	5.92	**0.016**	1,379	11.42	**0.001**	1,347	0.02	0.897	1,383	1.11	0.293

Table 4. Correlations between dominant frequency (DF) or trill number (TN), and development time, male age, male and female mass, and temperature. *Z* and *P*-values are for the comparison of r_s between inbred and outbred males. *P*-values significant at the 0.05 level are in bold.

	DF				TN			
	r_s inbred (*P*,*N*)	r_s outbred (*P*,*N*)	*Z*	*P*	r_s inbred (*P*,*N*)	r_s outbred (*P*,*N*)	*Z*	*P*
Development time	0.004 (0.951, 199)	-0.129 (**0.041**, 252)	1.400	0.162	-0.031 (0.660, 199)	-0.030 (0.635, 252)	0.010	0.161
Male age	-0.110 (0.123, 199)	0.021 (0.739, 252)	1.377	0.169	-0.073 (0.303, 199)	-0.076 (0.231, 252)	0.032	0.976
Male mass	-0.319 (**<0.001**, 185)	-0.196 (**0.003**, 230)	1.326	0.185	-0.276 (**<0.001**, 185)	-0.014 (0.830, 230)	2.707	**0.007**
Female mass	-0.121 (0.092, 194)	-0.061 (0.341, 244)	0.625	0.532	-0.065 (0.369, 194)	0.125 (0.051, 244)	1.969	**0.049**
Temperature	-0.004 (0.956, 196)	0.132 (**0.037**, 249)	1.422	0.155	0.028 (0.697, 196)	-0.181 (**0.004**, 249)	2.195	**0.028**

Block	Matings			
	Inbred 1	Outbred 1	Outbred 2	Inbred 2
1	A♂ A♀	A♂ B♀	B♂ A♀	B♂ B♀
2	C♂ C♀	C♂ D♀	D♂ C♀	D♂ D♀
3...	E♂ E♀	E♂ F♀	F♂ E♀	F♂ F♀
...33	Y♂ Y♀	Y♂ Z♀	Z♂ Y♀	Z♂ Z♀

Figure 1. The mating design showing Block 1 (comprising full sibling families A and B), Block 2 (comprising full sibling families C and D) and so on. There are two inbred and two outbred genotypes generated per block.

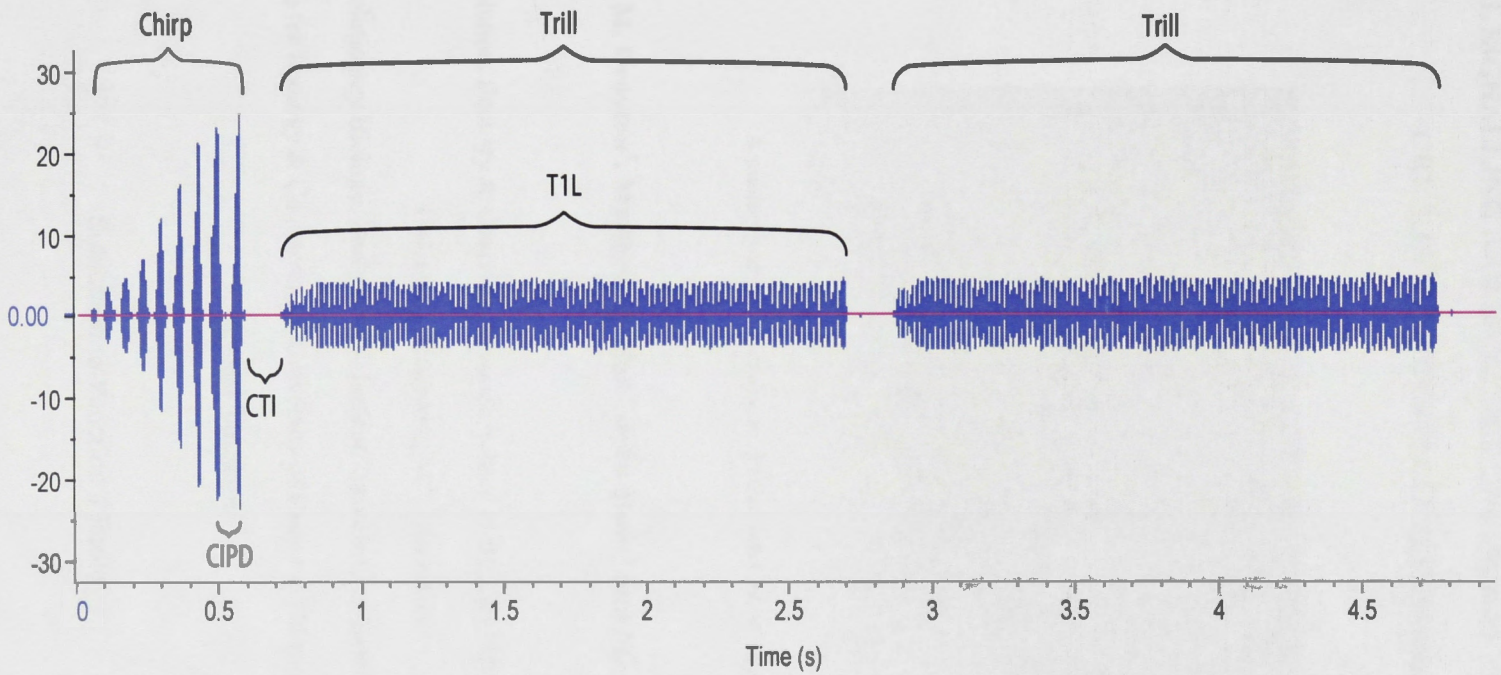
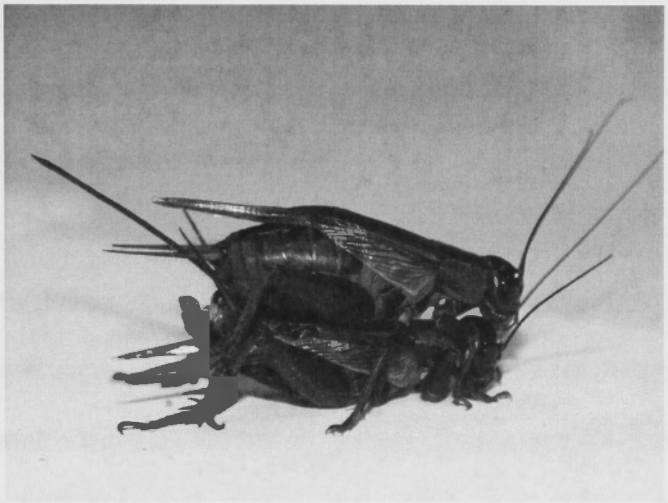


Figure 2. The courtship call of *T. commodus*. Each phrase consists of a single, amplitude-modulated chirp, followed by a variable number of trills. The phrase shown here has two trills. We measured: dominant frequency (DF), chirp pulse number (CPN), chirp inter-pulse duration (CIPD), chirp-trill interval (CTI), trill number (TN) and the length of the first trill (T1L). The vertical axis represents the amplitude of the sound produced.

CHAPTER 5

SEXUAL SIGNALLING AND IMMUNE FUNCTION IN THE BLACK FIELD CRICKET *TELEOGRYLLUS COMMODUS*



A mating pair of *T. commodus*. Photo: Alex McWilliam.

Jean M. Drayton[1], Matthew D. Hall[2], John Hunt[3] and Michael D. Jennions[1]

1. Evolution, Ecology & Genetics, Research School of Biology, The Australian National University, Canberra, ACT, Australia.

2. Evolutionary Biology, Zoologisches Institut, University of Basel, Basel, Switzerland

3. Centre for Ecology & Conservation, University of Exeter in Cornwall, Penryn, TR10 9EZ, United Kingdom.

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SUMMARY

1. The immunocompetence handicap hypothesis suggests that male condition, sexual trait expression and immune function are intimately linked, as only males with sufficient resources (i.e. high condition) can invest in costly sexual display and still maintain a strong immune system. The relationship between sexual signalling and immunity might, however, depend on the type of immune assay used and/or the nature of sexual selection acting on the male sexual trait(s).

2. Here we investigate how two assays of immune function in the field cricket, *Teleogryllus commodus,* are related to the specific components of male sexual signals and to certain multivariate combinations of these components that females most strongly prefer. In *T. commodus*, males produce both advertisement and courtship calls prior to mating. We measured the fine-scale structural parameters of both call types and also recorded nightly advertisement calling effort. We then measured two standard indices of immune function: the lysozyme-like activity of the haemolymph and haemocyte counts.

3. Our results reveal a weak, positive relationship between advertisement calling effort and lysozyme-like activity. There was, however, little evidence that any individual structural call component or the net multivariate attractiveness of either call type signalled immune function. The relationships between immunity and sexual signalling also did not differ between inbred and outbred males.

4. Our data suggest that it is unlikely that females assess overall male immune function using fine-scale features of male calls. There is, however, some evidence that a female mating preference for males that call more often will increase the likelihood of obtaining a mate with greater lysozyme activity. Given that the fine-scale structures of the courtship and advertisement calls of *T. commodus* do influence female choice, it appears that male call structure must signal beneficial aspects of male quality other than the current number of haemocytes or lysozyme activity.

KEY WORDS: Advertisement call, calling effort, courtship call, female choice, haemocytes, lysozyme, multivariate attractiveness

INTRODUCTION

Female mate choice based on male sexual ornaments and courtship displays is well documented, but the function of most mating preferences is poorly understood. Choosiness is selected for if preferred sexual traits signal aspects of male 'quality' that benefit choosy females (review: Kokko et al. 2006). Direct benefits accrue to a choosy female if non-random mating increases her longevity and/or fecundity. In addition, indirect benefits arise if there are paternal effects on a choosy female's progeny due to inheritance of genes (Hunt et al. 2004a) and/or paternal contributions (e.g. increased male care; Kelly and Alonzo 2009) that elevate offspring fitness. The mechanisms that allow sexual traits to function as reliable signals of male quality generally require that they are costly to produce or maintain (review: Kotiato 2001), and that higher quality males pay a smaller marginal cost to increase investment (Zahavi 1975; Grafen 1990; but see Getty 2006; Murai et al. 2009). A specific case study is related to a potential trade-off between sexual trait expression and immune system function, with the assumption that females gain direct or indirect benefits by choosing mates with more effective immune systems. The original hypothesis was formulated for vertebrates under the assumption that testosterone, which is needed to produce male secondary sexual traits, is an immunosuppressant (Folstad and Karter 1992; but see Boonekamp et al. 2008), but other mechanisms could also generate the required trade-off (Westneat and Birkhead 1998).

In insects an immunosuppressive cost of sexual signalling could reflect a resource trade off: sexual displays require resources such as energy or nutrition that might divert resources away from the immune system (Sheldon and Verhulst 1996). Alternatively, immune system activation could reduce investment into sexual signals (vertebrates: Boonekamp et al. 2008;

insects: Kerr et al. 2010). Only males with sufficient resources (i.e. in good condition) can invest in costly sexual display and maintain a strong immune system. If so, the expression of male sexual signals will, given certain assumptions (see Lawniczak et al. 2007), be positively correlated with immunocompetence. Invertebrates are popular subjects for studies of parasite-mediated sexual selection, as their immune systems are simpler than those of vertebrates, and can be assayed using well established protocols. Studies of invertebrates have reported phenotypic correlations between male sexual signals and measures of immune function that are both positive (beetles: Rantala et al. 2002; Pomfret and Knell 2006; crickets: Ryder and Siva-Jothy 2000; Rantala and Kortet 2003; Simmons et al. 2005; damselflies: Rantala et al. 2000; Siva-Jothy 2000; Contreras-Garduno et al. 2006; Gonzalez-Santoyo et al. 2010; spiders: Ahtiainen et al. 2004; scorpionflies: Kurtz 2007) and negative/absent (Rantala et al. 2000; Ryder and Siva-Jothy 2000; Siva-Jothy 2000; Rantala and Kortet 2003; Ahtiainen et al. 2004, 2005; Kurtz 2007; Ketola et al. 2009; Gonzalez-Santoyo et al. 2010; Gonzalez-Tokman and Cordoba-Aguilar 2010). Given these contrasting results, more data is needed to quantify the general relationship and, perhaps more importantly, to identify sources of variation in the direction of the relationship (e.g. does it depend on the immune trait, taxa or whether the sexual trait is actually subject to sexual selection?).

Here we test whether sexual traits act as signals of male immune function in the black field cricket, *Teleogryllus commodus* (Fig. 1). Males produce two acoustic signals prior to mating: an advertisement and then a courtship call. The advertisement call attracts sexually receptive females toward the male. Once a female has approached, the male starts to produce a courtship call to induce her to mate. Female choice exerts strong positive directional sexual selection for greater advertisement calling effort and moderate levels of

stabilizing and directional multivariate sexual selection on finer-scale advertisement call structure (Brooks et al. 2005; Bentsen et al. 2006). Furthermore, dietary manipulation has shown that advertisement calling effort is affected by nutrition (Hunt et al. 2004b; Maklakov et al. 2008). Importantly, inbreeding reduces calling effort (Drayton et al. 2010) and alters fine scale advertisement call structure (Drayton et al. 2007), indicating that both call effort and call structure may signal male fitness (given that inbreeding lowers fitness). It is therefore possible that advertisement calls signal key fitness components such as disease resistance. Indeed, the advertisement call structure of *T. commodus* seems to provide information about aspects of male immune function (Simmons et al. 2005). The fine-scale structure of the courtship call is under post-copulatory sexual selection because its influences how long a female will retain a spermatophore after mating (Hall et al. 2008), which determines the number of sperm transferred (Bussiere et al. 2006; Hall et al. 2010). Again, inbreeding affects courtship call structure (Drayton et al. 2011), indicating that courtship calls might signal fitness, and immune function, to females. There is also evidence from the congeneric *T. oceanicus* that courtship calls signal aspects of immune function (Tregenza et al. 2006), although this does not appear to be the case in another cricket, *Gryllodes sigillatus* (Ketola et al. 2009). Crucially, however, these studies only examined the relationship between individual components of the courtship call and immune function. This is biologically problematic as selection (e.g. that due to female choice) rarely acts on single call traits in isolation (review: Blows 2007). To determine if male calls signal immune function to females during mate choice, it is necessary not only to examine the univariate relationships between call components and immunity, but also to quantify multivariate combinations of call traits that are the direct target of mate choice and see how they are related to immune function.

We investigated whether advertisement or courtship calling signals aspects of male immune function in *T. commodus*. We recorded two measures of male immune function: lysozyme-like activity in the haemolymph and haemocyte counts. Lysozyme is an antibacterial enzyme that hydrolyses bacterial cell walls. Its activity is assayed by measuring the rate at which an individual's haemolymph clears a bacterial suspension (e.g. Rantala and Roff 2005). Haemocytes are cells in the haemolymph, and a subset of these are involved in immune responses by transporting molecules to infection sites, by ingesting foreign particles, or by encapsulating parasites (Korner and Schmid-Hempel 2004; Lawniczak et al. 2007). We first tested for relationships between 11 fine-scale characteristics of advertisement or courtship calls and male immune function. The five advertisement call traits that we measured were previously used in a selection analysis of *T. commodus* to quantify multivariate sexual selection due to female mate choice (Brooks et al. 2005). Similarly, the six courtship call traits were also previously used to quantify multivariate sexual selection arising from cryptic female choice for spermatophore retention time (i.e. male insemination success) (Hall et al. 2008). We utilized these selection analyses in conjunction with the measured call traits to estimate the multivariate attractiveness of each male's calls. We then tested whether attractiveness signals male immune function. We also tested for any relationship between the time spent advertisement calling each night (hereafter 'calling effort') and immunity. Males who call more often are more attractive to females (Bentsen et al. 2006). Finally, we have already shown that inbreeding between full siblings affects both immune function (Drayton and Jennions 2011) and male calling (Drayton et al. 2007; 2010; 2011). Inbreeding might therefore decrease the reliability of male calling as a signal of immune function by, for example, disrupting the usual proximate mechanism that determines the appropriate allocation of resources to sexual signals and immune function. Consequently, we tested whether the relationship between calling and

immunity differed between inbred ($F = 0.25$) and outbred males. In sum, we asked three key questions:

1) Do fine-scale structural features of courtship and/or advertisement calls signal male immune function (lysozyme activity and/or haemocyte count)?

2) Do the combinations of male call characteristics that most strongly influence female choice (i.e. the multivariate attractiveness of each call type) and/or total calling effort act as signals of male immune function?

3) Does inbreeding weaken the relationship between call traits and immunity? If not, the previous two questions can be addressed with a pooled dataset.

MATERIALS AND METHODS

GENERAL BREEDING PROTOCOLS

We collected and bred from ~70 wild caught gravid females in May 2006, at Smith's Lake, New South Wales, Australia. Their offspring were reared to adulthood and we then randomly paired a male and female (both virgins), ensuring that they did not have the same wild caught mother. Each of the resultant full-sibling families were reared in a separate 43x30x13 cm plastic tub with dry cat food (KiteKat Krunch, Uncle Ben's, Raglan, Australia) and water provided *ad libitum*. Crickets were maintained at 26-28°C on a 12:12 photoperiod.

The full-sibling families were grouped into pairs, hereafter known as blocks ($N = 33$ blocks). The two families in each block were always unrelated. In each block, brothers and sisters from both full sibling families were mated to create two inbred genotypes ($F = 0.25$). Outbred genotypes ($F \approx 0$, given panmixis in the wild population) were created by reciprocal matings between families in the block. For example, in a block consisting of full sibling families A and B, inbred progeny were generated by mating brothers and sisters from family A, and brothers and sisters from family B, while outbred progeny were generated by mating an A family male with a B family female, and a B family male with an A family female (Fig. 2). This design generated four offspring genotypes per block (two inbred; two outbred). After mating females were provided with moist cotton wool ("egg pads") for egg laying. Egg pads were checked every three days for emerging nymphs. Upon emerging, nymphs from the same pairing were reared communally for 20 days in 9x9x5cm plastic tubs with food and some moist cotton wool. After 20 days, each nymph was transferred to an individual 9x9x5cm container with a small tube of water plugged with cotton wool, a piece of cat food and a cardboard egg cup for shelter. Nymphs were not transferred immediately after emerging because they were small enough to be crushed by the water tube. The density of nymphs during the communal stage was similar in inbred and outbred groups. It is unlikely that communal reared nymphs competed for resources because food and water was provided *ad libitum*. Once crickets were set up individually, food and water was replaced every 10 days. All the measured males were >10 days post maturation to ensure that they were sexually mature. We have previously shown using a much larger dataset that male age is not correlated with lysozyme levels or hemocytes counts (Drayton and Jennions 2011).

MEASURING IMMUNE FUNCTION

We used a sterile pin to make a small puncture under the pronotum to collect 2µl of haemolymph with a Gilson pipette, which was added to 8 µl of phosphate-buffered saline (PBS: 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in 1L distilled water, pH 7.4), and frozen to induce cell lysis. The samples were later thawed and 90µl of a *Micrococcus lysodeikticus* solution (3mg/ml PBS) added. We then loaded samples into a microplate spectrophotometer (PowerWave 340, Bio-Tek Instruments Inc.) to measure lysozyme activity at 490nm and 30°C as the rate of change in optical density from 0 to 80 minutes. Several control samples (a PBS and *Micrococcus lysodeikticus* solution) were loaded on each plate to control for variation among assays. The rate of change in optical density for each haemolymph sample was calculated as the sample slope minus the control slope. This was multiplied by -1 so that a greater value indicates higher lysozyme-like activity. We also collected another 2µl of haemolymph from the pronotum puncture and mixed it with 8µl of anticoagulant (3.92g NaOH, 8.532g NaCl, 6.328g EDTA, 8.616g citric acid in 1L distilled water). This mixture was expelled onto a Neubauer haemocytometer and we estimated the number of haemocytes per ml of haemolymph. Lysozyme activity and haemocyte counts are both repeatable (lysozyme: $r_I = 0.74$, $n = 161$; haemocytes: $r_I = 0.52$, $n = 56$, both $P < 0.001$), and are positively correlated across individuals ($r = 0.56$, $P < 0.001$, $n = 675$) (see Drayton and Jennions 2011). Males were weighed before haemolymph extraction and were 23.0 ± 0.10 (± SE) days post-maturation.

THE ADVERTISEMENT CALL

Males call using a stridulatory apparatus consisting of a file and scraper on the forewings. The most basic call unit is a pulse, produced by closing the wings once (Kavanagh 1987).

There are two types of sound pulses in *T. commodus* advertisement calls. The first are longer, more intense and grouped into chirps. The second are shorter, softer and grouped into trills. Chirps and trills are arranged into the main repeated unit of the call, a phrase, which is a chirp followed by one to several trills (Bentley and Hoy 1972; Hill et al. 1972) (Fig. 3).

Males were set up in individual, acoustically isolated 9x9x5cm recording chambers with a condenser microphone mounted in the lid that was powered by a 9 volt battery. We recorded calls with a digital recorder (MicroTrack 24/96, M-Audio, Irwindale, CA, USA). Chambers were checked in the evenings for calling males. If a male was heard calling, we recorded him for 1-2 minutes, and he was then weighed. Recorded males were 23.0 ± 0.5 days post maturation. Advertisement calls were recorded opportunistically, and males can not be made to call on demand. So, despite intensive efforts, our sample sizes for advertisement call structure are lower than those for calling effort and courtship call structure.

Calls were analyzed using Raven Pro 1.3 (Cornell Lab of Ornithology, www.birds.cornell.edu/raven). We measured five parameters of five randomly chosen phrases from each male: dominant frequency (DF), the interval between the last trill pulse of the selected phrase and the first chirp pulse of the next [inter-call duration (ICD)], the number of pulses per chirp [chirp pulse number (CPN)], the duration of the interval between the last two pulses in the chirp [chirp inter-pulse duration (CIPD)] and the number of trills in a phrase [trill number (TN)] (Fig. 3). Calls were filtered before analysis to remove ambient noise below 3kHz and above 6kHz. Call parameters measured in this way are highly repeatable (Drayton et al. 2010). We therefore calculated a mean value of DF,

ICD, CPN, CIPD and TN for each male. We measured advertisement call structure and lysozyme-like activity for 50 males, and call structure and haemocyte counts for 55 males.

THE COURTSHIP CALL

The courtship call also consists of pulses that are grouped into chirps and trills that are arranged into repeated phrases. Each phrase consists of a single, amplitude-modulated chirp, followed by several trills that are consistent in intensity (Hall et al. 2008) (Fig. 4). Males were set up in individual 9x9x5cm recording chambers with a condenser microphone mounted in the lid. To induce a male to call, a randomly chosen virgin stock female was introduced into the chamber. We recorded 1-2 minutes of courtship calling using the MicroTrack recorder (see above). The male and female were then weighed. Males were 20.8 ± 0.2 days post maturation. Using the same protocol described for advertisement calls we measured six call parameters from five randomly chosen phrases per male: dominant frequency (DF), chirp pulse number (CPN), the chirp inter-pulse duration (CIPD), the duration of the interval between the chirp and the first trill [chirp-trill interval (CTI)], trill number (TN) and the length of the first trill [trill 1 length (T1L)] (Fig. 4). Again, these call parameters are highly repeatable (Drayton et al. 2011), so we calculated a mean value for each parameter per male. We measured courtship call structure and lysozyme-like activity for 204 males, and courtship call structure and haemocyte counts for 197 males.

CALLING EFFORT

Male nightly calling effort was measured using a custom-built electronic monitoring device (see Drayton et al. 2010). In brief, the device consists of 128 recording chambers

(9x9x5cm) with a condenser microphone mounted in the lid, each housing one male. The device samples the chambers throughout the night. During sampling, the microphone of one chamber is activated. If the sound level is >10 dB above the background noise a 1 representing that the male is calling is recorded, otherwise, a 0 is recorded. The microphone is then de-activated and the microphone in the next chamber is activated. Each recording chamber is sampled 10 times per second. If a male was recorded as calling for any of the 10 sampling events/second, he was recorded as calling for that second. Nightly calling effort was defined as the total number of seconds called per night. Calling effort was measured for 10 hours/night (19h00- 05h00) at 26-28°C. Males were weighed before they were put into a chamber. Males were 14.4 ± 0.02 days post maturation, and calling effort was recorded for 3.2 ± 0.01 nights. We have previously shown that calling effort is repeatable (Drayton et al. 2010). We therefore calculated mean nightly calling effort for each male. We measured calling effort and lysozyme-like activity for 511 males, and calling effort and haemocyte counts for 408 males.

ESTIMATING MULTIVARIATE CALL ATTRACTIVENESS

Both the courtship (Hall et al. 2008) and advertisement (Brooks et al. 2005) call of *T. commodus* are subject to strong sexual selection due to female choice in our study population. Hall et al. (2008) showed that the time until an unguarded female removes a male's spermatophore (thus terminating sperm transfer) can be predicted based on the structure of his courtship call. The strongest vector of sexual selection on male courtship call structure, $\mathbf{m}_7$, is characterized by stabilizing selection (Fig. 3 in Hall et al. 2008) and is described by the equation:

$$\mathbf{m_7} = (0.310 \times \text{CPN}) + (0.447 \times \text{CIPD}) + (0.103 \times \text{CTI}) + (-0.544 \times \text{TIL}) + (-0.618 \times \text{TN}) + (-0.124 \times \text{DF}) + (-0.010 \times \text{BS})$$

(Eq. 1)

Thus by substituting our courtship call parameters and body size (BS) into this equation, we can derive an $\mathbf{m_7}$ score for each male in our study. Furthermore, as sexual selection along this vector is stabilizing, we can solve for $Y = ax^2 + bx + c$, where $a = -0.860$ (λ in Table 3 of Hall et al. 2008), $b = 0.027$ ($\boldsymbol{\theta}$, linear selection along this vector) and $c = 1.122$ (the intercept from the quadratic regression of $\mathbf{m_7}$ on relative fitness). For courtship calls, Y is our best estimate of a male's multivariate post-copulatory attractiveness.

Likewise, Brooks et al. (2005) showed in laboratory-based binary choice trials that females prefer the structure of certain advertisement calls. The strongest vector of sexual selection on the advertisement call, $\mathbf{m_5}$, is subject to both linear and stabilizing selection, as convex selection is asymmetrical over the range of call structure components tested (Fig. 2 in Brooks et al. 2005). This relationship is described by the equation:

$$\mathbf{m_5} = (0.068 \times \text{CPN}) + (0.244 \times \text{CIPD}) + (-0.543 \times \text{TN}) + (0.758 \times \text{ICD}) + (0.258 \times \text{DF}) \quad \text{(Eq. 2)}$$

Again, we can substitute our advertisement call parameters into this equation to obtain an $\mathbf{m_5}$ score for each male and solve for $Y = ax^2 + bx + c$ to estimate each male's multivariate pre-copulatory attractiveness. Here, $a = -0.119$, $b = -0.160$ (λ and $\boldsymbol{\theta}$ respectively in Table 3 of Brooks et al. 2005) and $c = 1.038$. Our approach to construct a multivariate measure of attractiveness is similar to that of Jang and Greenfield (2000).

STATISTICS

To characterize the relationship between immunity and male sexual signals we ran multiple regressions with call properties or male attractiveness as continuous predictor variables and inbreeding treatment as a categorical variable. Before analysis we transformed average calling effort, trill number (TN) and inter-call duration (ICD) using natural logarithms, and lysozyme activity, haemocyte counts, chirp to trill interval (CTI) and first trill length (T1L) using square root transformations. We scaled our immune measures to relative estimates by dividing by the transformed mean, and standardized all other traits (mean = 0, SD = 1). The use of relative immune estimates and standardized predictor traits makes it easier to directly interpret differences between regression coefficients.

We then used partial *F*-tests to compare the fit of more complex regression models to reduced alternatives (Chenoweth and Blows 2005). We evaluated the potential for linear relationships between immune measures and the predictor traits of interest (linear compared to intercept only model) and, based on the addition of linear by inbreeding treatment interaction terms, we tested if the relationships differed between inbred and outbred males (interaction compared to linear only model). All analyses were originally performed with block as a random factor. However, as block had no effect on the regression coefficients, we report here the simplified analyses that do not include this term.

Finally, given that inbreeding did not affect the relationship between male calling and immunity (see Results), we did not conduct separate analyses for inbred and outbred males when answering questions 1 and 2. The inbreeding term was, however, always retained in the final models because we have previously shown that inbreeding can affect immune

parameters (see Drayton and Jennions (2011) for results and *P*-values for the effect of inbreeding on immune function using a larger dataset that includes non-calling males).

All analyses were conducted in JMP (Version 8.2, SAS, NC, USA). All test are two-tailed at $\alpha = 0.05$.

RESULTS

1. DOES FINE-SCALE CALL STRUCTURE SIGNAL MALE IMMUNE FUNCTION?

There was little evidence for a relationship between specific call structure characteristics and either immune measure. Male courtship call characteristics were only weakly associated with either immune measure. For lysozyme activity there was no evidence for a linear relationship with the six courtship call characteristics ($F_{6, 196}$=1.085, P=0.372), while for haemocyte counts, there was a slight trend towards a linear association with the six courtship call characteristics ($F_{6, 189}$=2.059, P=0.060). Only trill number (TN) was associated with a decrease in lysozyme activity, while haemocyte count was positively associated with chirp pulse number (CPN) (Table 1). For the advertisement call, there was little evidence for a linear relationship between the five call characteristics and either lysozyme activity ($F_{5, 43}$=0.544, P=0.742) or haemocyte count ($F_{5, 48}$=1.323, P=0.270). Only dominant frequency (DF) was positively related to an increase in haemocyte count (Table 2).

2. DOES CALL MULTIVARIATE ATTRACTIVENESS OR CALLING EFFORT SIGNAL IMMUNE FUNCTION?

The above analyses indicate that it is unlikely that any single characteristic of courtship or advertisement calls can be used by females to assess a male's immune status. Female choice in *T. commodus* is, however, based on the composite effect of a suite of male sexual traits that determine his attractiveness. We therefore need to test for a relationship between the net value of the combination of male sexual traits that females most strongly prefer and his immune status. The multivariate attractiveness of the courtship call predicted neither lysozyme activity ($F_{1, 202}$=1.394, P=0.239) nor haemocyte count ($F_{1, 195}$=1.848, P=0.176). The same was true for the advertisement call (lysozyme: $F_{1, 48}$=0.021, P=0.887; haemocyte count: $F_{1, 53}$=0.132, P=0.718). Calling effort was not significantly related to haemocyte count ($F_{1, 405}$=1.432, P=0.232). There was, however, a significant positive relationship between calling effort and lysozyme activity ($F_{1, 508}$=5.957, P=0.015) (Table 3).

3. DOES INBREEDING WEAKEN THE RELATIONSHIP BETWEEN CALLING AND IMMUNITY?

Male inbreeding status did not change the relationships between either call type and lysozyme activity or haemocyte counts. For the courtship call, there was no difference between inbred and outbred males in how the six call characteristics were related to each immune assay (lysozyme: $F_{6, 190}$=0.901, P=0.495; haemocyte: $F_{6, 183}$=0.418, P=0.866). The same was true for the five call characteristics of the advertisement call (lysozyme: $F_{5, 38}$=0.319, P=0.899; haemocyte: $F_{5, 43}$=0.581, P=0.714). Similarly, inbreeding did not change the relationships between the multivariate attractiveness of courtship or advertisement calls and either immune measure (courtship: lysozyme: $F_{1, 200}$=0.453, P=0.502; haemocyte: $F_{1, 193}$=0.142, P=0.707; advertisement: lysozyme: $F_{1, 46}$=0.495,

P=0.485; haemocyte: $F_{1, 51}$=0.507, P=0.480). Finally, there was no effect of inbreeding on the relationship between male nightly calling effort and lysozyme activity ($F_{1, 507}$=0.071, P=0.791) or haemocyte count ($F_{1, 404}$=2.249, P=0.116).

DISCUSSION

The immunocompetence handicap hypothesis (Folstad and Karter 1992) implies that the expression of sexually selected ornaments/displays by males will be positively correlated with their ability to combat diseases by maintaining a high functioning immune system. A trait that signals immunocompetence is a potentially important reason for females to engage in mate choice, because parasites and disease are major sources of natural selection (e.g. Møller et al. 2009). Mating with males with greater resistance could have direct benefits (i.e. lower the risk of disease or parasite transfer while mating) or indirect benefits if disease resistance is heritable. Indirect benefits will be gained if the offspring of more attractive males are of above average immunocompetence (e.g. Simmons and Roberts 2005; but see Simmons et al. 2010), and immunocompetence is positively correlated with net fitness (Kokko et al. 2006). There is some evidence for additive genetic variation in measures of immune function in insects (e.g. Kurtz and Sauer 1999; Simmons and Roberts 2005; Luong and Polak 2007; Gershman et al. 2010; Moreno-Garcia et al. 2010; Simmons et al. 2010). This could be due to genes that directly enhance immune function, or arise indirectly via genes that have a positive effect on body condition (Westneat and Birkhead 1998). There is also recent evidence from an insect that females can use cuticular hydrocarbons to choose males with genotypes that are complementary to their own with respect to enhancing the immunocompetence of their offspring (Ali and Tallamy 2010).

We found that neither courtship nor advertisement call characteristics of male *T. commodus* act as signals of two major components of the immune system. The only aspect of sexual signaling that did provide information about immune function (namely lysozyme activity) was male calling effort. Interestingly, calling effort is known to be under strong sexual selection through female choice (Bentsen et al. 2006). In addition, inbreeding did not decrease the reliability of the relationship between the expression of male sexual signals and immunity. It therefore seems unlikely that females can assess male immune function by paying attention to fine-scale features of calls. There is, however, some evidence that a female mating preference for males that call more often increases the likelihood of obtaining a mate with greater lysozyme activity.

CALLING EFFORT

In *T. commodus* the expression of calling effort, a preferred male trait, is moderately, but significantly, positively related to lysozyme-like activity in the haemolymph. There was, however, no such relationship with haemocyte count, even though haemocyte count is strongly correlated with lysozyme activity ($r = 0.56$, Drayton and Jennions 2011). A potential proximate mechanism generating a positive correlation between calling effort and lysozyme-like activity is that both traits are condition-dependent. In general, calling is energetically costly for crickets (Prestwich and Walker 1981; Kavanagh 1987), and in *T. commodus* advertisement calling effort is dependent on nutrition (Hunt et al. 2004b; Maklakov et al. 2008). Calling effort is also negatively affected by inbreeding, which tends to reduce body condition (Drayton et al. 2010). It is currently unknown whether lysozyme-like activity is affected by diet and/or resource assimilation (i.e. condition) in *T. commodus*. Several studies have, however, shown that maintaining and upregulating the immune

system is nutritionally and energetically demanding for insects (e.g. Feder et al. 1997; Siva-Jothy and Thompson 2002; Freitak et al. 2003; Rantala et al. 2003; Jacot et al. 2005). If the expression of lysozyme-like activity is condition-dependent, there is a trade off between resources allocated to lysozyme activity and calling effort (Sheldon and Verhulst 1996), and consequently a negative relationship might be expected. Variation among males in resource acquisition and assimilation ability (i.e. condition) is, however, typically high (Rowe and Houle 1996; see also Tomkins et al. 2010) and this often results in positive phenotypic relationships between condition-dependent traits at the population level (Box 2 in Reznick et al. 2000) because, for example, only individuals in good condition, can invest in high levels of lysozyme production and maintain a high calling effort. Future studies that both manipulate male condition using experimental diets (see Maklakov et al. 2008, Zajitschek et al. 2009) and measure immune function are therefore a high priority.

CALL STRUCTURE

We found little evidence that fine scale structural components of male calls were related to immune function either when considered individually, or as multivariate trait combinations that are known to influence net male attractiveness to females. In contrast, other studies with crickets have reported that the structure and/or attractiveness of advertisement calls (e.g. Ryder and Siva-Jothy 2000; Simmons et al. 2005; but see Simmons et al. 2010) and courtship calls (e.g. Rantala and Kortet 2003; Tregenza et al. 2006; but see Ketola et al. 2009) signal aspects of immunity (for phenotypic correlations between sexual traits and immune measures in other insects see Lawniczak et al. 2007). In particular, Simmons et al. (2005) found that fine scale advertisement call structure provides some information about male immune function in another population of *T. commodus*. There were, however, several

differences between our study and Simmons et al. (2005) that do not make them directly comparable. First, they recorded advertisement calls from males from a field population, who are exposed to a range of natural parasites and pathogens that could induce differences in immune function due to variation in parasite loads or past exposure to disease. We used laboratory reared males not exposed to parasites and measured innate immune function. Second, benign laboratory conditions, where there is *ad libitum* food and water, might mask the correlations between call structure and immune function seen in the field that are mediated through variation in body condition associated with differences in resource acquisition (Westneat and Birkhead 1998). Third, Simmons et al. (2005) measured a different aspect of male immune function, namely encapsulation rate.

Although the call traits that we measured influence female choice in our study population (Brooks et al. 2005; Bentsen et al. 2006; Hall et al. 2008), we used the results of previous selection analyses to predict the multivariate attractiveness of advertisement and courtship calls. We are therefore assuming that fitness surfaces are stable across time. Although we worked on the same population of *T. commodus* as used in these previous selection studies, our study was carried out several years later. We therefore cannot exclude the possibility, although we consider it unlikely, that there was no correlation between multivariate attractiveness and immune function because the fitness surfaces have changed over time.

IMMUNE MEASURES

Haemocyte counts and lysozyme-like activity have both been used as a proxy for immunity and disease resistance in other invertebrates (e.g. Rolff 2001; Ahtiainen et al. 2004; Rantala and Kortet 2004; Simmons et al. 2005; Simmons and Roberts 2005; Tregenza et al. 2006;

Simmons et al. 2010). It is important to note, however, that larger measured immune responses do not always indicate greater resistance against actual disease (Adamo 2004a;b). In most studies of parasite-mediated sexual selection it is unknown whether the reported immune measure accurately reflects actual disease resistance. The same is true in *T. commodus* for haemocyte counts and lysozyme-like activity. We have previously shown that inbreeding (which lowers fitness) actually increases one of our immune function measures (haemocyte counts) demonstrating that the correlation between fitness and immune function is not necessarily linear and might involve an optimal level of defense (Drayton and Jennions 2011). It appears, however, that the elevated lysozyme-like activity of males who call more is associated with higher fitness as males with greater calling effort attract more females (Bentsen et al. 2006), which is likely to be a major component of net fitness. Given our previous findings that inbreeding affects immune function (Drayton and Jennions 2011) and calling effort (Drayton et al. 2010), we tested whether the relationship between sexual signals and immune function differs between inbred and outbred males. This might occur, for example, if inbreeding affects the acquisition and/or allocation of resources (e.g. energy, nutrients) between sexual signaling and the immune system. Our results showed, however, that inbreeding did not affect any of the relationships between call structure, attractiveness or calling effort and the two immune measures. One interpretation of this finding is that inbreeding affects general body condition but not resource allocation. Given condition-dependence, this would explain how inbreeding can affect the absolute level of immune function (Drayton and Jennions 2011) and calling effort (Drayton et al. 2010), but does not alter the relationship between the two traits (this study).

It is worth noting that there are two general approaches to measuring immune function, either by measuring baseline levels (pre-challenge e.g. Simmons and Roberts 2005;

Tregenza et al. 2006) or by measuring levels after an immune challenge is administered (induced: e.g. Adamo 2004a). In this study, we measured baseline levels. While it would have been informative to also investigate induced immunity, we had to balance the pursuit of large sample sizes needed for statistical power, against the logistical constraints of measuring both baseline and induced immunity. There is evidence that baseline and induced immune measures are correlated in *Teleogryllus*: pre-challenge haemocyte counts correlate with the degree of encapsulation of a nylon filament insert in both *T. oceanicus* and *T. commodus* (Simmons et al. 2005; Simmons and Roberts 2005; Tregenza et al. 2006). The relationship between pre- and post challenge lysozyme-like activity in *T. commodus* is currently unknown. It is plausible that baseline levels reflect immunocompetence as a high baseline level of lysozyme could facilitate a more rapid immune response against invading pathogens. In scorpionflies, baseline lysozyme-like activity is positively correlated with the phagocytic capacity of the haemolymph (which aids in defence against bacteria and fungal spores) (Kurtz et al. 2000).

In summary, we found little evidence that females can assess a male's immune function based on the structure of the courtship or advertisement call that he produces. There was, however, a relationship between calling effort and lysozyme-like activity. More studies are needed before we can conduct a meta-analysis to test whether or not sexual traits in insects tend to signal male immune function (see the limited set of studies listed in the *Introduction*). It is important that both significant and non-significant phenotypic correlations continue to be reported to avoid any publication bias in the scientific literature. Finally, given that the fine-scale structures of the courtship and advertisement calls of *T. commodus* do influence female choice, and that females presumably gain direct or indirect

benefits from mate choice, male calls must signal some aspects of male quality other than the number of haemocytes or lysozyme-like activity.

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Table 1: The linear regression coefficients describing the relationships between properties of the courtship call and the two different immune assays.

	Lysozyme activity			**Haemocyte count**		
Courtship call	β	*SE*	P-value	β	*SE*	P-value
CPN	0.038	0.031	0.234	0.078	0.034	**0.022**
CIPD	0.022	0.032	0.478	-0.033	0.034	0.330
CTI	-0.030	0.032	0.356	-0.028	0.035	0.414
T1L	-0.092	0.050	0.066	-0.023	0.051	0.658
TN	-0.093	0.046	**0.044**	-0.063	0.048	0.188
DF	0.017	0.030	0.582	0.034	0.032	0.296

Table 2: The linear regression coefficients describing the relationships between the properties of the advertisement call and the two different immune assays.

	Lysozyme activity			**Haemocyte count**		
Advertisement call	β	*SE*	P-value	β	*SE*	P-value
CPN	-0.077	0.086	0.371	-0.066	0.053	0.216
CIPD	0.033	0.075	0.667	0.033	0.052	0.528
TN	0.010	0.064	0.873	0.016	0.049	0.749
ICD	-0.088	0.113	0.435	-0.060	0.053	0.262
DF	0.103	0.096	0.287	0.108	0.054	**0.050**

Table 3: The linear regression coefficients describing the relationships between the two different immune assays and three traits that have been shown to affect male mating/fertilization success. For a given immune assay, each coefficient was estimated using separate regressions, but represented here within a single table for simplicity.

	Lysozyme activity			**Haemocyte count**		
Attractiveness measures	β	*SE*	P-value	β	*SE*	P-value
Courtship call attractiveness	0.019	0.016	0.239	0.022	0.016	0.176
Advertisement call attractiveness	-0.072	0.504	0.887	0.153	0.422	0.718
Calling effort	0.046	0.019	**0.015**	0.026	0.022	0.232

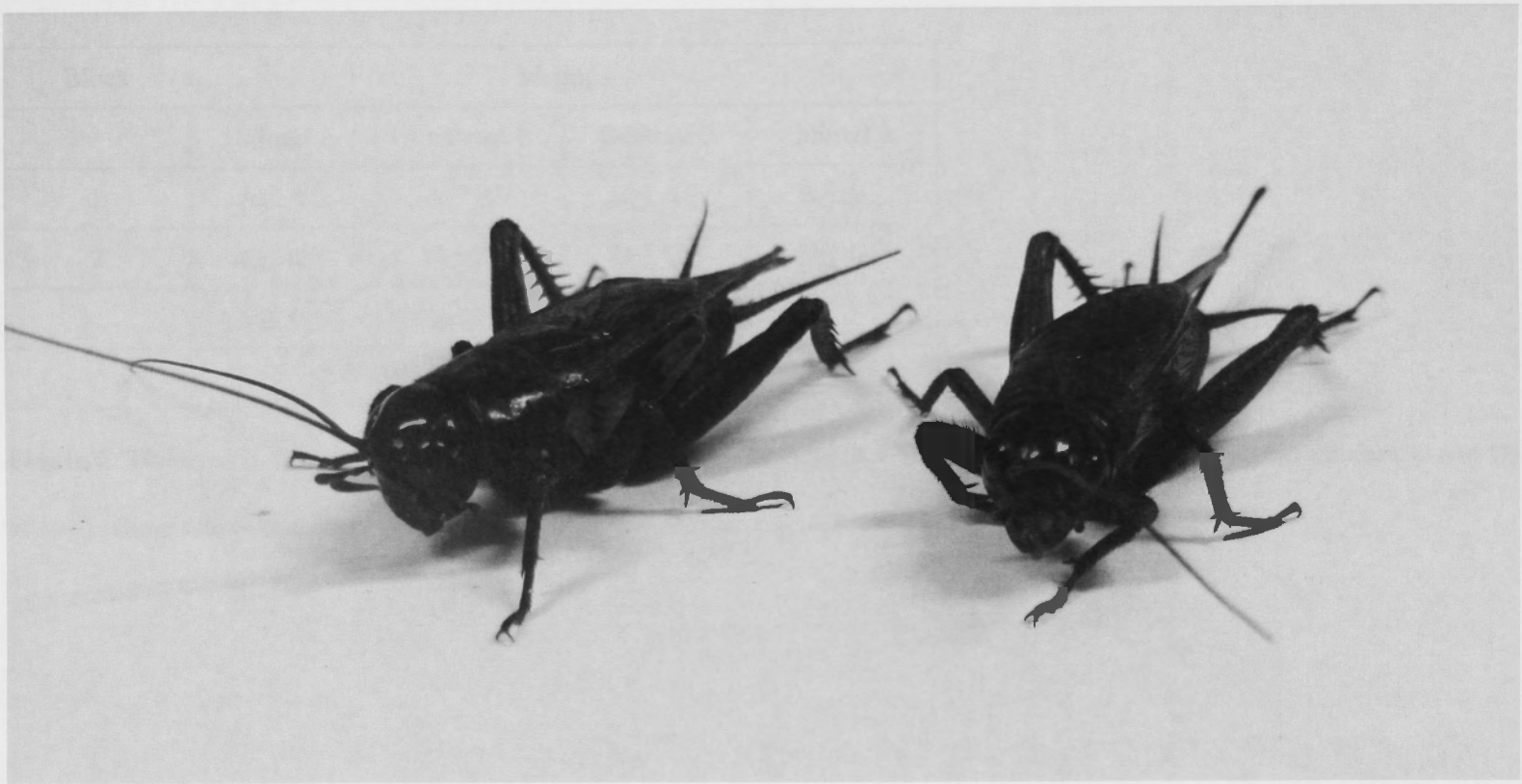

Figure 1. A *Teleogryllus commodus* male (left) and female (right).

Block	Matings			
	Inbred 1	Outbred 1	Outbred 2	Inbred 2
1	A♂ A♀	A♂ B♀	B♂ A♀	B♂ B♀
2	C♂ C♀	C♂ D♀	D♂ C♀	D♂ D♀
3...	E♂ E♀	E♂ F♀	F♂ E♀	F♂ F♀
...33	Y♂ Y♀	Y♂ Z♀	Z♂ Y♀	Z♂ Z♀

Figure 2. The mating design with Block 1 (comprising full sib families A and B), Block 2 (comprising full sib families C and D) etc. There are two inbred and two outbred genotypes generated per block.

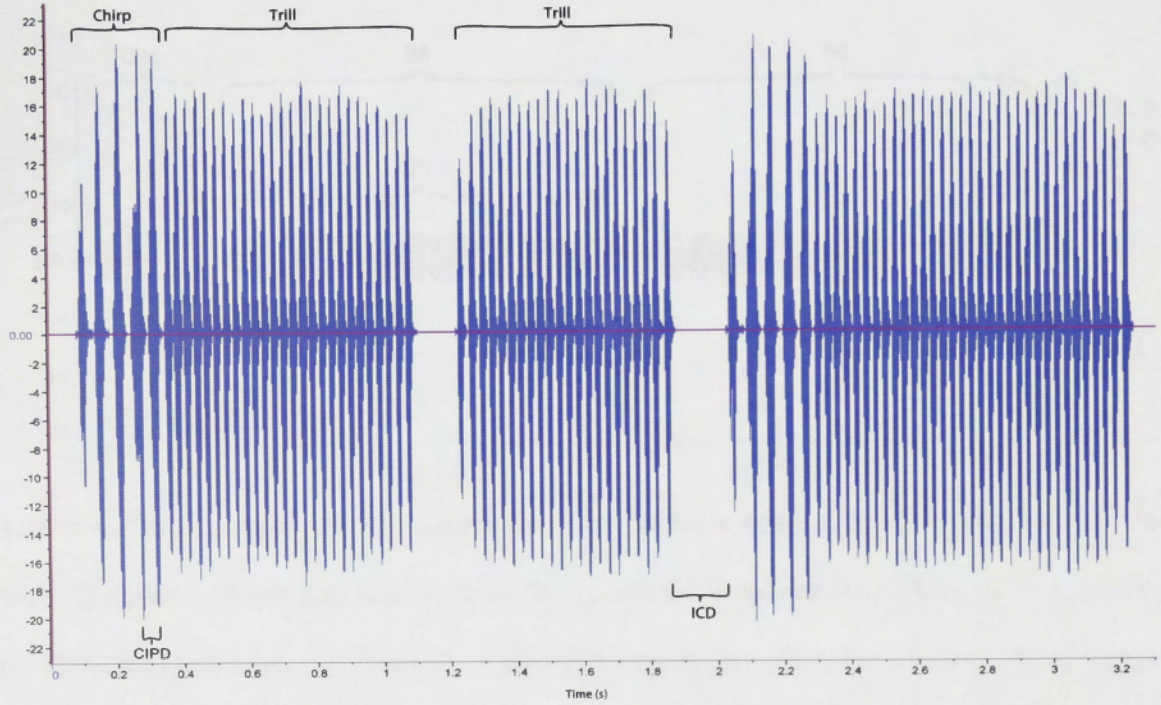

Figure 3. The advertisement call of *T. commodus*. Each phrase consists of a single chirp followed by a variable number of trills (the phrase in the figure has two trills). We measured the dominant frequency (DF), the number of pulses per chirp (CPN), the chirp inter-pulse duration (CIPD), the inter-call duration (ICD), and the number of trills in a phrase (TN). The vertical axis represents the amplitude of the sound produced.

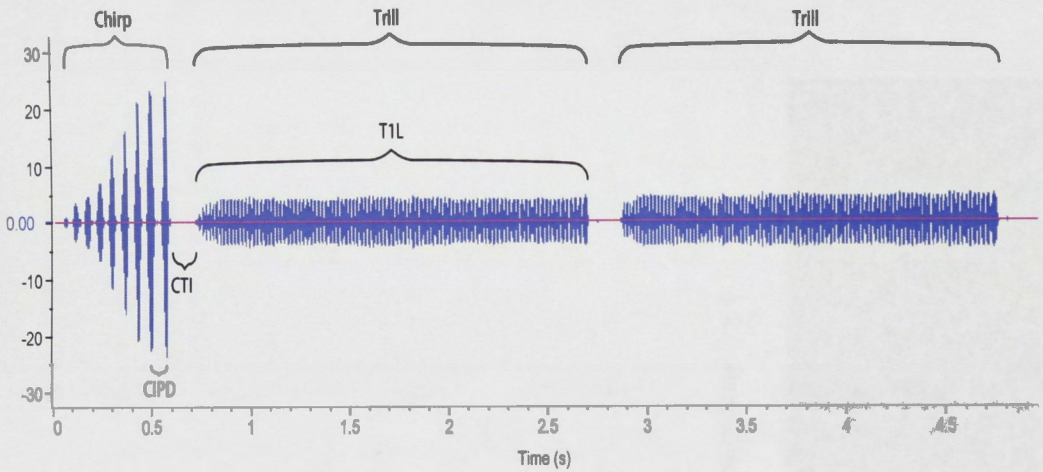

Figure 4. The courtship call of *T. commodus*. Each phrase consists of a single, amplitude-modulated chirp, followed by a variable number of trills. The phrase shown here has two trills. We measured: dominant frequency (DF), chirp pulse number (CPN), chirp inter-pulse duration (CIPD), chirp-trill interval (CTI), trill number (TN) and the length of the first trill (T1L). The vertical axis represents the amplitude of the sound produced.

CONCLUDING REMARKS

A *T. commodus* male. Photo: Hendrik Maat.

I tested the honesty of sexual signals in the field cricket *Teleogryllus commodus* using a series of experiments in which I either tested for correlations between male sexual traits and measures of male quality, or experimentally reduced male quality/fitness (by inbreeding) and then investigated the effects of this reduced quality on sexually selected traits. The sexual traits that I examined (namely advertisement calling and courtship) are known to influence female choice (Brooks et al. 2005; Bentsen et al. 2006; Hall et al. 2008). Presumably, females obtain benefits by preferring certain calls/males because mating preferences persist in *T. commodus*. It is therefore plausible that these sexual traits signal aspects of male quality to females.

Although the structure of the courtship call influences female post-copulatory choice in *T. commodus* (i.e. spermatophore retention time), I found no strong evidence that courtship signals aspects of male quality to females. Inbreeding in this species is known to reduce fitness traits such as egg hatching success, adult lifespan and nymph survival (Drayton et al. 2007; see Chapter 4), therefore inbred individuals should have reduced fitness. The structure and multivariate post-copulatory attractiveness of the courtship call was relatively unaffected by inbreeding. When I injected males with bacterial lipopolysaccharides (LPS) to induce an immune response, they were no less successful in courting and mating with females compared to control males. Furthermore, there was no difference in how long females retained the spermatophores of immune-challenged and control males. This indicates that an immune challenge did not affect the courtship call, or other pre- or post-copulatory mate choice cues (e.g. cuticular pheromones). I also found no strong evidence for a relationship between immune function, and courtship call structure or multivariate attractiveness. Consequently, it seems that courtship by *T. commodus* males does not signal

immune function (at least in the context of the experiments reported here) or fitness traits such as condition, vigor or heterozygosity (which should be reduced by inbreeding). However, because courtship calls do influence female choice, it is likely that courtship does signal other aspects of male quality to females that were not investigated in my experiments.

In contrast, advertisement calling seems to be a reliable signal of fitness components in *T. commodus*. I found a 30% reduction in advertisement calling effort with inbreeding, indicating that calling effort (a trait which is under positive directional sexual selection, Bentsen et al. 2006) is a reliable signal of condition and/or heterozygosity. I also found that calling effort might be used by females to obtain males with higher immune function (lysozyme-like activity). Although, as Chapter 2 demonstrates, the relationship between fitness and measured immune function might not be straight forward. Previous studies have shown that calling effort is affected by nutrition (Hunt et al. 2004; Maklakov et al. 2008). These studies and my results suggest that advertisement call effort is therefore a reliable signal of aspects of male quality in *T. commodus*. The fine scale structure of the advertisement call affects female choice (Brooks et al. 2005) and we have previously found substantial effects of inbreeding on advertisement call structure in another population of *T. commodus* (Drayton et al. 2007), indicating that call structure could reveal fitness. In the current population, however, neither the structure of the advertisement call nor call attractiveness was affected by inbreeding. In addition, call structure (either univariate traits or an estimate of multivariate attractiveness) was unrelated to immune function. I therefore found little evidence that advertisement call structure signals male quality to females.

In sum, my results show that fine scale aspects of calls do not signal the male qualities that were investigated in the current experiments. Because fine scale call structure is known to influence female choice, the structure of calls must signal other, as yet unknown, beneficial qualities to females. In contrast, *T. commodus* females that prefer males based on advertisement call rate (Bentsen et al. 2006) are using a signal which is correlated with components of male fitness. If male fitness components are heritable, this will increase female fitness by improving offspring performance.

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