Inbreeding and courtship calling in the cricket *Teleogryllus commodus*

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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.

Introduction

Males of many species possess multiple sexually selected ornaments that females may use when evaluating prospective mates (Moller & 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 & Rence, 1978; Campbell & Shipp, 1979; Evans, 1988). Female preferences for advertisement calls have been well studied (e.g. Hedrick, 1986; Stout & McGhee, 1988; Wagner, 1996; Wagner & Reiser, 2000; Holzer *et al.*, 2003; Brooks *et al.*,

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2005; Bentsen *et al.*, 2006; Wagner & 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 & 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 stabilizing selection on advertisement calls (e.g. Brooks *et al.*, 2005; Bentsen *et al.*, 2006). At a more proximate level, stabilizing 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 & 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 & Kolluru, 1998; Kotiaho, 2001) and females (e.g. Martin & Wagner, 2010)? It appears, however, that the courtship call is important to induce female mounting behaviour (Crankshaw, 1979; Burk, 1983; Adamo & Hoy, 1994; Libersat *et al.*, 1994; Balakrishnan & 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 & 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 & 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 & Eckhardt, 2001; *G. lineaticeps*: Wagner & 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 & Waller, 2002; Armbruster & Reed, 2005). This reduction in fitness mainly arises because of the unmasking of partially or fully recessive deleterious alleles, as well as the loss of advantageous heterozygosity (Charlesworth & Charlesworth, 1999; Charlesworth & Willis, 2009). 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 and/or assimilate the energetic and nutrient resources that are available to be allocated to sexual signalling (i.e. lowers condition *sensu* Rowe & 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 & 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 & 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 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 (Bussière *et al.*, 2006; Hall *et al.*, 2010). Hall *et al.* (2008) characterized the major axes of multi-variate sexual selection on courtship call structure using spermatophore attachment times as the measure of fitness. They found significant nonlinear 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 any aspect of male quality that is 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 multi-variate 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 Smiths Lakes, NSW, Australia. 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 $43 \times 30 \times 13$ 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 geno-types. 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

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♀

Fig. 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.

and sisters from family B, whereas 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 3 days for emerging nymphs. Upon emerging, nymphs from the same pairing were reared communally for 20 days in $9 \times 9 \times 5$ cm plastic tubs with food and a piece of moist cotton wool. After 20 days, each nymph was transferred to an individual container ($9 \times 9 \times 5$ cm, 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 ($\pm$ 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 develop-ment 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 sexu-ally 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 consist-ing of a file and scraper on the forewings. The most basic
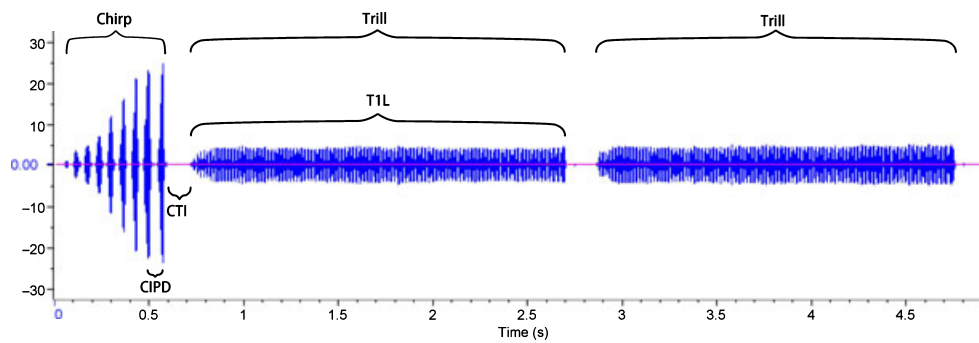
Fig. 2 The courtship call of *T. commodus*. Each phrase consists of a single, amplitude-modulated chirp, followed by a variable number of trills. The phrase shown here has two trills. We measured: dominant frequency (DF), chirp pulse number (CPN), chirp inter-pulse duration (CIPD), chirp-trill interval (CTI), trill number (TN) and the length of the first trill (T1L). The vertical axis represents the amplitude of the sound produced.

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 $9 \times 9 \times 5$ cm 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 min of the courtship call using a digital recorder (MicroTrack 24/96; M-Audio, Irwindale, CA, USA). A power unit, containing a 9-V 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 & 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 randomized.

Courtship calls were analysed using Raven Pro 1.3 sound analysis software (Cornell Laboratory of Ornithology, Ithaca, NY, USA; http://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 3 kHz and above 6 kHz.

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 standardized 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 nonparametric, 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 & 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 characterize 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 & 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 & Arnold, 1983) were then used to estimate the vectors of linear selection gradients (β) and the matrix of quadratic and correlational selection gradients (γ), with the reported quadratic regression coefficients doubled to obtain quadratic selection gradients (Stinchcombe *et al.*, 2008). Finally, a canonical rotation of the γ matrix was used to detect the major axes of nonlinear sexual selection (Phillips & Arnold, 1989). This analysis produced an **M** matrix with seven 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 standardized, multiplied by the respective trait loadings of the eigenvectors and then summed 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.

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)	$\chi^2_{450} = 1823.283$†	< 0.001	0.840
Trill number (TN)	$\chi^2_{450} = 1376.254$†	< 0.001	0.758

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

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 (Tables 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

Table 2 Mean ± SE for inbred and outbred males, δ (% change with inbreeding) and Hedge's d for the six courtship call parameters.

	Mean ± SE			
Call parameter	Inbred ($N = 199$)	Outbred ($N = 252$)	δ	Hedges d ± SE
Chirp inter-pulse duration (CIPD) (ms)	29.3 ± 0.5	28.6 ± 0.4	**−2.4**	**−0.097 ± 0.095**
Chirp-trill interval (CTI) (ms)	146 ± 5.0	143 ± 3.5	−2.1	−0.056 ± 0.095
Trill 1 length (T1L) (s)	2.723 ± 0.092	2.668 ± 0.081	−2.1	−0.042 ± 0.095
Chirp pulse number (CPN)	9.302 ± 0.145	9.495 ± 0.115	2.0	0.100 ± 0.095
Dominant frequency (DF) (Hz)	3978.55 ± 9.32	3947.48 ± 7.84	**−0.8**	**−0.243 ± 0.095**
Trill number (TN)	3.084 ± 0.122	3.078 ± 0.106	−0.2	−0.004 ± 0.095

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

Table 3 The effect of inbreeding and five covariates on four courtship call parameters: CIPD, CTI, T1L and CPN.

	Chirp inter-pulse duration (CIPD)												Chirp pulse number (CPN)		
	Inbred			Outbred			Chirp-trill interval (CTI)			Trill 1 length (T1L)					
	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

P-values are from the final model if the term was significant, or when it alone was added to the final model if nonsignificant.
P-values significant at the 0.05 level are in bold.

Table 4 Correlations between dominant frequency (DF) or trill number (TN) and development time, male age, male and female mass and temperature.

	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**

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.

between inbred and outbred males. Heavier inbred males had a significantly lower TN, whereas the TN of outbred males was significantly lower at higher temperatures. Finally, outbred male courtship calls directed at heavier females had a marginally nonsignificant 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: $71.9 \pm 2.0\%$, inbred survival: $64.5 \pm 2.1\%$, $\delta = 10.3\%$, 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, $N = 852$, $\delta = 4.6\%$, Hedges $d = 0.182 \pm 0.051$; inbred males: 611.9 ± 5.8 mg, $N = 764$, outbred males: 634.6 ± 5.4 mg, $N = 856$, $\delta = 3.6\%$, 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, $N = 867$, $\delta = −2.9\%$, 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, $N = 868$, $\delta = −1.2\%$, Hedges $d = −0.051 \pm 0.050$). 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 $d = −0.061 \pm 0.095$,

$\mathbf{m_7}$: $F_{1,27} = 0.201$, $P = 0.658$, Hedges $d = 0.039 \pm 0.095$). 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 nonsignificant trend for inbreeding to lower maturation weight (δ: 3.6–4.6%), 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 & Charlesworth, 1987; Lynch & Walsh, 1998). Traits that are more closely related to fitness are expected to be under stronger selection which depletes additive genetic variation (Mousseau & Roff, 1987; DeRose & Roff, 1999; Blows & 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 & Walsh, 1998; Roff, 1998; DeRose & 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 nonsignificant. 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: 13.4–31.5%). 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 & Waller, 2002; Armbruster & Reed, 2005), inbred individuals can be unambiguously assigned as low phenotypic quality relative to outbred individuals. If male sexually selected traits reflect the 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 & 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 & 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 precopulatory 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 & Walsh, 2002; Konior *et al.*, 2005; Gage *et al.*, 2006; Fitzpatrick & 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 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 the 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 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 & 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 & 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 & Eckhardt, 2001). Likewise, female crickets *G. lineaticeps* prefer courtship calls with a higher chirp rate but this trait was not affected by nutrition (Wagner & 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 & 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 & 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 & 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 (Bussière *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 precopulatory sexual selection and will influence whether a female will mount a male (e.g. Wagner & Reiser, 2000; Tregenza *et al.*, 2006). It is possible that inbreeding will affect measures of precopulatory 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 carried out approximately 2 years later than Hall *et al.* (2008). We therefore cannot 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 & Mackay, 1996; Szulkin & 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 & 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, because of 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 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 the 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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