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

Jean M. Drayton,[1,2] Richard N. C. Milner,[1] John Hunt,[3] and Michael D. Jennions[1]

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

[2]*E-mail: jean.drayton@anu.edu.au*

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

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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 nonlinear 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: **Call structure, calling effort, heterozygosity, male attractiveness, selection analysis, sexual selection.**

For many years, theoretical models have been based on the assumption that female mate choice in nonresource-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 nonadditive 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 will 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 a

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 that 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 affects 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 behavior (van Oosterhout et al. 2003; Mariette et al. 2006), reduces sexual coloration (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 postcopulatory 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 Lakes, Australia. Specifically we predict that if such mate choice cues reveal condition and/or relative heterozygosity then 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 stabilizing 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 nonlinear 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 Lakes (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, Uncle Ben's, Raglan, Australia) 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, 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. 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 $9 \times 9 \times 5$ cm plastic tubs with food and a piece of moist cotton wool. They were then transferred to individual containers ($9 \times 9 \times 5$ cm) 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 ($\pm$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 ($9 \times 9 \times 5$ cm). 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, 1 represents that the male is calling is recorded. Otherwise, 0 represents no calling is recorded. The microphone of that chamber is then deactivated 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 per night. Calling effort was measured for 10 h per night (1900h–0500h) at 26–28°C. Males were 12–23 days post maturation (14.4 ± 0.02 days), and their calling effort was recorded over two to five 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 both elytra. 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 9 × 9 × 5 cm recording chambers with a condenser microphone mounted on the lid. We recorded calls using a digital recorder (MicroTrack 24/96, M-Audio, Irwindale, CA). A power unit, containing a 9-volt battery powered the microphone. Chambers were checked through out the evening for calling males. If a male was heard calling, we recorded 1–2 min 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 each male: dominant frequency (DF), the interval between the last trill pulse of the selected phrase and the first chirp pulse of the next (intercall 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 interpulse 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, Logitech, Fremont, CA, one speaker faced into, the

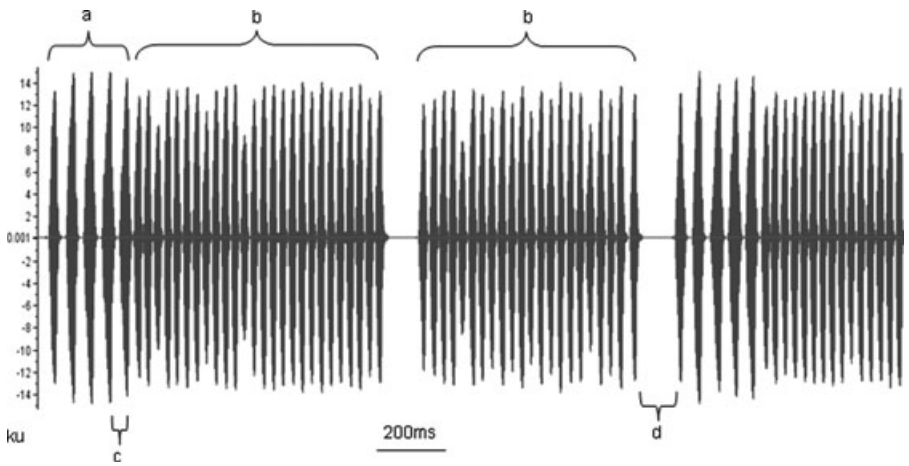

Figure 1. The advertisement call of male *T. commodus*. Louder pulses repeated at a lower rate are group into chirps (a) and softer pulses repeated at a higher rate are grouped into trills (b). Each phrase consists of a single chirp followed by a variable number of trills (the phrase in the figure has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

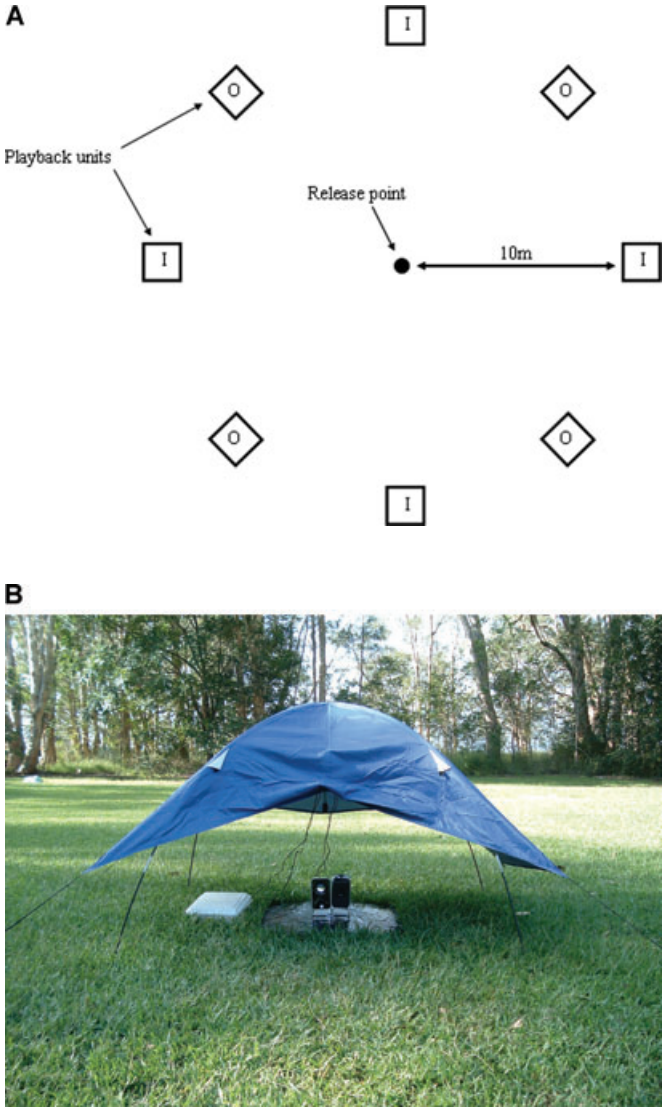

Figure 2. Set up of the field experiment. (A) Schematic diagram showing the placement of the playback units around the arena. The calls of inbred (I) and outbred (O) males were played in an alternating pattern around the circumference of the arena (B) one playback unit, showing the tent, speakers, and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

other out of the arena), a digital audio player (SanDisk Sansa c200, Sandisk, Quarry Bay, Hong Kong), and a small tent (for rain protection) covering the unit. The speakers were placed on a 60 × 60 cm piece of black Coreflute that was coated with sticky insect trap coating (TangleFoot, The Tanglefoot Company, Grand Rapids, MI) 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 60 dB at 80 cm using a sound level meter (Digitech QM-1589, DigiTech, Sandy City, UT) (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 (<5 km, 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 2100 h and 2200 h and ended at 0530 h. 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 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., eight 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 nonparametric 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 standardized 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 analysis of variance (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 nonparametric 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 datapoints). 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 datapoint 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. All tests are two-tailed and $\alpha = 0.05$.

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 nonlinear 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 nonlinear 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 nonlinear 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 reran the analysis on the full dataset to generate linear selection gradients ($\boldsymbol{\beta}$) and the $\boldsymbol{\gamma}$ 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 $\boldsymbol{\beta}$ and $\boldsymbol{\gamma}$. Relative fitness values were randomized across the calls and selection gradients recalculated using the Monte Carlo option in PopTools (version 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 $\boldsymbol{\gamma}$ matrix (Phillips and Arnold 1989). This produced an **M** matrix containing five eigenvectors $\mathbf{m_{1-5}}$ (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 $\boldsymbol{\theta}$ and $\boldsymbol{\lambda}_i$ (i.e., the eigenvalue), respectively. The values of $\boldsymbol{\theta}$ and $\boldsymbol{\lambda}_i$ 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 $\boldsymbol{\gamma}$ matrix, we tested the significance of $\boldsymbol{\theta}$ and $\boldsymbol{\lambda}_i$ 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 vs. outbred) and a second ANOVA on this shuffled dataset 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 nonindependence is ignored. In contrast, say, two-choice mating experiments in the laboratory often explicitly take into account nonindependence 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 nonparametric 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. 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,

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 (−.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 interpulse duration, ICD=intercall 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 phrases 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	χ^2_{97}=464.424†	<0.001	0.979
TN	χ^2_{97}=393.908†	<0.001	0.908

CIPD=chirp interpulse duration, ICD=intercall duration, CPN=chirp pulse number, DF=dominant frequency, TN=trill number. r_I: intraclass correlation coefficient.

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

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 NONLINEAR 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 ($F_{5,58} = 1.123$, $P = 0.357$),

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 nonsignificant (see text). *P*-values significant at the 0.05 level are in bold.

	CIPD			ICD			CPN		
	df	*F*	*P*	df	*F*	*P*	df	*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.

or correlational sexual selection ($F_{10,38} = 0.582$, $P = 0.818$), so we estimated sexual selection gradients on the combined dataset. There was significant linear selection favoring lower dominant frequency (DF), shorter ICD, and higher CPN. The only significant nonlinear selection was negative quadratic selection (stabilizing) on CIPD (Table 5).

Canonical rotation of the $\boldsymbol{\gamma}$ matrix of nonlinear 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 $\mathbf{m_2}$ (favoring a combination of higher CPN, higher TN and a lower DF: Fig. 4A) and along $\mathbf{m_4}$ (favoring 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 stabilizing 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).

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.

CALLING EFFORT AND INBREEDING

We found that one generation of full-sibling mating substantially reduced male nightly calling effort. Although this could be due to the effect of inbreeding at loci that directly code for calling behavior (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

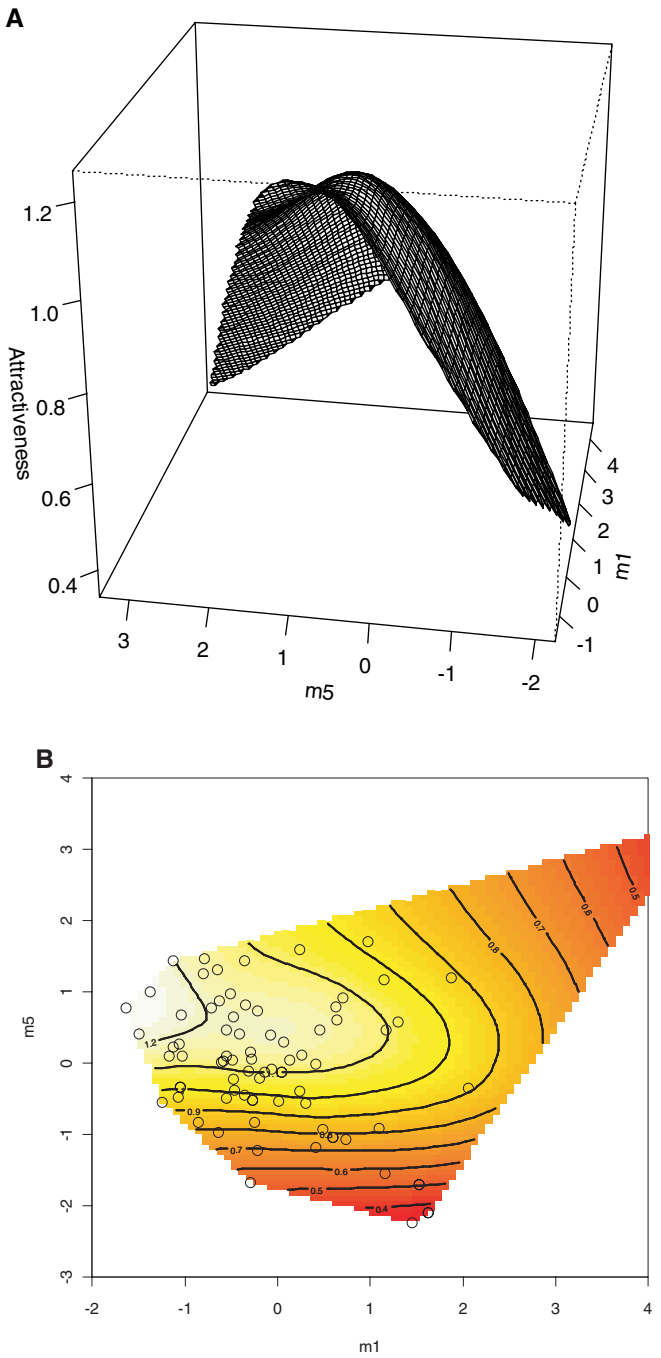

Figure 3. Thin-plate spline visualization of the fitness surface along the two major axes of nonlinear selection, m_1 and m_5. (A) Perspective view, (B) contour view with datapoints overlaid. Yellow regions indicate higher fitness.

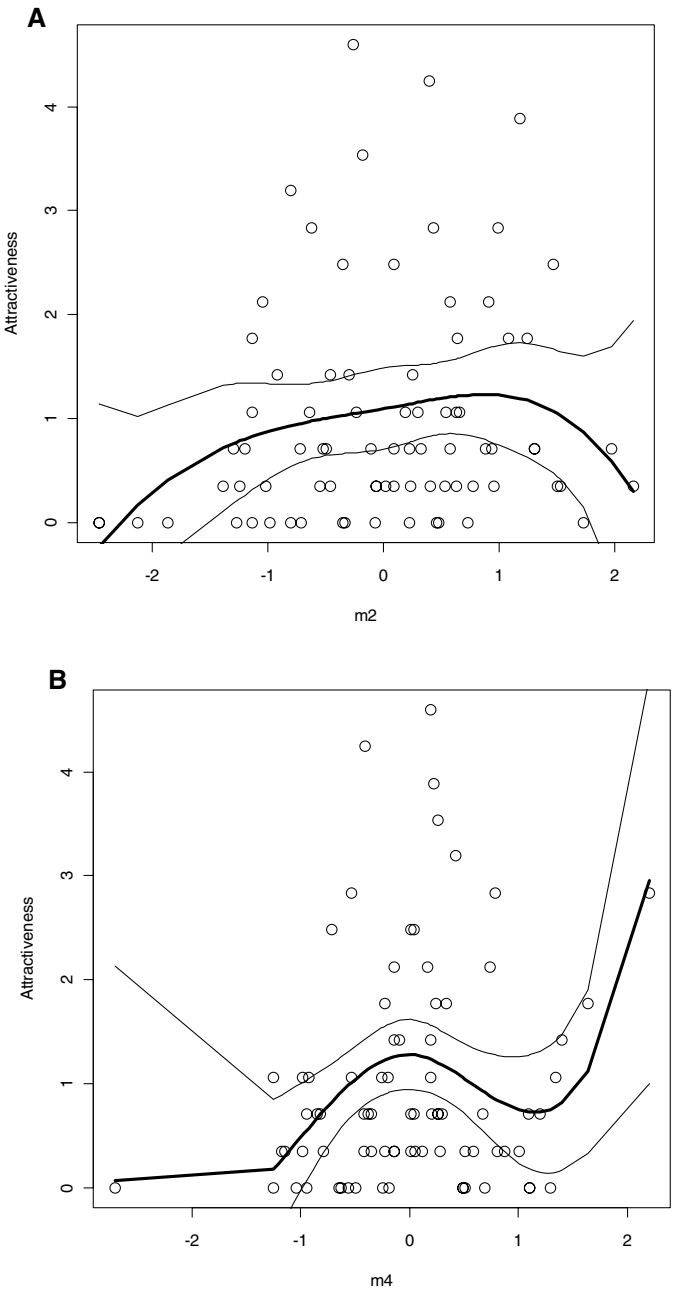

Figure 4. Univariate cubic spline visualization of sexual selection operating on (A) m_2 and (B) m_4 with datapoints overlaid. 95% confidence intervals are also provided.

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

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.

	CIPD	DF	ICD	TN	CPN	Selection	
						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.

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 behavior 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 TN 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

Although 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 favored a lower dominant frequency (DF), shorter 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 5- min 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 interbout 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 interbout intervals (less time "off air") overrides selection for shorter 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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