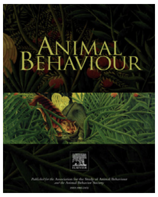
Animal Behaviour 133 (2017) 169–187

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav



Mate choice intensifies motor signalling in *Drosophila*

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ARTICLE INFO

Article history:
Received 2 April 2017
Initial acceptance 30 May 2017
Final acceptance 14 August 2017
Available online 14 October 2017
MS. number: 17-00292

Keywords:
courtship song
Drosophila pseudoobscura
experimental evolution
interpulse interval
mate choice
motor performance
sexual selection

Mate choice has the potential to act on the evolution of motor performance via its direct influence on motor sexual signals. However, studies demonstrating this are rare. Here, we performed an in-depth analysis of *Drosophila pseudoobscura* courtship song rate, a motor signal under mate choice in this species, and analysed the response of this signal to sexual selection manipulation using experimental evolution. We show that manipulating the opportunity for sexual selection led to changes in song production rate and singing endurance, with males from the polyandrous populations producing faster song rates over longer time periods than males from monogamous populations. We also show that song rate was correlated with estimates of overall courtship vigour. Our results suggest that the action of mate choice on a motor signal has affected male motor performance displayed during courtship. We consider potential selective benefits associated with changes in motor performance, including condition-dependent signalling, and discuss the implications of these results for the study of motor signals under sexual selection.

Motor performance, or vigour (Darwin, 1859, 1871), is the ability of an individual to repeatedly perform energetically costly motor acts (Byers, Hebets, & Podos, 2010). As this ability often has drastic fitness consequences (e.g. determining the ability to escape predators, forage or capture preys), its evolution is often driven by natural selection (Byers et al., 2010; Irschick & Garland, 2001). Yet, sexual selection also has the potential to affect the evolution of motor performance, when mate choice or mate competition targets motor signals (i.e. signals involving any kind of sustained muscular activity such as threat displays, courtship displays such as dances, or acoustic and vibratory signals; Bonduriansky, 2011; Husak & Fox, 2008). Because such signals typically require high-speed muscle contractions that are energetically costly to produce (Lailvaux & Irschick, 2006), they have the potential to be reliable indicators of a signaller's overall motor capacities, and thus of the individual's current condition (Byers et al., 2010; Clark, 2012; Lailvaux & Irschick, 2006; Oufiero & Garland, 2007). Hence, by directly influencing the evolution of a given motor signal, sexual selection may lead to a correlated increase in the overall motor capacities of signallers (Byers et al., 2010; Clark, 2012; Mowles & Ord, 2012; Ryan, 1988).

Although potential links between motor sexual signals and motor performance have received significant attention in the recent literature (Byers et al., 2010; Irschick, Meyers, Husak, & Le Gaillard, 2008; Mowles & Ord, 2012), their investigation has so far been restricted to two issues: the link between motor signals involved in mate competition and overall motor performance (Andersson, 1996; Byers et al., 2010; Lailvaux & Irschick, 2006) and the link between motor signals involved in mate choice and non-motor measures of mate condition (e.g. offspring production, growth rate, etc.; Irschick, Meyers, Husak, & Gaillard, 2008). For example, a link between male dominance display and running endurance has been shown in *Anolis* lizards (Perry, Levering, Girard, & Garland, 2004), and a correlation between male song structure and offspring survival was found in the zebra finch, *Taeniopygia guttata* (Woodgate, Mariette, & Bennett, 2012). Yet, mate choice for motor signals may also affect the evolution of overall mate motor performance (Byers et al., 2010; Clark, 2012; Mowles & Ord, 2012; Ryan, 1988; Ryan & Keddy-Hector, 1992). Numerous studies have shown that mate choice could drive the evolution of motor signals,

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https://doi.org/10.1016/j.anbehav.2017.09.014

but evidence for a correlated effect on overall motor performance is still lacking (Byers et al., 2010; Fusani, Barske, Day, Fuxjager, & Schlinger, 2014; Mowles & Ord, 2012).

A suitable approach to investigate this question is to determine how mate choice affects a motor signal over evolutionary time, and then examine whether these changes also result in changes in aspects of overall motor performance. As a widely studied acoustic mating signal, the pulse production rate of *Drosophila* male courtship song is a prime candidate for such a study, for multiple reasons. First, *Drosophila* courtship song consists of a series of repeated pulses created by rapid wing vibrations, obtained via high-speed contractions of thoracic muscles (Ewing, 1977, 1979; Shirangi, Stern, & Truman, 2013). The rate at which these pulses are produced (commonly reported as the interpulse interval, or IPI, representing the inverse of pulse rate) is thus likely to be a physically challenging motor trait. Next, the song pulse rate is a key target of female choice in several *Drosophila* species. It is involved in sexual isolation between *Drosophila melanogaster* and *Drosophila simulans*, and in intraspecific mate choice in *D. melanogaster*, *Drosophila montana* and *Drosophila pseudoobscura* (Bennet-Clark & Ewing, 1969; Debelle, Ritchie, & Snook, 2014; Kyriacou & Hall, 1982; Ritchie, Halsey, & Gleason, 1999; Veltsos, Wicker-Thomas, Butlin, Hoikkala, & Ritchie, 2012; Williams, Blouin, & Noor, 2001). The fact that song pulse rate is a target of female choice has been further demonstrated by showing the coevolution of pulse rate and female preference for pulse rate in experimental populations of *D. pseudoobscura* (Debelle et al., 2014). Finally, a direct action of male–male competition on pulse rate evolution is improbable. Courtship song is a near-field acoustic signal produced within 2.5–5 mm of the female's head (Bennet-Clark, 1971, 1998), rendering its accurate reception by surrounding male competitors unlikely (Morley, Steinmann, Casas, & Robert, 2012). Hence, and although playing artificial courtship songs to males in playback experiments triggers male locomotion (Eberl & Tauber, 2002; von Schilcher, 1976), varying pulse rate does not affect male courtship behaviour (Talyn & Dowse, 2004). Therefore, *Drosophila* song pulse rate has all the necessary characteristics to be a suitable candidate for this study.

Yet, how song pulse rate relates to the evolution of male motor performance has so far not been investigated. This may be because the rate of *Drosophila* courtship song is commonly considered to be static, that is, stable in time and independent of male motor capacities. Like many other acoustic signals, pulse rate is thus usually measured at a single time point or averaged over the entire courtship sequence (Tauber & Eberl, 2003; but see a notable exception in Arthur, Sunayama-Morita, Coen, Murthy, & Stern, 2013). That restricted view of this motor signal makes it impossible to know how much this trait depends on a male's motor capacities (Irschick & Garland, 2001). Another important aspect in studying the action of mate choice on motor performance evolution is to measure how the trait under mate choice is associated with other traits. By targeting pulse rate, mate choice could lead to a correlated response on other motor traits (Gerhardt & Brooks, 2009; Lande & Arnold, 1983), and therefore investigating these associations is essential to understand how mate choice may influence the evolution of motor performance beyond pulse rate.

In this study, we examined closely the production of a motor signal involved in mate choice, and quantified how manipulating the opportunity for sexual selection (Jones, 2009) influences the evolution of motor performance that is displayed during courtship. For that purpose, we studied the production of *D. pseudoobscura* pulse rate over the duration of courtship, and explored its response to a long-term experimental manipulation of the opportunity for sexual selection in *D. pseudoobscura* populations (>100 generations of experimental evolution of elevated polyandry or enforced monogamy). An analysis of these experimental lines performed after 30 generations of selection has found that mean pulse rate had responded to sexual selection manipulation, and had become faster in males from polyandrous lines compared to monogamous lines (Snook, Robertson, Crudgington, & Ritchie, 2005). This previous study, however, was performed on a restricted number of individuals and limited to the examination of average pulse rate. Consequently, it did not allow the investigation of potential differences in pulse rate production over time, which is necessary to study overall motor performance. Here, we performed an in-depth study of pulse rate production over time in our experimental lines after much longer evolution, and analysed the effect of sexual selection manipulation on motor signalling.

Our main prediction was that an increased opportunity for sexual selection will lead to the evolution of more intense male signalling characteristics and thus to an increased motor performance. For this end, we looked at the detailed structure of pulse rate production over courtship time, to uncover potential sources of motor performance difference between males. We then compared pulse rate production between the sexual selection treatments, to study whether pulse rate production responded to sexual selection manipulation. Finally, we examined associations between pulse rate and other motor courtship traits, to test whether pulse rate may be correlated with overall courtship vigour.

METHODS

Courtship Song Description

The courtship behaviour of *D. pseudoobscura* has been described in detail elsewhere (Brown, 1964; Ewing & Bennet-Clark, 1968). Courtship song is produced by the vibration of one or both male wing(s), and consists of two main components: a low-repetition rate song (LRR) and a high-repetition rate song (HRR; Fig. 1). LRR consists of high-amplitude polycyclic pulses and is generally produced first, while the male orients in the direction of the female and approaches her, by flicking one or both wing(s) in a scissoring movement. Once the male has reached the female, he extends the wing that is nearest the female's head to 90° and vibrates it rapidly, producing a burst of HRR, characterized by a high number of low-amplitude polycyclic pulses and an increase in pulse rate (i.e. a shorter duration between two consecutive pulses in a burst of song, and thus a shorter interpulse interval). The male will then generally attempt to mount the female and copulate. If the female refuses to mate, the male will start another courtship sequence, including another round of song bursts. As HRR pulse rate is the main target of female preference in this species (Debelle et al., 2014; Snook et al., 2005; Williams et al., 2001), we focus on HRR song in this paper (but provide a similar analysis of LRR song in Appendix 1).

Sexual Selection Treatments

An ancestral wild-caught population of *D. pseudoobscura*, a naturally polyandrous species (more than 80% of wild-caught females have been shown to be inseminated by up to two males at any given time; Cobbs, 1977), was used to create the selection lines. The establishment and maintenance of the selection lines are described in detail elsewhere (Crudgington, Beckerman, Brüstle, Green, & Snook, 2005). In brief, from an ancestral population derived from Tucson (AZ, U.S.A.), four replicates (replicate 1, 2, 3 and 4) of two sexual selection treatments were initiated. To modify the opportunity for sexual selection at each generation, the adult sex ratio in vials was manipulated by either confining one female with a single male (‘monogamy’ treatment; M) or one female with six males (‘elevated polyandry’ treatment; *E*) in vials. Both intra- and intersexual selection were relaxed in the monogamy treatment

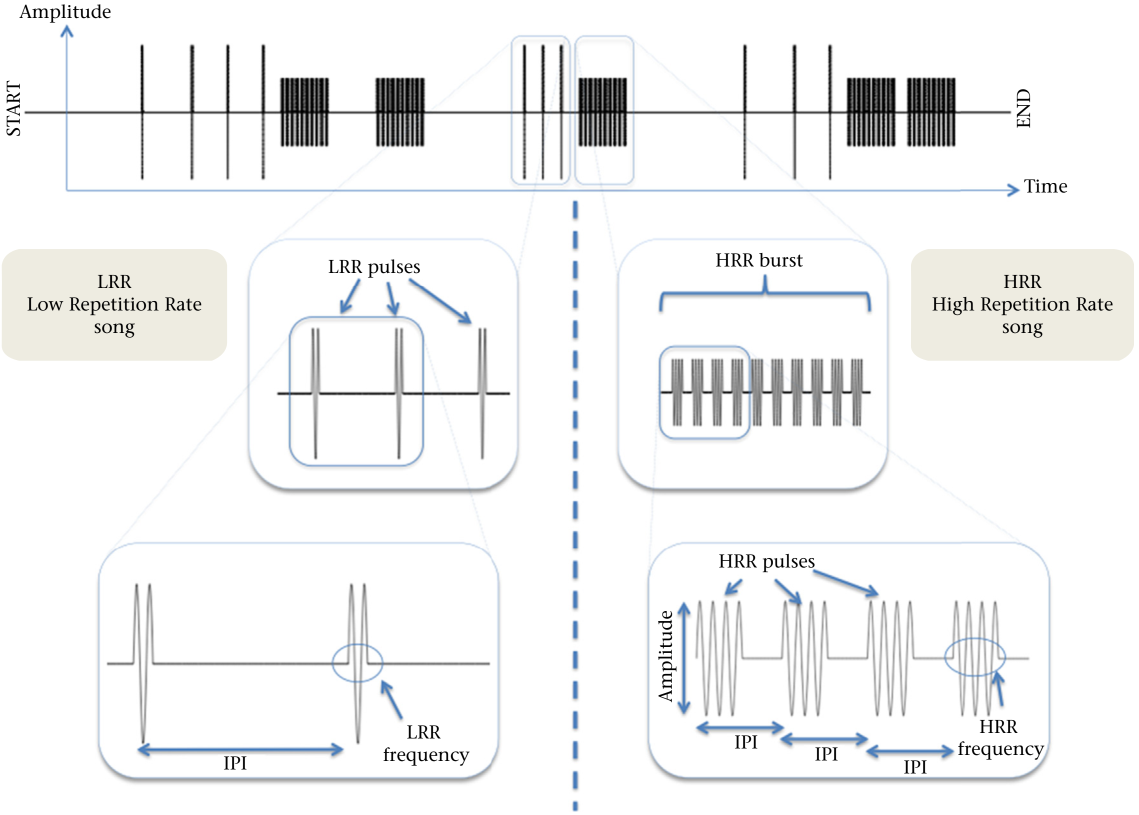

Figure 1. Representation of *D. pseudoobscura* courtship song. Both LRR (low-repetition rate) and HRR (high-repetition rate) song are represented. Each burst of song is composed of multiple pulses, each separated by a certain time interval, the interpulse interval (i.e. the inverse of pulse rate). As the interpulse interval represents the amount of time between two consecutive pulses, a short interpulse interval means that a male beats his wings rapidly (i.e. fast pulse repetition rate), whereas a long interpulse interval means that a male beats his wings slowly (i.e. slow pulse repetition rate). HRR frequency represents the intrapulse frequency of a pulse of HRR. In our analysis, we refer to 'burst position' as the position of an HRR burst in the courtship sequence (e.g. the first burst produced, the second burst produced, the third burst produced), and to 'pulse position' as the position of a pulse within a burst of HRR (e.g. the first pulse produced, the second pulse of a burst, the third pulse of a burst).

whereas both types of sexual selection were increased in the polyandry treatment. As previously reported, effective population size was successfully equalized between the treatments (*Ne* > 100 for all the populations; Snook, Brüstle, & Slate, 2009). At each generation and in each population independently, offspring were collected and then pooled. A random sample of this pool was used to establish the next generation using the appropriate sex ratios. This protocol thus proportionally reflects the relative offspring production across all families. Standard vials (2.5 mm × 80 mm) were used to maintain the selection lines, with a 28-day generation time. Bottles (57 mm × 132 mm) were used to maintain the ancestral population, with an equal sex ratio of adult flies. Therefore, a total of eight selection lines (M1, M2, M3, M4 and E1, E2, E3, E4) and one ancestral population were maintained and kept at 22 °C on a 12:12 h light:dark cycle, using standard food media and added live yeast.

Experimental Flies

The flies used in this experiment were from the following generations: 111 and 112 for E1 and M1, 110 and 111 for E2 and M2, 109 and 110 for E3 and M3, 107 and 108 for E4 and M4. To generate the experimental flies, 50 reproductively mature adults of each selection line (25 males and 25 females) were used as parents and kept in mass cultures, providing a common mating set-up for the parents of both sexual selection treatments. The resulting larvae were raised in controlled density vials (100 first-instar larvae per food vial), to standardize the larval rearing environment and relax selection. The flies were collected and sexed on the day of emergence, using CO_2 anaesthesia. Males from each population were kept in yeasted food vials of 10 individuals from the day of emergence to day 4, and then transferred to individual yeasted food vials the day before the recording. We used ancestral females for male courtship song recording to standardize female response. Ancestral females were collected and kept in vials of 10 individuals until used for the song recording experiment. Ancestral females were mated to ancestral males the day before the experiment to reduce their receptivity and prevent them from mating with the focal recorded male within the 5 min of the trial. Female receptivity is drastically reduced in the 24 h following a mating, and thus the probability of remating for the ancestral females used in this experiment would be nearly zero (Crudgington et al., 2005; Snook, 1998). This method forces males to continuously court females, therefore facilitating detailed study of song production over time. All males and females used in this experiment were 5 days old and thus reproductively mature (Snook & Markow, 2001). Henceforth, reference to polyandrous or monogamous does not mean current mating situation in any experiments, but refers to the experimental sexual selection treatment from which flies were derived.

Courtship Song Recording

Recordings were performed during the flies' morning photoperiod (Noor, 1998). Courtship song was recorded by confining one virgin selection line male with a mated ancestral female for 5 min in a transparent chamber (15 mm × 4 mm) in an Insectavox

(Gorczyca & Hall, 1987). Recordings took place over the course of 12 days. All eight lines were randomized across and within days of recording. Each male was recorded only once, and 60 males were recorded per selection line. The Insectavox was connected to a Toshiba Satellite Pro S300-117 laptop, and sound was recorded using Audacity (v. 1.3.11). All songs were digitized after filtering with a Fern EF5-04 filter, band-passed between 100 and 800 Hz. After the experiments, recordings were manually prepared for software analysis by silencing parts of the recording without song using Audacity (v. 1.3.11). Recordings were then analysed using a custom script from the software DataView (Heitler, 2007), allowing the detection of the position of each ‘song event’ (pulses and bursts) in a recording. Intrapulse frequency for both LRR and HRR songs was obtained using a fast Fourier transform (FFT) in DataView (FFT duration = 16 ms, FFT window = hamming, percentage overlap = 50%).

Temperature and Body Size

To understand more extensively how pulse rate is related to male motor capacities, it is informative to examine how it covaries with two key bioenergetic factors that can affect acoustic communication in insects: temperature and body size (Bailey, 1991; Bennett, 1990; Gillooly & Ophir, 2010). Temperature, which strongly influences muscle contraction rate, is tightly associated with motor power and endurance in ectotherms via its effect on metabolic rate (Gillooly, Brown, West, Savage, & Charnov, 2001), and has a major impact on the temporal components of acoustic signals in insects (Bailey, 1991), including *Drosophila* courtship song traits (Noor & Aquadro, 1998; Ritchie & Gleason, 1995; Ritchie & Kyriacou, 1994; Ritchie, Saarikettu, Livingstone, & Hoikkala, 2001). Likewise, body size, a target of sexual selection, is positively correlated with motor performance, notably due to the increased power provided by larger muscles (Biewener, 2003; Carrier, 1996). Thus, including these variables in our analyses will give a better understanding of how much pulse rate production depends on male motor capacities, and thus of how the physiological properties of *Drosophila* courtship song can have an impact on its evolution as a sexual signal.

As the light within the Insectavox generates inevitable random small variations in temperature, we examined in detail how song traits vary with these minor changes in temperature. Temperature was measured within the chamber every 10 s (±0.01 °C) using a Testo 735-1 thermometer (Testo Limited, Alton, U.K.) and recorded for each burst of song in each recording. This temperature variation was then included as a covariate in the song analyses (temperature was either calculated for each burst in the case of HRR pulse rate or averaged over all bursts for the other traits).

To estimate how body size could associate with pulse rate production, the size of the singing male was included in the analyses. The length of wing vein IV of each individual was measured after the experiment (wing vein length has been shown to be a good estimator of body size in *Drosophila* species (e.g. Crudgington et al., 2005; Gilchrist, Huey, & Serra, 2001; Robertson & Reeve, 1952; Sokoloff, 1966). Wings were mounted in a 30% glycerol–70% ethanol medium, images taken using a Motic camera and Motic Images Plus 2.0 software (Motic Asia, Hong Kong) and then measured with ImageJ (v. 1.44e; Abramoff, Magalhães, & Ram, 2004).

Courtship Traits Analysis

The different courtship traits analysed in this study are represented in Fig. 1. All the statistical analyses were performed in R (v. 3.3.2; R Core Team, 2017).

We first tested for differences between the sexual selection treatments in body size and in their probability of producing song. As HRR interpulse interval (i.e. the inverse of pulse rate) is not constant over time but lengthens as courtship time increases (see Fig. A2 in Appendix 2), we then conducted a detailed analysis of pulse rate production over courtship time, and compared pulse rate production between treatments. Finally, we performed multivariate analyses on all courtship traits to study phenotypic correlations between pulse rate and other courtship traits, and to test whether sexual selection manipulation modified these associations.

Differences in body size and singing probability between treatments

Potential differences in body size between the sexual selection treatments were analysed using a univariate linear mixed model (LMM), in which the sexual selection treatment of the recorded male (E or M) was included as a fixed effect, and the male replicate population (M1, M2, M3, M4, E1, E2, E3 or E4) included as a Gaussian random effect nested within sexual selection treatment. The model was fitted using maximum likelihood estimation, with a Gaussian error distribution. The difference in the probability of singing (i.e. the probability of a male producing at least one burst of HRR during the 5 min recording) between the sexual selection treatments was analysed using the same model structure but fitting a generalized linear mixed model (GLMM) for the binomial family. Both models were fitted using the package spaMM (Rousset & Ferdy, 2014) and estimates were compared to zero using parametric bootstraps which were consistent with results from model comparison using asymptotic likelihood ratio tests.

Analysis of pulse rate production over time

To distinguish between HRR interpulse and interburst interval (i.e. the interval of time between the last pulse of a burst and the first pulse of the following burst), an upper threshold was determined visually by plotting the distribution of the duration between two pulses (threshold = 55 ms; the average HRR interpulse interval is approximately 38 ms in *D. pseudoobscura*, Noor & Aquadro, 1998; Snook et al., 2005). To allow sufficient HRR interpulse interval values for each burst, we only included recordings with at least 10 interpulse interval values (i.e. the overall average number of HRR pulses per burst for both E and M males is 17; see Fig. A3 in Appendix 2 for more details).

Variation in individual interpulse interval values along the courtship sequence was analysed by fitting a univariate LMM, using the function glmmPQL() of the MASS package (Venables & Ripley, 2002). This enables correcting for temporal autocorrelation between consecutive interpulse interval values within a burst. We thus included in the model a fourth-order autoregressive moving-average (corARMA) function for autocorrelation, using the pulse position in a burst (i.e. 1, 2, 3, etc.) as a time covariate, and the burst identity (1735 levels) nested within replicate (eight levels) as a grouping factor (nlme package; Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2016). We also included two covariates indicating the position of the interpulse interval value within the courtship sequence, the burst position in the recording (i.e. 1, 2, 3, etc.) and the pulse position within a burst (i.e. 1, 2, 3, etc.), to test for a lengthening of interpulse interval over courtship duration (both within bursts and along bursts; see Fig. 1 for more details). The interactions between sexual selection treatment and the two event position covariates (burst position and pulse position) were included in the model, as well as their three-way interaction. This allowed us to assess how interpulse interval variation changes depending on the quantity of song already produced, and to test whether interpulse interval variation over time is consistent between the two treatments. The interaction between temperature and burst position was also added, to test for an effect of temperature on interpulse interval lengthening over time (the interaction between temperature and pulse position in the burst could not be included in the model, as the mean duration of a burst, <3 s, did not allow enough time for the recorded temperature to vary). The significance of the different fixed effects was extracted from the summary table of the glmmPQL fit, which provides the t test results comparing estimates to zero. The same pulse rate production model was also fitted while including individual body size as an additional covariate (see Table A8 in Appendix 3).

Multivariate response of courtship song to treatment

Because multivariate analyses require the different dependent variables to present the same number of observations, we performed the multivariate analysis using only the mean interpulse interval value of the first HRR burst produced (E and M males produced on average 14 bursts of HRR song in a recording; see Fig. A3 in Appendix 2 for more details). Performing such averaging also precludes the need to consider the temporal autocorrelation that exists between successive pulses. To analyse whether interpulse interval and the other courtship traits jointly responded to sexual selection manipulation, we fitted a multivariate LMM on song data. In a multivariate LMM, the different response variables are transformed into a single univariate response variable by creating a vector that considers all observations across the different response variables sequentially (Christensen, 2001). A fixed-effect factor is then used to indicate the correspondence between these observations and the original response variables. We assessed the fixed effects of the mean temperature during a recording and sexual selection treatment on five courtship traits: the mean interpulse interval of the first burst of song, the mean amplitude, the mean intrapulse frequency, the total number of bursts produced and the singing latency (i.e. the time it took a male to produce its first burst of song). All response variables were log-transformed for normalization and then converted to z-scores, to facilitate model convergence. The estimates we provide in the tables correspond to the direct output from the model fit. In the text, we untransformed the estimates back to the original scale of the response variable. To do this, we calculated the exponential of the sum of (1) the product of the standard deviation of the log of the original variable and the corresponding estimate and (2) the log of the mean of the original variable.

The model was fitted using the MCMCglmm package (Hadfield, 2010). We ran MCMC chains for 100 000 iterations (burn-in phase), followed by 5 million iterations during which parameter estimates were sampled every 5000 iterations. This sampling scheme resulted in 1000 recorded estimate values for each parameter and for each model. This was sufficient to ensure that the autocorrelation between successive estimates was always lower than ±0.07. All tests on estimates or quantities derived from estimates (e.g. correlations, see below) for this model are based on the analysis of the distribution of the 1000 records associated with a given parameter. Details about the specification of the prior distributions are given in Appendix 4.

We allowed for the effects of the different covariates to differ between courtship traits. The number of estimated fixed-effect parameters was thus 15 ($[1+2]\times 5$). We estimated the variances and covariances between the response courtship traits using random effects. We computed these covariance matrices for each selection treatment (i.e. $[5 \text{ variances} + 10 \text{ different covariances}] \times 2 = 30$ (co)variances). We also estimated the variance between replicates separately for each courtship trait (i.e. five variances) considering the identity of the replicate as a random effect. We assumed the covariance between model residuals to be null, as no dependence between observations is expected with the random structure considered. The significance of the different fixed effects was extracted from the summary table of the MCMCglmm fit. Here, the P value is computed as twice the minimum between the probabilities that estimates sampled along the MCMC chains are either greater or lower than zero. The same model was also run while including individual body size as an additional covariate (see Table A9 in Appendix 3), with 20 estimated fixed-effect parameters ($[1+1+2]\times 5$).

Estimating the variances and covariances of courtship traits allowed us to calculate the correlations between courtship traits for each treatment. Using this approach offers the advantage of estimating correlations that are not confounded by the variables included in the model as fixed (e.g. temperature) or random effects (e.g. the replicate). We then examined the significance of each individual correlation estimate, and tested for differences between the treatments, to examine whether the associations between courtship traits have changed as a result of sexual selection treatment. The significance test of these correlations was based on the analysis of estimates along the MCMC chains, as explained previously.

In all figures, the mean fixed-effect estimates, hereafter referred as ‘predicted values’ of the mixed models, are represented. Predicted values were adjusted to 22 °C, the temperature at which all populations are maintained. The 95% confidence intervals (CI) were computed for the two univariate LMMs as ±1.96 standard errors around the predicted values, with the standard error being derived from the covariance matrix of parameter estimates for fixed effects. For the GLMM, CIs were computed similarly, but at the scale of the linear predictor (i.e. before the transformation from logit to probabilities). For the multivariate LMM, CIs were computed as quantiles of the posterior distribution of parameter estimates along the MCMC chains. Although technically, intervals obtained this way present statistical properties that can differ from CIs (e.g. Rousset, Gouy, Martinez-Almoyna, & Courtiol, 2017; they are called credibility intervals), we refer to both types as CIs.

Predictions

First, given that energetically costly repeated motor signals are predicted to advertise the signaller's condition (Mowles & Ord, 2012), we expected pulse rate to depend on courtship effort, and thus on the quantity of song already produced by a male. For similar reasons, as motor performance should correlate positively with both temperature and body size, particularly for traits likely to act as indicators of mate condition (Clark, 2012), we also expected pulse rate to be associated with temperature and body size. Then, if pulse rate production has been affected by sexual selection manipulation, we expected to observe faster pulse rates and a shallower slope of decline in pulse rate (i.e. a less pronounced lengthening in interpulse interval) in polyandrous males compared to monogamous males. Finally, for pulse rate to be used as an indicator of motor performance, fast pulse rates should be positively correlated with overall courtship vigour estimates (i.e. here estimated by the other motor courtship traits measured).

Ethical Note

Our design minimized the stress imposed on the individuals used in this experiment. Stress at the larval stage was prevented by controlling for larval density. At adulthood, individuals were transferred in new vials with fresh food and medium adult density. A mouth aspirator was used to gently handle live individuals throughout all the steps of the experiment. The experimental time was only 5 min long, after which flies were anaesthetized with CO_2 and rapidly killed in ethanol for wing measurement.

RESULTS

Differences in HRR Singing Probability Between Treatments

The probability of singing differed significantly between the sexual selection treatments (Table 1), with monogamous males having a lower probability of singing than polyandrous males (Fig. 2).

Analysis of HRR Pulse Rate Production Over Time

This analysis, based on all bursts produced, identified changes in interpulse interval variation between, and within, bouts of courtship. The interpulse interval value lengthened between consecutive bursts, meaning that the rate at which males produced pulses decreased more and more as the male produced song (Table 2, Fig. 3). Interpulse interval also lengthened within a burst, meaning that pulse rate progressively decreased during a burst too (Table 2, Fig. 4).

Temperature

As expected, temperature was strongly associated with courtship traits (Tables 2, 3). Interpulse interval was negatively associated with temperature, meaning that interpulse interval was longer at lower temperatures, as indicated by both the pulse rate production LMM and the multivariate LMM. In the latter case, interpulse interval shortened by 0.32 ms (95% CI = −0.07 − −0.57, $P = 0.012$) when temperature increased by 1 °C (Table 3).

The pulse rate production LMM (Table 2, and see Appendix 3 Table A8 for its equivalent with body size included) also showed that the progressive shortening observed in interpulse interval was strongly correlated with temperature, with lower recording temperatures being associated with an even more pronounced lengthening in interpulse interval over courtship time (i.e. a steeper decrease in pulse rate; Fig. 5a).

Body size

Males from polyandrous lines were larger on average than males from monogamous lines (Table 1, Fig. 5b). When body size was included in the pulse rate production LMM (Appendix 3 Table A8), we observed a negative effect of body size on interpulse interval. Body size significantly influenced interpulse interval both within and between bursts, meaning that larger males produced song with a shorter interpulse interval and maintained this short interpulse interval for a longer time than smaller males (Fig. 5c, Appendix 3 Table A8).

The multivariate LMM also revealed that, when body size was included in the model, interpulse interval shortened with increasing body size, with an increase in wing size of 1 standard deviation being associated with a reduction of 0.31 ms in interpulse interval (95% CI = −0.03 − −0.62, $P = 0.046$; Appendix 3 Table A9). Amplitude increased with increasing body size as well, with an increase in wing size of 1 standard deviation being associated with an increase in amplitude of 11.8 units (95% CI = 2.2–21.4, $P = 0.024$; Appendix 3 Table A9).

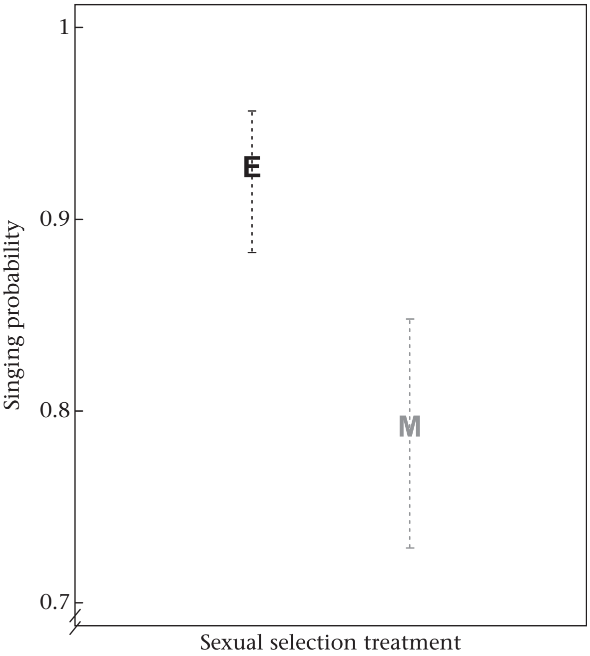

Figure 2. Differences between the sexual selection treatments in singing probability (the probability of producing high-repetition rate song). Model estimates are given in Table 1. The letters represent the fitted values predicted by the mixed model depending on male sexual selection treatment (E = polyandrous males, M = monogamous males). Dashed lines show 95% confidence intervals.

Evolutionary Response to Sexual Selection Manipulation

The pulse rate production LMM showed a significant effect of sexual section treatment on interpulse interval, with polyandrous males producing a shorter interpulse interval (i.e. a faster pulse rate) than monogamous males (Table 2, Figs 3 and 4). The model also showed a significant interaction between sexual selection treatment and the quantity of song already produced by a male (i.e. the burst and pulse positions in the courtship sequence), showing that the decrease in pulse rate in polyandrous males was shallower than in monogamous males. Therefore, pulse rate differed between the sexual selection treatments, and this difference gradually widened the more males beat their wings to produce song.

Although body size was significantly different between the sexual selection treatments, including body size in the pulse rate

Table 1
Summary tables for the fitted GLMM analysing HRR singing probability and the univariate LMM analysing body size

Model parameters	Factor level	HRR singing probability				Body size			
		β^a	Lower CI	Upper CI	*P*	β	Lower CI	Upper CI	*P*
Treatment	E	1.20	0.56	1.85	<0.001	0.023	0.010	0.035	<0.001
Intercept		1.35	0.98	1.71	<0.001	1.08	1.07	1.09	<0.001
Inter-replicate variance		0.038				0.000066			
Residual variance		–				0.00062			

In both models, sexual selection treatment was tested as a fixed effect, and replicate was included as a random effect. The following elements are specified: the model estimate of each variable (β), the lower and upper limit of the estimate's 95% confidence interval (CI), and *P* value of the test comparing the estimate to zero (*P*). HRR = high-repetition rate song, Treatment = sexual selection treatment (E = polyandrous; the monogamous treatment M was used as the reference level). $N = 471$ recordings for HRR probability and $N = 355$ recordings for body size.

[a] Given that the HRR singing probability GLMM used a binomial error distribution, the given estimates for this model are on a logit scale.

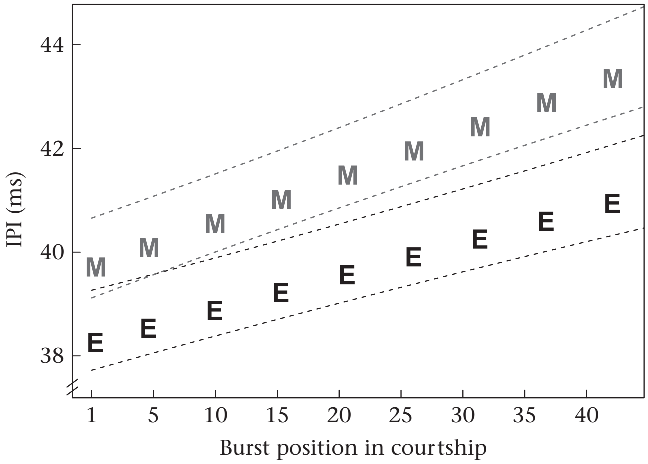

Figure 3. Changes in interpulse interval production in high-repetition rate song along bursts in the courtship sequence, depending on sexual selection treatment, as predicted by the fitted univariate pulse rate production LMM. The figure shows the changes in the mean interpulse interval value during a 40-burst courtship sequence of song for monogamous (grey) and polyandrous (black) males. Model estimates are given in Table 2. The letters represent the fitted values predicted by the mixed model depending on male sexual selection treatment (E = polyandrous males, M = monogamous males). Dashed lines show 95% confidence intervals.

production LMM showed a difference between the sexual selection treatments that was independent from the effect mediated by body size, with polyandrous males showing again more endurance than monogamous males (Appendix 3 Table A8).

Analysis of the Associations Between Courtship Traits

The multivariate LMM showed that polyandrous males started to produce song earlier than monogamous males (mean difference in song latency: 5.7 s; 95% CI = 3.5–7.7, $P = 0.001$; Table 3). All other courtship traits did not show a significant difference between the sexual selection treatments (Table 3).

Table 4 presents the correlations (r) between courtship traits for each sexual selection treatment extracted from the fit of the multivariate LMM (Table 3), while Table 5 examines whether these associations differed between the sexual selection treatments ($r_{E}–r_{M}$). The equivalents of these two tables for the multivariate LMM with body size included as a covariate are shown in Appendix 3 (Tables A10 and A11). Two of the 10 correlations between courtship traits changed as a result of selection (Fig. 6, Table 5), and both were associated with interpulse interval. Interpulse interval was correlated with almost all other courtship traits in the polyandry treatment (i.e. with amplitude, latency and the total number of bursts produced; Table 4), but only with amplitude in the monogamy treatment. Faster pulse rates were thus associated with shorter singing latencies, louder songs and more bursts produced.

The multivariate LMM identified three significant associations between courtship traits that did not differ between sexual selection treatments (compare Tables 4 and 5). In addition to the correlation between interpulse interval and amplitude, it found similar associations between the sexual selection treatments between intrapulse frequency and amplitude, and between latency and the total number of bursts produced (Table 4). The last associations found were a small positive correlation between amplitude and the total number of bursts produced, and between amplitude and latency; however, these were only significant for polyandrous males, and did not differ significantly between the sexual selection treatments (Table 5).

DISCUSSION

In this study, we tested the hypothesis that mate choice influences the evolution of motor performance and predicted improved motor performance in populations subjected to more intense sexual selection. We performed a detailed analysis of the production of *D. pseudoobscura* song pulse rate, a motor signal

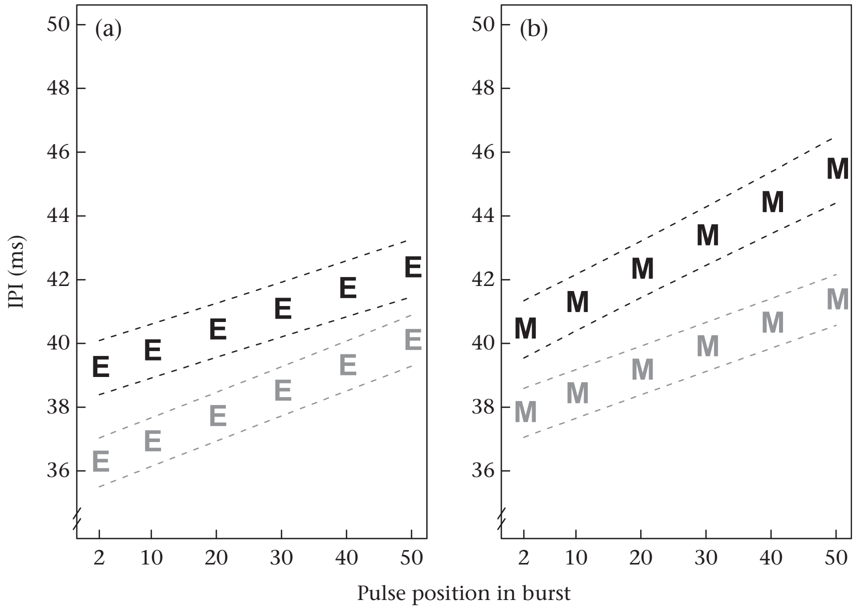

Figure 4. Changes in interpulse interval production in high-repetition rate song along pulses within a burst, depending on sexual selection treatment, as predicted by the fitted univariate pulse rate production LMM. The figure shows the changes in individual interpulse interval values along pulses at the beginning of courtship (burst 1; grey) and after 40 bursts of song (burst 40; black), for males of (a) polyandrous and (b) monogamous males. Model estimates are given in Table 2. The letters represent the fitted values predicted by the mixed model depending on male sexual selection treatment (E = polyandrous males, M = monogamous males). Dashed lines show 95% confidence intervals.

Table 2
Summary table for the fitted univariate LMM analysing the HRR pulse rate production between and within HRR bursts

Model parameters	Factor level	β	Lower CI	Upper CI	*P*
Treatment	E	−1.57	−2.64	−0.501	0.026
Temperature		−0.71	−0.81	−0.62	<0.001
BP		0.69	0.51	0.87	<0.001
PP		0.92	0.87	0.98	<0.001
Treatment∗BP		−0.10	−0.32	0.11	0.339
Temperature∗BP		−0.30	−0.40	−0.20	<0.001
Treatment∗PP		−0.093	−0.17	−0.018	0.013
BP∗PP		0.11	0.039	0.17	0.002
Treatment∗BP∗PP		−0.16	−0.24	−0.076	<0.001
Intercept		39.27	38.52	40.01	<0.001
Inter-replicate variance		0.56			
Interburst variance (nested within replicate)		3.47			
Residual variance		4.92			

The following elements are specified: the model estimate of each variable (β), the lower and upper limit of the estimate's 95% confidence interval (CI), and the *P* value of the test comparing the estimate to zero (*P*). BP = burst position, PP = pulse position, HRR = high-repetition rate song, IPI = interpulse interval, treatment = sexual selection treatment (E = polyandrous; the monogamous treatment M was used as the reference level), burst position = the position of the burst in the recording, pulse position = the position of the pulse in the HRR burst. The autocorrelation parameters are $\varphi_1 = 0.23$, $\varphi_2 = 0.12$, $\varphi_3 = 0.058$ and $\varphi_4 = 0.031$. $N = 35\,206$ individual interpulse interval values. The same model was fitted with body size as a covariate (Appendix Table A8).

Table 3
Summary table for the fitted multivariate LMM analysing HRR traits

Trait	Model parameters	Factor level	β	Lower CI	Upper CI	*P*
IPI	Treatment	E	−0.49	−1.10	0.12	0.092
	Temperature		−0.14	−0.24	−0.028	0.012
	Intercept		3.41	1.14	6.10	0.010
Amplitude	Treatment	E	−0.21	−0.57	0.18	0.262
	Temperature		0.083	−0.032	0.188	0.158
	Intercept		−1.79	−4.24	0.78	0.176
Frequency	Treatment	E	−0.196	−0.771	0.43	0.446
	Temperature		0.076	−0.024	0.20	0.172
	Intercept		−1.65	−4.20	0.89	0.218
Total number of bursts	Treatment	E	0.21	−0.31	0.74	0.398
	Temperature		0.047	−0.078	0.15	0.414
	Intercept		−1.16	−3.84	1.45	0.378
Latency	Treatment	E	−0.51	−0.77	−0.25	0.001
	Temperature		0.080	−0.061	0.19	0.192
	Intercept		−1.56	−4.25	1.39	0.254

The following elements are specified: the model estimate of each variable (β; here the posterior mean), the lower and upper limits of the estimate's 95% credibility interval (CI), and the *P* value of the test comparing the estimate to zero (*P*). IPI = interpulse interval, treatment = sexual selection treatment (E = polyandrous; the monogamous treatment M was used as the reference level), latency = the time taken to sing the first burst of HRR song. Covariances between the response variables of the model are provided as correlations in Table 4. Estimated variances between replicates were $\sigma^2 = 0.17$ for IPI, $\sigma^2 = 0.04$ for amplitude, $\sigma^2 = 0.14$ for intrapulse frequency, $\sigma^2 = 0.12$ for the total number of bursts and $\sigma^2 = 0.01$ for latency. Note that all responses are expressed as z-scores of the log-transformed value of the original measurements, but temperature was not altered. Estimates in the table are thus not expressed on the original data scale, but in z-scores of log values (see Methods). The means and standard deviations of the log of the original variables are as following: IPI (mean = 3.63, $\sigma = 0.06$), amplitude (mean = 5.85, $\sigma = 0.20$), frequency (mean = 5.56, $\sigma = 0.09$), total number of bursts (mean = 2.35, $\sigma = 0.84$), latency (mean = 9.38, $\sigma = 1.32$). $N = 280$ recordings. The same model was fitted with body size as a covariate (Table A9).

under mate choice in this species. We also analysed the response of this motor signal to sexual selection manipulation via experimental evolution. We showed that song pulse rate decreased with the amount of song a male had already produced, and was associated with body size and recording temperature, indicating a potential for pulse rate to act as an indicator of male condition. Consistent with this, manipulating the opportunity for sexual selection led to the evolution of faster pulse rates and improved song production endurance in males from polyandrous lines compared to males from monogamous lines. Finally, we showed that pulse rate was correlated with estimates of overall courtship vigour, particularly in polyandrous males. In total, these results suggest that selection on song pulse rate by females led to the evolution of increased courtship vigour displayed during courtship, indicating a potential correlated response of overall male motor capacities.

Pulse Rate Production

Our results show that pulse rate was not constant over courtship duration, but progressively declined as a male continued to beat his wings. Although this pattern has previously been reported in the courtship songs of two other *Drosophila* species (*D. melanogaster*: Bernstein, Neumann, & Hall, 1992; Dow, 1978; Ewing, 1983; Wilson, Burnet, Eastwood, & Connolly, 1976; *D. simulans*: Bernstein et al., 1992), its relevance and implications for sexual selection have not yet been considered. In *D. pseudoobscura*, pulse rate appeared to decrease progressively both within a burst of song and along the burst sequence. The pattern observed in our study suggests that males start producing song with a fast pulse rate but cannot sustain this as courtship progresses (and particularly for males that evolved under monogamy conditions). Repetitive signals are thought to provide a useful measure of mate quality to the receivers, both via the average rate at which they are produced and via the variation in this rate (i.e. increase or decrease) over courtship time (Kotiaho et al., 1998; Mowles & Ord, 2012). As producing a song with a fast pulse rate can be a physically challenging task, requiring both sustained motor power and motor endurance and thus pushing males to their maximum capacities, variation in the ability of males to maintain a given pulse rate over time has the potential to accurately reflect mate condition.

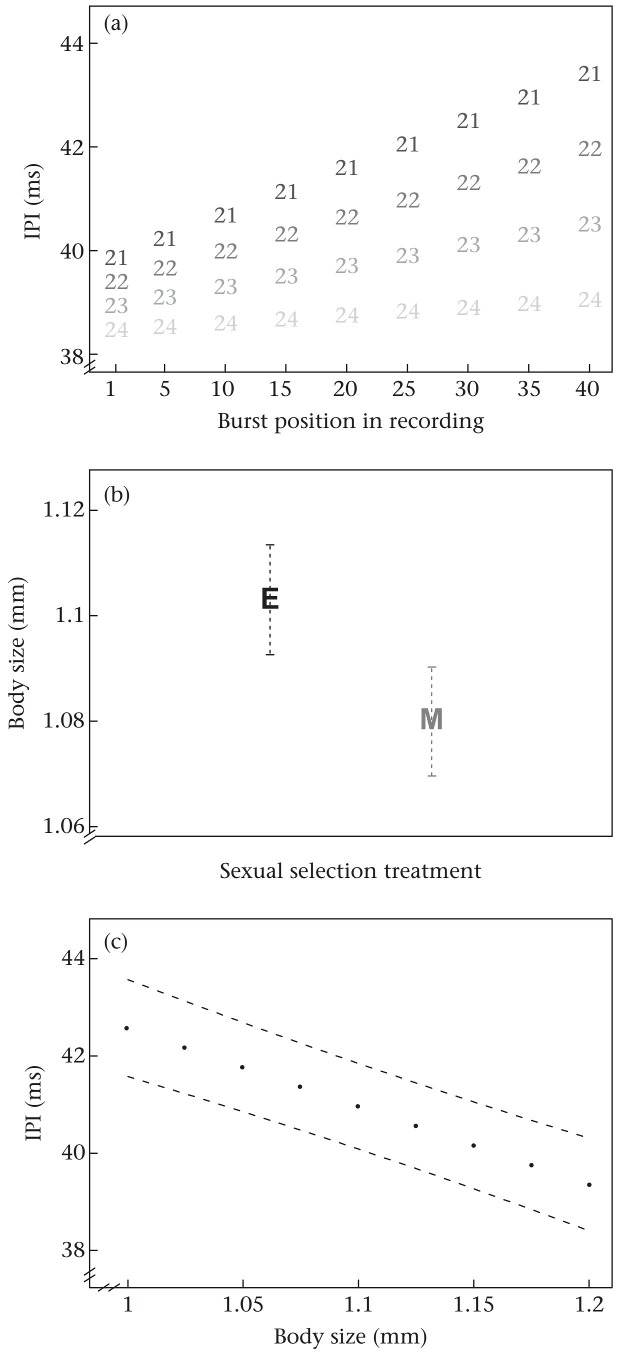

Figure 5. Body size and temperature effects on interpulse interval production in high-repetition rate song, as predicted by the fitted univariate pulse rate production and body size LMMs: (a) the effect of recording temperature variation on interpulse interval variation along bursts (estimated for four recording temperatures: 21, 22, 23 and 24 °C); (b) the average body size difference between the treatments; and (c) the effect of body size on interpulse interval. Model estimates for (a) and (c) were extracted from the univariate pulse rate production LMM that included body size as a covariate (Table A8), while (b) is based on the univariate body size LMM presented in Table 1. The symbols represent the fitted values predicted by the mixed models depending on male sexual selection treatment (E = polyandrous males, M = monogamous males), body size (circles) or temperature (21, 22, 23 and 24). Dashed lines show 95% confidence intervals.

Pulse rate production was associated with both temperature and body size variation. The effect of temperature on acoustic signalling is common through its effect on metabolic rate and has already been shown in many species (Gillooly et al., 2001; Gillooly & Ophir, 2010), including *Drosophila* species (Noor & Aquadro, 1998; Ritchie & Gleason, 1995; Ritchie & Kyriacou, 1994; Ritchie et al., 2001). In addition to this effect, we showed that temperature was associated not only with mean pulse rate, but also with pulse rate decrease over time, indicating that both power output and endurance are temperature dependent. These results suggest that pulse rate probably relies strongly on male physiological state (Lailvaux & Irschick, 2006). Pulse rate also correlated positively with body size, this effect being unsurprising as motor power often covaries positively with body size (Biewener, 2003; Carrier, 1996). The influence of body size on pulse rate has rarely been investigated in *Drosophila* species, sometimes only via correlations between body size and ‘raw’ pulse rate data (i.e. not temperature corrected), which failed to find an association between size and rate (Hoikkala, Aspi, & Suvanto, 1998; Partridge, Ewing, & Chandler, 1987). The positive influence of body size on pulse rate found here indicates that larger males are able to produce a faster pulse rate than smaller males, suggesting that body size influences motor power. This effect could potentially be due to variation in thoracic muscle size, and lead to a higher power output (i.e. a faster pulse rate) of larger males. Indeed, thorax volume is positively correlated with flight wing beat frequency in *D. melanogaster* (Curtsinger & Laurie-Ahlberg, 1981). Overall, these results suggest pulse rate can reflect both male motor power and endurance to females, potentially making it an evolutionary driver of overall male motor capacities (Clark, 2012).

Effect of Sexual Selection on Courtship Song Evolution

Males from polyandrous lines were not only more likely to produce song and produce song faster, but also to maintain a fast rate for longer than males from the monogamous lines, demonstrating that manipulating sexual selection had a significant impact on male motor performance during courtship. Our results are consistent with previous work suggesting that polyandrous females prefer faster male pulse rates (Debelle et al., 2014; Williams et al., 2001). Signals with an increased energy content have been shown to be under directional female preference in several species (e.g. in frogs: Gerhardt & Brooks, 2009; Ryan, 1988; in wolf spiders: Shamble, Wilgers, Swoboda, & Hebets, 2009; in crickets: Simmons, Thomas, Simmons, & Zuk, 2013), with females typically preferring louder song, higher calling rate and higher pulse repetition rate (Clark, 2012; Mowles & Ord, 2012). The fact that the ability to sustain a fast pulse rate was affected by sexual selection manipulation suggests that selection by females towards fast pulse rates led to the evolution of males delivering songs with increased motor power and sustained intensity (i.e. more endurance), and indicates that pulse rate may be used as an indicator of male motor performance by females.

We found, after ca. 110 generations of selection, a difference in average pulse rate in the same direction as in the preliminary song study (conducted after 30 generations of selection; Snook et al., 2005). The comparable difference in pulse rate between males from polyandrous and monogamous lines after a further 80 generations of selection (1.54 ms between the polyandry and monogamy treatments in Snook et al., 2005; 1.57 ms in the current study, see Table 2) could indicate that pulse rate evolution has reached stable equilibrium conditions between sexual and viability selection (Hine, McGuigan, & Blows, 2011; Kirkpatrick, 1996), but could also mean that genetic variation for faster pulse rates has been depleted in the polyandrous lines. Two studies using artificial selection on pulse rate in *D. melanogaster* showed a lower evolutionary response towards faster pulse rates (Ritchie & Kyriacou, 1996; Turner & Miller, 2012), suggesting reduced expressed genetic variation for fast pulse rates in this environment, an expected result if selection has persistently acted in this direction.

Table 4
Correlation matrix between courtship traits for the two sexual selection treatments

	IPI		Amplitude		Frequency		Bursts		Latency	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
IPI	1	–	−0.41	<0.001	−0.11	0.192	−0.38	<0.001	0.28	0.002
Amplitude	−0.31	<0.001	1	–	−0.29	<0.001	0.24	0.008	−0.17	0.038
Frequency	0.07	0.470	−0.42	<0.001	1	–	0.20	0.028	−0.09	0.298
Bursts	0.09	0.354	0.07	0.410	−0.04	0.656	1	–	−0.29	<0.001
Latency	−0.05	0.594	−0.06	0.504	−0.06	0.544	−0.39	<0.001	1	–

The following elements are specified: the correlation coefficient (*r*) and the *P* value (*P*). HRR = high-repetition rate song, IPI = interpulse interval, bursts = the total number of HRR bursts produced, latency = the time taken to sing the first burst of HRR song. These correlations were derived from the variances and covariances estimated by the multivariate LMM (see Table 3). Since correlation matrices are symmetric, correlation values for polyandrous males and monogamous males are shown above and below the diagonal, respectively.

Table 5
Differences in courtship trait correlations between the sexual selection treatments (r_{E}–r_{M} from Table 4)

	IPI		Amplitude		Frequency		Bursts		Latency	
	r_{E}–r_{M}	*P*	r_{E}–r_{M}	*P*	r_{E}–r_{M}	*P*	r_{E}–r_{M}	*P*	r_{E}–r_{M}	*P*
IPI	–	–								
Amplitude	−0.10	0.358	–	–						
Frequency	−0.18	0.144	0.13	0.242	–	–				
Bursts	−0.47	<0.001	0.16	0.188	0.24	0.090	–	–		
Latency	0.33	0.008	−0.11	0.344	−0.03	0.814	0.10	0.402	–	–

The following elements are specified: the corresponding correlation coefficients for polyandrous (r_{E}) and monogamous males (r_{M}) and the *P* value (*P*). HRR = high-repetition rate song, IPI = interpulse interval, bursts = the total number of HRR bursts produced, latency = the time taken to sing the first burst of HRR song.

Male body size responded to the variation in sexual selection opportunity, with males from the polyandrous lines being overall larger than males from the monogamous lines. Body size commonly responds to precopulatory sexual selection among species (Andersson, 1996; Blanckenhorn, 2000; Thornhill & Alcock, 1983) and affects male mating success in several *Drosophila* species (including *D. pseudoobscura*), with larger males winning more aggressive encounters, delivering more courtship and mating faster (Ewing, 1961; Partridge, Ewing et al., 1987; Partridge & Farquhar, 1983; Partridge, Hoffmann, & Jones, 1987). As body size also influences pulse rate, any pulse rate difference between the experimental evolution treatments could thus be explained by size differences. However, even after controlling for the effects of body size (see Appendix 1 and 3), sexual selection treatments still differed in their pulse rate production pattern, indicating that traits other than body size had diverged between the treatments and contributed to the differences in motor signalling between them.

Mate Choice Driving the Evolution of Motor Performance

Males from polyandrous lines were more vigorous than males from monogamous lines. Indeed, males from polyandrous lines have an enhanced mating capacity and a higher courtship frequency relative to males from monogamous lines (Crudgington, Fellows, Badcock, & Snook, 2009; Crudgington, Fellows, & Snook, 2010). Our study also showed that males from polyandrous lines started producing song faster, produced a faster pulse rate, and had a higher endurance than males from monogamous lines. In theory, male–male competition could contribute to this observed increase in male motor capacities. A direct effect of male–male competition on the evolution of pulse rate seems unlikely, however, as courtship song is a near-field sound (Bennet-Clark, 1971, 1998) and pulse rate value does not affect other males' behaviour (Talyn & Dowse, 2004). Conversely, pulse rate affects male mating success in no-choice assays in this species (Williams et al., 2001), coevolved with female preference for pulse rate in our experimental lines (Debelle et al., 2014) and is correlated with other courtship motor traits. This suggests that the action of mate choice on pulse rate is actively involved in the observed evolutionary motor changes in our experimental lines.

Drosophila courtship song has so far only been linked to non-motor selective benefits (i.e. high intrapulse frequency in *D. montana* is associated with higher male mating success and higher offspring survival; Hoikkala et al., 1998; Ritchie et al., 2001). Our results support the idea that *Drosophila* courtship song could also signal motor performance. Although our analysis focused on courtship-related traits, motor performance expressed during courtship is likely to reflect an individual's overall motor performance (Byers et al., 2010; Clark, 2012; Lailvaux & Irschick, 2006; Oufiero & Garland, 2007). In a context of strong sexual selection, the selection of fast-singing males by females could thus also influence the evolution of other motor characteristics (e.g. flying ability, competitive ability, etc.; Byers et al., 2010).

Contrary to what we observed in the polyandrous lines, reducing the opportunity for sexual selection in the monogamous lines was associated with lower singing probability, a longer singing latency and the inability to maintain a fast pulse rate. This suggests that these traits are costly and could be selected against in the absence of mating competition. Males from monogamous lines also had a lower courtship frequency than males from polyandrous lines (Crudgington et al., 2010). As courting (without mating) has been shown to reduce male longevity in *D. melanogaster* (Cordts & Partridge, 1996), these results overall suggest that intense courtship song could impose an important fitness cost to males in a monogamous context, which may have resulted in the reduced investment in courtship song observed in populations under relaxed sexual selection (Crudgington et al., 2005, 2010).

In conclusion, our results suggest that the pulse rate has the potential to be an indicator of male condition to females, and that the action of female choice on this motor signal affected male motor performance during courtship in our replicated experimental populations. In natural populations, female selection of male courtship motor performance could thus have an

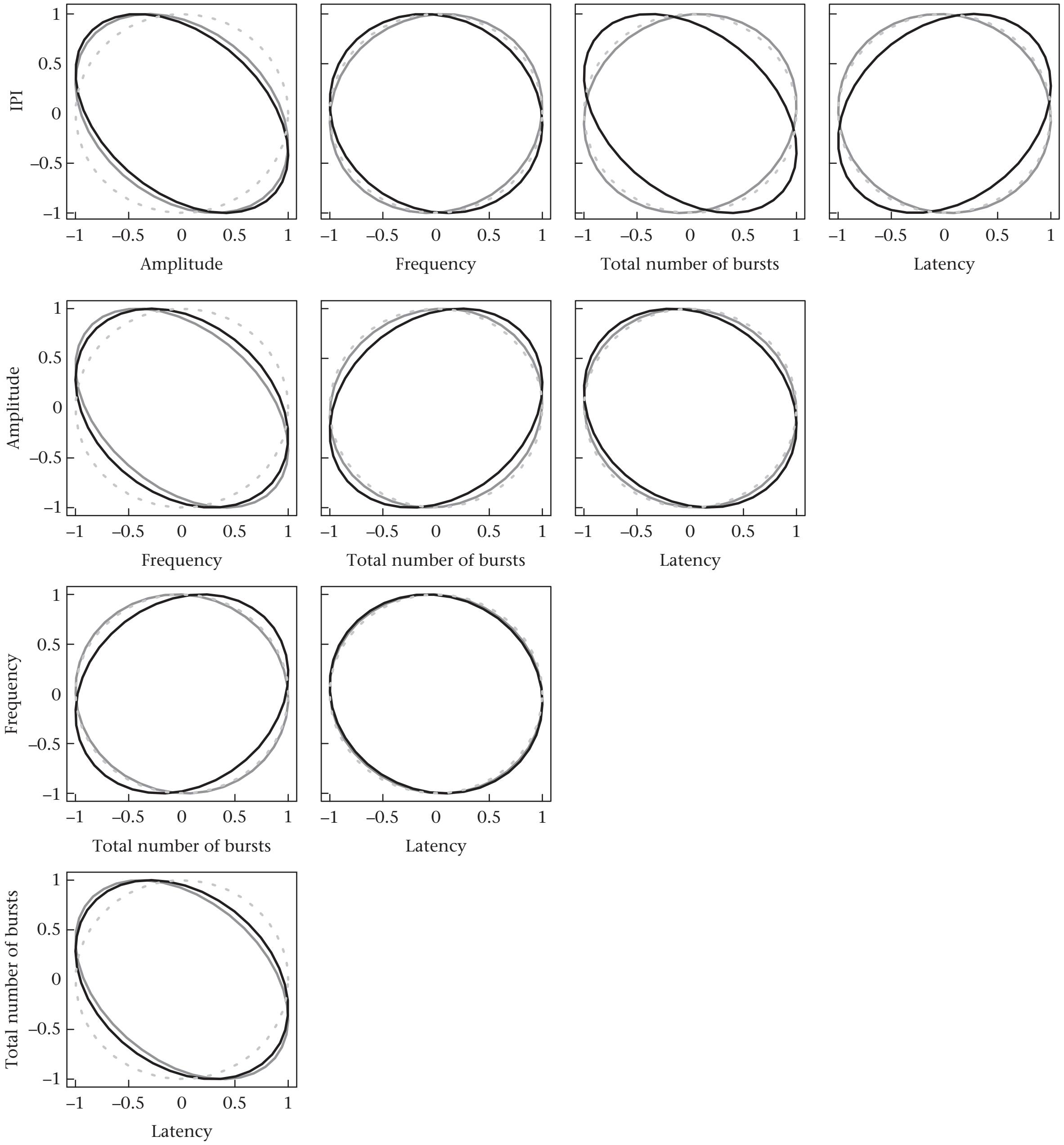

Figure 6. Correlation ellipses between courtship traits for polyandrous (black) and monogamous (dark grey) males. This figure is a graphical representation of the correlation values provided in Table 4. The dotted light grey circle represents a null correlation ($r = 0$). The stronger the correlation, the narrower the ellipse becomes.

impact on the evolution of motor performance exhibited in contexts other than courtship. This work contributes to the limited number of studies providing evidence that sexual selection via mate choice of motor signals may also drive the evolution of mate motor performance (Byers et al., 2010; Mowles & Ord, 2012). Further work in this and other systems should quantify the selective benefits gained by an increased motor performance in mating and nonmating contexts (e.g. standard locomotion, foraging, escaping predators), and investigate what evolutionary changes lead to enhanced motor signals (e.g. morphological, anatomical, physiological), to gain a better understanding of the influence of sexual selection on the evolution of motor performance.

Acknowledgments

We are grateful to Paris Veltsos for advice on recording *Drosophila* courtship song and Bill Heitler for his DataView script allowing the extraction of song parameters. This work was funded by the Marie Curie Initial Training Network ‘Understanding the evolutionary origin of biological diversity’ (ITN-2008- 213780 SPECIATION; M.G.R.), and by a US National Science Foundation grant (R.R.S.) (DEB-0093149) and NERC grants (R.R.S. and M.G.R.; NE/B504065/1, NE/D003741/1, NE/I014632/1). We thank all the participants of this network for fruitful comments and discussions about this project, and particularly Anneli Hoikkala for her helpful insight throughout the project.

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Appendix I. LRR Song Analyses

Methods

LRR song was analysed similarly to HRR song. To distinguish between interpulse and interburst intervals, an upper threshold was also determined visually by plotting the distribution of the duration between two pulses (LRR threshold = 482 ms, the mean LRR interpulse interval is approximately 220 ms in our populations; Snook et al., 2005).

Differences in singing probability between treatments

The difference in the probability of singing LRR (i.e. the probability of a male producing at least a single pulse of LRR) between the sexual selection treatments was investigated using a Fisher's exact test on the pooled replicates, due to the distribution of LRR data (as polyandrous males always produced LRR in all replicates, but monogamous males do not, model parameters could not be estimated by a linear model as maximum likelihood estimates do not exist for this particular pattern of data; Albert & Anderson, 1984).

Multivariate response of courtship song to treatment

LRR interpulse interval did not vary over the length of courtship (see Table A1), and therefore values were averaged over the entire length of each recording, and the resulting mean LRR interpulse interval was used for statistical modelling. We fitted a multivariate LMM on LRR song traits with the same structure as that for HRR song, to test for a response of the mean LRR interpulse interval (of the entire recording in this case), the mean LRR intrapulse frequency, the total number of LRR pulses produced and the LRR singing latency to sexual selection manipulation (Table A2). The number of estimated fixed-effect parameters were 12 ($[1 + 2] \times 4$), with $[4 + 6] \times 2 = 20$ (co)variance parameters. We also estimated the variance between replicates separately for each trait (four variances) as random effects.

We also tested the significance of correlations between song traits (i.e. LRR interpulse interval, LRR intrapulse frequency, the total number of LRR pulses produced and LRR latency), as well as the differences in song trait correlations between sexual selection treatments.

The same model was also run with individual body size as a covariate (Table A3). The number of estimated fixed-effect parameters was this time 16 ($[1 + 1 + 2] \times 4$), with $[4 + 6] \times 2 = 20$ (co) variance parameters.

Results

Differences in LRR singing probability between treatments

The probability of singing LRR song differed between treatments, with monogamous males having a lower probability than polyandrous males (Fisher's exact test; polyandrous males: 0 recordings without LRR song out of 231 recordings; monogamous males: 11 recordings without LRR song out of 230 recordings; $P < 0.001$).

Evolutionary response to sexual selection manipulation

The multivariate LMM did not identify any significant response of LRR traits to selection sexual treatment (Table A2).

Temperature

The multivariate LMM showed that LRR interpulse interval shortened with temperature (Table A3). Increasing temperature by one degree reduced the interpulse interval by 3.28 ms (95% CI = −0.67 − −6.05, $P = 0.02$). Both LRR singing latency and LRR intrapulse frequency significantly increased with temperature, with an increase in temperature of one degree resulting in a latency increase of 14.5 s (95% CI = 302–2558, $P = 0.01$), and an LRR intrapulse frequency increase of 4.18 Hz (95% CI = 1.20–7.18, $P = 0.01$).

Body size

Including body size in the multivariate LMM did not change the results of the model (Table A3). LRR interpulse interval shortened with body size, with an increase of 1 standard deviation in wing size being associated with an interpulse interval reduction of 4.72 ms (95% CI = −1.52 − −7.53, $P = 0.004$).

Analysis of the associations between courtship traits

The multivariate LMM also revealed that LRR interpulse interval was positively correlated with the total number of LRR pulses produced (Table A4), but the correlation reached significance only for monogamous males. As with HRR song, we found a significant negative association between LRR latency and the total number of LRR pulses produced for both treatments (Table A4). There was no significant impact of the sexual selection treatment on the correlations between LRR traits (Table A5, Fig. A1).

Including body size in the multivariate LMM generated very similar results (Tables A6, A7).

Table A1

Correlations between LRR IPI and courtship duration in eight randomly chosen songs, one from each of the eight replicated populations

Song	*r*	*P*
M1	0.081	0.438
M2	0.19	0.365
M3	0.36	0.113
M4	−0.17	0.437
E1	−0.30	0.161
E2	0.023	0.904
E3	0.28	0.235
E4	−0.16	0.395

The Spearman correlation coefficient (r) and its associated *P* value (*P*) are given. E = polyandrous, M = monogamous, 1–4 refers to the replicate population of the song example.

Table A2

Summary table for the fitted multivariate LMM analysing LRR song

Song trait	Model parameters	Factor level	β	Lower CI	Upper CI	*P*
IPI	Treatment	E	−0.080	−0.71	0.54	0.798
	Temperature		−0.11	−0.20	−0.016	0.022
	Intercept		2.59	0.39	4.68	0.020
Frequency	Treatment	E	−0.27	−0.85	0.24	0.246
	Temperature		0.14	0.044	0.23	0.001
	Intercept		−2.95	−5.47	−0.92	0.006
Total number of pulses	Treatment	E	0.14	−0.083	0.39	0.220
	Temperature		−0.077	−0.16	0.027	0.094
	Intercept		1.69	−0.68	3.63	0.11
Latency	Treatment	E	−0.25	−0.60	0.19	0.206
	Temperature		0.13	0.019	0.21	0.010
	Intercept		−2.75	−4.80	−0.35	0.014

The following elements are specified: the model estimate of each variable (β; here the posterior mean), the lower and upper limits of the estimate's 95% credibility interval (CI), and the *P* value of the test comparing the estimate to zero (*P*). IPI = interpulse interval, treatment = sexual selection treatment (E = polyandrous; the monogamous treatment M was used as the reference level), latency = the time taken to sing the first pulse of LRR song. Covariances between the response variables of the model are provided as correlations in Table A4. Estimated variances between replicates were $\sigma^2 = 0.20$ for IPI, $\sigma^2 = 0.12$ for intrapulse frequency, $\sigma^2 = 0.01$ for the total number of bursts and $\sigma^2 = 0.06$ for latency. Note that all responses are expressed as z-scores of the log-transformed value of the original measurements, but temperature was not altered. Estimates in the table are thus not expressed on the original data scale, but in z-scores of log values (see Methods). The means and standard deviations of the log of the original variables are as following: IPI (mean = 5.34, σ = 0.14), frequency (mean = 6.19, σ = 0.06), total number of pulses (mean = 3.36, σ = 0.78), latency (mean = 8.90, σ = 1.38). $N = 415$ recordings. The same model was fitted with body size as a covariate (Table A3).

Table A3

Summary table for the fitted multivariate LMM analysing LRR song (with body size included in the model)

Song trait	Model parameters	Factor level	β	Lower CI	Upper CI	*P*
IPI	Treatment	E	0.056	−0.61	0.67	0.838
	Temperature		−0.11	−0.21	−0.023	0.020
	Body size		−0.16	−0.26	−0.052	0.004
	Intercept		2.58	0.30	4.72	0.026
Frequency	Treatment	E	−0.24	−0.82	0.26	0.298
	Temperature		0.14	0.039	0.23	0.010
	Body size		0.0040	−0.096	0.11	0.922
	Intercept		−3.01	−5.29	−0.84	0.012
Total number of pulses	Treatment	E	0.068	−0.17	0.31	0.592
	Temperature		−0.077	−0.17	0.019	0.096
	Body size		0.10	−0.0090	0.20	0.082
	Intercept		1.71	−0.58	3.72	0.106
Latency	Treatment	E	−0.26	−0.63	0.13	0.192
	Temperature		0.13	0.029	0.22	0.001
	Body size		0.014	−0.087	0.12	0.814
	Intercept		−2.82	−4.83	−0.58	0.008

The following elements are specified: the model estimate of each variable (β; here the posterior mean), the lower and upper limits of the estimate's 95% credibility interval (CI), and the *P* value of the test comparing the estimate to zero (*P*). IPI = interpulse interval, treatment = sexual selection treatment (E = polyandrous; the monogamous treatment M was used as the reference level), latency = the time taken to sing the first pulse of LRR song. Covariances between the response variables of the model are provided as correlations in Table A6. Estimated variances between replicates were $\sigma^2 = 0.23$ for IPI, $\sigma^2 = 0.13$ for intrapulse frequency, $\sigma^2 = 0.01$ for the total number of bursts and $\sigma^2 = 0.06$ for latency. Note that all responses are expressed as z-scores of the log-transformed value of the original measurements. Wing size was also transformed into z-scores, but temperature was not altered. Estimates in the table are thus not expressed on the original data scale, but in z-scores of log values (see Methods). The means and standard deviations of the log of the original variables are as following: IPI (mean = 5.34, σ = 0.14), frequency (mean = 6.19, σ = 0.06), total number of pulses (mean = 3.36, σ = 0.78), latency (mean = 8.90, σ = 1.38). $N = 415$ recordings.

Table A4
Correlation matrix between LRR song traits for the two sexual selection treatments

	IPI		Frequency		Pulse number		Latency	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
IPI	1	–	−0.14	0.088	0.07	0.38	0.06	0.428
Frequency	−0.14	0.06	1	–	0.06	0.466	−0.01	0.900
Pulse number	0.22	0.002	0.03	0.650	1	–	−0.23	0.001
Latency	0.07	0.358	−0.06	0.512	−0.35	<0.001	1	–

The following elements are specified: the correlation coefficient (r) and the *P* value (*P*). LRR = low-repetition rate song, IPI = interpulse interval, latency = the time taken to sing the first pulse of LRR song. These correlations were derived from the variances and covariances estimated by the multivariate LMM (see Table A2). Since correlation matrices are symmetric, correlation values for polyandrous males and monogamous males are shown above and below the diagonal, respectively.

Table A5
Differences in LRR song trait correlations between the sexual selection treatments (r_{E}–r_{M} from Table A4)

	IPI		Frequency		Pulse number		Latency	
	r_{E}–r_{M}	*P*	r_{E}–r_{M}	*P*	r_{E}–r_{M}	*P*	r_{E}–r_{M}	*P*
IPI	–	–						
Frequency	0.00	0.988	–	–				
Pulse number	−0.15	0.152	0.03	0.802	–	–		
Latency	−0.01	0.978	0.04	0.684	0.12	0.224	–	–

The following elements are specified: the corresponding correlation coefficients for polyandrous (r_{E}) and monogamous males (r_{M}) and the *P* value (*P*). LRR = low-repetition rate song, IPI = interpulse interval, latency = the time taken to sing the first pulse of LRR song.

Table A6
Correlation matrix between LRR song traits for the two sexual selection treatments (with body size included in the model)

	IPI		Frequency		Pulse number		Latency	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
IPI	1	–	−0.13	0.100	0.09	0.238	0.06	0.450
Frequency	−0.15	0.052	1	–	0.06	0.482	−0.01	0.900
Pulse number	0.21	0.004	0.03	0.682	1	–	−0.23	0.004
Latency	0.09	0.256	−0.05	0.556	−0.35	<0.001	1	–

The following elements are specified: the correlation coefficient (*r*) and the *P* value (*P*). LRR = low-repetition rate song, IPI = interpulse interval, latency = the time taken to sing the first pulse of LRR song. These correlations were derived from the variances and covariances estimated by the multivariate LMM (see Table A3). Since correlation matrices are symmetric, correlation values for polyandrous males and monogamous males are shown above and below the diagonal, respectively.

Table A7
Differences in LRR song trait correlations between the sexual selection treatments (r_{E}–r_{M} from Table A6) (with body size included in the model)

	IPI		Frequency		Pulse number		Latency	
	r_{E}–r_{M}	*P*	r_{E}–r_{M}	*P*	r_{E}–r_{M}	*P*	r_{E}–r_{M}	*P*
IPI	–	–						
Frequency	0.01	0.868	–	–				
Pulse number	−0.12	0.262	0.02	0.886	–	–		
Latency	−0.03	0.808	0.04	0.722	0.12	0.252	–	–

The following elements are specified: the corresponding correlation coefficients for polyandrous (r_{E}) and monogamous males (r_{M}) and the *P* value (*P*). LRR = low-repetition rate song, IPI = interpulse interval, latency = the time taken to sing the first pulse of LRR song.

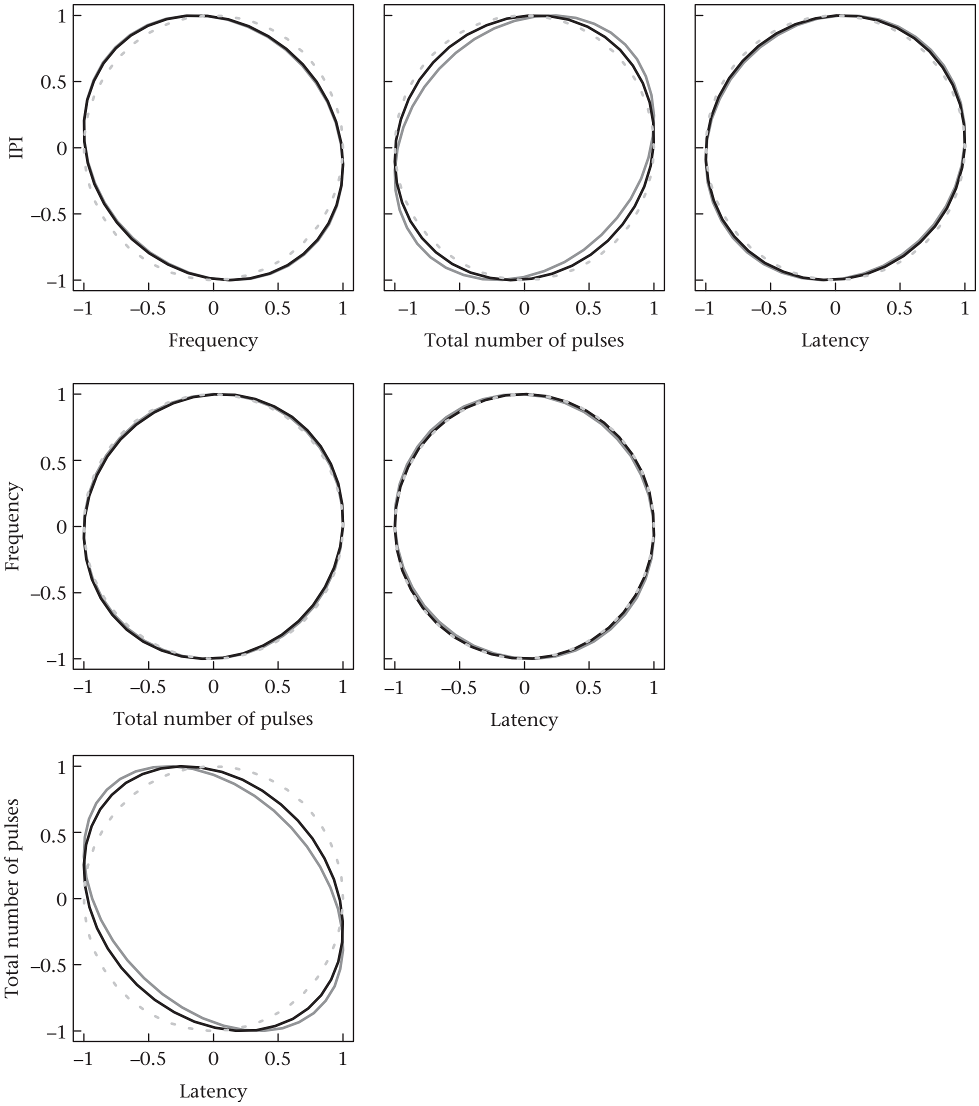

Figure A1. Correlation ellipses between low-repetition rate song traits for polyandrous (black) and monogamous (dark grey) males. This figure is a graphical representation of the correlation values provided in Table A4. The dotted light grey circle represents a null correlation ($r = 0$). The stronger the correlation, the narrower the ellipse becomes.

Appendix II. HRR Interpulse Interval Variation Over Time

Figure A2 shows a pattern in pulse rate production over courtship time, by illustrating how HRR interpulse interval lengthened as courtship time increased. Figure A3 shows the range of the distribution of the burst and pulse numbers, depending on sexual selection treatment and recording temperature.

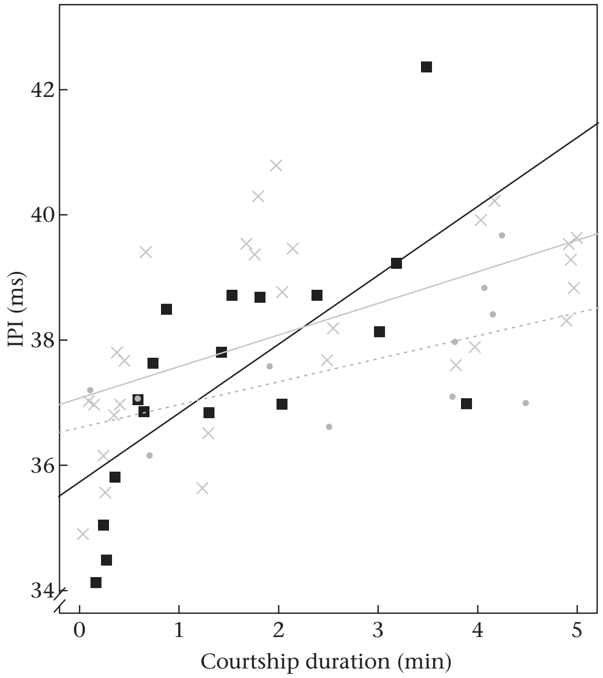

Figure A2. Example of interpulse interval lengthening along high-repetition rate song bursts, over courtship duration. Three random songs are represented (black, dark grey and grey symbols), with each data point showing the mean interpulse interval value of a single burst of song. A trend line showing the relationship between interpulse interval and courtship duration is shown for each song, for illustration purposes only. Spearman correlations: black squares: $r_S = 0.69$, $P = 0.002$; grey circles: $r_S = 0.59$, $P = 0.057$; crosses: $r_S = 0.57$, $P = 0.001$.

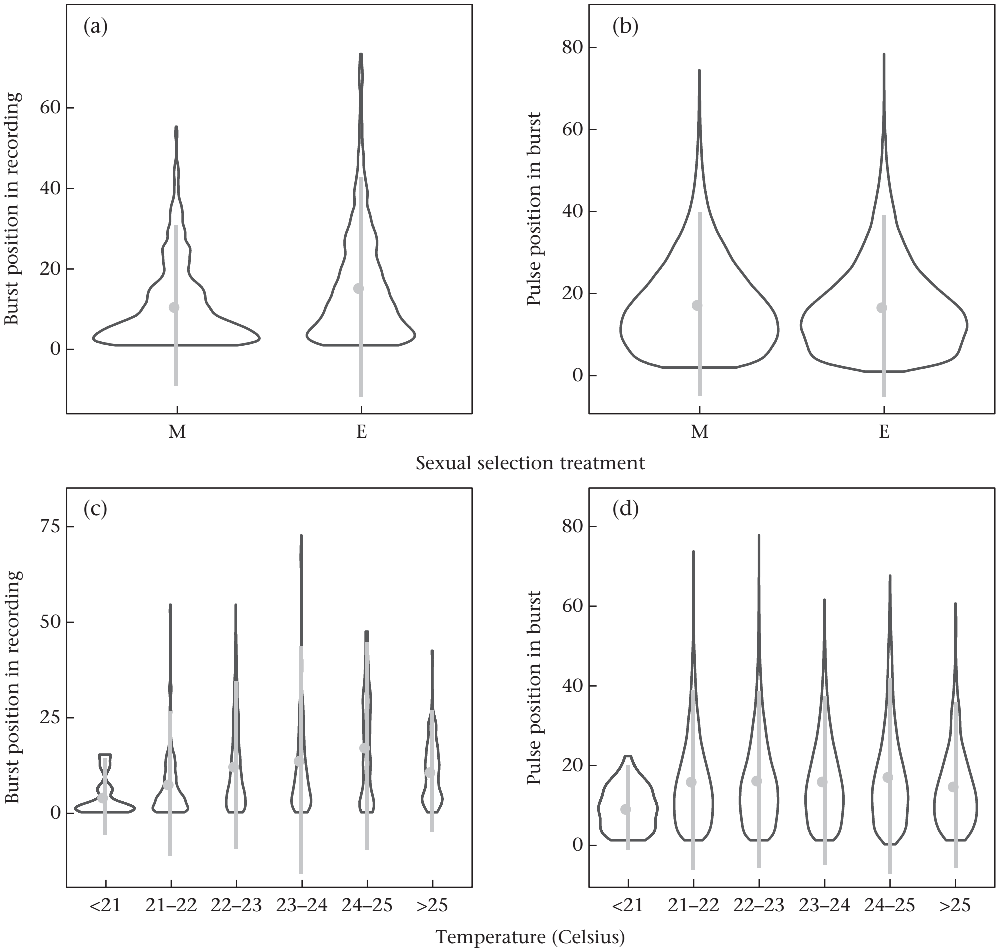

Figure A3. Violin plots showing the distribution of (a) burst number and (b) pulse number depending on sexual selection treatment, and (c) burst number and (d) pulse number depending on recording temperature distribution. The means (grey circles) ±1 SD (vertical grey bars) are represented. E = polyandrous males, M = monogamous males.

Appendix III. HRR Song Analyses (With Body Size Included)

Running analyses of HRR interpulse interval while including body size as a covariate showed that, although body size had a significant effect on courtship song production, the effect of sexual selection treatment also remained significant (see Tables A8–A11).

Table A8

Summary table for the fitted univariate LMM analysing the HRR pulse rate production between and within HRR bursts (with body size included in the model)

Model parameters	Factor level	β	Lower CI	Upper CI	*P*
Wing size		−0.42	−0.53	−0.31	<0.001
Treatment	E	−1.17	−2.39	0.046	0.102
Temperature		−0.75	−0.85	−0.65	<0.001
BP		0.69	0.51	0.87	<0.001
PP		0.92	0.86	0.98	<0.001
Treatment∗BP		−0.055	−0.27	0.16	0.609
Temperature∗BP		-0.3	−0.40	−0.20	<0.001
Treatment∗PP		−0.09	−0.16	−0.015	0.015
BP∗PP		0.11	0.042	0.18	0.001
Treatment∗BP∗PP		−0.16	−0.24	−0.079	<0.001
Intercept		39.04	38.20	39.89	<0.001
Inter-replicate variance		0.73			
Interburst variance (nested within replicate)		3.32			
Residual variance		4.91			

The following elements are specified: the model estimate of each variable (β), the lower and upper limit of the estimate's 95% confidence interval (CI), the *P* value of the test comparing the estimate to zero (*P*). BP = burst position, PP = pulse position, HRR = high-repetition rate song, IPI = interpulse interval, treatment = sexual selection treatment (E = polyandrous; the monogamous treatment M was used as the reference level), burst position = the position of the burst in the recording, pulse position = the position of the pulse in the HRR burst. The autocorrelation parameters are $\varphi_1 = 0.23$, $\varphi_2 = 0.12$, $\varphi_3 = 0.058$ and $\varphi_4 = 0.031$. $N = 35\,206$ individual interpulse interval values.

Table A9

Summary table for the fitted multivariate LMM analysing HRR song (with body size included in the model)

Song trait	Model parameters	Factor level	β	Lower CI	Upper CI	*P*
IPI	Treatment	E	−0.41	−1.014	0.26	0.178
	Temperature		−0.14	−0.26	−0.045	0.016
	Body size		−0.13	−0.27	−0.014	0.046
	Intercept		3.51	1.032	5.89	0.008
Amplitude	Treatment	E	−0.32	−0.74	0.11	0.128
	Temperature		0.095	−0.018	0.21	0.106
	Body size		0.16	0.042	0.30	0.024
	Intercept		−2.02	−4.63	0.57	0.138
Frequency	Treatment	E	−0.11	−0.63	0.52	0.678
	Temperature		0.069	−0.057	0.19	0.266
	Body size		−0.092	−0.22	0.040	0.180
	Intercept		−1.52	−4.12	1.38	0.296
Total number of bursts	Treatment	E	0.21	−0.37	0.74	0.432
	Temperature		0.050	−0.064	0.16	0.440
	Body size		0.010	−0.10	0.14	0.862
	Intercept		−1.22	−3.52	1.58	0.404
Latency	Treatment	E	−0.47	−0.74	−0.18	0.002
	Temperature		0.076	−0.041	0.19	0.202
	Body size		−0.054	−0.17	0.073	0.392
	Intercept		−1.48	−4.18	1.12	0.246

The following elements are specified: the model estimate of each variable (β; here the posterior mean), the lower and upper limits of the estimate's 95% credibility interval (CI), and the *P* value of the test comparing the estimate to zero (*P*). IPI = interpulse interval, treatment = sexual selection treatment (E = polyandrous; the monogamous treatment M was used as the reference level), latency = the time taken to sing the first burst of HRR song. Covariances between the response variables of the model are provided as correlations in Table A10. Estimated variances between replicates were $\sigma^2 = 0.2$ for IPI, $\sigma^2 = 0.05$ for amplitude, $\sigma^2 = 0.13$ for intrapulse frequency, $\sigma^2 = 0.12$ for the total number of bursts and $\sigma^2 = 0.01$ for latency. Note that all responses are expressed as z-scores of the log-transformed value of the original measurements. Wing size was also transformed into z-scores, but temperature was not altered. Estimates in the table are thus not expressed on the original data scale, but in z-scores of log values (see Methods). The means and standard deviations of the log of the original variables are as following: IPI (mean = 3.63, $\sigma = 0.06$), amplitude (mean = 5.85, $\sigma = 0.20$), frequency (mean = 5.56, $\sigma = 0.09$), total number of bursts (mean = 2.35, $\sigma = 0.84$), latency (mean = 9.38, $\sigma = 1.32$). $N = 280$ recordings.

Table A10
Correlation matrix between HRR courtship traits for the two sexual selection treatments (with body size included in the model)

	IPI		Amplitude		Frequency		Bursts		Latency	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
IPI	1	–	−0.39	<0.001	−0.12	0.138	−0.37	<0.001	0.26	0.002
Amplitude	-0.3	<0.001	1	–	−0.27	<0.001	0.23	0.008	−0.15	0.076
Frequency	0.06	0.560	−0.41	<0.001	1	–	0.21	0.018	-0.1	0.260
Bursts	0.07	0.436	0.09	0.314	−0.04	0.650	1	–	−0.29	0.002
Latency	−0.04	0.614	−0.07	0.490	−0.05	0.584	−0.39	<0.001	1	–

The following elements are specified: the correlation coefficient (*r*) and the *P* value (*P*). HRR = high-repetition rate song, IPI = interpulse interval, bursts = the total number of HRR bursts produced, latency = the time taken to sing the first burst of HRR song. These correlations were derived from the variances and covariances estimated by the multivariate LMM (see Table A9). Since correlation matrices are symmetric, correlation values for polyandrous males and monogamous males are shown above and below the diagonal, respectively.

Table A11
Differences in HRR courtship trait correlations between the sexual selection treatments ($r_{\mathrm{E}}–r_{\mathrm{M}}$ from Table A10) (with body size included in the model)

	IPI		Amplitude		Frequency		Bursts		Latency	
	$r_{\mathrm{E}}–r_{\mathrm{M}}$	*P*	$r_{\mathrm{E}}–r_{\mathrm{M}}$	*P*	$r_{\mathrm{E}}–r_{\mathrm{M}}$	*P*	$r_{\mathrm{E}}–r_{\mathrm{M}}$	*P*	$r_{\mathrm{E}}–r_{\mathrm{M}}$	*P*
IPI	–	–								
Amplitude	−0.09	0.426	–	–						
Frequency	−0.18	0.142	0.14	0.218	–	–				
Bursts	−0.44	<0.001	0.14	0.290	0.25	0.042	–	–		
Latency	0.31	0.012	−0.09	0.484	−0.05	0.700	0.10	0.360	–	–

The following elements are specified: the corresponding correlation coefficients for polyandrous (r_{E}) and monogamous males (r_{M}) and the *P* value (*P*). HRR = high-repetition rate song, IPI = interpulse interval, bursts = the total number of HRR bursts produced, latency = the time taken to sing the first burst of HRR song.

Appendix IV. Prior Definition for Fitting the Multivariate LMM

In both MCMCglmm models (the one with body size and the one without), we retained the default settings for the prior distributions for fixed effects. In contrast, we set identity matrices as prior specification for all other prior distributions and we used a degree of belief of 0.001 for the priors used in variance estimations only (i.e. weakly informative improper prior) and a degree of belief equal to the number of response variables plus one (i.e. proper prior) for the prior used in covariance matrix estimations. This structure follows the recommendations of the package instructions.