



Original Article

Signal reliability and multivariate sexual selection in a lek-breeding amphibian

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In leks, females are often attracted to males bearing costly secondary sexual traits that are thought to honestly signal male genetic quality. The Good Genes Hypothesis predicts 1) consistent male attractiveness across a variety of environments, 2) directional selection on male attractiveness, and 3) a positive correlation between paternal attractiveness and offspring fitness. We tested these predictions in a natural population of the Italian tree frog, *Hyla intermedia*. We recorded the calling activity of all males at a small lek for 2 breeding seasons and we carried out an incomplete factorial breeding experiment to test for an association between paternal attractiveness and offspring fitness. Because we found no evidence for temperature-dependent genotype-by-environment interactions in calling properties, we provided evidence that male attractiveness was consistent across different thermic conditions (first prediction). Moreover, in 1 breeding season, we showed a statistically significant association between mating success and call rate, call duration, and spectral properties, suggesting that multivariate directional selection did act on these properties (second prediction). However, we found no conclusive evidence for either accepting or rejecting our third prediction. Indeed, although paternal call rate showed a marginally significant, negative correlation with offspring age at metamorphosis, the other call properties did not show any statistically significant association with offspring fitness. These results suggest that, in our study population, alternative mechanisms, such as male–male competition for signal detectability, might be a stronger driving force for the evolution of male costly displays than good genes.

Key words: female choice, good genes, multivariate sexual selection, signal reliability.

INTRODUCTION

In lek-breeding species, female choice often favors males with the most elaborate sexual displays, even when choosing those mates does not convey any apparent direct benefit to the female (Andersson 1994; Höglund and Alatalo 2014). Several hypotheses have been proposed to explain the evolution of these preferences. Preferences might have resulted from sensory or cognitive biases that preceded and drove the evolution of the preferred trait (Ryan et al. 1990), or they might have coevolved with the preferred trait either antagonistically, for resistance to male manipulation (Holland and Rice 1998), or mutualistically, for the indirect benefits of increased offspring reproductive value (Kokko et al. 2002). In the latter case, indirect benefits might arise either as the side effect of the positive genetic correlation between female preferences and male displays (the Sexy Sons Hypothesis, Lande 1981) or as the direct effect of the positive genetic correlation between female preferences, male displays, and male genetic qualities (the Good Genes Hypothesis, GGH). According to the GGH, the preferred traits are

honest indicators of the signaler's breeding value for total fitness (Hunt et al. 2004), whose reliability is guaranteed either by their morphophysiological constraints or by the differential costs they impose on signalers of different qualities (Zahavi 1975; Grafen 1990; Smith and Harper 2003).

The GGH makes 3 empirically testable assumptions: 1) male attractiveness should be consistent and largely independent of the signaling environment (i.e., it is not subjected to genotype-by-environment interactions [GEIs]); 2) attractiveness should bias male reproductive success and be under directional selection (Brooks et al. 2005; Gerhardt and Brooks 2009); and 3) attractiveness should correlate positively with offspring fitness (Kokko 2001). Despite the intense empirical work of the last 3 decades, there is still scarce evidence for these theoretical predictions and the relative importance of the GGH in explaining the evolution of female choice is still a debated issue in sexual selection theory (Kotiaho and Puurtinen 2007).

Lek-breeding anurans are a particularly suitable model for testing the first prediction derived by the GGH, which postulates consistent male attractiveness across different environmental conditions. The advertisement call of frogs and toads is one of the most energetically demanding trait in the animal world (Wells 2001)

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and variation in its temporal and spectral properties is known to explain a large amount of the observed variation in females' mating decisions (Gerhardt and Huber 2002). The expression of most call properties is temperature-dependent (Pough et al. 1992) and among-male variation in temperature-dependent reaction norms may negatively affect the signaling potential of the trait. Indeed, if GEIs in the expression of an acoustic trait occur, and if the reaction norms intersect within the range of calling temperatures, then no genotype will perform best in all thermic conditions (Hunt et al. 2004). Because the GGH predicts that such an inconsistent trait is unlikely to evolve as a reliable indicator of male genetic quality (reviewed in Holman and Kokko 2014), an empirical test against the hypothesis would be to seek for GEIs in traits that are supposed to play an important role in mate-quality assessment.

Lekking anurans are suitable models also for testing the second prediction of the GGH, which postulates attractive traits to be under directional selection. In anurans, female preferences for male calling traits can be easily described by playback experiments under controlled laboratory conditions (Ryan 2001; Gerhardt and Huber 2002). By studying the shape of the female preference functions over variation in male call properties, these experiments have shown that anuran advertisement calls are likely to convey multiple messages (Gerhardt 1992): some acoustic traits have been found to be under female stabilizing preferences and to be important for species recognition, whereas other acoustic properties have been found to be under directional preferences and, thus, to be important for mate-quality assessment (Gerhardt 1991). In most playback experiments, female preferences have been described by manipulating a single acoustic property at a time. Because acoustic properties are often highly inter-correlated with each other, the preferences observed in the lab might not accurately describe selection under natural conditions (Gerhardt and Brooks 2009). For this reason, evidence for directional selection on male attractiveness might be obtained by describing the fitness surface of male advertisement calls (Lande and Arnold 1983) and by providing evidence that the preferred traits reside on fitness peaks.

Finally, amphibians can be easily mated and reared under controlled conditions, which makes them optimal models to explore the third GGH prediction, regarding the indirect genetic benefits of choosing females. This prediction can be tested by comparing the fitness of maternal half-siblings sired by males of different genetic quality and by seeking for evidence of correlations between attractive paternal traits and offspring fitness. So far, data suggesting that preferred males sire fitter offspring have been found for several species

of arthropods (Moore 1994; Jia and Greenfield 1997; Alatalo et al. 1998; Watson 1998), birds (Møller 1994; Sheldon et al. 1997), fish (Reynolds and Gross 1992; Barber et al. 2001), and amphibians (Welch et al. 1998; Doty and Welch 2001; Welch 2003; Briggs 2013). However, the net genetic benefit arising from female choice is usually weak (Møller and Alatalo 1999) and the correlation between paternal quality and offspring fitness is sometimes absent (Barber et al. 2001; Evans et al. 2004) or even negative (Wedekind et al. 2008).

In this paper, we test the GGH using as a model the Italian tree frog, *Hyla intermedia*. Males of this species spend several nights at the chorus, where they engage in highly energetically demanding acoustic competitions (Castellano et al. 2002). The advertisement call is a short pulse-modulated call, repeated rhythmically in long series (call groups). It is composed of 6–10 pulses, whose intensity increases linearly during two-thirds of its duration and with energy concentrated in 2 quasi-harmonically related spectral components (Castellano and Rosso 2006, Figure 1). Playback experiments showed directional preferences for both the call rate and the number of calls per call group (Castellano and Rosso 2007; Castellano 2010) and stabilizing preferences for call frequency and pulse rate (Castellano and Rosso 2006; Rosso et al. 2006). A previous work on the same population evaluated the effects of mating effort, in terms of number of nights spent at the lek (attendance) and amount of time spent calling per night (calling effort) and demonstrated that both attendance and calling effort significantly affected male mating success, but that only the former was correlated with offspring fitness, suggesting that good genes arise from female-mediated endurance rivalry, rather than from female choice itself (Botto and Castellano 2016). Here, we focus on call quality and we investigate the effect of temperature on call reliability, by analyzing the temperature-dependent reaction norms of calling males. Furthermore, we use the data about the mating success of the recorded males to describe the call fitness surface and the pattern of selection on univariate and multivariate calling properties. Finally, we describe the results of a breeding experiment to explore the pattern of association between the calling properties of males and some fitness components of their offspring.

MATERIALS AND METHODS

Field activities

The study was carried out over 2 consecutive breeding seasons, from 22 April to 27 June in 2013 and from 20 April to 20 June in 2014. The breeding site, situated in a sparsely inhabited rural area

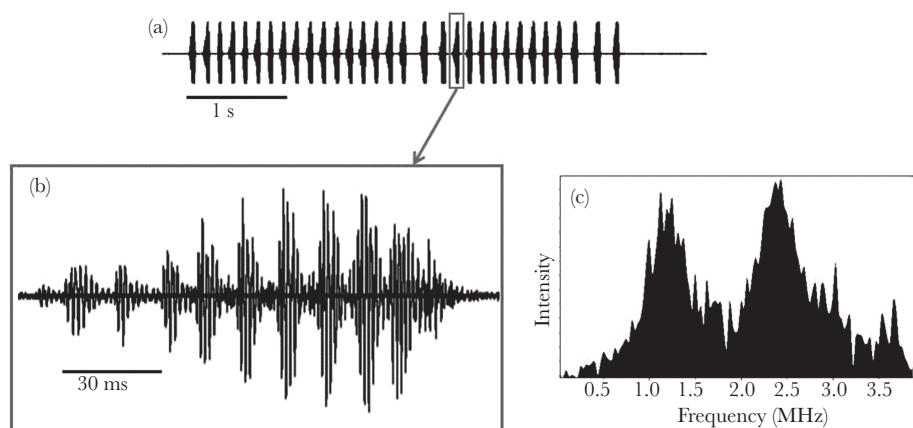


Figure 1

The advertisement call of the Italian tree frog: (a) a time wave of a call group; (b) the time wave of a single call; and (c) the power spectrum of the call.

in central Piedmont (Northwest Italy), was a $5 \times 1 \times 0.3$ m artificial pond obtained by aligning five 1-m^2 pools (Bestway model 56217B, Shanghai, China). The pond has been regularly used by tree frogs as a breeding site for a few years before the onset of the experiments described here.

Each night, we recorded the identity, the position, and the mating success of all males and females at the breeding site. Untagged tree frogs were kept overnight in individual fauna boxes. On the following morning, they were anesthetized with a 2‰ solution of tricaine methanesulfonate (MS222 Sandoz, Sandoz Inc., Princeton, NJ) and marked with unique subcutaneous alphanumeric tags (Northwest Marine Technology Inc., Shaw Island, WA) injected in the tarsal area on the left hind leg.

Male mating success was defined as the number of matings gained by a male during the entire breeding season. During our regular nightly inspections, we captured all the detected pairs and assessed the male and female identities without separating them from the amplexus. We then transferred the pairs in separated fauna boxes to complete egg deposition. Eggs were left to hatch in these fauna boxes and tadpoles were released in a nearby water body (distance ≈ 0.4 km), a few days after hatching. The accuracy of our assessment of male mating success was remarkably high, because, during the 2 breeding seasons, only 2 females escaped our detection and spawned in the breeding site.

Recording apparatus and acoustic analyses

For the whole duration of the 2 breeding seasons, we recorded the calling activity at the lek using a multichannel recording apparatus. We set up 4 fixed recording points. By representing the pond as a rectangle with vertices at (0, 0), (0, 1), (5, 0), and (5, 1), the recording points had coordinates (2, 0), (4, 0), (1, 1), and (3, 1). At each point, about 50 cm above the ground, we placed 2 Sony ECM-T6 condenser microphones (Sony Corporation, Tokyo, Japan) pointing toward the center of the pond, respectively, with a 45° and a 135° orientation relative to the border of the breeding site. To improve directionality, we placed a 25×25 cm wood panel covered with a 30-mm thick pyramid polyurethane acoustic foam between, above, and behind the 2 microphones. The 8 microphones were connected to an 8-input/8-output audio interface M-Audio Fast Track 8R (Avid Technology Inc., Burlington, MA). Multichannel recordings (sample rate = 44.1 kHz) were carried out using the open source software KRISTAL Audio Engine (Kreatives.org). Each night, we monitored the lek from dusk to the end of male activity, and we carried out 92 eight-channel recordings (49 in 2013 and 43 in 2014), during which the chorus activity lasted from 11 to 190 min (median = 79 min).

The analysis of the recordings was divided into 2 phases. The first phase aimed at separating single calls and at calculating the call rate for each call group. A detailed description of the methods employed at this stage is presented in more details in [Botto and Castellano \(2016\)](#) and in the [Supplementary Materials](#) of this paper. In short, after a resampling the calls at 11 025 Hz, we assigned each call to a focal male. In most cases, a channel had its own focal male, recorded at a much higher intensity than the chorus background noise, and the identity of such individuals could be unequivocally identified by using the field notes. In other cases, however, 2 or more males happened to call close to each other and equally distant from the nearest microphone, at least for a portion of the recording session. In these cases, we took advantage of the partial overlapping of the recording space of different microphones to assign the calls to different males (see below). We then used a custom-designed Matlab program (The

Mathworks Inc., Asheboro, NC) to automatically cut the recordings in separate 60-s wav files, whose name retained information about their temporal position relative to the start of the recording session. The 60-s files were processed with a Matlab graphical user interface (GUI) program that allowed the operator to analyze the calling activity of up to 3 males on a single channel, provided that at least one of them was recorded with an appropriate signal-to-noise ratio on another channel. When 2 males (e.g., m1 and m2) were recorded at similar intensity on a channel (e.g., C1), it was impossible to discriminate between them by setting the intensity threshold. However, if one of these males (e.g., m2) was recorded also on a second channel (e.g., C2), at an intensity that allowed effective discrimination, we subtracted from the C2 peak array (m2) from the C1 peak array (m1 + m2): in other words, we filtered m2 out of C1. This Matlab GUI also allowed us to identify, for each channel, single calls and to save them in separate wav files of predefined length (90–120 ms), centered about the call peaks. Each file contained a single tree frog call and its name retained information about the temporal position of the buffer. The program also originated a txt file listing the temporal position (described as the sample number) of all the peaks for each 60-s portion. We then concatenated all the txt files of a male recording session and assigned each call to a new call group if the interval from the previous call was longer than 1 s. From the concatenated txt files, we computed the call rate (as number of calls in a call group minus one, divided by the duration of the call group) for each call group emitted by a given male during the recording session.

In the second phase of acoustic analyses, we processed the previously created call files to measure several spectral and fine-scale temporal call properties, as described in [Castellano et al. \(2009\)](#). In short, we used a second GUI, either with a semiautomatic or fully automatic procedure. In the semiautomatic procedure, the program sequentially opened, in random order, the files contained in the directory of the call files to be analyzed. The program then computed the envelope, the peak array, and the call array (a binary vector identifying the signal segment corresponding to the call) and plotted the signal time wave, the peak and the call array and, in a separate window, the call power spectrum. In this phase, visual inspection of the calls was crucial; if the program failed in correctly identifying the temporal and spectral structure the call, the operator could either discard it or modify the setting and repeat the analysis. When the program correctly defined the onset, offset, and the location of all pulses, the operator would choose to continue the analysis and the program proceeded by measuring the following several fine-scale parameters (defined in [Castellano and Rosso 2006](#) and in [Table 1](#)): 1) total call duration, 2) first–last pulse, 3) rise time, 4) number of pulses, 5) root mean square, 6) modulation index, 7) intensity range, 8) fundamental frequency, and 9) dominant frequency.

The semiautomatic procedure was repeated until the sample of accepted analyses contained 150 calls per male. Every time a call was accepted, the program updated the mean and standard deviation (SD) of all the acoustic properties in the accepted sample. Because the file list had been randomly permuted, the first 150 calls accepted represented a random sample of the entire recording session. The automatic procedure did not allow the operator to decide whether to reject or to accept a call for the analysis. It was the program that made such decisions by evaluating the probability that a call would have belonged to the populations of calls that had been previously analyzed with the semiautomatic procedure. Operatively, the program carried out, for each acoustic property, a *t*-test for the

Table 1
Definition of fine-scale acoustic properties cited in the text

Acoustic property	Definition
Total call duration (s)	Total duration of the call from the onset of the first pulse to the end of the last pulse
First–last pulse (s)	Time gap from the instant of maximum amplitude of the first pulse to the instant of maximum amplitude of the last pulse
Rise time (s)	Time gap between the onset and the instant of maximum amplitude of the call
Number of pulses	Number of pulses in the call
Pulse rate (s ⁻¹)	Number of pulses minus one, divided by the duration of the call
RMS	Integral of the normalized amplitude envelope
Modulation index	Varies from 0 (no modulation) to 1 (complete modulation). Computed using the following algorithm: $MI = \frac{1}{N} \sum_i \frac{\text{peak}(i) - \text{saddle}(i)}{\text{peak}(i)}$
Intensity range (dB)	Gap between the maximum and the minimum intensities of the call
F0 (Hz)	Fundamental frequency
FD (Hz)	Dominant frequency

comparison of a single observation with the sample mean (Sokal and Rohlf 1995, p. 227–229). The program accepted a call only if none of the statistical tests showed a null-hypothesis probability lower than 0.01. Once a call was accepted, the program updated the sample means and SDs. The program terminated when all the calls on the list had been processed. The output file showed the list of all files processed (either accepted or rejected) together with the results of the analyses (only for the accepted files).

In total, we analyzed 16486 call groups over the 2 breeding seasons. The mean number of calls recorded per each individual recording session was 3050 (range = 12–16899 calls, sum = 689302). About 40% of the recorded calls were further analyzed and their fine-scale temporal and spectral structures were described.

Breeding experiment

We set up a breeding experiment with incomplete factorial design, to disentangle maternal and paternal effect on offspring development (Roff 1997). Over the 2 years, we used 9 quartets composed by 2 males and 2 females. Within each quartet, the 2 males were each mated to the 2 females to produce 4 families. Because in 2 quartets (1 in 2013 and 1 in 2014) we were able to obtain only 2 of the 4 possible families, our breeding experiment involved 32 full-sib families, each with 2 replicates of 15 tadpoles. Because even slight variations in tadpole density might have had dramatic effects on tadpole growth and development (e.g., Wilbur 1976; Cadeddu and Castellano 2012), the number of tadpoles in each replicate was checked daily. During the first 2 weeks of the 2013 breeding experiment, a few tadpoles died because of a predation event, and 5 others died because of accidental errors during the manipulation. Those tadpoles were immediately replaced with full-sibs from the reserve stock.

In the 2 years, experimental matings involved randomly selected males and were carried out exclusively during the peak of breeding activity to ensure that tadpoles hatched synchronously and were faced with comparable rearing conditions. Each replicate was

transferred to 40-L plastic tanks placed on a sunny lawn. Tanks were randomly swapped once a week to avoid differential larval growth due to different levels of sun irradiation (Smith-Gill and Berven 1979). Tadpoles were fed ad libitum and the water in the tanks was changed weekly to avoid algal bloom formation.

Every week, we randomly captured 8 tadpoles from each tank, placed them in a Petri dish, lined with graph paper, and photographed them using a Sony Cyber-Shot DSC-HX200V camera (Sony Corporation). From the pictures, after calibration, we measured tadpoles' SVL with the open source software ImageJ (Rasband WS, ImageJ, U.S. National Institutes of Health, Bethesda, MD, <http://imagej.nih.gov/ij/>, 1997–2014). These weekly measures were then used to fit growth curves and estimate growth rate for each replicate (see below). At metamorphosis, froglets were captured, anesthetized with a 1‰ solution of MS222 Sandoz, and measured to the nearest 0.01 mm.

We considered 3 interrelated life-history traits that are known to affect fitness. The first 2, body size (SVL) and age at metamorphosis (AGE), were directly measured on the first 10 froglets that completed metamorphosis within each replicate, whereas the third trait, the larval growth rate (GROWTH), was estimated by fitting the within-replicate averages of weekly tadpoles' SVL (l_t) and age (t) to the von Bertalanffy growth equation, $L_t = L_\infty \cdot (1 - e^{-\text{GROWTH}(t-t_0)})$, where L_∞ was set at 1.688 cm (observed maximum body size) and $t_0 = 0$.

Ethic statement

The “Regione Piemonte – Direzione Ambiente – Settore Sostenibilità e recupero ambientale, bonifiche” provided the authorization for tree frog capture and handling (permits no. 152 and 153 issued on 25 February 2013).

Data set preparation and statistical analyses

Our first aim was to describe the temperature-dependent linear reaction norms of call acoustic properties and to test for among-male differences in either their intercept or slope. To this purpose, we restricted the sample to the males with more than 4 recordings per breeding season and with a range in body temperatures larger than 3 °C. For each recording, we calculated the call group means of all the acoustic properties and averaged them over the entire session so that we obtained a data set with 155 recordings of 14 different males. Three of these 14 males were recorded in both the 2013 and the 2014 breeding seasons. Because the body size of these males increased in 2014 and because body size is known to affect several spectral and temporal acoustic properties in tree frogs (Castellano and Rosso 2006; Rosso et al. 2006), we did not pool the recordings, but, to avoid pseudoreplication, we analyzed only the year with the highest number of recording sessions. Each acoustic property was then used as a dependent variable in a linear model (analysis of covariance [ANCOVA]), where male identity, body temperature, and their interaction were the predictors, and year of study was a random factor introduced to control for differences between the 2 breeding seasons.

Our second aim was to evaluate the selection pressures acting on males' advertisement calls. First of all, we tested the hypothesis that mating success did affect the acoustic properties of males' advertisement call. We selected all the males that were recorded at least twice both before and after their first mating episode within a season ($N = 10$), and we performed an analysis of variance comparing their pre- and postmating calls. Because we found no

evidence for a mating effect on calling performance (see Table S1 in Supplementary Materials), the selection analysis was carried out on the entire sample of recorded males for which we had complete information about their calls and independent of their mating history ($N = 51$, about 78.4% of the entire male breeding population). We followed a 3-step procedure.

The first step was to provide a quantitative description of the male average advertisement call. Because the acoustic variables were found to be significantly affected by body temperature and male identity, but not by their interaction (with only one exception, see Results), we used standard linear regression techniques to adjust all the acoustic properties to the average temperature of 17.4 °C. Furthermore, because preliminary analyses showed significant differences between the 2 breeding seasons in several temperature-adjusted acoustic properties, we adjusted the properties by including the factor “year” as an offset. Finally, for each male (within a breeding season), we calculated the mean values of all his temperature-adjusted properties. Due to the between-year differences, the call properties of those males that were recorded in both breeding seasons ($N = 8$) were temperature-adjusted, for each year, independently.

The second step was to reduce the dimensionality of the data set, by finding the directions that best explained the among-male variation in call acoustic properties. We identified 3 sets of highly intercorrelated variables (Castellano et al. 2002): 1) the set of gross-scale temporal properties, which included call rate only; 2) the set of fine-scale temporal properties, controlled by the contraction of either the trunk or the arytenoid muscles (Castellano and Rosso 2006); and 3) the set of spectral properties (Rosso et al. 2006). On the second and the third groups of variables, we carried out principal component analyses (PCAs) so that we could describe call variation by means of only 4 variables: call rate; 2 orthogonal components of fine-scale properties; and a component of spectral properties. The 2 PCAs were carried out, first, on the complete (2-year) data set and then, separately, for the 2013 and the 2014 data set. Because the canonical loadings of the 2013 components correlated positively with their 2014 analogues (PC1: Pearson's $R = 0.974$, $P < 0.001$; PC2: $R = 0.894$, $P = 0.003$; PC3: $R = 1$, $P < 0.001$), we used the PCAs derived from the complete data set.

The third and last step was to estimate, for each breeding season, the strength and the nature of the selective forces acting on call variables. At the seasonal level, we measured the mean and variance of male mating success and computed the opportunity-of-selection index for the 2 breeding seasons ($MS = \text{mating success}$; $I_s = \text{VAR}(MS) / \overline{MS}^2$) (Arnold and Wade 1984). At the nightly level, we measured the operational sex ratio (OSR; mm:ff) of all nights with at least 1 female at the lek and computed the seasonal means. For each year, then, we estimated the factors affecting male mating success by performing a generalized linear model (GLM; Poisson error distribution, logit link function) that included adjusted call rate and of the 3 PCs as dependent variables. For the breeding seasons in which significant effects of the predictors were observed, the results were visualized in R using the `Tps` function in the package “fields,” choosing the value of the smoothing parameter that minimized the GCV score, as described in Brooks et al. (2005).

For a rigorous estimation of linear and nonlinear selective forces, for each year, we used the procedure described by Blows and Brooks (2003). We computed relative fitness as the ratio between individual seasonal mating success and mean seasonal mating success and transformed the 4 predictive variables to zero means and unit variances. Then, we used a linear model to estimate the

standardized linear regression gradients (β_i), using a linear model that included only the linear terms. A second linear model including the linear terms, the quadratic terms, and all the possible interactions between the linear terms was run to estimate quadratic (γ_{ii}) and correlational regression gradients (γ_{ij}). We used the double-linear regression method (Bisgaard and Ankenman 1996) to further investigate selective pressures acting on the acoustic properties of male calls. Following Stinchcombe et al. (2008), quadratic selection gradients (γ_{ii}) were doubled to obtain quadratic gradients sensu Lande and Arnold (1983), in order to avoid underestimation of nonlinear selection (Phillips and Arnold 1989; Blows and Brooks 2003). For each year, by performing a canonical rotation of the γ -matrix, we derived \mathbf{M} , the matrix of the 4 eigenvectors \mathbf{m}_i , and $\mathbf{\Lambda}$, the vector of the 4 eigenvalues, λ_i , each describing the strength of nonlinear selection along the i -esim eigenvector. The strength of directional selection (θ) and the significance of both directional and nonlinear selection were tested by fitting a new full second-order model. Because our response variable (relative fitness) was not normally distributed, we inferred null-hypothesis probabilities by using a randomization procedure based on the random shuffling of the response variable: after 9999 permutations, the null-hypothesis probabilities were estimated as the relative frequencies of trials in which the absolute values of the regression coefficients were equal to or larger than those actually observed (2-tailed tests) (Reynolds et al. 2010).

Our third aim was to explore the hypothesis that the most attractive males sired tadpoles with superior fitness. In the study population, paternal effects were observed on 2 of the 3 fitness-related traits: age at metamorphosis and larval growth rate (Botto and Castellano 2016). Therefore, we looked for correlations between paternal attractiveness and each of these 2 fitness-related traits, by carrying out a series of general linear models, whose independent categorical factors were year, quartet (nested within year), and dam identity (nested within quartet and year), and which included as covariates either the call rate, the call duration (PC1), the call modulation (PC2), or the spectral properties (PC3) of sires' advertisement calls. To avoid pseudoreplication, these analyses were carried out using the mean values in each replicate.

RESULTS

Population structure and male mating success

During the 2 breeding seasons, we captured 65 males (29 only in 2013, 28 only in 2014, and 8 in both years) and analyzed the fine-scale structure of the calls of 51 of them (24 in 2013 and 27 in 2014), with an average of 4.4 recordings per male (range = 1–18). The number of reproductive females that visited the lek was 46 (27 in 2013 and 19 in 2014), 6 of them spawned twice during the same breeding season (5 in 2013 and 1 in 2014). The population sex ratio (males/females) was lower in 2013 (sex ratio = 1.37) than in 2014 (sex ratio = 1.89). Consequently, the mean male mating success of recorded males was higher (2013: mean = 0.86; range = 0–4 matings) and the opportunity of selection (2013: $I_s = 1.52$) lower in the first than in the second breeding season (2014: mean mating success = 0.56; range = 0–3 matings, $I_s = 2.73$). However, when we analyzed the breeding population at a nightly level and considered only those nights when at least 1 female was at the lek (2013: $N = 13$; 2014: $N = 11$), we found that the nightly OSR was, on average, higher in 2013 (OSR = 5.6, range = 2–14) than in 2014 (OSR = 3.2, range = 1–8; U Mann–Whitney = 34.5, $P = 0.034$),

because the number of nights that males spent at the lek was larger in 2013 (mean = 5.2, standard error [SE] = 0.81) than in 2014 (mean = 3.6, SE = 0.72), although the differences were not statistically significant (U Mann–Whitney = 551.5, $P = 0.197$).

Effects of temperature on the call acoustic properties

Table 2 shows the results of the ANCOVAs on the acoustic properties of males recorded at least 4 times throughout a single breeding season and with body temperature spanning at least 3 °C. Results show a statistically significant effect of temperature on all variables. They show also a significant effect of male identity on most of the fine-scale temporal properties (call duration, first–last pulse, number of pulses, pulse rate) and on the spectral properties (fundamental and dominant frequency), whereas male–temperature interaction was statistically significant only for the number of pulses per call.

Table 2

Results of ANCOVAs between call acoustic properties (dependent variables) and body temperature and male identity (independent variables)

	Temperature (°C)		Male ID	Temperature × male ID
	<i>B</i>	<i>P</i>	<i>P</i>	<i>P</i>
Call rate (1/s)	0.34	<0.001***	0.430	0.569
Call duration (s)	0.00	<0.001***	0.003*	0.972
First–last pulse (s)	0.00	<0.001***	0.006	0.920
Rise time (s)	0.00	<0.001***	0.547	0.997
Number of pulses	−0.05	0.045	<0.001***	0.015
Pulse rate (1/s)	5.83	<0.001***	0.002*	0.433
Modulation index	−0.02	<0.001***	0.641	0.290
RMS	0.00	0.008	0.654	0.753
Intensity range (dB)	0.01	0.036	0.720	0.097
Fundamental frequency (Hz)	5.99	0.002	0.001**	0.407
Dominant frequency (Hz)	14.41	0.001**	0.009**	0.241

When the interaction term was not statistically significant, it was removed and the model was reran to test for main effects. The table shows both the raw probabilities of the null hypothesis (statistically significant values are indicated in italic) and its statistical significance based on the experimentwise error rate $\alpha' = 1 - (1 - \alpha)^{1/k}$ (where $k = 11$ is the number of statistical tests, Dunn–Sidak method). The experimentwise error rates considered are, respectively, $P < \alpha' = 0.0046$ (*), $P < \alpha' = 0.0009$ (**), and $P < \alpha' = 0.000045$ (***)

Table 3

Descriptive statistics of call acoustic properties of the entire male population versus the males used as sires in the breeding experiment

	Entire male population					Sires				
	<i>N</i>	Mean	Min	Max	SD	<i>N</i>	Mean	Min	Max	SD
Call rate (1/s)	51	5.424	4.161	6.537	0.505	16	5.398	4.942	5.851	0.273
Call duration (s)	51	0.067	0.058	0.079	0.004	16	0.069	0.059	0.076	0.004
First–last pulse (s)	51	0.055	0.046	0.067	0.005	16	0.057	0.048	0.064	0.004
Rise time (s)	51	0.047	0.031	0.059	0.005	16	0.048	0.032	0.055	0.005
Number of pulses	51	7.989	5.948	9.25	0.57	16	8.126	6.385	8.868	0.567
Pulse rate (1/s)	51	128.7	105.375	154.376	9.459	16	126.803	111.933	143.177	7.686
Modulation index	51	0.73	0.593	0.846	0.046	16	0.731	0.694	0.802	0.034
RMS	51	0.288	0.233	0.4	0.032	16	0.28	0.241	0.303	0.015
Intensity range (dB)	51	0.793	0.71	0.859	0.033	16	0.81	0.765	0.848	0.019
Fundamental frequency (Hz)	51	1176.5	1021.76	1407.63	81.966	16	1153.32	1083.89	1255.86	48.715
Dominant frequency (Hz)	51	2423.83	2166.97	2862.31	153.293	16	2380.89	2177.31	2575.59	107.603

Table 3 shows the descriptive statistics of the temperature-adjusted acoustic variables for the whole male population. Because the fine-scale temperature-adjusted properties are still highly inter-correlated with each other (Table 4), we identify the main directions of variation by running 2 PCAs on the set: the fine-scale and the spectral properties, respectively. The PCA on the fine-scale properties identifies 2 principal components (PC1 and PC2, Table 3), which cumulatively explain 68.6% of the variance. PC1 is positively correlated with the duration of the call (i.e., call duration, first–last pulse, and rise time) and it is related to the pattern of contraction of thoracic muscles. PC2 is positively correlated with pulse rate and pulse number and negatively with the modulation index (Table 4) and it describes the pattern of contraction of arytenoid muscles. The second PCA on the call spectral properties identifies one principal component (PC3), which explains 90.6% of the variance and is positively correlated with both the fundamental and the dominant frequency (Table 4).

Male mating success and selective pressures acting on acoustic properties

In 2013, male reproductive success was not affected by any of the acoustic properties considered; in 2014, instead, it was positively affected by call rate ($B = 3.276$, SE = 0.998, $z = 3.284$, $P = 0.001$) and PC1 ($B = 1.045$, SE = 0.369, $z = 2.831$, $P = 0.005$), and negatively affected by PC3 ($B = -0.782$, SE = 0.322, $z = -2.431$, $P = 0.015$). These between-year differences were statistically significant for call rate (absolute differences in slopes: $B = 3.481$, SE = 1.216, $P = 0.001$), marginally significant for PC1 (absolute differences in slopes: $B = -0.885$, SE = 0.469, $P = 0.059$), but not statistically significant for PC3 (absolute differences in slopes: $B = 0.497$, SE = 0.451, $P = 0.271$). Figure 2 shows the 2014 relative fitness surface described by 2 of the 3 traits (standardized call rate and PC1), which were found to be associated with male mating success.

The standardized linear selection gradients (β_i) and the matrices of standardized quadratic and correlational gradients (γ) for the 2 breeding seasons are presented in Table 5; the \mathbf{M} matrices of eigenvectors and their associated linear (θ_i) and quadratic coefficients (λ_i) of selection are presented in Table 6. In accordance with the results of the GLMs, in 2013, there is no evidence for linear or nonlinear selection on any eigenvector. In 2014, instead, we observed positive linear selection on \mathbf{m}_5 ($P = 0.009$ after randomization), a component that is positively loaded by both call rate and PC1

Table 4 Pearson's correlation coefficients between temperature-adjusted call properties and canonical loading of call properties on the each of the 3 PCs

	Call rate	Call duration	No. of pulses	First-last pulse	Pulse rate	Rise time	RMS	Intensity range	Modulation index	Fundamental frequency	Dominant frequency	PC1 (call duration)	PC2 (call modulation)	PC3 (call frequency)
Call rate	1	-0.554***	-0.331*	-0.602***	0.344*	-0.417**	0.227	-0.246	-0.269	0.165	0.233	—	—	—
Call duration		1	0.529***	0.934***	-0.473***	0.684***	-0.413**	0.085	0.201	-0.123	-0.287*	0.914	0.012	—
No. of pulses			1	0.619***	0.401**	0.681***	-0.028	0.162	-0.219	-0.26	-0.311*	0.577	0.769	—
First-last pulse				1	-0.442**	0.776***	-0.439**	0.197	0.278*	-0.172	-0.297*	0.968	0.047	—
Pulse rate					1	-0.112	0.406**	-0.062	-0.559***	-0.082	0.027	-0.453	0.812	—
Rise time						1	-0.366**	0.226	0.095	-0.059	0.837	0.318	—	
RMS							1	-0.355*	-0.232	-0.163	-0.161	-0.585	—	
Intensity range								1	0.003	-0.092	-0.139	0.309	—	
Modulation index									1	0.391**	0.218	-0.692	—	
Fundamental frequency										1	0.812***	—	—	
Dominant frequency											1	—	—	
												0.952	—	—
													0.952	—

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

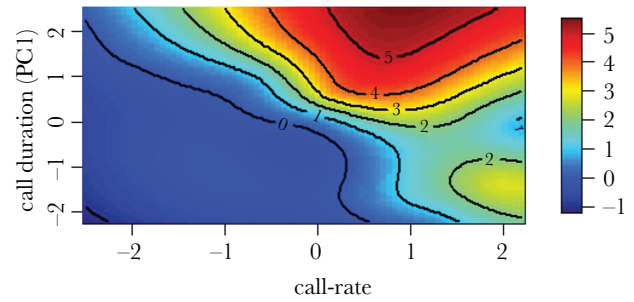


Figure 2 The contour plot of the fitness surface along 2 traits acoustic subject to significant directional selection, standardized call rate, and standardized PC1.

and negatively loaded by PC3 (Table 6), and that is positively correlated with male relative fitness ($B = 0.863$, $SE = 0.244$, $z = 3.539$, $P = 0.002$), as shown in Figure 3. Alike in 2013, also in 2014, there is no evidence for a significant nonlinear selection.

Correlation between paternal call attractiveness and offspring fitness

Table 3 shows the descriptive statistics of the temperature-adjusted call properties computed on the subsample of males used as sires in the breeding experiment. The males used as sires in the breeding experiment were chosen randomly at the beginning of the breeding season and are quite representative of the entire male population. Indeed, we observed no significant difference between the sires and the whole population with regards to call rate ($U = 813$, $P = 0.98$), PC2 ($U = 727$, $P = 0.41$), and PC3 ($U = 967$, $P = 0.16$); however, the sires showed lower-than-average values for PC1 ($U = 547$, $P = 0.01$). Although males with higher call rate, producing longer calls, and calling at lower frequencies were proved to be most attractive to females at least in some conditions, our models provided no conclusive evidence for the hypothesis of a positive correlation between paternal call attractiveness and offspring fitness. Indeed, we only observed a marginally significant negative correlation between offspring AGE at metamorphosis and paternal call rate ($B = -1.544$, $SE = 0.726$, $t = 2.002$, $P = 0.051$), and no other indications that tadpoles sired by attractive males grew faster or reached metamorphosis earlier than the tadpoles sired by less attractive males. In contrast, and quite unexpectedly, we found a significant association between PC2 and both GROWTH ($B = 0.0024$, $SE = 0.0007$, $t = 3.151$, $P = 0.007$) and AGE ($B = -1.181$, $SE = 0.474$, $t = -2.493$, $P = 0.025$): males with higher-than-average pulse rates and pulse number and with a lower-than-average modulation index were found to sire offspring that grew faster and metamorphosed earlier than average, although we found no evidence for sexual selection on these acoustic traits.

DISCUSSION

The GGH assumes 1) consistent male attractiveness across a variety of signaling environments, 2) directional selection on male attractiveness, and 3) a positive correlation between paternal attractiveness and offspring fitness. Our results provided support for the first 2 assumptions; evidence for the latter, instead, was relatively scarce. Indeed, we observed that temperature did not affect the reliability of male advertisement calls in the Italian tree frog, *H. intermedia*. In addition, in one of the 2 breeding seasons, we observed linear

Table 5

Standardized linear selection gradients (β) and γ matrices of standardized quadratic and correlational selection gradients, for each breeding season

2013						2014					
	β	γ					β	γ			
		Call rate	PC1	PC2	PC3			Call rate	PC1	PC2	PC3
Call rate	-0.075	0.144				Call rate	1.288**	1.836			
PC1	0.144	-0.478	-1.528			PC1	0.915*	1.190	1.410		
PC2	-0.100	0.085	-0.068	0.336		PC2	-0.178	-0.021	0.490	-0.232	
PC3	-0.192	-0.134	-0.675	0.041	-0.904	PC3	-0.519*	-0.641	-1.131	0.289	-0.492

PC1, call duration; PC2, amplitude modulation; PC3, call frequency.

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$.

Table 6

M matrices of eigenvectors deriving from canonical rotation of the γ matrices for the 2013 (a) and 2014 (b) breeding season

	M				Selection	
	CR	PC1	PC2	PC3	θ_i	λ_i
a) 2013						
m_1	-0.555	0.184	-0.808	-0.065	0.027	0.413
m_2	0.786	-0.195	-0.586	-0.002	-0.126	0.199
m_3	-0.169	-0.481	-0.064	0.858	-0.068	-0.502
m_4	-0.211	-0.834	-0.007	-0.510	0.475	-2.063
b) 2014						
m_5	0.688	0.652	0.063	-0.310	1.917**	3.251
m_6	0.692	-0.625	-0.324	0.154	0.326	0.626
m_7	-0.216	0.076	-0.841	-0.490	-0.102	-0.118
m_8	0.001	0.420	-0.428	0.800	-0.025	-1.238

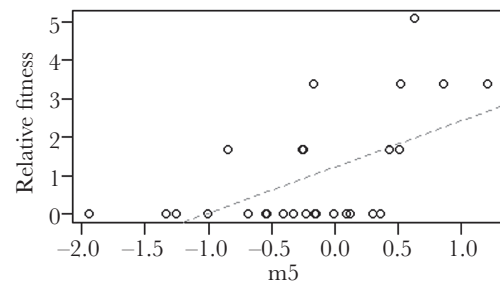
The last 2 columns of each table reports the linear (θ_i) and quadratic selection gradients (λ_i) along each eigenvector. Quadratic selection gradients (λ_i) correspond to the eigenvalue along the corresponding eigenvector. PC1, call duration; PC2, amplitude modulation; PC3, call frequency.

** $P < 0.01$.

selection on 3 acoustic properties (call rate, call duration, and call frequency). However, of these 3 properties, only call rate showed a positive and marginally significant association with offspring fitness, whereas call modulation (PC2), which was not found to affect male mating success, did show a significant association with both the age at metamorphosis and the growth rate of tadpoles.

Effects of temperature on the expression and reliability of male advertisement call

Our findings suggest that temperature affects the expression, but not the reliability of male advertisement calls in *H. intermedia*. The influence of thermic conditions on muscular contraction is a common feature among poikilothermic species: in general, higher temperatures allow for a faster contractile activity, but also increase the energetics of calling activity, posing intrinsic constraints to call duration and call rate (Pough et al. 2003). In most of the acoustic properties considered (the only exception being the number of pulses per call), individual temperature-dependent linear reaction norms differed in the intercept, but not in the slope, suggesting that no temperature-dependent GEI occurred in our study population, within the range of temperatures naturally encountered throughout the breeding season. In other words, the observed differences in call properties depended on males identity (i.e., on their genetic makeup) but the relative ranking of male call attractiveness remained consistent across a variety of thermic conditions.

**Figure 3**

The plot shows the positive correlation between m_5 and relative fitness.

Interestingly, male identity affected both the spectral properties, which are related to the resonance of vocal folds, and the temporal properties, which are related to the duration and to the amplitude modulation of calls, but it did not affect call rate. Unlike the spectral and fine-scale temporal properties, call rate is a highly dynamic component of the tree frog advertisement call and males are known to adjust their call rates in relation to the calling activity of the other males in the chorus (Gerhardt and Huber 2002; Castellano et al. 2009). The lack of a statistically significant effect of male identity on the relationship between body temperature and call rate, thus, might be due to the random error introduced by the uncontrolled effect of the social context on male identity.

Male reproductive success and sexual selection on acoustic properties

The 2 breeding seasons showed some intriguing differences. Our results, indeed, show that the strength and direction of selection varied between the 2 breeding seasons. Such temporal variation in the strength and direction of selection is not an uncommon finding in studies of the selective pressures acting on natural population (see, e.g., Grant and Grant 2002). A recent review of several studies about selection on a variety of traits revealed that the strength and direction of selection vary considerably across years and also suggests that the shape of selection may often change from linear to nonlinear—or vice versa—from 1 year to the following (Siepielski et al. 2009). Although much of this variation might be due to sampling errors (Kingsolver et al. 2012), temporal dynamism in selection may be caused by a variety of causes (Siepielski et al. 2009). In our case, in 2013, the population sex ratio (mm:ff) and the opportunity of selection were smaller than in 2014, but the nightly OSR was larger, because, in 2013, males spent more nights at the lek than in 2014. Because mate competition occurs on a nightly basis, competition and, consequently, selection should be expected

to increase with an increase in nightly OSR. But this is not what we observed. Indeed, in 2013, we found no evidence for either linear or nonlinear selection on call acoustic properties, whereas in 2014, we did. This counterintuitive result suggests that, in the study population, the opportunity for mate choice did not increase linearly with the number of prospective mates, possibly because of the high level of background noise that might impair the accuracy of female mate choice (Bishop et al. 1995). In this population, in fact, the breeding pond was small (only 5 m²) and the density of chorusing males was often high. In a previous work on this population (Botto and Castellano 2016), we analyzed the effect of calling effort on the within-chorus mating success. We showed female preferences for males with higher-than-average calling effort, but we showed also that these preferences decreased with an increase in chorus size (Botto and Castellano 2016). Whatever the reasons for the interannual variation in selection, in 2014, we found that females mated preferentially with males with higher-than-average call rate, longer-than-average call duration, and lower-than-average call frequency. Coherently, the analysis of selective pressures on male acoustic properties, after canonical rotation, revealed a strong positive directional selection on the eigenvalue \mathbf{m}_5 , which was positively loaded by both call rate and temporal acoustic properties and negatively loaded by spectral properties. The substantial irrelevancy of the canonical rotation and the lack of latent variables suggest that the variables that we chose were convenient and adequate to describe the phenomenon under study.

The 3 acoustic traits under selection can all be interpreted as reliable indicators of male quality. Directional preferences on call rate and call duration have been a common finding in univariate studies of acoustic communication in frogs (Arak 1983; Klump and Gerhardt 1987; Ryan and Keddy-Hector 1992; Welch et al. 1998; Castellano et al. 2009) and insects (Snedden and Sakaluk 1992; Crnokrak and Roff 1995; Bentsen et al. 2006) and have also been described in a recent multivariate study by Gerhardt and Brooks (2009). Calling activity in tree frogs is one of the most energy-demanding activities in the animal world (Wells 2001), and both call rate and call duration are considered to be reliable, condition-dependent signals of male quality (Welch et al. 1998; Holzer et al. 2003; Hunt et al. 2004). Spectral acoustic properties, in general, are negatively correlated with body size (Bradbury and Vehrencamp 2011), so that larger individuals produce sounds with lower frequency. In amphibians, growth is indefinite and strongly influenced by the environmental conditions and by the age of the individual (Duellman and Trueb 1986). The observed preference for males calling at lower frequencies may thus be favored by selection because call frequency, being an index of body size, may convey information about the caller's genetic quality and, ultimately, about its ability at surviving and at efficiently finding and metabolizing food (Wilbur et al. 1978; Friedl and Klump 2005).

In the second breeding season, thus, female preferences seemed to ultimately favor males bearing traits indicating higher-than-average genetic quality; such males, according to the GGH, should provide the females with indirect benefit, by producing fitter offspring (Welch et al. 1998). But is there any evidence that, in our study population, males with attractive calls sire fitter offspring?

Good genes, female choice, and male–male competition

The results of our breeding experiment provided little evidence for a positive correlation between paternal attractiveness and offspring fitness and, thus, for indirect genetic benefit for the choosing

females. Actually, we did find some evidence that calls might convey information about the genetic quality of callers, because we observed that males with a higher-than-average pulse rate and with a higher-than-average number of pulses tended to sire offspring that grew faster and metamorphosed earlier than average. However, these acoustic traits were not found to affect mating success and, thus, to be under sexual selection. The only attractive trait that was associated with offspring fitness was call rate, but the association was statistically feeble ($P = 0.051$).

These results are consistent with those obtained by analyzing the association between the nightly calling effort of sires (the fraction of time spent calling) and the quality of their offspring (Botto and Castellano 2016). The nightly calling effort was found to correlate positively with lek attendance and both were positively correlated with male mating success. However, alike call duration and call rate, calling effort did not show a positive association with offspring quality, whereas lek attendance did. Lek attendance and the quantity and the rate of calling are the components of male mating effort. The mechanism responsible for the association between lek attendance and mating success is male–male competition by endurance rivalry, whereas the mechanism that links the quality and the quantity of calling to mating success is female choice. In our population, thus, it seems that sexual selection favors males with good genes mainly through male–male competition rather than female choice.

But, then, why do females show preferences for traits that do not convey reliable information about the heritable quality of mates? In addition to the GGH, there are 2 alternative (but not-mutually exclusive) hypotheses for the evolution of female preferences. The first hypothesis suggests that the indirect benefits of choosing females are due to the attractive (rather than good) genes of sexy mates (Kokko et al. 2002) and it predicts heritability of attractive traits and genetic correlation between male traits and female preferences (Lande 1981). The second hypothesis does not assume any indirect genetic benefits and suggests that preferences might arise from selection for signal detectability (Ryan and Cummings 2013). In an elegant commentary, Kotiaho and Puurtinen (2007) underlined how several studies supporting the GGH failed to convincingly distinguish active female choice (which is expected to be energetically costly) from passive attraction for more detectable signals (which can be considered to be virtually cost-free) and to quantify the magnitude of indirect benefits correlated with paternal attractiveness. Given the moderate net genetic benefits associated with the preferred males (Møller and Alatalo 1999), they argued that good genes might not be such a strong driving force for the evolution of female preferences as it has been often assumed. What at first thought might seem active female choice, could in fact be the consequence of a perceptual bias for those call properties that are more effective in stimulating the female neuro-auditory system (Ryan et al. 1992) and, therefore, more easily detectable. According to this hypothesis, female preferences for high call rates and high calling effort might simply reflect a general feature of neuro-sensory systems that predisposes receivers to give more attention to more conspicuous stimuli. Males compete against each other for female attention and the costs of highly redundant signals can be better explained as the efficacy costs of male–male competition rather than as the strategic costs imposed by females to make signals reliable indicators of signaler qualities.

As fuzzy is the boundary between active and passive choice (Castellano et al. 2004; Kotiaho and Puurtinen 2007), so it may be the distinction between male–male competition and female choice,

in lekking species. In leks, male–male competition occurs at 2 distinct temporal levels: at the nightly level, males compete for attractiveness within the chorus, and at the seasonal level, they compete for chorus attendance. We argue that the strong nightly competition forces all males to call at their best when at the lek. This reduces the among-male variation in calling performance and, consequently, the amount of information that calling performance can convey about the signaler's quality. However, if the nightly competition costs are higher for low- than for high-quality males, low-quality males will be forced to reduce their already low competition potential for lek attendance. This would increase the among-male variation in lek attendance and, incidentally, it would also increase the association between lek attendance and male genetic quality (Castellano *et al.* 2009). In conclusion, our study suggests that, in our population, sexual selection is more likely to favor good genes, not through the direct mechanism of female choice, but through male–male competition for lek attendance, which is however driven by female preferences for attractive traits.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at <http://www.behco.oxfordjournals.org/>

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