Female fowl (*Gallus gallus*) do not prefer alarm-calling males

David R. Wilson[1] **& Christopher S. Evans**

(Centre for the Integrative Study of Animal Behaviour, Macquarie University, Sydney,
NSW 2109, Australia)

Summary

Phenotypic traits associated with reproductive outcomes are often thought to be under sexual
selection. In fowl, *Gallus gallus*, the rate at which males produce anti-predator alarm calls is
an excellent correlate of their mating and reproductive success. However, two different models can explain this relationship. Calling, like many costly traits, may be attractive to females.
Alternatively, males that have recently mated may invest in their mates by increasing alarm
call production. Although previous work provides strong support for the male investment hypothesis, the two hypotheses are not mutually exclusive. In this study, we tested the mate
attraction hypothesis by manipulating male alarm calling rates in three separate mate choice
experiments. The first experiment was conducted in a highly controlled laboratory setting.
There, we used video playback techniques to present females with simulated males that differed only in their alarm calling responses to simulated predators. In the second experiment,
females were presented with two live males in a naturalistic outdoor setting. One male's vocal output was supplemented with his own pre-recorded alarm calls, and the other male's was
not. In the third experiment, we combined the realistic spatial scale of an outdoor context
with the stringent experimental control offered by video playback. The male stimuli used in
this experiment differed in their propensity to produce four intercorrelated vocal signals that
are each correlated with male mating and reproductive success. These included aerial alarm
calls, ground alarm calls, food calls, and crows. Results from the three experiments consistently showed that females do not prefer alarm-calling males, suggesting that male alarm
calling is not a sexually selected signal.

Keywords: alarm signal, mate choice, mate investment, sexual selection, video playback.

Introduction

In many species, females enhance their inclusive fitness by choosing to mate with males that advertise a preferred quality (Darwin, 1871). In some cases, preferred qualities are readily apparent and afford females direct and immediate benefits (Searcy, 1979). Examples include the male's ability to provide material benefits, such as food (Thornhill, 1976) and territory (Alatalo et al., 1986). In other cases, preferred qualities cannot be assessed directly, and must instead be assessed indirectly through correlated traits. For example, at the time of mating, females cannot directly assess the quality of a male's genes, his fecundity, or his propensity to provide future parental care (Andersson, 1994). Instead, females assess correlates of these traits, which are often manifested by males as conspicuous ornaments, brilliant plumage, complex calls, or elaborate visual displays (Maynard Smith, 1956; Zahavi, 1975; Ryan, 1980; Hamilton & Zuk, 1982).

Identifying which traits are under selection by female mate choice is challenging evolutionary biologists. A necessary prerequisite is that variation in a trait should predict variation in reproductive success under natural conditions (Darwin, 1871). Observing animals in the wild, where all relevant factors are present, thus, is an ideal method for identifying candidate traits (Andersson, 1994). Observation alone, however, cannot disentangle the cause and effect of reproductive success, or the relative contributions of female choice and alternative mechanisms of sexual selection (Halliday, 1983; Andersson, 1994). To address these, mate choice experiments are necessary. These typically present females with a choice between two or more males that differ in their expressions of a particular trait. The males in these tests are unable to interact with each other, but the female can interact with each of them. This ensures that one male does not threaten or suppress the others (e.g., Houck, 1988), and that the female can choose freely between them.

Despite offering greater control than observational studies, mate choice experiments have several limitations. For example, most prevent males from interacting, yet females in the wild may actually choose males according to the outcomes of competitive interactions (Mennill et al., 2002). Similarly, the short duration of most mate choice tests may prevent females from assessing facultative traits, thereby forcing females to rely on readily available, but less reliable, secondary cues (Sullivan, 1990, 1994). As many traits are intercorrelated, it can also be difficult to ascertain the precise criteria on

which females rely. Manipulating just the trait of interest can overcome this (Alatalo et al., 1986), but manipulations per se may introduce additional artefacts. For example, female fowl, *Gallus gallus*, typically prefer males with large combs (Parker & Ligon, 2003). When the male's comb is manipulated, however, hens completely ignore it and rely instead on less-preferred secondary traits (Zuk et al., 1992). Another challenge is that mate choice can be both state- (e.g., hunger, Lesna & Sabelis, 1999) and context-dependent (e.g., season, Chaine & Lyon, 2008), yet controlled experiments often diminish the natural variability in these important factors. Finally, the expression of choice is difficult to measure in a mate choice apparatus. Association and mating are common proxies, but these do not account for the effects of sperm competition and cryptic female choice (Halliday, 1983).

Fowl are an ideal system for the study of female mate choice. Males exhibit conspicuous red ornaments on their heads, and the size of their ornament reflects their underlying resistance to internal parasites (Zuk et al., 1990b, 1995). Females offered a choice between two tethered males mate preferentially with the one exhibiting the larger ornament, suggesting that females are seeking high-quality genes for their offspring (Zuk et al., 1990a). This finding has been replicated in over a dozen experiments (reviewed in Parker & Ligon, 2003). However, it is based on an unnatural context in which males are unable to interact with each other, and in which females are given only minutes to evaluate prospective mates that were previously unfamiliar to them (Collias et al., 1966; Collias & Collias, 1967; Sullivan, 1990; Parker & Ligon, 2003).

In the wild, fowl live in stable social groups in which females have months or years to evaluate prospective mates (Collias et al., 1966; Collias & Collias, 1967; Sullivan, 1990). Males form pronounced dominance hierarchies, and females obtain direct benefits from the most dominant males (Pizzari, 2003). Under these more natural conditions, a male's ornamentation completely fails to predict his mating and reproductive success (Wilson et al., 2008). Instead, dominance and an intercorrelated suite of signalling behaviours are most important (Wilson et al., 2008), and the single best predictors of mating and reproductive success are the rates at which males produce aerial and terrestrial alarm calls, respectively. These are referential signals that warn conspecifics about avian and terrestrial predators (Evans et al., 1993). Both sexes produce terrestrial alarm calls, whereas only males accompanied by a conspecific audience produce aerial alarm calls (Karakashian et al., 1988).

The relationship between male dominance and mating success is expected, as dominance affords males preferential access to females through the exclusion of rivals (Pizzari, 2003). In contrast, the relationship between alarm calling and mating/reproductive success is surprising, and can potentially be explained by two non-mutually exclusive hypotheses. The male investment hypothesis suggests that increased calling reflects investment in mates and prospective offspring by males that have achieved recent mating success. Wilson & Evans (2008) provide strong support for the male investment hypothesis; they manipulated male mating success and showed that males that were permitted to mate produced approximately 30% more alarm calls than control males, which were prevented from mating. The mate attraction hypothesis suggests that alarm calling is attractive to females because it provides them with immediate information about nearby predators. Furthermore, calling is potentially costly for males, as it attracts the attention of nearby predators (Wood et al., 2000). Calling could, therefore, provide females with additional indirect information about a male's underlying ability to avoid predation (Zahavi, 1975).

In the present study, we tested the mate attraction hypothesis by conducting three complementary experiments in which male alarm calling was manipulated and female choice observed. In the first experiment, we used video playback to present females with simulated males that differed only in their production of aerial alarm calls during simulated attacks from avian predators. While this approach offers stringent experimental control, it potentially introduces artefacts caused by the small spatial scale and the simulated males. In a second experiment we, therefore, presented females with two live males in a large outdoor arena. Using acoustic playback techniques, one male was supplemented with his own pre-recorded aerial alarm calls, and one male was not. In the third experiment, we combined the stringent control of video playback with the realistic spatial scale of an outdoor context. The male stimuli used in this experiment differed in their propensity to produce four intercorrelated vocal signals that are each correlated with male mating and reproductive success (Wilson et al., 2008). These included aerial alarm calls, ground alarm calls, food calls, and crows. In each experiment, we predicted that females would prefer the male that alarm called at a higher rate.

Methods and results

General methods

Subjects were sexually mature fowl (*Gallus gallus*) derived from a population of freely interbreeding golden Sebrights bantams. This strain is morphologically distinct from the ancestral red jungle fowl. Their behavioural repertoire, however, is highly conserved, as comparisons between red jungle fowl and other domesticated strains have revealed few differences (McBride et al., 1969; Väisänen et al., 2005). Unlike many domesticated strains, however, Sebrights have not been artificially selected for rapid growth or egg production.

Experiments 1, 2 and 3 were conducted during the breeding seasons (September–March) of 2005/2006, 2006/2007 and 2007/2008, respectively. Prior to each experiment, subjects were housed individually indoors to standardize their recent mating experience (see Evans & Evans (1999) for details of indoor housing conditions). The duration of holding ranged from 2 to 4 weeks, but was the same for all individuals in any given experiment.

The same general design was used in each experiment. Briefly, female subjects were placed one at a time between two live or two video males that differed systematically in one or more traits. The male expressing higher levels of the trait of interest was the experimental stimulus, and the other male was the control stimulus. Females could approach either stimulus male, so measures of association were used to test whether females discriminated between the males and whether they potentially preferred one male to the other. Each male was used in only one experiment, whereas each female was potentially used in more than one experiment. Details of each experiment, including the number of subjects and stimuli, the method of manipulating traits of interest, and the duration and number of trials, are summarized in Table 1. They are described in detail below.

Prior to each experiment, we measured the morphology of each stimulus male using the methods outlined in Wilson et al. (2008). Briefly, we measured each male's body weight, and then photographed him in right side profile against a ruled background using a Canon EOS 300 digital camera (6.5 megapixels resolution). From the photographs, we used NIH ImageJ software (version 1.33u) to measure comb length and the combined surface areas of the comb, wattles, ear lappets, and red facial skin. For each stimulus male, we also characterized his vocal behaviour, which was scored from the stimulus videos (experiments 1 and 3) or the trial recordings (experiment 2) us-

Table 1. Summary of the setups used in three female mate choice experiments.

Variable	Experiment 1	Experiment 2	Experiment 3
No. of male stimuli	5	8	9
No. of unique male dyads used	10	4	30
Stimulus traits manipulated	Aerial alarm	Aerial alarm	Aerial alarm, ground alarm, food call, crow
Method of manipulation	Video playback	Acoustic playback	Video playback
Length of choice arena	1.1 m (laboratory)	6.5 m (outside)	13 m (outside)
No. of female subjects	20	32	30
No. of trials per subject	8	1	1
Duration of each trial	50 min	24 h	15 h
Duration of playback/trial	50 min	12.5 h	15 h
Response variables	Spatial association, orientation	Spatial association, roosting, copulation	Spatial association, roosting

ing JWatcher event recording software (version 1.0). Variables of interest included the number of crows, ground alarm calls, aerial alarm calls, and food calls, though not all vocalizations were observed in all experiments. Table 2 summarizes the phenotypes of the male stimuli used in each experiment.

Experiment 1 methods

Subjects were 20 females, which were each presented with two life-size video males in a highly controlled laboratory environment. Male stimuli differed systematically only in their propensity to produce aerial alarm calls during simulated aerial predator attacks.

Stimuli

Video stimuli were generated from five males, which were audio- and video-recorded between 9 and 25 November 2005. Our objective was to obtain from each male a minimum of 4 h of useable footage and 16 high-quality recordings of aerial alarm calls. An individual's recording sessions each lasted for approximately 1 h and were separated by at least 2 days.

During recording, males were confined inside a wire cage (1.12 m across the front, 0.45 m deep, 0.73 m high) within an anechoic sound chamber (Am-

Table 2. Description of male stimuli viewed by N female fowl, *Gallus gallus*, in three mate choice experiments.

Variable	Experiment 1 ($N = 20$)			Experiment 2 ($N = 32$)			Experiment 3 ($N = 30$)		
	Experimental	Control	Difference	Experimental	Control	Difference	Experimental	Control	Difference
Crows/h	38 ± 24.5	38 ± 24.5	0 ± 38.8	10 ± 7.7	9 ± 7.3	1 ± 11.5	20 ± 0.0*	2 ± 0.0*	18 ± 0.0*
Ground alarms/h							4 ± 0.0*	0 ± 0.0*	4 ± 0.0*
Aerial alarms/h	5 ± 0.0*	0 ± 0.0*	5 ± 0.0*	13 ± 4.2*	6 ± 3.8*	7 ± 4.7*	7 ± 0.0*	1 ± 0.0*	6 ± 0.0*
Food calls/h	—	—		1 ± 0.7	1 ± 0.6	0 ± 0.7	2 ± 0.0*	0 ± 0.0*	2 ± 0.0*
Weight (g)	1240 ± 145	1240 ± 145	0 ± 230	1270 ± 164	1270 ± 164	0 ± 103	1400 ± 163	1400 ± 163	0 ± 247
Ornament area (cm^2)	32 ± 2.5	32 ± 2.5	0 ± 4.0	33 ± 6.5	33 ± 6.5	0 ± 4.8	30 ± 4.1	30 ± 4.1	0 ± 6.1
Comb length (cm)	8 ± 0.3	8 ± 0.3	0 ± 0.5	8 ± 1.1	8 ± 1.1	0 ± 0.9	7 ± 0.8	7 ± 0.8	0 ± 1.2

Means (±SD) are shown for the experimental and control stimuli, as well as for the differences between them. Missing values indicate behaviours that were not expressed by stimuli in that particular experiment. *Significant difference (paired t tests: all $p < 0.0001$). All other $p > 0.5$.

plisilence, model 10070). The cage had an artificial grass mat and was lit by two 100-watt projection lamps (Aspherics®, model DLH4) placed 1.5 m apart and 1.0 m from the front of the cage. Video was shot with a Sony 3 CCD high-definition video camera (model HVR-Z1P; format HDV1080/50i) placed 1 m in front of the cage. The camera's optical zoom was set such that footage appeared precisely life-size when viewed on the displays used for playbacks. Sound was monitored in stereo using twin Sennheiser microphones (models MKH 40-P48 and MKH 20-P48; frequency response range $20–2 \times 10^4$ Hz, ± 1 dB deviation) that were connected to the camera and placed 0.3 m from either end of the cage. Audio and video signals were recorded digitally (audio 16 bits/48 kHz; video HDV/1080i50) to the hard drive of a Macintosh computer using Apple's QuickTime Pro software (version 7.1.5).

Prior to the first recording session, we calibrated our system by recording 30 s of continuous white noise that was broadcast through a StudioPhile speaker (model BX5) from the centre of the cage at 85 dB SPL (measured at a distance of 1 m with a RadioShack sound level meter, model 33-4050, C weighting, slow response). After this initial calibration step, the gain on the recording system was not adjusted to ensure that all stimuli were recorded at the same level. In addition, the amplitude of recorded signals was not adjusted during subsequent editing, which further ensured a consistent recording level across all stimuli.

Aerial alarm calls were evoked from males by presenting them with videos of raptor silhouettes on an overhead monitor (Lowe, model 8672 2P, 100-Hz refresh rate) at 10 min intervals. Raptor videos were played using Final Cut Pro software (version 3.0) on a Macintosh computer, and were converted to analogue signals using a Canopus converter (model ADVC110). The raptor sequences were constructed in the context of a previous study (Evans et al., 1993), and were known to evoke natural anti-predator responses, including crouching, fixating upwards, alarm calling, seeking cover, and fleeing. In each presentation, the raptor made four alternating passes across a white background at a rate of 8.8 body lengths per second. To minimize habituation, eight different renditions of the raptor were used, varying in terms of its apparent size (either 4.5° or 6.8° subtended at the subject's eye) and the corner of the monitor from which it originated (Evans et al., 1993). Finally, because aerial alarm calls are produced only in the presence of a conspecific

audience (Karakashian et al., 1988), a hen was placed in a cage immediately beside the camera. She was excluded from subsequent playback trials.

Footage from the five males was imported for editing into Final Cut Pro software (version 4.5) on a Macintosh computer. Unusable footage was deleted, including when the male was laying down or pacing rapidly within his cage. The remaining footage was then arranged into four playback sequences per male that satisfied the following criteria:

1. Sequences were exactly 52 min long (the first and last minutes provided time to introduce and remove females during playbacks).
2. Responses to predator presentations, denoted by crouching and the production of an alarm call, occurred at exactly 11, 21, 31 and 41 min within each sequence.
3. Only responses to predators that included high-quality aerial alarm calls were used.
4. The male's position and movement across-adjoining clips within a sequence were made as seamless as possible and were improved by applying a 4-frame cross-dissolve transition.
5. Sound generated by the audience hen was replaced with ambient sound chamber noise.
6. Footage was used only once.

After editing, the 20 sequences (4 sequences × 5 males) were duplicated. Within each duplicate, we replaced the alarm calls in the audio track only with ambient sound chamber noise. We did not remove the corresponding video because aerial alarm calls do not have an obligatory visual component. Indeed, in the majority of alarm calling events, males either remain motionless or simply roll their head and fixate upwards (Evans et al., 1993). For every alarm call that was replaced in the duplicate sequence, a corresponding edit of identical duration (but containing no signal) was made to the audio track of the original sequence to control for possible editing effects. The original 20 sequences became the experimental stimuli and the 20 duplicates (with alarm calls excised) became the control stimuli. All sequences were then given a 10-s prelude of a black screen and a 150 Hz high-pass audio filter that reduced background noise. They were then exported in their native format to digital videotape (Sony, model DVM60PRO). The final 40 playback sequences represented two treatments from each of five males that differed systematically only in their inclusion of aerial alarm calls.

Playbacks

We adopted a randomised complete block design with repeated measures. The 20 subjects were assigned at random to one of three groups (2 groups of 7, 1 group of 6); each group was tested daily during a different 8-day period (group 1, 16–24 December 2005; group 2, 2–10 January 2006; group 3, 15–23 January 2006). Within a given group, a given female was always tested at the same time each day. All tests were conducted in either the morning (0730–1130 h) or the late afternoon (1530–1830 h), which corresponds to the periods of peak reproductive activity in fowl (Cheng & Burns, 1988).

A total of 10 unique male dyads could be constructed from the five available male stimuli. Each male dyad was assigned at random to two female subjects. For one of these subjects (selected at random), one video male became the experimental stimulus and the other video male the control stimulus. For the second female subject, the experimental roles of the two male stimuli were reversed. This ensured that all aspects of male phenotype, other than the production of aerial alarm calls, were balanced perfectly between the two treatments across the entire experiment. Treatment positions (i.e., left or right of the female) were assigned at random to each subject and were maintained throughout that subject's 8-day playback series. For a given female, each male's four exemplars were played in a random order over the first 4 days of playback, and were then re-broadcast in the same order over the final 4 days.

During playbacks, females were held in the same wire cage and sound chamber that were used to record males. However, two black transect lines were added to the artificial grass mat to divide the cage into three equal sections. Also, the subject was lit with two incandescent lamps (60 W) used in place of the projection lamps. Male stimuli were presented at life-size on two Sony flat panel plasma displays (model PFM-42X1; 105.8 cm measured diagonally; 1024×768 lines of resolution), which were each placed facing the subject at 30 cm from either end of the cage. This viewing distance is important for effective video playback (Dawkins, 1996; Smith & Evans, 2008). Audio corresponding to each stimulus male was broadcast in stereo from two StudioPhile speakers (model BX5) placed at either end of each video display (i.e., four speakers in total). Because males had been audio-recorded in stereo, our playbacks were able to simulate a dynamic audio source that corresponded to the position of the stimulus male as he moved back and forth across the monitor. Although it is possible that the female perceived

the stereo playback as two distinct sound sources, we believe that this is unlikely, as the two audio channels were perfectly synchronized and the female was unable to approach either speaker. Finally, the same raptor silhouettes used to elicit alarm calls from males were played to female subjects on the overhead monitor at precisely the moments when the male stimuli appeared to respond to them. Each subject, therefore, experienced an overhead predator stimulus and two males responding to that stimulus (only one of which produced an alarm call) in synchrony, four times per day, over eight consecutive days.

Prior to commencing playback trials, we calibrated the playback system by broadcasting the previously recorded white noise from the playback speakers. We then adjusted the playback level until the white noise measured precisely 85 dB SPL at a distance of 1 m (i.e., the same level used during recording). Following calibration, the playback level was not adjusted for the remainder of the experiment, which ensured that all vocalizations were broadcast at a natural level and at precisely the same level at which they were recorded. Finally, we estimated the absolute amplitude of our playback stimuli by measuring the sound pressure level of 5 crows selected at random from each of the 5 stimulus males. The average sound pressure level of these crows (± 1 standard deviation) was 97 ± 1 dB SPL (measured at a distance of 1 m), which is consistent with the levels reported for domestic fowl (95–100 dB SPL at a distance of 1 m) by Brackenbury (1978).

Trials commenced by simultaneously broadcasting the two male stimuli from two Sony high definition decks (model HVR-M10P) that were connected to the playback equipment via a conduit panel in the chamber wall. The transition from the 10-s prelude of the black screen to the footage of the stimulus males provided the cue necessary for synchronizing the raptor playbacks. Immediately following this transition, the female was placed inside the cage and the chamber door was closed. Data collection began exactly 1 min after the transition. The trial ended exactly 50 min later and the female was removed during the final minute of playback.

Subjects were monitored using a Panasonic video camera (model WV-CL320) and a Sennheiser microphone (model MKH 40 P 48), which were connected to a Canopus converter (model ADVC110) via the chamber's conduit panel. The converter was attached to a Macintosh computer, which recorded the subject's behaviour during the following 50 min using QuickTime Pro software (version 7.1.5).

Analysis

Female behaviour was scored from video using JWatcher event recording software (version 1.0). For each female, we scored the total time spent in each third of the cage during each of her eight 50-min sessions. The middle third of the cage was considered an area of no preference, while the end thirds were considered areas of preference for the corresponding males. We also scored the total time spent orienting towards each stimulus male. The female was considered orienting towards a male when the longitudinal axis of her body was directed more towards him than towards the other male.

   Due to the non-independence between times spent orienting towards each stimulus, we expressed orientation as the daily difference between the two measures (i.e., experimental–control). Similarly, time spent in the ends of the cage corresponding to the experimental and control stimuli were non-independent, so we expressed spatial association as the daily differences between the two measures (i.e., experimental–control). Orientation and spatial association were then tested for changes over the 8-day playback series using repeated measures ANOVA. The intercept in this model is based on the average response over the 8 days and tests whether the female's average response (experimental–control) deviates significantly from zero. Effect sizes for both the deviation from zero and the change over time are estimated using partial η^2 values. Although side biases were not expected due to the symmetrical design of the playback apparatus, orientation and spatial association with respect to side (i.e., left–right) were also analysed using repeated measures ANOVA. Finally, we used multiple linear regression to test whether morphological and behavioural differences between the experimental and control stimuli, other than those created by experimental manipulations, had any relationship with female orientation or spatial association. The assumptions of parametric tests were met in all cases.

Experiment 1 results

We detected no side biases in experiment 1. Time spent facing left and time spent facing right did not differ significantly from each other on any of the 8 test days (repeated measures ANOVA: deviation from zero: $F_{1,19} = 0.725$, $p = 0.405$; change over time: $F_{7,133} = 1.532$, $p = 0.162$). Similarly, time spent in the left third of the cage did not differ significantly from time spent in the right third of the cage on any day (deviation from zero:

$F_{1,19} = 2.181$, $p = 0.156$; change over time: $F_{7,133} = 1.503$, $p = 0.171$). Females did not orient preferentially towards the experimental stimulus male (deviation from zero: $F_{1,19} = 1.654$, $p = 0.214$, partial $\eta^2 = 0.080$; change over time: $F_{7,133} = 1.997$, $p = 0.060$, partial $\eta^2 = 0.095$; Figure 1a) or associate preferentially in his third of the cage (deviation from zero: $F_{1,19} = 0.003$, $p = 0.957$, partial $\eta^2 < 0.001$; change over time: $F_{7,133} = 0.710$, $p = 0.664$, partial $\eta^2 = 0.036$; Figure 1b). Finally, unmanipulated behavioural and morphological differences between experimental and control stimuli, including differences in crowing rates, comb length, ornament size, and body weight, did not predict patterns of female orientation
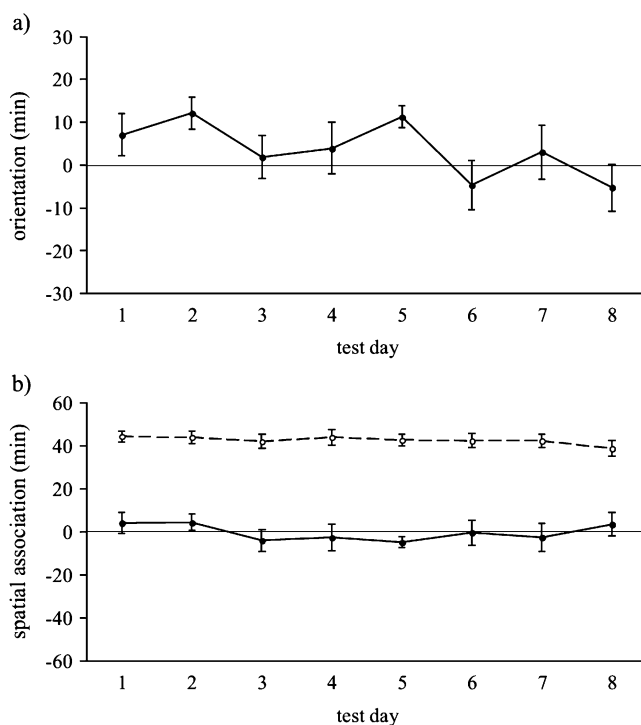

Figure 1. Mate choice behaviour of 20 female fowl in the first experiment. Each female was tested for 50 min on each of 8 days (abscissa). (a) Shown for each day is the mean ± SE difference (experimental–control) between time spent orienting towards the experimental male and time spent orienting towards the control male. (b) Shown for each day is the mean difference (experimental–control) between time spent in the experimental male's end of the cage and time spent in the control male's end of the cage (solid circles and lines). Also shown for each day is the total time spent in the two preference zones (open circles, hatched lines).

(multiple linear regression: $F_{4,15} = 0.154$, $p = 0.958$, $R^2_{adjusted} = -0.217$) or spatial association ($F_{4,15} = 0.089$, $p = 0.985$, $R^2_{adjusted} = -0.237$) when these were averaged across the eight test days.

Experiment 2 methods

Subjects were 32 females, which were each presented with two live males over a 24-h period in a large outdoor aviary. One male was supplemented with his own pre-recorded aerial alarm calls (experimental stimulus) and the other male was not (control stimulus).

Stimuli

Stimuli were eight live males from which we had recorded aerial alarm calls in the context of a previous study (Wilson & Evans, 2008). During recording, each male had been housed with a female in one of six outdoor pens ($l \times w \times h = 3.5 \times 1.5 \times 1.5$ m). The pens had transparent wire roofs, so alarm calls were evoked by natural stimuli flying overhead. Vocalizations were acquired using Behringer C-2 studio condenser microphones (frequency response 20–2×10^4 Hz, ± 12 dB) attached (inverted) to the centre of each pen roof. Signals were digitised using an 8-channel, 24 bit/96 kHz interface (FirePod, PreSonus Audio Electronics) and recorded as multichannel WAVE files (16 bit, 44.1 kHz) using Boom Recorder software (version 7.5, VOSGAMES).

For each male, 100 alarm calls, chosen for their high signal-to-noise ratio, were extracted from 100 different alarm calling bouts from within the raw recordings using Raven Interactive Sound Analysis Software (version 1.3 Pro, Cornell Lab of Ornithology Bioacoustics Research Program). From the 30 s preceding each call, we also extracted a segment of ambient noise, which was the same length as the subsequent call and which contained only background noise. In each case, the ambient noise was paired with its corresponding alarm call as the second channel in a single stereo file. Each stereo file hence contained background noise in both channels, but an alarm call in only the first. Following extraction, all stereo files were high-pass filtered (200 Hz) and then normalized using Peak Pro software (version 5.2). Finally, silence lasting 7, 7.5 or 8 min (selected at random) was appended to the end of every file so that, when the files were played sequentially, the alarm calls were broadcast at a variable and realistic rate. This variable calling rate was

based on the mean call rate (±1 standard deviation) of 18 alpha males observed in outdoor social groups between 1999 and 2006 (Wilson et al., 2008).

Playbacks

Choice tests were conducted in an outdoor aviary (9.0 m long, 3.0 m wide, 2.8 m high) that was divided lengthwise into three compartments. The outer compartments housed the males and measured 1.25 m in length, whereas the middle compartment housed the female and measured 6.5 m in length. The entire aviary had an open wire construction. However, the roof and exterior walls of the end compartments were covered with opaque shade cloth that prevented the male occupants from viewing much of their surroundings. This was important because it minimized the number of alarm calls produced by males in response to external stimuli, thereby affording greater experimental control over their apparent alarm calling rates. The interior walls separating each compartment were also covered with shadecloth, though this was removed along the bottom metre to allow visual contact between male and female occupants.

Aviary compartments were designed to house birds for a minimum of 24 h. They were each provided with food and water, as well as with sheltered perches for roosting along both sides of each interior wall (i.e., four perches in total). The perches were only 0.75-m high, so females could roost adjacent to and within view of either male. The female was also provided with a small enclosure (1.7 m long, 0.9 m wide, 0.8 m high) in the centre of her compartment, which provided her with shelter and a 'no-choice' roosting site.

The 32 females were assigned at random to four groups of eight that were tested sequentially in a randomised complete block design between 26 January and 23 March 2007. Each group was tested with a different pair of stimulus males and no male was used with more than one group. Males were paired so as to minimize the morphological differences between them. Furthermore, for each of the eight females within a given group, the positions (i.e., left or right of the female) and treatments (i.e., experimental or control) of the two males were assigned at random in a fully balanced factorial design. Each male, therefore, spent two trials per treatment in each of the aviary's two end-compartments.

Trials began 1 h after sunrise by placing the stimulus males into their assigned compartments. The female was then placed into the enclosure within the middle compartment, where she was confined until 1 h before sunset.

During this time, she could view and listen to both males, but could not approach either of them. Throughout this period, we supplemented the experimental male's alarm calling rate by broadcasting his own pre-recorded alarm calls at intervals averaging 7.5 min. Because aerial alarm calls are individually distinctive (Bayly & Evans, 2003), and because subjects were held equidistant between the two stimulus males during playbacks (approximately 3.25 m from each male), we assume that subjects could reliably associate alarm calls with their corresponding males.

For each female, the experimental male's 100 pre-recorded alarm calls were played in a random order (calls were used only once per female) using iTunes software (version 7) on a Macintosh computer. Calls were converted to analogue signals using a Digidesign MBox converter (24 bits/48 kHz) and were amplified using a Behringer Ultra Linear Reference Amplifier (model A500). Alarm calls were broadcast at natural amplitude (76 dB SPL, measured at a distance of 1 m) from a Bose outdoor speaker (model Free Space 51), which was located centrally along the back wall of the experimental male's compartment. The amplitude was based on our subjective assessment of alarm call levels produced by free-living birds. Background noise corresponding to each alarm call (i.e., the second channel of each stereo file) was broadcast simultaneously from an identical speaker located in the control male's compartment. Although these speakers are omnidirectional, the shadecloth covering the surrounding walls was acoustically transparent and so should have prevented reverberation. More importantly, we broadcast all stimuli in situ before beginning the experiment and could detect no reverberation or other acoustic artefacts while standing beside the female's enclosure.

At 1 h before sunset, playbacks were terminated and a remote latching mechanism was used to release the female from her enclosure into the larger central compartment. For the remainder of the evening, she was free to approach, inspect, and roost adjacent to either male. After birds had selected their final roosting locations, we selected one of the two males at random and used a remote latching mechanism to open a door in the wall between his compartment and the female's. In no case did this cause the birds to descend from their perches. The following morning, when the birds did come down to the ground, the female was free to interact and copulate with the released male until the next trial commenced (approximately 1.5 h).

Male stimuli were monitored throughout the first day of each trial using Behringer C-2 studio condenser microphones attached to their compartments. Audio signals were digitised using a Digidesign MBox converter

(24 bits/48 kHz) and were recorded as stereo WAVE files (16 bit, 44.1 kHz) using Boom Recorder software (version 7.5, VOSGAMES). In addition, the female was video-recorded during the entire time in which she was released from her enclosure using an infrared video camera (All Things Sales & Services, model MINI-M33HR) attached to the roof of her compartment. Illumination at night was provided by infrared light emitting diodes (850 nm wavelength; model IR36-PCB) attached to the four corners of her compartment. Video signals were digitised using a Canopus converter (model ADVC110) and were recorded to disk using QuickTime Pro software (version 7.1.5) on a Macintosh computer.

Analysis

Each female's behaviour was scored from video during the 90 min that followed her initial release (i.e., until 30 min after sunset) using JWatcher event recording software (version 1.0). For each female, we scored the total time spent within 1.5 m of each male's compartment, which was denoted by posts on the outer aviary walls. In all cases, the female selected her final roosting perch within this time and did not descend from it until the following morning. The next morning, we counted all copulations between the female and the released male prior to the start of the subsequent trial.

Spatial association data from the 90-min test period were divided into nine 10-min time bins using JWatcher event recording software (version 1.0). For each time bin, we expressed female spatial association as the difference between time spent within 1.5 m of the experimental stimulus male and time spent within 1.5 m of the control stimulus male. However, spatial association scores in the final four time bins became dichotomous due to females selecting their final roosting sites. These data were hence excluded from measures of spatial association and were used instead to determine females' roosting preferences. Spatial association data from the first five time bins had continuous, but skewed distributions that could not be corrected using data transformations. Changes in spatial association over the first five time bins were, thus, tested using a non-parametric Friedman test. This test is based on individual ranks, however, and does not include an intercept. To test if females associated preferentially with either male, we compared the total time spent with each male during the first 50 min of the test period using a Wilcoxon signed-ranks test. This test is comparable to the intercept provided in repeated measures ANOVA and tests whether females spent more

or less time with experimental males. Although side biases were not expected due to the symmetrical design of the playback apparatus, spatial association with respect to side (i.e., left–right) was also analysed. To examine female roosting preferences, we used a χ^2 goodness-of-fit test to determine whether females were more likely to roost with experimental as opposed to control males. Similarly, we used a 2×2 contingency table (experimental vs. control; mated vs. not mated) and Fisher's exact test to assess female mating preferences on the following morning. Finally, we used multiple regression to test whether morphological and behavioural differences between experimental and control males, other than those created by experimental manipulations, were related to female spatial association or female roosting preferences.

Experiment 2 results

We found no evidence of a side bias in experiment 2. Time spent in the left section of the aviary did not differ significantly from time spent in the right section during the first 50 min of testing (deviation from zero, Wilcoxon signed ranks test: $Z = -0.262$, $N = 32$, $p = 0.793$; change over time, Friedman test: $\chi_4^2 = 4.216$, $N = 32$, $p = 0.378$). Similarly, subjects did not spend more time with experimental males than with control males during this time (deviation from zero, Wilcoxon signed ranks test: $Z = -0.711$, $N = 32$, $p = 0.477$, partial $\eta^2 = 0.017$; change over time, Friedman test: $\chi_4^2 = 2.432$, $N = 32$, $p = 0.657$, partial $\eta^2 = 0.007$; Figure 2). Females also did not roost preferentially beside experimental males (χ^2 goodness of fit test: $\chi_1^2 = 0.125$, $N = 32$, $p = 0.724$; Figure 2) or mate preferentially with them on the morning of their release. Indeed, females mated with only two of the 15 released control males and only four of the 17 released experimental males (Fisher's exact test: $N = 32$, $p = 0.659$). Finally, none of the unmanipulated behavioural and morphological differences between experimental and control males, including differences in food calling, crowing, comb length, ornament size, or body weight, were related to female spatial association (multiple linear regression: $F_{5,26} = 0.520$, $p = 0.788$, $R^2_{\text{adjusted}} = -0.102$) or roosting preference (multiple logistic regression, whole model likelihood ratio: $\chi_5^2 = 1.211$, $N = 32$, $p = 0.976$, $R^2 = 0.037$).

Experiment 3 methods

Subjects were 30 females, which were each presented with two life-size video males in a large outdoor enclosure. The two male stimuli differed sys-
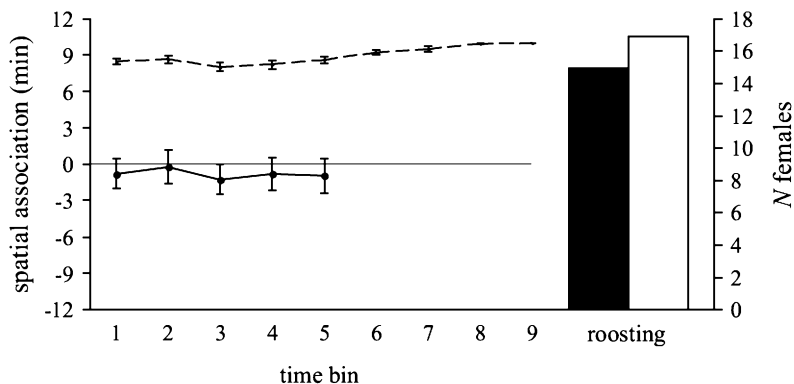
Figure 2. Mate choice behaviour of 32 female fowl in the second experiment. Females were released from the central enclosure 1 h before sunset and were allowed to approach either stimulus male for the following 90 min. Shown for each 10-min interval along the abscissa is the mean ± SE difference (experimental–control) between time spent within 1.5 m of the experimental male and time spent within 1.5 m of the control male (solid circles and lines). Note that data are not presented during the final four intervals because females had already selected their final roosting sites. Also shown for each interval is the total time spent in the two preference zones (open circles, hatched line). Shown on the right are the number of females that roosted beside the experimental (filled bar) and the control male (open bar).

tematically in their propensity to produce four intercorrelated vocal signals that are also correlated with male mating and reproductive success (Wilson et al., 2008). Variables included aerial alarm calls, ground alarm calls, crows and food calls.

Stimuli

A total of nine males were audio- and video-recorded between 2 and 27 October 2007. From each male, we obtained a minimum of 3 h of useable footage, which included at least 60 crows, 21 aerial alarm calls, 13 bouts of ground alarm calls, and seven bouts of food calls. Recording sessions lasted for approximately 1 h and employed the same recording apparatus and methods as described in experiment 1. In addition, a Sony flat panel plasma display (model PFM-42X1) was placed 40 cm beside the male's cage and was used to present males with videos of a terrestrial predator. Similarly, a remotely operated food dispenser was placed immediately behind the cage and was used to deliver live mealworms to the male. Finally, in contrast to experiment 1, audio was recorded with only one microphone (Sennheiser, model MKH 40-P48), which was attached (inverted) to the centre of the cage roof.

For each male, we elicited aerial alarm calls with videos of raptor silhouettes (see experiment 1 for details), ground alarm calls with a 60-s video of a raccoon (*Procyon lotor*: see Evans et al. (1993) for details), and food calls with two live mealworms (see Smith & Evans (2008) for details). Stimuli were presented at 10-min intervals in a random order until the required number of each behaviour was achieved. There was no need to elicit crows, as they were produced spontaneously throughout recording sessions.

Footage from the nine males was imported for editing into Final Cut Pro software (version 5) on a Macintosh computer. Unusable footage was deleted, including when the male was laying down or pacing rapidly within his cage. For each male, the remaining footage was then arranged into one 3-h playback sequence that satisfied the following criteria:

1. Each sequence contained in a random order 60 crows, 21 aerial alarm calls, 13 bouts of ground alarm calls and 7 bouts of food calls (call rates are one standard deviation above the population mean, as described in Wilson et al. (2008); calls within bouts were separated by less than 5 s and bouts were separated by at least 2 min).
2. Only responses to stimuli that included high quality alarm calls and food calls were used.
3. The male's position and movement across-adjoining clips within a sequence were made as seamless as possible and were improved by applying a 4-frame cross-dissolve transition.
4. The male's position and posture at the beginning and the end of the sequence were similar so that the sequence could be looped without obvious motion artefact.
5. Sound generated by the audience female was replaced with ambient sound chamber noise.
6. Footage was used only once.

After editing, the nine sequences were duplicated and the behaviours of interest within the duplicates reduced to one standard deviation below the population mean, as described in Wilson et al. (2008). Specifically, the 21 aerial alarm calls were reduced to two by replacing their audio component with ambient sound chamber noise (note that aerial alarm calls do not have an obligatory visual component). Similarly, the 13 ground alarm calls were reduced to 0, the 60 crows to 7, and the 7 food calls to 0 by removing the relevant audio and video components. For each signal removed, we also

removed a segment of equal duration (but containing no signal) from the original sequence to control for possible editing effects. The original nine sequences became the experimental stimuli and the nine duplicates became the control stimuli. All sequences were given a 200 Hz high-pass audio filter to reduce background noise and were then exported for playback as QuickTime files (audio 16 bits/48 kHz; video DVCPRO50/720p50).

Playbacks

Trials were conducted in a long outdoor enclosure (11 m long, 1.2 m deep, 1.0 m high). It had an earth substrate and an open wire construction that permitted its occupants to view their surroundings. The central 4 m of the enclosure was covered with metal roofing and contained food, water, and perches for roosting. Attached to each end of the enclosure was a wooden shelter (2.2 m long, 1.2 m deep, 1.0 m high) that contained straw bedding and perches for roosting in its front half (i.e., the end closest to the wire enclosure) and the equipment necessary for playbacks in its rear half. The front and rear halves of each wooden shelter were separated by a transparent wire partition.

Of the 36 unique male dyads that could be constructed from the nine video male stimuli, 30 were randomly selected for use in playbacks. Each of these was assigned at random to a different female subject. For each subject, one video male (selected at random) became the experimental stimulus and the other video male the control stimulus. The positions (left or right of the female) of the two video male stimuli were assigned at random.

The 30 subjects were tested sequentially between 6 November 2007 and 28 February 2008. Trials began at 0700 h by broadcasting the video male stimuli on two Sony flat panel plasma displays (model PFM-42X1; 105.8 cm measured diagonally; 1024×768 lines of resolution), which were placed along the rear walls of the two wooden shelters (1 m from the wire partition separating the two halves of the shelter). Stimulus files were played using QuickTime Pro software (version 7.1.5) running on two Macintosh computers (Mac Mini, 1.66 GHz Intel Core Duo) that were connected to and concealed behind the two video displays. For each video male, the corresponding audio was converted to an analogue signal using a Behringer converter (model FCA202, 24 bits/96 kHz) and was broadcast at natural amplitude (see experiment 1 for details of sound pressure level calibration) from a

forward-facing directional StudioPhile speaker (model BX5) that was placed immediately behind the video display.

Immediately after playbacks had commenced, the subject was placed into the centre of the wire enclosure. For the remainder of the day, she could explore the wire enclosure, enter the wooden shelters at either end, and approach the video males to a minimum distance of 1 m. The video sequences, which were each 3 h in length, were looped continuously throughout the day. Playbacks were terminated at 2200 h, which, in all cases, was at least 30 min after sunset. This provided sufficient time for females to select their final roosting sites.

Throughout the trial, subjects were monitored using two infrared video cameras (model Maxi-Day/Night, 720×576 lines of resolution) located in the centre of the wire enclosure. The cameras faced the two video males and provided complete coverage of the enclosure. They also provided infrared illumination, allowing them to operate at night when subjects were choosing their roosting locations. The two video signals were multiplexed using a 4-channel digital video recorder (model DVMR-AVMP4) and were recorded as a single video image using a Canopus converter (model ADVC110) and QuickTime Pro software (version 7.1.5) running on a Macintosh computer.

Analysis

Behaviour was scored from video using JWatcher event recording software (version 1.0). For each female, we scored the total time spent in each of the two wooden shelters.

Spatial association data from each 15-h trial were divided into fifteen 1-h time bins. For each time bin, we calculated the difference between time spent in the experimental video male's wooden enclosure and time spent in the control video male's wooden enclosure. However, spatial association data became dichotomous in the final five time bins due to females selecting their final roosting sites. Data from this period were hence excluded from measures of association and were used instead to determine female roosting preferences. During the first 10 time bins, data had continuous, but skewed distributions, thus, preventing parametric analyses. Female spatial association data were, therefore, analysed over the first 10 time bins using the nonparametric methods described in experiment 2. To explore female roosting preferences, we used a χ^2 goodness-of-fit test to determine whether females were more likely to roost with experimental or control males. Finally, we

used multiple regression to test whether morphological and behavioural differences between experimental and control males, other than those created by experimental manipulations, had any effect on female spatial association or female roosting preferences.

Experiment 3 results

In experiment 3, we found no evidence of a side bias. Time spent by the subject in the left wooden enclosure did not differ significantly from time spent in the right wooden enclosure over the first 10 h of testing (deviation from zero, Wilcoxon signed ranks test: $Z = -0.545$, $N = 30$, $p = 0.586$; change over time, Friedman test: $\chi_9^2 = 6.607$, $N = 30$, $p = 0.678$). Similarly, time spent in the experimental video male's wooden enclosure did not differ significantly from time spent in the control video male's wooden enclosure (deviation from zero, Wilcoxon signed ranks test: $Z = -0.545$, $N = 30$, $p = 0.586$, partial $\eta^2 = 0.006$; change over time, Friedman test: $\chi_9^2 = 5.481$, $N = 30$, $p = 0.791$, partial $\eta^2 = 0.010$; Figure 3). Furthermore, females did not roost preferentially beside experimental males
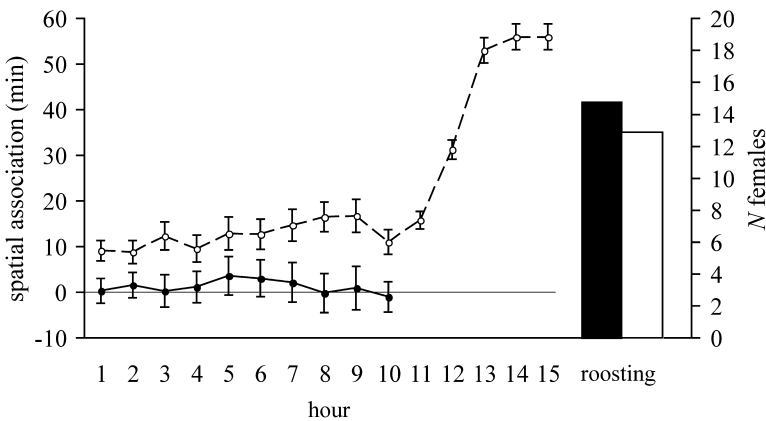

Figure 3. Mate choice behaviour of 30 female fowl in the third experiment. Each female was allowed to approach either stimulus male over a 15-h period. Shown for each 1-h interval along the abscissa is the mean difference ± SE between times spent in each male's wooden enclosure (experimental–control) (solid lines and circles). Note that data are not presented for the final five intervals because females had already selected their roosting sites. Also shown for each interval is the total time spent in the two preference zones (open circles, hatched line). Shown on the right are the number of females that roosted beside the experimental (filled bars) and the control male (open bar).

(χ^2 goodness-of-fit test: $\chi_1^2 = 0.143$, $N = 28$, $p = 0.705$; Figure 3; note that two females roosted in the middle section and were hence excluded from this analysis). Finally, unmanipulated differences between experimental and control males, including differences in comb length, ornament size, and body weight, did not account for patterns of spatial association (multiple linear regression: $F_{3,26} = 0.866$, $p = 0.471$, $R_{adjusted}^2 = -0.014$), or female roosting preferences (multiple logistic regression, whole model likelihood ratio: $\chi_3^2 = 1.814$, $N = 28$, $p = 0.612$, $R^2 = 0.063$).

Discussion

We tested whether female fowl were attracted to alarm-calling males in three separate mate choice experiments. In each experiment, females were presented with an experimental stimulus male that had his alarm-calling rate increased, and a control stimulus male that had his alarm-calling rate reduced. In all cases, females failed to express a preference for either experimental or control stimuli, suggesting that females were not attracted to alarm-calling males. The mate attraction hypothesis is hence unable to explain the observed relationship between male alarm calling and reproductive success (Wilson et al., 2008). Instead, it appears that this relationship is explained exclusively by males investing in their mates (Wilson & Evans, 2008).

Findings were consistent across the three experiments, suggesting that female indifference was not an artefact produced by the context or methodology of any one experiment. For example, female indifference cannot be attributed to the use of video stimuli, as females were also indifferent towards the live stimuli presented in experiment 2. Furthermore, video playback is known to elicit biologically appropriate responses from fowl (Evans et al., 1993; Evans & Evans, 1999; Smith & Evans, 2008) and has been used successfully to demonstrate female mate choice in a variety of other taxa (e.g., reviewed in Rosenthal, 1999). In addition, the small spatial scale of experiment 1 cannot explain the results, as females also failed to express preference in the larger outdoor arenas. In experiments 1 and 2, alarm calling rates were manipulated independently and were hence incongruent with the rates of other vocalizations that are normally correlated with alarm calling. Experiment 3, however, manipulated the entire suite of intercorrelated behaviours that are known to predict male mating and reproductive success, including

rates of alarm calling, crowing, and food calling (Wilson et al., 2008). Despite this, females remained indifferent. Lack of preference also cannot be attributed to an overall disinterest in male stimuli, as females reliably chose to roost adjacent to males in experiments 2 and 3, despite the availability of roosting sites in the central sections of each arena. Another potential problem was caller reliability. In experiments 2 and 3, male alarm calls were not reliably associated with predator stimuli, and natural predator stimuli that the subjects could potentially see were not reliably associated with male alarm calls. It is, therefore, possible that female indifference resulted from female habituation to unreliable callers. In vervet monkeys (*Cercopithecus aethiops*) and Richardson's ground squirrels (*Urocitellus richardsonii*), for example, signal recipients show reduced vigilance in response to alarm calls from individuals that repeatedly issue false alarms (Cheney & Seyfarth, 1988; Hare & Atkins, 2001). In fowl, females do habituate to the food calls of unreliable males; they do not, however, habituate to the alarm calls of unreliable males (Gyger & Marler, 1988; Evans, unpublished data), which, together with our first experiment in which male alarm calls were reliably associated with predator stimuli, suggests that caller reliability does not explain female indifference.

Differences in male morphology did not predict any of the measures of female choice in any of the experiments. This contrasts with previous mate choice studies (but see Leonard & Zanette, 1998), which have often revealed a preference for males with large sexual ornaments (reviewed in Parker & Ligon, 2003). The discrepancy suggests that females in the present study employed different mate choice criteria than those used by females in previous studies. Variation in mate choice criteria could reflect differences between seasons (e.g., Chaine & Lyon, 2008) or populations (Endler & Houde, 1995). It could also reflect the duration of the assessment period available to females (Sullivan, 1990, 1994). In previous mate choice experiments, females were given between 20 and 120 min to evaluate males that were previously unfamiliar to them (reviewed in Parker & Ligon, 2003). Thus, females in those studies may not have had enough time to evaluate preferred facultative traits and may have relied instead on static morphological traits that could be readily assessed. In contrast, females living in stable social groups have more time to evaluate males and do not mate preferentially with males exhibiting large sexual ornaments (Wilson et al., 2008). The extended assessment period available to females in the current study (8–24 h) may, therefore, explain

why differences in male morphology did not predict any of our measures of female choice.

This study provides new insight into the evolution of anti-predator alarm calls. It is based on an observed relationship between a male's rate of alarm calling and his mating and reproductive success (Wilson et al., 2008). This relationship suggests either that females are attracted to alarm-calling males, or that males are alarm calling as a form of male investment. Wilson & Evans (2008) provide strong evidence for the male investment hypothesis, but that hypothesis is not mutually exclusive with the mate attraction hypothesis. Furthermore, alarm calling is an ideal cue upon which females could rely when selecting their mates (Zahavi, 1975; Andersson, 1994); it varies considerably among males (Wilson et al., 2008), provides females with valuable information about predators (Evans et al., 1993), and, although energetically inexpensive (Horn et al., 1995), is potentially costly for males to express due to the increased risk of attracting nearby predators (Wood et al., 2000). The present study provides the first definitive test of the mate attraction hypothesis for any system of alarm calls. Results suggest that male alarm calling is not a sexually selected signal.

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