Mating success increases alarm calling effort in male fowl, *Gallus gallus*.

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**WILSON & EVANS:** MATING INCREASES ALARM CALLING IN MALE FOWL

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Investment in mates and offspring typically reflects a tradeoff between survival and reproductive potential. Individuals should therefore invest according to potential fitness benefit. Males typically cannot ascertain their probability of paternity directly, but this can often be approximated from mating success. In fowl, mating frequency and fitness are both predicted by the rate at which males produce alarm signals. These novel phenomena suggest insights into the evolution of apparently altruistic signals. However, like virtually all studies examining the adaptive value of alarm calling, the relations between behaviour and its possible benefits are correlative. Demonstration of a causal relationship requires experimental evidence. There are two likely models of the relation between alarm calling and reproductive success. Calling, like other costly traits, might be attractive to females. Alternatively, calling might result from recent mating success and hence be a form of mate investment. Here, we test the latter possibility by manipulating the mating success of male fowl. Results show that males increase their alarm calling effort as a function of their recent mating success. This provides strong support for the mate investment hypothesis and is the first demonstration of a causal link between mating frequency and alarm calling.

Keywords: alarm signal; altruistic behaviour; animal communication; antipredator behaviour; mate investment
Investing in mates and offspring can be one of the most energetically demanding and time-consuming activities that animals engage in (Clutton-Brock & Godfray 1991). In providing critical resources, such as food, territory, and protection from predators, investors forego additional mating opportunities and risk both injury and death (Clutton-Brock 1991). These costs can be offset by increases in the probability of survival and future reproduction by recipients, but such fitness compensation is contingent upon young or prospective young being genetically related to the investor (Trivers 1972). Optimal investment is especially problematic for males, because paternity is often compromised by mate infidelity, sperm competition, and cryptic female choice (Møller & Birkhead 1993). Mate guarding can be surprisingly ineffective (e.g. Gowaty & Bridges 1991; Kempenaers et al. 1992), and males of only a few species have been shown to be capable of recognizing and investing selectively in their own offspring (Davies & Brook 1989; Davies et al. 1992).

Given that direct measures of paternity are typically unavailable, decisions about when and how much to invest can only depend upon a proxy (Trivers 1972). A male’s first mating affords him the possibility of siring offspring, but the probability of success is less certain. A comparative study involving 52 bird species found that paternal investment was positively associated with certainty of paternity (Møller & Birkhead 1993), as estimated from the relative frequency of extra-pair offspring. Investment strategies can also vary within a species. Female dunnock’s (*Prunella modularis*), for example, reside and mate with either one or two males, and males, provided they have mated with the female, provision her chicks with food (Burke et al. 1989). Unlike monogamous males, however, polyandrous males adjust their chick feeding effort according to the share of matings obtained by their competitors during the period in which the eggs are fertilized (Davies et al. 1992).
Identifying the adaptive utility of alarm calling is a classic problem in behavioural and evolutionary biology. Signallers endanger themselves by warning conspecifics of impending danger, yet they receive no obvious fitness payoff in return (Marler 1955; Sherman 1977; Alatalo & Helle 1990; Wood et al. 2000). Individual selection (Woodland et al. 1980), kin selection (Maynard Smith 1965) and reciprocal altruism (Trivers 1971) have all been invoked as explanations. However, empirical tests of these theories have been complicated by the challenge of quantifying signalling costs and controlling confounding factors in the field, such as the presence and proximity of kin, individual experience, and recent mating history.

Furthermore, calling may serve multiple functions, and functions may vary across species and contexts. Most inferences about call function have necessarily been based upon observations of the classes of callers and call recipients (e.g. sex; mating status; parental status; kinship; residency) present during predator encounters (Dunford 1977; Sherman 1977; Smith 1978; Schwagmeyer 1980; Woodland et al. 1980; Cheney & Seyfarth 1981; Hoogland 1983, 1996; Blumstein et al. 1997; Haftorn 2000). This approach identifies putative functions of calling based on the conditions conducive to each, but experimental manipulations of the relevant factors are lacking.

Here we focus on a subset of potential functions, that of warning mates and prospective offspring (Witkin & Ficken 1979). This makes the clear prediction that propensity to alarm call should depend on the signaller’s reproductive potential (Trivers 1972). Supporting evidence comes from studies of socially monogamous willow tits (Parus montanus). During the winter, males are more likely to alarm call if they can see their mate (Hogstad 1995). Willow tits form pair bonds that persist throughout the year, so a male that
protects his mate during the non-breeding season increases the probability that she will survive until spring and raise his offspring (Alatalo & Helle 1990; Hogstad 1995). Hogstad (1995) interprets this as evidence for mate investment. While the data are certainly consistent with such a function, they do not exclude an alternative explanation, which is that costly alarm calling (Alatalo & Helle 1990) functions instead to attract females (Zahavi 1975). The precise adaptive value of alarm calling hence remains unclear.

Fowl (Gallus gallus) are an ideal system for examining the function(s) of antipredator signalling. They live in stable social groups for most of the year and both sexes establish pronounced dominance hierarchies. During the breeding season, males defend territories and escort sexually receptive females (McBride et al. 1969). Furthermore, males emit functionally referential alarm calls that indicate the presence of terrestrial and aerial predators (Evans 1997; Evans et al. 1993). Remarkably, the rate at which males emit aerial alarm calls is the single best predictor of their mating success (Wilson et al. In press). This correlation has not been observed in any other species. It thus offers a potential novel insight into the evolution of aerial alarm calling.

In the present study, we manipulated the mating success of male fowl over a period of several weeks to test for a causal effect on the production of aerial alarm calls. To verify that any change was specific to antipredator signals, we also monitored crowing, an energetically cheap territorial signal. To evaluate whether changes in behaviour were categorical or continuous, we tracked every mating achieved and also measured the number of zygotes fertilized by each male. We predicted that because male fowl are highly polygamous and cannot recognize their own offspring (Ligon & Zwartjes 1995), they should
use mating frequency as a proxy for reproductive success and adjust alarm calling effort accordingly.

METHODS

Subjects were sexually mature fowl (*Gallus gallus*) derived from a population of golden Sebrights that had been interbreeding freely for several generations. This strain has not been artificially selected for rapid growth or egg production and, although morphologically distinct from junglefowl, their behavioural and vocal repertoires are very similar (Kruijt 1964; Collias 1987; Stevens 1991). Fowl are easily manipulated and tolerant of experimental conditions; they have proven to be an ideal system for studies of sexual selection and acoustic signalling (Evans & Marler 1991; Cornwallis & Birkhead 2008; Wilson et al. In press).

A total of 30 males and 30 females were assigned randomly to mixed-sex pairs. All birds were fitted with numbered leg bands to allow individual identification, and each was tested only once. For four weeks before entering the experiment, individuals were denied physical contact with the opposite sex to standardize their recent mating history and to ensure that female sperm storage tubules were evacuated (Lodge et al. 1971). During this time, hens were placed in a female-only outdoor aviary, and males were housed singly indoors to minimize the effects of social dominance (Parker & Ligon 2002). Birds not being tested were housed in an indoor colony (see Evans & Evans (1999) for details). Immediately before testing, we obtained morphometric data from each male, including body weight, tarsus length, and ornament size (see Wilson et al. (In press) for details).
Pairs of birds were housed in one of six adjacent outdoor pens (3.5 x 1.5 x 1.5-m l x w x h; Fig. 1a-c). Each pen had a dirt substrate to facilitate food searching, scratching, and dustbathing behaviour, a coop at one end that contained straw bedding, opaque walls to prevent visual contact with adjacent pens, and a transparent wire roof that afforded a view of the sky. Partitions between pens were sufficient to attenuate the calls of neighbouring males by approximately 8 dB. All alarm calls recorded were evoked by naturally occurring events, such as birds flying over the birds' pens (see Gyger et al. 1987). A removable partition divided the pen longitudinally (Fig. 1a, b) and prevented physical interaction and mating between the male and female occupants. This was constructed of galvanized mesh, which allowed the two birds to view each other and to interact vocally. Food, water, and a perch for roosting were available on both sides of the partition throughout the experiment.

We adopted a randomized complete block design, in which five cohorts (blocks) were tested sequentially in the six pens between 13 September 2006 and 6 March 2007. This period corresponds to the breeding season of fowl in the southern hemisphere. In each cohort, a pair of birds was assigned at random to each pen (males and females were assigned randomly to opposite sides of the partition) at approximately 1600 h. Data collection began the following morning and continued for 28 days (see Fig. 2a). During the first week, the male was prevented from mating so that we could measure his pre-mating calling effort (baseline period; Fig. 2a, b). We then manipulated male mating success during weeks two and three by either removing the wire partition to allow mating (experimental group, 3 per cohort, N\text{total} = 15 males) or leaving it in place to prevent mating (control group, 3 per cohort, N\text{total} = 15 males). Within each cohort, 3 pairs were assigned at random to each treatment. Whenever the partition was removed or installed in the experimental group
pens, we did a sham manipulation in the control group pens to control for disturbance.

Finally, in week four, we examined the effect of recent mating experience on male calling effort by re-installing the partition and returning all pairs to baseline conditions (Fig. 2a, b).

We recorded each male's vocal behaviour every day for 2 h beginning at local sunrise (determined using Geoscience Australia for latitude: -33° 50' 00" longitude: 151° 15' 00").

During this time, both subjects and predators are active, windspeed is low, and anthropogenic disturbance is minimal. Vocalizations from all six males were acquired simultaneously using Behringer C-2 studio condenser microphones (frequency response: 20 Hz - 20 kHz; pickup pattern: cardioid) suspended from the centre of each pen roof (Fig. 1a, b). Signals were digitized using an 8-channel, 24 bit/96 kHz interface (PreSonus FirePod) and recorded as separate channels within WAVE files (16 bit, 44.1 kHz sampling rate) using Boom Recorder software (version 7.5, VOSGAMES) on a Macintosh computer. A seventh channel comprising a mix of the other six was also created to facilitate subsequent scoring of vocal activity.

We recorded 56 h of audio from each of the 30 males, totalling 1680 h in all. Raw audio files were processed using automated sound detection software (ISHMAEL, © David K. Mellinger) which used an energy summation algorithm to extract all clips containing sound in at least one channel that exceeded a user-specified detection threshold for > 0.25 s and < 2.0 s. To improve selectivity, signal amplitude was calculated from the 700 - 1800 Hz band only. These detection criteria were based on previous measurements of aerial alarm call structure and ensured that all alarm calls were extracted (Bayly & Evans 2003). The resulting files contained all seven channels, plus 0.25 s preceding and following the vocalization(s).
Clips were collated according to recording day, and scored using Raven Interactive Sound Analysis Software (version 1.3 Pro, © Cornell Lab of Ornithology Bioacoustics Research Program). Although we specified an extremely sensitive detection threshold (0.02), we confirmed that ISHMAEL had extracted all of the relevant signals by periodically scoring vocalizations from the raw files. In all cases, results were identical.

Our response variables were the total number of aerial alarm calls and crows produced daily by each male. Crowing is an energetically inexpensive dominance-related signal directed towards other males (Chappell et al. 1995; Horn et al. 1995). We would consequently not expect crowing rate to vary as a function of recent mating experience. Comparisons between these two signals were designed to test the specificity of any detected change in alarm calling and, in particular, to distinguish this from a more global change in vocal activity.

We scored signals and signallers by simultaneously viewing the six pens’ audio channels as scrolling real-time spectrograms (512 sample, 50% overlap, Hamming window), while at the same time listening to the time-locked mix channel at approximately natural amplitude. Females do not produce crows or aerial alarm calls and so were excluded as possible signallers. When only one microphone detected a vocalization, the male in the pen corresponding to that microphone was deemed the caller. When multiple microphones detected the same vocalization, the pronounced amplitude differences between channels corresponding to adjacent pens allowed us to reliably identify the calling male.
We also obtained an estimate of reproductive potential for each male in the experimental group. A Panasonic colour CCTV security camera (model WV-CF212E) mounted on the back wall of each pen provided a complete view of the interior, which we recorded from 0.5 hours before sunrise until 0.5 hours after sunset using a D-Teg 8-channel digital video recorder (model SRXM5008-DVD, mpeg-4 compression, 12 frames per second, 720 x 288 lines of resolution). This corresponded to the entire period in which birds were active. For each male, we scored all copulations achieved during the two weeks in which he had access to the female. This amounted to an average of 201 h of footage for each of the 15 males in the experimental group, totalling 3015 h in all. Scoring this volume of footage was made possible by viewing it at 16x normal speed. We confirmed that we could accurately and reliably identify all copulations by periodically scoring footage at the normal speed. In all cases, the results were identical.

Birds were disturbed as little as possible during the experiment. We entered the pens at the end of each week to collect eggs and replenish food and water. Eggs were subsequently incubated at 38.3°C and 85% relative humidity. Incubation was stopped by chilling at 72 hours and eggs were tested for the presence of an embryo. The total numbers of copulations, number of eggs laid by the female, and number of eggs fertilized by the male provided us with three non-independent estimates of reproductive success.

We calculated the total number of alarm calls and crows produced weekly by males. For each vocalization, we considered each male's calling effort in week one to be 100%, and expressed calling in subsequent weeks relative to this baseline performance. Calling effort during weeks two and three was then compared between treatments using a repeated
measures ANOVA, with cohort as a blocking factor. To check whether differences between treatments in weeks two and three reflected differences in housing conditions, as distinct from the mating success of males, we performed a separate comparison of calling behaviour in week four, when both treatment groups had been returned to baseline conditions and males differed only in their recent mating experience. In all analyses, the data conformed to the parametric assumptions of normality and homoscedasticity.

For all comparisons, we describe the magnitude of differences between treatments using Cohen's $d$. Effect sizes larger than 0.8 are considered ‘large’ (Cohen 1988). Finally, we used linear regression to test whether the number of copulations, eggs produced, or number of embryos affected the magnitude of the change in male alarm calling effort. Effect sizes were calculated manually according to Cohen (1988); all other analyses were conducted using SPSS for Mac (software, version 16). Tests were 2-tailed, with an alpha level of 0.05.

RESULTS

Treatments effectively manipulated male mating and reproductive success. In the experimental group, males mated an average ($\pm$ SE) of 35.6 ($\pm$ 7.9) times and fertilized an average of 6.0 ($\pm$ 1.5) eggs out of a total of 12.1 ($\pm$ 2.0) eggs laid over the four weeks. Mating frequency and the number of eggs laid were correlated (Pearson correlation: $r_{15} = 0.584$, $p = 0.022$) and together were an excellent predictor of the number of eggs fertilized (multiple regression: $F_{2,12} = 7.682$, $p = 0.007$, $R^2_{\text{adjusted}} = 0.488$). No eggs were fertilized in the control group, although the total number of eggs laid in the control group was very similar
(12.5 ± 2.1, mean ± SE) and did not differ significantly from that in the experimental group (unpaired t-test: \( t_{28} = -0.114, p = 0.910 \)).

Males permitted to mate increased their alarm calling effort an average of 31.5% relative to control males during weeks two and three (Fig. 3a). This was reflected in a significant treatment effect \( (F_{1,20} = 14.211, p = 0.001) \) of large size (week 2: \( d = 1.1 \), region of nonoverlap between distributions = 58.9%; week 3: \( d = 0.9 \), region of nonoverlap between distributions = 51.6%). Importantly, the effect of being allowed to mate was also clearly apparent in week 4, after all males had been returned to identical housing conditions (Fig. 3a). Alarm calling in the experimental group was still 20.9% greater than in controls \( (F_{1,20} = 9.413, p = 0.006) \) and effect size was similar to that during the differential treatment phase \( (d = 0.9, \text{region of nonoverlap between distributions} = 51.6\%). \)

In contrast, crowing was unaffected by mating performance (ANOVA: all \( p \geq 0.35 \); effect sizes: all \( d < 0.4 \), all regions of nonoverlap between distributions < 27%; Fig. 3b), demonstrating that the effect of mating was specific to alarm calls. Miscellaneous grouping variables, including pen number and the side of the partition that the male was placed in, had no effect on the production of either call type (all \( p > 0.05 \)).

None of our measures of male reproductive success (number of copulations, eggs laid by the female, and eggs fertilized) predicted the magnitude of increase in alarm calling effort (simple linear regressions: all \( p > 0.15, R^2 < 0.15 \)). The experience of mating thus appears to have a categorical effect. Finally, we could find no strong relationship between
alarm calling effort and any aspect of male morphology (body weight, tarsus length, and ornament size; simple linear regressions: all $p > 0.25$, $R^2 < 0.1$).

**DISCUSSION**

We manipulated the mating and reproductive success of male fowl to examine their effects on alarm call production. In each week, males in the experimental group alarm called almost one-third more than controls, which were prevented from mating (Fig. 3a). Both current access to a receptive hen and recent experience of one were sufficient to induce this effect. This latter comparison is important because the only difference between the groups in week four was previous mating success. Furthermore, increases in alarm calling were specific. Mating had no effect on the rate of crowing (Fig. 3b), allowing us to reject the possibility of a more global increase in vocal activity. Similarly, differences between the treatments do not reflect an audience effect (Karakashian et al. 1988; Evans & Marler 1991, 1992). This is an immediate potentiation of aerial alarm calling in the presence of any adjacent conspecific and hence quite distinct from the much slower divergence between experimental and control groups in the present study (Fig. 3a), both of which effectively had a female audience throughout. We conclude that males selectively increased production of a costly signal in response to the possibility of reproductive success. This provides the first experimental evidence that mating has a causal effect on alarm calling effort.

Mating frequency and the number of eggs laid by a female were both excellent predictors of male reproductive success, yet alarm calling did not vary in relation to the magnitude of these potential cues. Instead, mating caused a categorical increase in alarm
calling effort. This is perhaps surprising, and may reflect the high certainty of paternity afforded by the absence of competitors in this experiment. Nevertheless, our experimental conditions were not unrealistic. Fowl live in social groups consisting of several males during the non-breeding season, but frequently form subgroups containing a single male and one or more females (Collias et al. 1966; Collias & Collias 1967). During the breeding season, males become highly territorial. During this time, flocks disperse and breeding pairs form for the nest building and egg laying periods (McBride et al. 1969). The female access treatment hence reproduced the social conditions under which reproductive success is naturally achieved.

Discrete investment in alarm calling effort could also reflect well-documented post-copulatory mechanisms that disrupt the link between mating and reproductive success. Females in large social groups, for example, selectively eject the sperm of non-preferred males (Pizzari & Birkhead 2000). Sperm competition (Froman et al. 2002) and differential sperm allocation (Pizzari et al. 2003) further diminish a male's certainty of paternity. Females can store the sperm of preferred males (Brillard 1993; Pizzari & Birkhead 2000), so a single mating may fertilize many eggs or none at all. The probability of extrapair fertilizations could be so high under these conditions that a male would have no way of estimating his probability of paternity and would, instead, invest on the basis of a threshold function reflecting the mere possibility of paternity (i.e. whether or not he had mated at all).

We suggest two possible mechanisms underlying the observed increase in alarm calling effort. First, calling may reflect endocrine state. Testosterone affects the production of alarm calls in male fowl; that is, calling is abolished by castration and is reinstated by
androgen therapy (Gyger et al. 1988). Furthermore, it is well documented that territory establishment, mating, and mate guarding affect testosterone titre in other avian species (Moore 1982; Wingfield et al. 1990). The experience of mating in our study may therefore have caused a hormonal change in males that subsequently elevated alarm call rates. Note, however, that crowing is also testosterone dependent (Marler et al. 1962), yet this was unaffected (Fig. 3b). Second, increased alarm calling might reflect cognitive processes. Adult fowl are capable of individual discrimination (Guhl & Ortman 1953; Hauser & Huber-Eicher 2004). If males recalled their previous mating experiences, it would be possible for them to invest selectively in individuals with which they had mated. Further experiments will be required to distinguish between these two putative mechanisms.

The increase in alarm calling effort observed during the breeding season in this study is consistent with the mate investment hypothesis. During the breeding season, dominant males establish territories from which they exclude other males (McBride et al. 1969). Broody females (i.e. those incubating eggs or those accompanied by chicks) reside within these territories, but are unlikely alarm call recipients, as they remain solitary and distant from other adults. In contrast, sexually receptive females are each accompanied closely by a male throughout their nest-building and egg-laying periods, and are hence the probable beneficiaries of mating-induced alarm calls (McBride et al. 1969).

Our results emphasize the importance of direct benefits in the evolution of alarm signalling (see also Blumstein et al. 1997) and of apparently altruistic behaviour more generally. We acknowledge the possibility that alarm calling in fowl has multiple functions; continued calling by control males suggests strongly that it does. Nevertheless, we believe
this to be the first demonstration of a causal relationship between mating success and alarm
calling effort in any species.

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References


Figure 1.

(a) Schematic and (b) photograph of an experimental pen used to manipulate male mating success. (c) Six adjacent pens were used to test each of five cohorts of birds.
Figure 2.  
(a) Timeline and (b) experimental design used to test the male investment hypothesis.
Figure 3.

Effects of mating on (a) alarm calling and (b) crowing. Shown are both the experimental treatment (filled circles) and the control treatment (open circles) during the baseline (week 1), treatment (weeks 2 and 3), and post-treatment (week 4) periods.