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Mechanisms of mate investment in the polygamous fowl, *Gallus gallus*

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Abstract Male fowl (*Gallus gallus*) that have recently mated invest in their mates by producing antipredator alarm signals at a higher rate. It remains unclear, however, whether these males are investing judiciously in their mates, or responding more generally to recent mating success. Here, we manipulated each male's mating experience with two different females to test whether males invest selectively in their mates. For one week, males could interact with both females, but could mate with only one of them. In the second week, we removed either the mated or the unmated female and measured the male's rate of alarm calling. Males did not invest preferentially in their mates, suggesting that increased alarm calling is a more general response to recent mating experience. This relationship could be based on a relatively simple cognitive rule of thumb or on an underlying physiological mechanism. Testosterone and corticosterone are associated with reproduction and antipredator behaviour in other species, and so could provide the necessary physiological link in fowl. To test this, we measured plasma levels of testosterone and corticosterone before, during, and after mating. Results show that hormone levels did not change as a function of male mating status, and hence cannot provide the link between mating and calling behaviour. Instead, we suggest that a general cognitive mechanism is more likely to explain prudent mate investment in this species.

Keywords: alarm signal, cognition, communication, corticosterone, individual discrimination, male investment, mate investment, testosterone

Mating affects male investment in many species, but the mechanism providing the necessary link between mating and investment is not always clear (Møller 1988; Møller & Cuervo 2000). One possibility is that mating, or some correlate of mating, induces a physiological change in males that modulates their subsequent investment behaviour (Moore 1982; Hegner & Wingfield 1987; Berg & Wynne-Edwards 2001; Roney et al. 2003). Alternatively, a cognitive mechanism could provide the necessary link. For example, males could follow a simple rule of thumb, whereby they invest provided they have recently mated. A more complex cognitive mechanism is also logically possible. Males might monitor the mating behaviour of themselves, their mates, or their competitors, and then adjust their investment strategy according to likely payoffs (Moczek 1999). This seems probable in dunnocks (*Prunella modularis*), in which polygynous males adjust their chick-feeding effort according to the share of matings obtained by their competitors during the mating period weeks earlier (Davies et al. 1992).

We explored potential mechanisms of male investment in the polygamous fowl, *Gallus gallus*. Males in this species provide little or no parental care (McBride et al. 1969), but they do provision females with critical resources, such as food, vigilance, breeding territories, and protection from harassment by subordinate males (Pizzari 2003). In general, the precise role of provisioning remains unclear. It may function either as the cause (i.e., in mate attraction) or the consequence (i.e., in mate investment) of male mating success (Pizzari 2003; Wilson et al. 2008). A particularly well-understood example of provisioning - broadly defined - is the production of aerial alarm calls (Wilson et al. 2008; Wilson & Evans 2008). These distinctive vocalizations are uttered predominantly by males (Collias 1987) and are reliably and specifically associated with the presence of avian stimuli (Evans et al. 1993; Wilson & Evans 2010; Evans, unpublished data).

Alarm calls benefit females by warning them of impending danger (Collias 1987; Evans et al. 1993), but are potentially costly for males to produce since they attract the attention of nearby predators (Wood et al. 2000). The propensity to produce these calls is an excellent correlate of male mating success (Wilson et al. 2008), but females do not prefer alarm calling males (Wilson & Evans 2010). Rather, alarm calls function unambiguously in male mate investment. Wilson & Evans (2008) manipulated the mating success of 30 mixed-sex pairs of fowl held in outdoor enclosures, and showed that mating had a causal effect on alarm call production. Males that were permitted to mate produced approximately 30% more alarm calls than males that were prevented from mating. This effect persisted even when males could view, but no longer mate with, their female companions (Wilson & Evans 2008). As a result of mating-induced alarm calling, males probably benefit through increased survival of their mates and prospective offspring (Wilson & Evans 2008).

The mechanism linking male mating success to increased alarm call production in fowl remains unclear. Increased calling could reflect judicious investment in mates (Wilson & Evans 2008). Fowl are capable of discriminating between individuals (Guhl & Ortman 1953; Hauser & Huber-Eicher 2004) and adjusting their behaviour according to their prior experiences with those individuals (Evans unpublished data). It is hence possible that males discriminate between females and invest judiciously in their mates. This cognitive mechanism would be highly flexible and would allow males to avoid the unnecessary cost of investing in females that did not increase their fitness.

Increased alarm calling could also be a general response to recent mating experience (Wilson & Evans 2008). Although less flexible than discrimination-based calling, this strategy

could be highly effective, as there is a reliable spatiotemporal relationship between males and their mates during the nest-building and egg-laying periods (McBride et al. 1969). Male investment could hence be based on a relatively simple rule of thumb (Bouskila & Blumstein 1992). It could also be based on a physiological mechanism linking calling behaviour to mating success. Testosterone is known to affect alarm calling in male fowl, as calling is abolished by castration and is reinstated by androgen therapy (Gyger et al. 1988). Furthermore, testosterone levels are affected by reproductive activity in many other species (Moore 1982; Hegner & Wingfield 1987; Berg & Wynne-Edwards 2001; Roney et al. 2003; Villani et al. 2006; Peters et al. 2008), with maximum concentrations observed during the breeding season (Morton et al. 1990; Schraden 2008). Similarly, plasma levels of corticosterone are correlated with reproductive activity and antipredator behaviour in several species (Manzo et al. 1994; Tokarz et al. 1998; Leary et al. 2006; Thaker et al. 2009). It is therefore possible that mating induces a change in the plasma levels of either testosterone or corticosterone that causes a concomitant change in alarm call production.

We modified the experimental design used by Wilson & Evans (2008) to determine whether increased alarm calling reflects judicious investment in mates, or whether it is a more general response to recent mating success. To test the judicious mate investment hypothesis, we manipulated each male's experience with two different females. Males became familiar with both females, but could mate with only one of them. We then removed either the mated or the unmated female and observed the male's investment in the remaining hen. If males invest selectively in mates, then alarm calling should subside when the mate is removed and the male is left with the familiar non-mate. In addition, we tested the physiological basis of calling by measuring plasma levels of testosterone and corticosterone before, during, and after males

were permitted to mate. If increased alarm call production following copulation has an endocrine basis, then we should observe a mating-induced change in the plasma levels of one or both of these hormones.

METHODS

General

Subjects were sexually mature individuals derived from a colony of freely interbreeding golden Sebright bantams. This strain has not been artificially selected for rapid egg or meat production, and they exhibit a behavioural repertoire similar to that of ancestral red junglefowl (Kruijt 1964; Collias 1987). This is a well-established system for studies investigating sexual selection (Wilson et al. 2008; Wilson & Evans 2008) and animal communication (Evans et al. 1993; Gyger et al. 1987).

We used a total of 42 males and 63 females. Males were used only once to preserve independence of data, whereas females, which were not subjects in this experiment, were used in a maximum of two trials. For two weeks before entering a trial, both males and females were deprived of physical access to the opposite sex to standardize their recent mating experience and to ensure that female sperm storage tubules were empty (Lodge et al. 1971; Brillard 1993). During this time, birds were housed individually indoors in metal cages (1 x 1 x 0.5-m l x w x h). They were provided with *ad libitum* access to food and water, perches for roosting and straw for bedding, and had visual and vocal contact with other birds.

Experimental design

Each trial involved one male and two females and was conducted over a two-week period in one of six adjacent outdoor enclosures (3.5 x 1.5 x 1.5-m l x w x h; see Fig. 1 in Wilson & Evans 2008). One end wall and both sidewalls of each enclosure were constructed of opaque composite material, while the roof and other end wall had an open-wire construction. Individuals could thus view their surroundings, but not the occupants of adjacent enclosures. A removable partition dividing each enclosure longitudinally allowed us to control the male's ability to mate with his two female companions. Partitions were constructed of galvanized chicken wire that permitted birds on opposite sides to interact visually and vocally throughout the trial. Food, water, shelter, perches for roosting, straw for bedding, and friable earth for dustbathing were available *ad libitum* on both sides of the partition in every enclosure throughout the experiment.

We adopted a randomised complete block design, in which seven cohorts (blocks) of six males each were tested sequentially during the breeding season between 29 September 2007 and 22 January 2008. The 42 males were assigned at random to pairs of females, but with the constraint that they were not paired to their previous cagemates. The trios were then assigned at random to one of the six enclosures, and moved into it at approximately 1100 h on the day before data collection began. During the first week of data collection, the male could view and interact vocally with both females, but could mate with only one of them. Mating was controlled by placing females on either the same or the opposite side of the wire partition as the male. After one week in this condition, we removed one of the two females from each enclosure, and prevented the male from mating with the remaining female by placing her on the side of the partition opposite the male. Throughout the second week, he was thus

accompanied either by his mate (3 per cohort, $N_{total} = 21$) or by an equally familiar hen with which he had not mated (3 per cohort, $N_{total} = 21$).

Behaviour

We audio-recorded each male throughout the experiment following the methods detailed in Wilson & Evans (2008). Recordings began each day at sunrise (time of sunrise determined using Geoscience Australia website for latitude: $-33^{\circ} 50' 00''$ longitude: $151^{\circ} 15' 00''$) and continued for precisely two hours. During this time, subjects and potential aerial predators are active, wind speed is low, and anthropogenic disturbance is minimal. The six enclosures were recorded simultaneously using Behringer C-2 studio condenser microphones (frequency response: 20 Hz - 20 kHz; pickup pattern: cardioid) attached to the roof of each enclosure. Signals were digitised using an 8-channel interface (PreSonus FirePod) and were recorded as separate channels within WAVE files (16 bit, 44.1 kHz sampling rate) using Boom Recorder software (version 7.5, VOSGAMES). A seventh channel comprising a mix of the other six was also created to facilitate data scoring.

Each male was recorded for 28 hours, totalling 1176 hours in all. Prior to scoring, we processed sound files using automated sound detection software (ISHMAEL, © David K. Mellinger), which uses an energy summation algorithm to extract sounds from any channel that exceed a user-specified detection threshold (see details in Wilson & Evans 2008). Following detection, a new clip was created that contained the seven channels, the putative signal, and 0.25 s both preceding and following the signal. Extracted clips were organized by recording day, then collated and scored using Raven Interactive Sound Analysis software (version 1.3 Pro, © Cornell Lab of Ornithology Bioacoustics Research Program). Signals and signallers were

identified by viewing the six audio channels corresponding to the six enclosures as scrolling real-time spectrograms (512 samples, 50% overlap, Hamming window), while simultaneously listening to the time-locked mix channel at a natural amplitude.

For each male, we scored the total number of aerial alarm calls produced each day during the two-hour recording session. We also scored crowing, which is a territorial vocalization directed towards other males. Crowing is not affected by recent mating experience (Wilson & Evans 2008), and therefore provided a control to which potential changes in alarm calling rates could be compared. Females do not produce aerial alarm calls or crows and so could hence be excluded as possible signallers. When multiple microphones detected signals, the pronounced amplitude differences between adjacent enclosures allowed us reliably to identify the calling male.

The experimental design provided each male with access to a female for the first week, but did not guarantee that he mated with her during that time. We therefore estimated each male's reproductive success in week one by counting his copulations and the number of eggs laid by his mate. In this context, mating frequency and egg production together account for approximately 50% of the variance in the number of eggs fertilized (Wilson & Evans 2008). Copulations were recorded with a CCTV security camera (Panasonic, model WV-CF212E) mounted on the back wall of each enclosure. These provided a complete view of the interior, which we recorded daily 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). Birds were recorded each day for 3 hours in the morning (beginning 0.5 h before sunrise) and 4 hours in the evening (ending 0.5 h after sunset). These times correspond to periods of peak reproductive activity in

fowl. Unfortunately, copulations were not observed for 2 males in cohort 6 because a lightning strike destroyed the cameras.

Hormones

We measured changes in plasma levels of testosterone and corticosterone by obtaining three blood samples from each male in cohorts 2 - 7 (i.e., $N_{\text{males}} = 36$). The first sample was obtained immediately before the subject was placed into an enclosure, following the two-week period in which he was prevented from mating. The second sample was taken after the first week of data collection, following the 7-day period of unrestricted access to his mate. The final sample was drawn at the end of the second week of data collection, following the 7-day period in which the male was again prevented from mating. Blood samples from any given male were always taken at the same time of day (0815h-1035h), and males within a given cohort were always sampled in the same order to minimize intra-individual variation in putative disturbance effects. For each sample, we punctured the brachial vein with a 21-gauge needle and collected approximately 600 μl of blood in a heparinized tube. In all cases, we extracted the blood immediately after capture to minimize the effects of handling stress on hormone levels. Samples were placed immediately on ice and, within two hours, were centrifuged at 2500 rpm for 5 min. The plasma was aspirated and stored at -20°C for subsequent analysis.

Plasma samples were analysed at the School of Health Sciences, University of Wollongong. There, plasma levels of testosterone and corticosterone were measured using Cayman enzyme immunoassay kits (Cat. Nos. 582701 and 500651, respectively; Cayman Chemical, An Arbor, MI USA) following the methods described by Olsson et al. (2007). Prior to analysis, the appropriate plasma dilution was determined by pooling 10 μl of plasma from each

of 15 individuals, and then testing serial dilutions prepared from the pooled sample. Final dilutions of 100x for testosterone and 40x for corticosterone were selected because these best achieved binding between 40% and 70%, which corresponds to the most sensitive region of the testosterone and corticosterone standard curves.

Testosterone and corticosterone assay plates were prepared according to the manufacturer's specifications, with standards added in triplicate and diluted plasma samples added in duplicate. All samples from a given individual were run on the same plate to avoid inter-plate variations (interplate variation was 12.4% for testosterone and 17.2% for corticosterone), and each plate had an equal number of males from each experimental treatment. Absorbance was measured at 405 nm on a plate reader (Power-Wave 340, BioTek Instruments, VT) using KC Junior software (BioTek Instruments, VT) and was analysed with GraphPad Prism IV software. Reported concentrations are adjusted for dilution and sample recovery; mean recoveries were 90.4% for testosterone and 81.2% for corticosterone.

Analysis

For each male, we calculated the total number of aerial alarm calls and crows produced in each week. We considered a male's calling effort in week one, when he had access to a female, to be 100%. In week two, calling was expressed relative to this baseline performance. For each vocalization, call rates in week two were then compared between treatments using ANOVA. Cohort was entered into the model to account for possible seasonal variation in vocal activity. The magnitude of differences between treatments was described using Cohen's *d*, where effect sizes larger than 0.8 are considered 'large' and effect sizes smaller than 0.2 are considered 'small' (Cohen 1988).

To explore the physiological basis of male mate investment, we analysed factors affecting hormone levels using a linear mixed model approach with restricted maximum likelihood estimation. Main effects of mating status, experimental treatment, and cohort were entered as fixed factors, whereas subject was entered as a random factor to account for repeated measurements of the same individual. We accounted for putative disturbance effects by including bleed time as a covariate with fixed effects. A separate model was used for testosterone and corticosterone. We also used linear mixed models to test for possible relationships between hormone levels and calling rates during the mating and post-mating periods. Main effects of mating status, experimental treatment, and cohort were entered as fixed factors and subject as a random factor; bleed time, testosterone concentration, and corticosterone concentration were entered as covariates with fixed effects. A separate model was used for each vocalization. Note that the plasma levels of testosterone could not be determined for five samples from four males (3 pre-mating, 1 mating, 1 post-mating), so these five samples were excluded from all analyses involving testosterone. Statistics were calculated using SPSS for Mac (version 17.0), tests were 2-tailed, and results were considered statistically significant where $p \leq 0.05$.

RESULTS

In week one, our experimental design effectively manipulated each male's mating experience with two equally familiar female companions. Males never copulated with the hen to which they were denied access (hereafter 'familiar'), but always copulated at least one time with their

mates (average \pm SE, 6.55 ± 0.81 ; one-sample t -test, reference = 0: $t_{39} = 8.099$, $p < 0.001$). Also during week one, mates laid an average of $2.4 (\pm 0.3)$ eggs, which did not differ significantly from the number laid by the familiar hens (3.0 ± 0.3 ; paired-samples t -test: $t_{41} = -1.587$, $p = 0.120$). Thus, mating experience with the remaining hen was the only difference between the two experimental groups at the beginning of the second week.

The frequency of alarm calling in week 2 did not differ significantly between experimental males that remained with mates and control males that remained with familiar hens (ANOVA with cohort as blocking variable: $F_{1,28} = 0.260$, $p = 0.614$; effect size: $d = 0.16$; Fig. 1a). Similarly, crowing rates (ANOVA with cohort as blocking variable: $F_{1,28} = 0.009$, $p = 0.925$; effect size: $d = 0.03$; Fig. 1b) did not differ significantly between the two groups. We found no effect of mating status (pre-mating, mating, post-mating) or experimental treatment (mate or familiar hen) on plasma levels of testosterone (linear mixed model analysis: all $F \leq 1.595$, all $p \geq 0.210$; Fig. 2a) or corticosterone (all $F \leq 0.122$, all $p \geq 0.791$; Fig. 2b). Bleed time did not affect plasma levels of testosterone (linear mixed model analysis: $F_{1,92} = 0.015$, $p = 0.903$), but had a significant and positive effect on plasma levels of corticosterone ($F_{1,86} = 5.503$, $p = 0.021$). Finally, there were no significant relationships between hormone levels and the frequency of either alarm calling or crowing (linear mixed model analysis: all $F \leq 0.364$, all $p \geq 0.550$).

DISCUSSION

Recently mated male fowl produce approximately 30% more alarm calls than their unmated male counterparts (Wilson & Evans 2008; effect size: $d = 1.1$). Here we tested whether this

increased alarm call production reflects judicious investment in mates. To test this, we permitted each male to mate with only one of two familiar females over a one-week period. In the following week, we removed either the mate or the non-mate and observed the male's subsequent alarm calling behaviour. Results show that alarm calling declined in the second week (Fig. 2a), which probably reflects habituation to innocuous stimuli (e.g., non-predatory birds flying overhead) as males acclimatized to the outdoor enclosures (see also Fig. 3 in Wilson & Evans 2008). However, males did not invest preferentially in their mates. Remarkably, rates of alarm calling were virtually identical across the two experimental groups (Fig. 1a; Cohen's measure of effect size: $d = 0.16$). Together with Wilson & Evans (2008), this study provides compelling evidence that male fowl increase their alarm calling effort in response to recent mating experience, but that this investment is not specific to the individual females with which they have recently mated.

Instead, our findings suggest that increased alarm calling is a more general response to recent mating experience *per se*. This does not simply reflect the fact that males in both treatments were prevented from mating during the second week of trials. Previously, males that were permitted to mate continued to call at higher rates than control males that were prevented from mating, even after they were able to view, but no longer mate with, their female companions (Wilson & Evans 2008). Although less specific and less flexible than discrimination-based calling, we suggest that this more general investment strategy may be suitable for the fowl's mating system and functionally sufficient for male fowl to benefit. During the non-breeding season, fowl reside in mixed-sex social groups with pronounced dominance hierarchies in both sexes (Collias & Collias 1967; McBride et al. 1969). During the breeding season, however, dominant males become highly territorial and are escorted closely by one or

more sexually receptive females. Competition among males is intense, and approximately half of all males remain solitary or accompanied exclusively by other males (Collias & Collias 1967; McBride et al. 1969). Thus, a male that has mated is probably a territorial male that has continual access to one or more females, and any female that is in close proximity to him is likely to be his mate. Calling in response to recent mating success, or to a correlate of recent mating success (e.g., successfully defending a territory or having access to a female), may therefore be an optimal strategy by which territorial males enhance the survival of their mates and prospective offspring.

The mechanism underlying this effect may reflect a simple rule of thumb (Bouskila & Blumstein 1992), whereby males invest in nearby females provided they have recently achieved mating success. A physiological change could also provide the link between increased alarm calling and recent mating success. However, plasma levels of testosterone and corticosterone remained constant throughout the pre-mating, mating, and post-mating periods, and hence can be excluded as putative mechanisms. This result cannot be attributed readily to our experimental design. Using the same apparatus, sampling technique, and population of birds, Wilson & Evans (2008) showed a pronounced change in alarm calling effort over just two weeks. The mechanism linking mating to increased alarm calling must therefore be equally responsive, yet we did not observe a concomitant change in the titre of either hormone over a similar two-week period using a larger sample size. Although mating may affect other physiological parameters, our study provides strong evidence against the hypothesis that mating and calling are linked through a mutual change in plasma levels of testosterone or corticosterone. Instead, this study, together with our earlier work (Wilson & Evans 2008), suggests that mate investment follows a simple cognitive rule, whereby males produce more alarm calls following recent

mating success.

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Fig. 1:

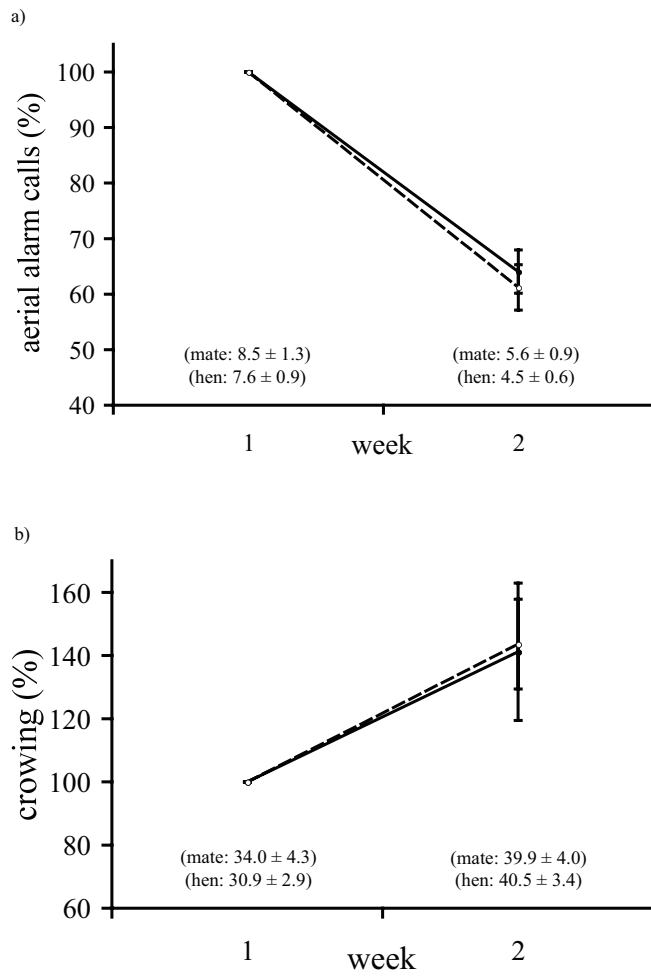


Fig. 1: Effects of a recent mate (solid circles) and a familiar hen (open circles) on the production (mean ± SE) of (a) alarm calls and (b) crows by 42 male fowl, *Gallus gallus*. Each call rate for each individual in week two was expressed as a percentage of the corresponding call rate observed for that individual in week one. Actual hourly call rates are presented in parentheses.

Fig. 2:

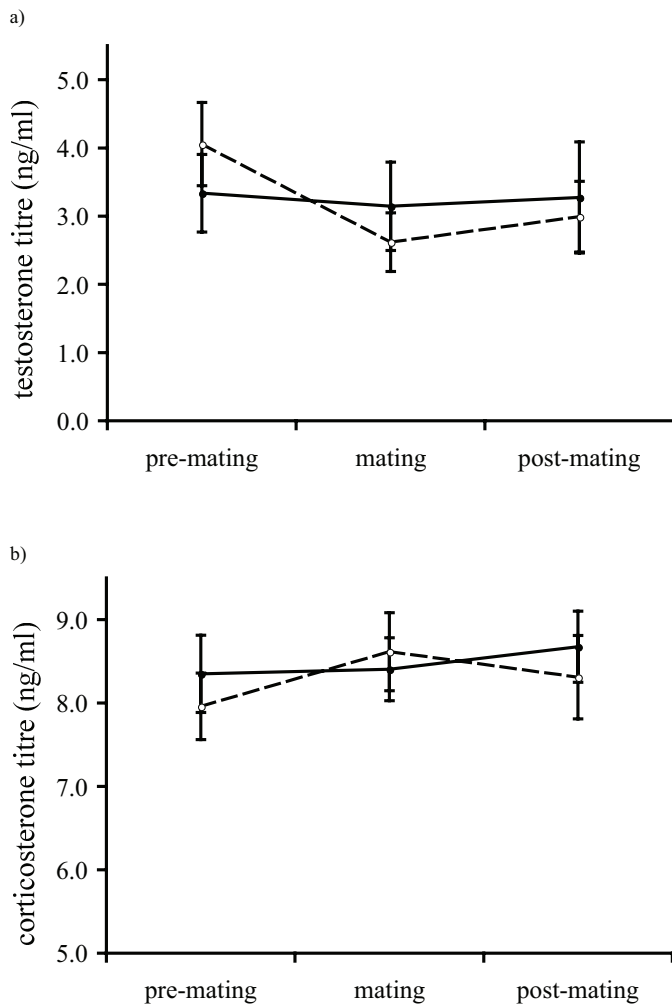


Fig. 2: Hormone levels of 36 male fowl, *Gallus gallus*, during the pre-mating, mating, and post-mating periods. Shown are plasma levels of (a) testosterone and (b) corticosterone. Experimental males that remained with their mates during the post-mating period are shown with solid circles, whereas control males that remained with familiar hens during the post-mating period are shown with open circles.