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## Adrenocortical response to mating, social interaction and restraint in the female Japanese quail

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### ABSTRACT

In some species, the act of mating may be the only occasion when a female judges the male and the course of the sexual encounter might influence forms of maternal investment that are mediated by hormonal pathways. Despite the growing interest in fitness consequences of acute adrenocortical responses, it has not been determined to what extent the act of mating provokes such responses in females. We used female Japanese quail (*Coturnix japonica*) to determine to what extent any increase in corticosterone (CORT) is specific to actually mating with a male compared to visual and auditory contact with a male, and compared to social interaction with a female. We also asked if greater mating experience decreases the CORT response. We assessed how body condition of the individuals and their behavior during testing contributed to the variation in CORT response. As an additional reference for CORT increase we used a restraint procedure which resulted in the highest CORT. Our results show that females mating with a male have plasma CORT concentrations significantly increased from the baseline, which is not the case in females interacting with a male without the possibility of mating. Greater previous mating experience does not affect the CORT change caused by sexual interaction. Interestingly, social interaction with a female elicited a similar CORT response as mating with a male, which suggests that the effect on CORT was due to direct social interactions per se and not specific to the act of mating. Behavior during social and sexual interactions did not predict the CORT response, but larger females exhibited lower baseline and response CORT concentrations. We suggest that the lesser adrenocortical response of larger females might be related to their better control of the course of mating and might help them to adjust their reproductive investment, as reported in earlier studies.

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### 1. Introduction

Stress hormones are one of the mediators of individual reaction to environmental and social factors and both chronic [1] and acute [2] CORT levels have been documented to vary in relation to fitness relevant cues. Baseline stress hormone levels are affected by factors such as season, resource availability, predation threat and social stress [e.g. 3–6]. It has also been shown that male attractiveness can affect female corticosterone (CORT) levels. For example, female Gouldian finches (*Erythrura gouldie*) exhibit chronically elevated corticosterone levels when paired with unattractive partners [7]. Concurrently, there is growing interest in fitness consequences of acute adrenocortical responses and the most often investigated relationships are those relating stress response to survival reviewed in [2]. Fewer studies examine the relationship between acute adrenocortical responses and reproductive performance. Among them, a study on mourning doves

(*Zenaidura macroura*) reported a negative relationship between CORT levels induced by a capture–restraint protocol and female parental effort, leading to decreased nestling body weight [8].

Comprehensive studies of acute adrenocortical responses use various standard stressors, such as tonic immobility, manual restraint, restraint in a crush cage, presentation of novel objects, isolation and change of cagemates, e.g. [9]. Such an approach is quite valuable especially in comparative studies, e.g. [10], yet it does not include some naturally occurring stressors, such as social and sexual interactions, which might also cause a significant adrenocortical response. Insight into variation in CORT responses to such stimuli could explain a range of behaviors exhibited during social interactions and sexual encounters and predict outcomes of those. However, surprisingly little is known about female stress hormonal levels under such circumstances. So far it has been shown that in female rats, levels of progesterone and the testosterone metabolite, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, but not CORT, differ between paced and non-paced mating [11]. To our knowledge no study has assessed how mating itself changes steroid concentrations in female birds. It is not known how directly or indirectly interacting with a male affects CORT and if any response depends upon prior mating

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experience. Nor is it known how interaction with the same vs. the opposite sex affects CORT levels.

Japanese quail (*Coturnix japonica*) seem to be a very suitable model organism to answer those questions. They exhibit very stereotypic mating behavior, yet variation in fertilization success is extremely high, even from confirmed inseminations [12]. There are indications that mating might be stressful for female quail, as copulations can be forced [12], aggressive males may injure females during mating [13] and sexually experienced females avoid aggressive males [14]. It has been shown that stress experienced by a laying bird has significant effects on offspring viability [15], development and resulting adult phenotype, possibly mediated by the transfer of maternal corticosterone (CORT) to the yolk [16]. Elevated maternal CORT concentrations have also been shown to bias offspring primary sex ratio in quail [17], further emphasizing potential fitness consequences of elevated CORT concentrations in response to mating.

The aim of this study was to determine to what extent any increase in CORT due to mating is specific to actually mating with a male compared to interacting with a male without an opportunity to mate, and compared to social interaction with a female. We also asked if greater previous mating experience decreases the CORT response. We assessed how body condition of the individuals and their behavior during testing contributed to the variation in CORT response. As an additional reference for CORT increase, we used a restraint procedure that has been established as being clearly stressful.

## 2. Methods

### 2.1. Animals

All birds used in the study were 3 month-old, sexually experienced birds raised in the laboratory. They were housed in one room in standard quail cages. Each bird was housed individually but had visual contact with other birds of the same sex. Tarsus length of all birds was measured to the nearest 0.1 mm prior to the experiment using electronic calipers. Body mass was measured using a Pesola balance after the last bleeding. All procedures were performed within one week and took place between 9:30 am–12:30 pm EST.

### 2.2. Experimental groups

There were thirty-four focal females for which we measured CORT levels. Twenty-four of the focal females used in this study had limited sexual experience consisting of a single mating which occurred three to four weeks earlier. They constituted the following experimental groups, named after the stimuli experienced by the focal female: female (F, N = 8), male behind the wall (MW, N = 8) and mating with male (MM, N = 8). Ten other focal females were very experienced sexually, as one month earlier they had mated on average (mean  $\pm$  SEM)  $12.4 \pm 1.2$  times. They formed the fourth experimental group, namely very experienced females mating with males (VEMM, N = 10).

The mating cage was located in an empty room across the hall from the colony room. It measured 28 cm high, 60 cm long and 45 cm wide, and was made of Plexiglas with a wire-mesh floor over the Plexiglas floor. It was divided in the middle with Plexiglas wall, so that birds could be separated.

Two minutes after a focal female was placed in the cage, another bird was added to the other side of the cage. The dividing wall was immediately removed for all experimental groups, except for the one in which the female was interacting with a male behind the wall (MW). Each test lasted 5 min, and in each case the stimulus bird was unfamiliar to the focal female. Blood samples (ca. 400  $\mu$ l) from each female were taken from the alar vein the day before the behavioral trial, to measure baseline hormone concentration, and 5 min after the

trial, at the same time of day on both days. In each case females were bled while housed in their home cage.

### 2.3. Reference CORT concentrations

To provide reference values of events that are known to be stressful (to produce a substantial acute elevation in CORT), 10 females randomly chosen from all experimental groups were restrained in a crush cage for 5 min [9], released to their home cage and blood sampled 7 min later. Those tests were performed four days after the behavioral trials. The 'crush-cage' was a black plastic box (15 cm length  $\times$  5 cm width  $\times$  10 cm height) closed at the top by a netting cover. Quail had very restricted movement in such a crush cage.

### 2.4. Hormone assay

CORT was measured using enzyme immunoassay kits from Cayman Chemical (Ann Arbor, MI) following ether extraction. Briefly, samples and cold-spiked controls were extracted twice using ether and snap freezing and subsequently evaporated by heating the samples to 30  $^{\circ}$ C and placing them under a stream of nitrogen gas. Recoveries, as determined by three samples each spiked with different CORT concentrations, averaged 95%. Reconstituted samples were assayed in duplicate, in a total of three 96-well plates. Each plate contained its own standard curve and constituted a separate assay. The mean intra- and inter-assay CVs were 9.1% and 18.0%, respectively. The CORT kit has a detection limit of 24 pg/ml and a cross-reactivity of less than 1% for 11 steroids tested, except 11-deoxycorticosterone (19.6%) and progesterone (1.01%).

### 2.5. Behavioral observations

All trials were videotaped and subsequently analyzed by a person blind to the group assignment of the birds. For females that encountered another female during the trial (group F), the following scores were assigned: 0 – highly antagonistic behavior between the females, including pecking, 1 – active avoidance of the female, spending most of the time on the opposite sides of the cage, 2 – female calm, neutral behavior, 3 – positive interactions between females, most of the time close to each other (less than 15 cm apart). For females that could interact with males through the Plexiglas wall (group MW), the following scores were assigned: 0 – female actively avoiding male, trying to get out of the cage, 1 – not active, staying mostly at the further side of the cage, 2 – female possibly interested in the male, spending most of the time close to the barrier, 3 – female pecking at the barrier, showing active interest. For females that had contact with the male (groups MM and VEMM), receptivity was assessed. The following scores were assigned: 0 – female highly unreceptive, constantly moves away from male and refuses to squat, 1 – female unreceptive, but calm and does not run away, 2 – female receptive, but does not encourage male for mounting, 3 – female clearly receptive, squats and does not move while mounted (see [18] for validation of that protocol).

For male behavior, latency to head grab of the female and total duration of mating attempts were measured in seconds. Number of struts, feather fluffs and crows were counted.

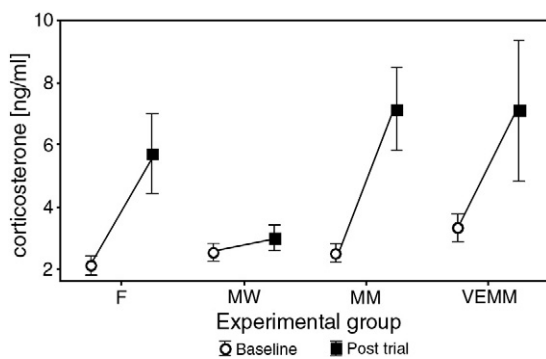
### 2.6. Statistical analyses

Variation in CORT concentrations was analyzed using ANOVAs and, if required, the tests assumed unequal variances. Within-individual comparisons were performed using Student's paired *t*-test. Relationships between CORT concentrations and female characteristics were assessed using Pearson correlation and between CORT concentrations and behavioral measures using non-parametric Spearman correlation. Analyses were performed with JMP, Version 7 (SAS Institute Inc., Cary, NC).

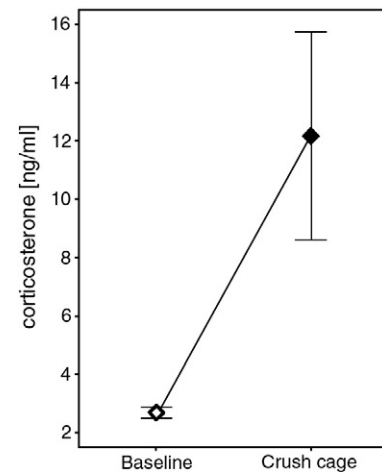
### 3. Results

Baseline CORT concentrations did not differ significantly between the groups ( $F_{(3, 33)} = 2.5$ ,  $p = 0.078$ , Fig. 1). The absolute increase from baseline concentration differed depending on the stimulus during testing (test assuming unequal variances,  $F_{(3, 15.58)} = 3.31$ ,  $p = 0.048$ ). When baseline concentrations were not taken into account, the differences between the groups in absolute CORT response were more pronounced (test assuming unequal variances,  $F_{(3, 14.10)} = 3.84$ ,  $p = 0.034$ , Fig. 1). Post hoc comparisons between females interacting with males via the barrier and the two groups of females allowed to mate reveal that mating females had significantly greater CORT levels (test assuming unequal variances,  $F_{(1, 19.83)} = 8.42$ ,  $p = 0.009$ , Fig. 1). Females interacting with females (F) showed a marginally significant increase in CORT levels compared to the baseline (paired  $t$  test,  $N = 8$ ,  $t = 2.2$ ,  $p = 0.06$ ), and their post-interaction levels were not different from those of females that had interacted with males (ANOVA,  $F_{(1, 24)} = 0.83$ ,  $p = 0.370$ ). Females that encountered the male behind the wall (MW) did not show a significant difference between baseline concentration and CORT concentration following testing (Fig. 1). Restraint in the crush cage significantly elevated CORT concentration compared to the baseline (paired  $t$  test,  $N = 10$ ,  $t = 2.56$ ,  $p = 0.030$ , Fig. 2) and this response was marginally greater (paired  $t$  test,  $N = 10$ ,  $t = 2.21$ ,  $p = 0.054$ ) but similar in scope to the increases in CORT that were associated with direct social interactions.

Baseline CORT concentration was negatively related to female body mass ( $r = -0.42$ ,  $F_{(1, 32)} = 6.92$ ,  $p = 0.013$ ) and tarsus length ( $r = -0.47$ ,  $F_{(1, 32)} = 8.97$ ,  $p = 0.005$ ). CORT concentration following experimental trials was negatively related to tarsus length ( $r = -0.36$ ,  $F_{(1, 32)} = 4.85$ ,  $p = 0.035$ ) irrespective of experimental group. Mass and size of the stimulus birds did not explain variation in CORT response. Focal females exhibited a range of behaviors during testing trials. Mean scores ( $\pm$  SEM) were  $1.4 \pm 0.3$  for females interacting with another female (group F),  $1.7 \pm 0.5$  for females interacting with a male through the wall (group MW), and receptivity scores were  $1.9 \pm 0.3$  for females mating with males (MM) and  $2.1 \pm 0.4$  for very experienced females mating with males (VEMM). Despite the variation in behavioral response to the test stimuli, the scores did not predict CORT response concentrations in any of the groups (F:  $r_s = -0.24$ ,  $p = 0.56$ ; MW:  $r_s = -0.09$ ,  $p = 0.84$ ; MM:  $r_s = -0.04$ ,  $p = 0.92$ ; VEMM:  $r_s = 0.23$ ,  $p = 0.53$ ). In the two experimental groups in which females were interacting with males (MM and VEMM) we quantified male sexual behavior and found it did not affect female CORT response (latency to head grab:  $r_s = 0.46$ ,  $p = 0.062$ , mating duration:  $r_s = 0.21$ ,  $p = 0.411$ , number of struts:  $r_s = -0.32$ ,  $p = 0.207$ , number of fluffs:  $r_s = -0.30$ ,  $p = 0.234$ ).



**Fig. 1.** Baseline and post trial plasma corticosterone concentrations (mean  $\pm$  SEM) in female Japanese quail in the four experimental groups: F: females interacting with another female ( $N = 8$ ), MW: females interacting with males behind the wall ( $N = 8$ ), MM: females mating with males ( $N = 8$ ), VEMM: very experienced females mating with males ( $N = 10$ ).



**Fig. 2.** Plasma corticosterone concentrations (mean  $\pm$  SEM) in female Japanese quail: baseline level and acute elevation (stress response) following restraint in the crush cage ( $N = 10$ ).

### 4. Discussion

Our results show that females mating with males have plasma CORT concentrations significantly increased from baseline, which is not the case in females interacting with males without direct physical contact or the possibility of mating (Fig. 1). The extent of the female's mating experience did not affect the CORT response associated with the sexual interaction (Fig. 1, VEMM group). Interestingly, social interaction with a female elicits similar CORT elevation to those when mating with a male. Behavior during social and sexual interactions does not predict CORT response, yet larger females exhibit lower CORT concentrations.

The absence of a CORT rise in females separated from the males by a barrier serves as a control for handling, moving to the novel cage and for the presence of male and thus rules out a role of those factors in producing the CORT elevations observed in other groups. However, visual and/or auditory contact with a male has been previously shown to affect female receptivity e.g. [19] and hormone concentrations, both circulating [20] and deposited to the eggs [21]. It would have been expected, therefore, that seeing the male through the barrier would affect female CORT concentrations. Our behavioral observations documented that stimulus males showed interest in the females; for instance they were strutting. Focal females clearly paid attention to the males and although they varied in response, at least half of them showed positive interest in the males.

We cannot tell whether CORT increase is a reaction specific to mating, as unrestricted interaction with stimulus birds, either male or female, causes a significant adrenocortical response. Therefore, it would have been informative to have another experimental group in which a focal female would have direct social interactions with a male but without possibility of mating. Furthermore, having virgin females tested in the same contexts as in our study would contribute to the understanding of how a broader range of sexual experience affects adrenocortical response following social interactions. Such questions merit future study.

CORT elevation following social and sexual encounters could be interpreted either as positive or negative. In the former case a CORT elevation would help prepare females for the energetic challenge of being with another bird and engaging in social behavior and the greater locomotor activity involved, reviewed by Adkins-Regan [22]. In the latter case, when interaction is aversive and stressful, a CORT rise might function to help the bird escape from another individual and to recover from the event. For example, female-male interaction in the Japanese quail might be traumatic if mating with a specific male is harmful for the female, e.g. [14]. Even mating with an unattractive male might cause a

stressful reaction, as indicated by elevated CORT concentrations in the eggs laid by peafowl females mated to males with reduced train elaboration [23]. In most cases female-female interactions observed in our study were avoidant or neutral while female-male interactions were mostly sexual. However, we found no relationship between specific behaviors of focal females and their CORT concentrations nor between male behavior and CORT response in females; thus the interpretation of observed CORT rise remains open. We do not know whether our results indicate lack of true relationships or our inability to detect them due to low power of the tests.

Our results contribute to understanding how female size/condition and CORT response affect her reproductive output. Recent study of the Japanese quail highlighted the significance of the context of mating and female body condition in shaping reproductive investment. Specifically, females mated in the context that predicted mating opportunity varied the number of fertilized eggs and egg mass depending on their own body condition [18]. On the other hand, Japanese quail exhibit a negative relationship between female adrenocortical stress responsiveness and embryonic viability [15]. Our current results clearly show that heavier and larger females exhibit lower baseline as well as post-mating CORT concentrations. Since the relationship between size and investment is dependent on the context of mating [18], we conclude that greater maternal investment might be more related to stress responsiveness than to size.

The negative relationship between CORT concentrations and body size observed here is consistent with studies in other avian species in the wild, e.g. [24,25], but see [8]. It is surprising that such a pattern is apparent in the present study even though the birds in our study were not food-limited as are free-living birds. Possibly, body size and baseline CORT concentrations are predetermined at the earlier stages of development. In line with such reasoning, male zebra finches selected for high acute CORT responses have smaller tarsus length than those selected for low acute CORT concentrations [26], yet that was not the case in similarly selected Japanese quail males, which do not differ in body weight [27].

Our study is the first to show that visual contact with the male does not elicit a CORT increase in females, even though sexual interactions with males do. Furthermore, we demonstrate that elevated CORT concentrations in response to social or sexual interaction with a conspecific bird are moderate. In fact, the low adrenocortical response of larger females might be related to their better control of the course of mating and might help them to adjust their reproductive investment. A larger sample size would be needed for more in-depth interpretation of the relationship between the specific behavior shown by birds during the tests and the CORT response following mating.

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