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Brain aromatase and circulating corticosterone are rapidly regulated by combined acute stress and sexual interaction in a sex specific manner

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Abstract

Neural production of 17 β -oestradiol via aromatisation of testosterone may play a critical role in rapid, non-genomic regulation of physiological and behavioural processes. In brain nuclei implicated in the control of sexual behaviour, sexual or stressful stimuli induce respectively a rapid inhibition or increase in preoptic aromatase activity (AA). Here, we tested quail that were either non-stressed or acutely stressed (15 min restraint) immediately prior to sexual interaction (5 min) with stressed or non-stressed partners. We measured nuclei-specific AA changes, corresponding behavioural output, fertilisation rates and corticosterone (CORT) concentrations. In males, sexual interaction rapidly reversed stress-induced increases of AA in the medial preoptic nucleus (POM). This time scale (<5min) highlights the dynamic potential of the aromatase system to integrate input from stimuli that drive AA in opposing directions. Moreover, acute stress had minimal effects on male behaviour suggesting that the input from the sexual stimuli on POM AA may actively preserve sexual behaviour despite stress exposure. We also found distinct sex differences in contextual physiological responses: while males did not show any effect of partner status, females responded to both their stress exposure and the male partner's stress exposure at the level of circulating CORT and AA. In addition, fertilisation rates and female CORT correlated with the male partner's exhibition of sexually aggressive behaviour suggesting that female perception of the male can affect their physiology as much as direct stress. Overall, male reproduction appears relatively simple – sexual stimuli, irrespective of stress, drives major neural changes including rapid reversal of stress-induced changes of AA. In contrast, female reproduction appears more nuanced and context specific, with subjects responding physiologically and behaviourally to stress, the male partner's stress exposure, and female-directed male behaviour.

Keywords

Oestrogens; acute stress; sexual behavior; sex differences; medial preoptic nucleus

INTRODUCTION

Actions of testosterone in the brain often depend on its local conversion to oestrogens via the enzyme aromatase. For example, changes in physiological and behavioural variables such as sexual behaviour (e.g. 1), parental behaviour (2), aggression (e.g. 3), nociception (4), cognition (5) and neuroprotection (5) are controlled through the binding of oestrogens to

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their cognate nuclear receptors which then act as transcription factors (6). Recent studies have also demonstrated that oestrogens can act rapidly at the level of the cell membrane (7). Prior studies have demonstrated that aromatase activity (AA) can be rapidly modulated both *in vitro* (8) and *in vivo* (9–11). Rapid modulation of AA may therefore produce fast changes in local oestrogen concentrations matching the time scale required for membrane-initiated effects of oestrogens.

Because birds express much higher concentrations of aromatase than mammals, most studies demonstrating rapid *in vitro* and *in vivo* changes in aromatase activity (AA) have focused on avian species. Interestingly, it was recently found that while both sexual interactions and stress rapidly induced changes in AA, the directionality of the changes was opposite in brain nuclei commonly associated with the control of sexual behaviour and fertility. Specifically, in the males medial preoptic nucleus POM, stress induces an increase in AA while sexual interaction induces a decrease and in the ventromedial nucleus of the hypothalamus and the tuber (VMN/Tub), stress induces an increase or no change while sexual interaction induces a decrease. In females, there is a similar directionality in the POM but in the ventromedial nucleus of the hypothalamus and the tuber (VMN/Tub), stress induces a decrease while sexual interaction induces a slight increase or no change (1, 9, 10, 12). Together these data suggested that the targeted changes in AA may serve as a potential link between the stress response system and the neural control of sexual behaviour.

Such a link is often expected to be inhibitory due to the assumption that reproductive effort is suspended immediately following exposure to an acute stressor (13). Although acute stress is noted to decrease reproductive behaviour in mammals (14, 15), amphibians (16) and birds (17), the literature on this phenomenon is limited and studies have suggested that it is not universal (18). Additionally, the mechanisms that may underlie a connection between the physiological stress response and reproductive behaviour remain understudied.

When exposed to two equally rapid stimuli (stressful or sexual) that have been shown to have opposing effects on AA, the directionality of preoptic AA fluctuations may depend on whether or not the effects of one stimulus type prevails over the other in the brain. Here we investigated the interplay between the effects of these two types of stimuli on nuclei and sex-specific changes in AA. We additionally assessed the indirect effects of stress on AA by testing the effect of partner stress exposure. To quantify potential downstream effects of neural changes, we also measured male and female sexual behaviour and fertility rates. To investigate these questions, we compared, in both sexes, the effect of 15 min of acute restraint stress and 5 min of sexual interaction alone or in combination on behaviour, fertility rates and AA measured in four discrete brain nuclei that are known to express the highest levels of aromatase activity and where previous studies demonstrated significant changes in enzymatic activity after exposure to stress or to a sexual partner (10, 12). In addition, individual stress perception (either in direct response to restraint stress or in response to partner behavior in the arena) was quantified by measuring circulating corticosterone (CORT) concentrations. We used a two-by-two experimental design such that all individuals were either stressed or non-stressed immediately prior to sexual interaction with a stressed or non-stressed partner.

We predicted that the changes in AA and CORT would either reflect the prevalence of input from one stimulus type (stressful or sexual) or a combinatorial profile. We also anticipated that such changes in AA would be reflected in behavioural output and fertility. For example, the persistence of stress-induced AA changes despite the sexual stimuli was predicted to mediate inhibitory effects of stress on behaviour and fertility, but prevalence of sexually-induced AA changes would allow maintenance of sexual behaviour and fertility despite stress exposure. Finally, we expected that, given the sex differences demonstrated in nuclei-

specific AA responses, sex differences would also be observed in the responses to combined stimuli and pairing context.

MATERIALS AND METHODS

Subjects

A total of 119 quail (*Coturnix japonica*) were used (females, $n = 59$ and males, $n = 60$) either as experimental ($n = 40$ for each sex that were allowed to interact sexually for 5 min, see below) or control birds ($n = 19$ – 20 for each sex that never interacted in the test arena). This species has been well established as an important model species for the study of the aromatase system (overview provided in 19). Birds were obtained from a local breeder at 5 weeks of age and were between 13 and 15 weeks of age during the experimental period. All experimental subjects were individually housed starting at 7 weeks of age and maintained on a long day photoperiod (16L:8D). Both males and females were kept gonadally intact. All animals were provided food and water *ad libitum* and experiments complied with the Belgian laws on the "Protection of experimental animals" and were approved by the Ethics Committee for the Use of Animals at the University of Liège (Protocol #1027).

Experimental procedures

All experiments were run between the hours of 0900 and 1200. We were interested in two main questions in addition to behavioural responses: fertilisation effects and changes in brain AA. Unfortunately, the need for tissue collection for the latter analysis precluded us from examining both questions in one round; therefore, the identical protocol for behaviour was run twice with a two-week interval with birds being redistributed evenly but randomly between each group for the second round and male and female pairs were changed. Based on prior data, effects of pairing on AA were not expected to last beyond 2 hours (12) and our previous study indicated that effects of acute stress on both brain AA and plasma corticosterone concentration last less than 30 min (10). We had therefore every reason to expect that the conservative selection of a two-week interval between the two rounds of measures would ensure their independence. This was confirmed by statistical analyses of the results (see below). The two-week interval was additionally required to allow egg collection and tests of fertility after the first round of manipulations.

For each round, experimental birds were randomly assigned to one of two treatment groups (stressed or non-stressed) and then one of two pairings (stressed or non-stressed partner) such that the pairings followed a two-by-two design (Fig. 1). From hereon, these groups will be referred to as follows: control groups are either non-stressed (ns) or stressed only (S); paired groups are described as both stressed (S+S), stressed individual with non-stressed partner (S+ns), non-stressed individual with stressed partner (ns+S), and both non-stressed (ns+ns). Birds in the stress treatment group were exposed to 15 minutes of restraint in a restraint cage (10 cm in diameter $\times$ 16 cm in length); for a detailed protocol see (10). Immediately following the stress or rapidly following removal from the home cage (for the non-stressed group), both males and females were introduced simultaneously into the breeding arena (60 $\times$ 40 $\times$ 50 cm) and allowed to freely interact for 5 min during which their behaviour was recorded. In addition, two control groups were never allowed to interact with partners of the opposite sex and were killed for brain collection after being exposed to 15 min of stress (S group) or immediately after removal from their home cage (ns group).

Behavioural interaction - round 1—Pairs of males and females were run two at a time. The order of the pairings (S+S, ns+ns, etc.) tested alternated such that timing across test day and across days was evenly distributed for all treatments and pair groups. Immediately following the behaviour tests, all birds were returned to their home cage. Eggs were

collected from each female's cage beginning on day 1 (day of testing = day 0) and ending on day 10. Eggs were incubated immediately (38°C, 60% humidity) and assessed on days 12–15 (thus ending the incubation period) for fertilisation and sex. Sex of the embryos was determined by visual inspection or PCR-based sexing for embryos in which we were unable to definitively assign a sex (see below). All eggs with evidence of embryonic differentiation were successfully sexed.

Behavioural interaction - round 2—At least two weeks after the first round, birds were tested again and no additional handling occurred during this interlude. Pairs of males and females were run one at a time to minimise the time required to collect brain and blood samples at the conclusion. Immediately following the behaviour tests, birds were removed from the arena and immediately sacrificed by decapitation. Animals were not anaesthetised to minimise potential neurochemical changes and decrease the exposure time to additional stressors. Trunk blood was collected for steroid analysis and brains were rapidly dissected from the skull and snap frozen on dry ice (average time from removal from arena to ice < 90s). Blood was collected, without heparin, in Eppendorf® tubes and immediately placed at 4°C. After allowing the blood to clot overnight, the blood was centrifuged for 10 minutes at 9,300 g to separate the serum. Serum was then stored at –80°C until assayed.

Behavioural analysis

Males—All behavioural interactions were recorded with a video camera and we reviewed the videotape at a later point to score the male behaviour. All scoring was done blindly. We recorded both the latency and the frequency of four well-documented male behaviours that are part of the full complement of the typical copulatory sequence in quail. These behaviours have been thoroughly described elsewhere (20, 21). Specifically, we measured neck grabs (NG), mount attempts (MA), mounts (M), and cloacal contact movements (CCM). We also made note of the time elapsed before each behaviour was displayed (latency), the last behaviour performed and the elapsed time between the last behaviour and the end of the testing period. To avoid redundancy, only analyses of MA and CCM frequencies and latencies will be reported in the results since analysis of NG and M always leads to nearly identical conclusions.

Females—All behavioural interactions were recorded with a video camera and we reviewed the videotape at a later point to score the female behaviour. The frequency of each of the following behaviour patterns were measured (as reported in 22): squat, crouch, stand up, short avoid, long avoid, pecking, and hopping/darting. Squat and crouch behaviors are both considered to indicate positive receptivity and were combined for analysis due to the small number of squats viewed. Stand up, short avoid, and long avoid all indicate negative receptivity of decreasing degrees such that stand up (simply standing upright with extended legs to discourage male mount attempts) is the least negative while long avoid (female runs away and continues to run for 4s) is considered the most negative (see 22 for full description). For each pair, we also calculated the relative percentages of each behavioural display by the female relative to the total number of times the male approached her during the 5 min test.

PCR-based sexing

We used the method described previously using primers against a sequence present on the female (WZ) specific W chromosome is absent from male (ZZ) samples (23). An additional sequence (18S) common to both sexes was also amplified to ensure that negative reactions are really due to the absence of the W specific sequence in male samples rather than a failure of the PCR reaction. Briefly, a piece of embryonic tissue (most often a hind limb) was soaked in digestion buffer (EDTA 20 mM, SDS 2%, proteinase K 100 mg/ml, Tris 50 mM

pH 8.5) at 55°C overnight. Genomic DNA was extracted by phenol/chloroform separation. After precipitation in cold acetate/ethanol, the DNA pellet was reconstituted in water (200 µl) and 0.5 µl of this material was subjected to PCR. The PCR protocol comprised denaturation for 4.5 min at 95°C followed by 40 cycles of incubation at 95°C for 30 s, 56°C for 30 s and 72°C for 30 s. A final extension step of 72°C for 5 min was carried out for all reactions. The reaction was performed in a final volume of 25 µl containing Green GoTaq® reaction buffer (Promega, Madison, WI, USA), 0.2 mM dNTP Mix (Promega, Madison, WI, USA), 0.26 µM 18S primers (Forward: 5' AGCTCTTCTCGATTCCGTG 3'; Reverse: 5' GGGTAGACACAAGCTGAGCC 3' (24) (Eurogentec S.A., Seraing, Belgium), 0.8 µM Wpkci primers (Forward: 5'-TTGGGCATTTGAAGATTGTC-3'; Reverse: 5'-GTCTGAAGGGTCTGAGGGT-3'; Eurogentec S.A., Seraing, Belgium) and GoTaq® DNA polymerase (Promega, Madison, WI, USA). PCR products together with molecular size standards were fractionated by electrophoresis on a 3% agarose gel.

Micropunches

Our method of isolating brain nuclei to assess region-specific AA followed the identical protocol presented in previously (10, 25) using a modified Palkovits punch technique (26) validated for use in quail (27). Since only six nuclei are known to contain high densities of aromatase-positive neurons and since only four of these nuclei demonstrate changes in AA following either acute restraint stress (10) or sexual interactions (12), the following brain nuclei were targeted for collection: medial preoptic nucleus (POM), medial portion of the bed nucleus of the stria terminalis (mBST), ventromedial and tuberal hypothalamus (VMN and Tub, respectively). The distribution of aromatase expression in these regions was described previously (28). Micropunches were obtained from 200 µm coronal sections beginning at the caudal end of the tractus septopallio-mesencephalicus (TSM). Positioning was determined using the quail atlas as presented in (29). All 200 µm sections for each animal were kept frozen until punched. Micropunches were obtained using a specialised brain punch apparatus (Leica Biosystems #39443001RM) and two heads with nominal diameters of 1.00mm and 0.75mm depending on the nuclei size. The protein measured for AA calculations demonstrated that these punch needles provided similar punch sizes as those reported previously (10, 25). Punches were expelled into a frozen Eppendorf® tube and all punches from a same nucleus in a given individual were pooled in a same tube (a given nucleus could indeed be observed and punched on several successive sections). Samples coming from different nuclei and from different birds were however analysed individually. Micropunches were kept frozen throughout and stored at -80°C until assayed.

AA assay

Micropunches were homogenised in 120 µL of ice cold buffer (150 mM KCl, 1mM Na-EDTA, 10 mM Tris-HCl, pH 7.2) prior to assaying using a glass pestle specific for 1.5 mL Eppendorf tubes. Homogenised samples were then refrozen on dry ice and stored at -80° C.

We determined aromatase activity by measuring the production of tritiated water following the conversion of [1β - ^3H]-androstenedione into oestrone (30, 31). This assay has been thoroughly described and validated in published studies, most recently adapted for micropunches (25). Individual samples (one sample per nucleus per individual) were run in duplicate and randomly distributed between seven assays, each containing two internal controls (HPOA homogenates expressing high [male] or low [female] AA). The intra-assay coefficient of variability was less than 3% while the inter-assay coefficient of variation was less than 4.5%.

AA results were corrected for the amount of tissue obtained for each micropunch sample by analyzing protein concentrations with a commercially available Coomassie Plus Protein

Assay reagent (Pierce, Rockford, IL). For this assay, we used 10 μL of the micropunch homogenates (duplicates of 5 μL). With this correction, we are expressing AA as a measure of pmol of aromatisation product $\times \text{h}^{-1} \times \text{mg protein}^{-1}$.

Corticosterone Enzyme Immunoassay

CORT was assayed using Enzyme Immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI) validated for quail (10). We first extracted CORT from the protein component of the serum using dichloromethane and dried samples at 37°C under a constant stream of nitrogen gas. Samples were reconstituted to a 1:10 (20 μL serum) or 1:20 (10 μL serum) dilution using 200 μL EIA buffer supplied with the kit. Samples were run in duplicate and randomly assigned to one of three plates, run in one assay. For each plate, both the intra-assay coefficient of variability was less than 5% and inter-assay coefficient of variation was less than 15%. Sensitivity of the EIA, determined from the lowest reliable value detectable when samples were diluted at a 1:10 dilution factor, was greater than 0.15 ng/mL.

Statistical Analysis

Behavioural data, AA and CORT concentration measurements were analyzed with Prism, version 4.0b (2004; GraphPad Software, Inc., San Diego, CA) using two-way ANOVAs with the stress status of the sex considered and the stress status of the partner as independent variables. When appropriate, additional paired comparisons were carried out using the post-hoc Bonferroni test. Fertilisation data were analyzed using Chi-Square analysis or Mann Whitney U test comparing proportion of non-fertilised eggs to fertilised eggs in non-stressed and stressed females. Individual comparisons, eggs fertilised versus male behaviour and female CORT versus male behaviour were compared by linear regression analyses. Chi-Square tests and linear regression analyses were run using JMP, version 5 (SAS Institute, 2005, Cary, NC).

Identical to procedures explained previously (see 10), AA data points were removed if histological analysis revealed that punches had been performed at an erroneous anatomical location (each punch quantified on a four-point qualitative scale from 0 to 3 with only those with totals of zero or near zero removed), if technical issues were noted for the assay (e.g. very low protein content in the AA assay or if results could be considered as outliers (more than two standard deviations away from the mean for each group)). Less than 6% of data points in each analysis were removed for these reasons and such removals were evenly distributed between experimental groups and sexes. Result for one sample in the CORT assay had to be discarded after we discovered that liquid volume in the assay tube was for unexplained reasons lower than expected. All data are presented by their mean $\pm$ SEM.

RESULTS

Behavioural changes

In order to definitively ascribe functionally relevant effects of acute stress on sexual behaviour, we expected to observe clear, significant, and consistent effects on latency and the ability to exhibit a full copulatory sequence (CCM). Specifically, we predicted that following acute stress exposure, we would observe increases in latency and decreases in the ability to exhibit CCM.

As illustrated in Table 1, our data did not meet this prediction. Specifically, in the first round, we only found significant differences between groups for the latency to perform a CCM such that there was a significant effect of female treatment ($F_{1,36} = 4.400$, $p = 0.043$) and a significant interaction ($F_{1,36} = 4.2502$, $p = 0.047$) but no stress effect ($F_{1,36} = 0.010$, p

= 0.922). Post-hoc analysis revealed that this effect is mainly explained by the fact that non-stressed males copulated faster when paired to a non-stressed female rather than a stressed female. For the latency to MA, there was no significant effect of male or female treatment or interaction between these two factors ($F_{1,36} = 1.057$, $p = 0.311$). In contrast, in round 2, a similar effect on CCM latency was not observed: latencies were not significantly affected by treatments or their interaction ($F_{1,36} = 1.701$, $p = 0.200$). If anything, the latency data showed a completely opposite profile with non-stressed males displaying a shorter CCM latency when paired to stressed females rather than with non-stressed females. Stressed males also took more time to mount attempt than non-stressed males, but this trend did not reach significance (Stress: $F_{1,36} = 3.047$, $p = 0.089$; female treatment: $F_{1,36} = 0.254$, $p = 0.617$; Interaction: $F_{1,36} = 0.232$, $p = 0.633$).

The analysis of behavioural frequencies did not reveal any stress effect on any component of the copulatory sequence in the first round of behavioural tests ($F_{1,36} = 1.920$, $p = 0.174$). In the second round, however, stressed males displayed lower MA frequencies than non-stressed males (MA: $F_{1,36} = 8.872$, $p = 0.005$) and post-hoc tests revealed that the effect was driven by the non-stressed males paired with stressed females. This difference was not observed for CCM (CCM: $F_{1,36} = 2.504$, $p = 0.122$) indicating that there was no efficiency difference between stressed and non-stressed males. No interaction between male stress and female status was found for any behaviour ($F_{1,36} = 2.504$, $p = 0.122$). Since we ran the individuals in two separate rounds of behavioural tests, we also examined the effect of prior treatment (stress or non-stressed) on behavioural outcomes in the second round. Using three-way ANOVAs with the male treatment, female treatment, and prior test group as variables, we found that the only significant effect of prior test group was for MA. There was no significant effect of prior male treatment on MA in the second round ($F_{1,32} = 0.425$, $p = 0.519$) and the significant interaction was driven by prior male treatment and second round female treatment ($F_{1,32} = 5.975$, $p = 0.02$). Post-hoc analysis demonstrated that males who were stressed during the first round showed much higher MA frequencies when paired with a stressed female as compared to a non-stressed females in the second round. This indicates that only male perception of the female mate is potentially altered by previous stress exposure but that actual MA output is not affected by prior stress exposure. It should also be noted (and is discussed further below) that prior stress treatment did not have significant effects on CORT or AA levels measured during the second round of behavior tests.

As illustrated in Table 2, in females, we found no significant effects of stress or partner stress status on behavioural frequencies in either round 1 ($F_{1,36} = 2.937$, $p = 0.095$) or round 2 ($F_{1,36} = 2.583$, $p = 0.117$) with the exception of a significant effect of the stress status of the female on the percentage of female stand ups in response to male approaches ($F_{1,36} = 4.762$, $p = 0.036$). This behavior was also the only behavioural component that was affected by prior stress exposure such that there was an interaction between prior treatment and female treatment in second round ($F_{1,32} = 6.199$, $p = 0.018$). However, because the female behaviour is relatively dependent on the male she is paired with (e.g. number of stand ups may simply reflect the number of approaches from the male), subtle effects on behaviour are difficult to observe statistically but might translate into effects on the fertilisation success.

Overall, stress did not seem to affect behavioural endpoints in females. And, in males, few significant effects were detected. However, since these differences do not achieve the main criteria set forth for exhibiting functionally relevant effects (consistency across tests for changes in latency and specific changes in total number of CCM), overall, the data demonstrate that there is no clear effect of acute stress on male sexual behaviour.

Fertilisation rates

Nearly all females laid consistently across the days of egg collection with the exception of 4 individuals that did not lay any eggs (2S+ns, 1ns+S, 1S+S) and data from these subjects were removed from further analysis of egg laying performance and fertilisation rates. Overall, few females laid fertilised eggs but the highest number of females laying fertilised eggs belonged to the ns+ns group. Numbers of females laying at least one fertilised egg were as follows: both sexes stressed - 3 out of 9 laying females; stressed female, non-stressed male - 2 out of 8 laying females; non-stressed female, stressed male - 3 out of 9; and both sexes non-stressed - 6 out of 10. These differences are not statistically significant ($X^2 = 2.76$, $p = 0.43$). The total numbers of fertilised vs. non-fertilised eggs laid over 10 days by stressed females versus non-stressed females were statistically different ($X^2_{1,386} = 13.38$; $p = 0.0003$; Fig. 2A). This measure suffers, however, from some degree of pseudo-replication since the different eggs laid by a given female are not independent events. A more stringent test of these differences assessing whether the percentage of fertilised eggs laid by stressed females was actually lower than in non-stressed females only suggested the presence of a difference but it was not statistically significant (One tailed Mann-Whitney U-test: $U=126.5$, $p=0.0702$).

Numbers of fertilised eggs laid by each female also negatively correlated to the number of neck grabs and mount attempts received from males (Neck grabs: $R^2 = 0.43$, $p < 0.0001$; Mount attempts: $R^2 = 0.42$, $F_{1,25} = 18.75$; $p = 0.0002$; Fig. 2B), behaviours that may be viewed negatively by the female because of their injurious potential (32). These data suggest that fertilisation is regulated by the female status (decreased fertilisation if stressed) as well as by the behaviour of the male towards her (decreased fertilisation with greater numbers of neck grabs and mount attempts). Interestingly, nearly all females (both stressed and non-stressed) that received fewer than 10 mount attempts from the male laid fertilised eggs. We should note that in the regression analysis, we removed non-laying females and pairs in which males did not demonstrate any CCM ($n = 9$) thus having no chance of fertilising eggs. However, since we cannot distinguish whether decreased fertilisation results from pre-copulatory behaviour (thus denying males opportunity to achieve an effective CCM) or post-copulatory regulation, we also ran an analysis including males that did not demonstrate any CCM. It appears that even these males who did not achieve CCM showed a relatively high number of mount attempts and neck grabs and, when included in the analysis, the correlation was maintained (Neck grabs: $R^2 = 0.42$, $p < 0.0001$; Mount attempts: $R^2 = 0.38$, $p < 0.0001$).

Changes in AA

AA levels were analyzed by separate two way ANOVA for each brain nucleus with the sex as one factor and the different experimental ($n = 4$; S+S, S+ns, ns+S, ns+ns) and control ($n = 2$; ns and S) groups as the other factor. Repeating the findings from prior studies, there were significant sex differences in AA in all brain nuclei studied with males consistently showing higher AA than females: POM ($F_{1,95} = 5.84$, $p = 0.018$), mBST ($F_{1,95} = 25.95$, $p < 0.0001$), VMN ($F_{1,100} = 7.49$, $p = 0.007$), and Tub ($F_{1,100} = 17.35$, $p < 0.0001$).

POM and mBST—In the POM (Fig. 3A), there was a significant effect of groups on AA ($F_{5,95} = 6.91$, $p < 0.0001$) with no significant interaction between groups and sex ($F_{5,95} = 1.04$, $p = 0.39$). Because we found both group and sex effects, we ran Bonferroni post-hoc tests comparing AA of all groups. In **males**, the stress control group significantly differed from all groups except the S+S group, and the ns control group was not significantly different from any group of paired males (S+S, S+ns, ns+S, ns+ns). These results suggest that being in the arena with the female brought stress-induced increases in AA back to non-stressed control levels but did not result in changes for non-stressed, paired individuals. In

females, the post-hoc tests revealed that all stressed groups (S, S+S, S+ns) had elevated AA compared to ns birds while groups with non-stressed females (ns+S, ns+ns) did not differ from the ns controls.

To discriminate between the direct effects of stress on the female from the indirect effects due to the partner status, we ran a 2-way ANOVA specifically on data from the 4 groups that had been paired with female stress status and male partner stress status as the independent variables. We found a significant effect of the female stress status ($F_{1,35} = 5.37$, $p = 0.03$) but no effect of male partner stress status ($F_{1,35} = 2.57$, $p = 0.12$) and no interaction effect between the variables ($F_{1,35} = 0.01$, $p = 0.91$).

In the mBST (Fig. 3B), there was a significant group effect ($F_{5,95} = 5.18$, $p = 0.0003$) but no significant interaction between sex and group ($F_{5,95} = 1.35$, $p = 0.24$). Post-hoc analyses surprisingly indicated that, in males, the groups that were significantly different were the S+ns and ns+S as compared to the ns+ns group. In females, post-hoc tests identified no significant difference.

To summarize, in the male POM, AA is increased by stress (as expected from prior studies) but pairing with a female results in a rapid return to baseline activity levels and there is no decrease of AA following pairing with female only. In the female POM, although the non-significant group*sex interaction implies that changes are similar to those detected in males, qualitative analysis of the data and post-hoc tests suggest that the stressed females have elevated AA that remain above baseline levels despite male pairing. In the male mBST, no paired group differed significantly from the baseline levels although individual and partner status may affect AA in this nucleus.

VMN and Tub—In the VMN (Fig. 3C), there was a significant effect of group ($F_{5,100} = 3.01$, $p = 0.014$) and a significant group by sex interaction ($F_{5,100} = 2.9$, $p = 0.017$). In the Tub (Fig. 3D), there was a strong trend suggesting an effect of group ($F_{5,100} = 2.11$, $p = 0.070$) and a significant group by sex interaction ($F_{5,100} = 2.54$, $p = 0.030$). In **males**, Bonferroni post-hoc tests indicate that both in the VMN and Tub there was no stress-induced changes in AA, but that in all groups paired with a female AA was decreased compared to the ns control group. The significant interaction seen in the VMN and Tub suggested that **females** respond differently from males to stress and mate pairing. Indeed, stress numerically decreased AA in the VMN and Tub (as expected from prior experiments) and AA remained low in the VMN of stressed females but returned to baseline in the Tub of stressed females when they were mated. However, none of these comparisons were statistically significant in Bonferroni post-hoc analyses.

To discriminate between the direct effects of stress on the female from the indirect effects due to the partner status, we ran a two way ANOVA with female stress status and male partner stress status as the independent variables. In the VMN, the female stress status significantly affected AA ($F_{1,33} = 5.21$, $p = 0.03$) but there was no effect of male partner stress status ($F_{1,35} = 0.43$, $p = 0.52$) or interaction ($F_{1,35} = 0.24$, $p = 0.63$). In the Tub, none of these effects were found significant ($F_{1,35} = 0.60$, $p = 0.44$). These analyses further confirm that in the VMN AA in stressed females is different from non-stressed females regardless of the status of the partner while this difference is not present in the Tub.

Arena effect in males—Because we observed significantly different AA in the brain of males sacrificed immediately after the 15 min stress compared to males sacrificed 5 min (the duration of the sexual interaction with the female) after the 15 min stressor, we wondered whether this effect was specifically female-related or reflected a potential “recovery” due to the simple fact of being in the arena for 5 min after stressor cessation. With 18 additional

subjects, we compared AA in males stressed for 15 min and immediately sacrificed ($n = 8$) to males stressed for 15 min, placed in the empty arena for 5 min, and then sacrificed ($n = 10$). We found no significant differences in AA between these groups for all nuclei studied: POM (stress = $60.6 \pm 6.5 \text{ pmol} \times \text{h}^{-1} \times \text{mg protein}^{-1}$; stress+arena = $65.8 \pm 5.8 \text{ pmol} \times \text{h}^{-1} \times \text{mg protein}^{-1}$; $t = -0.63$, $df = 16$, $p = 0.54$), BST (stress = 31.6 ± 3.2 ; stress+arena = $28.6 \pm 2.9 \text{ pmol} \times \text{h}^{-1} \times \text{mg protein}^{-1}$; $t = 0.69$, $df = 16$, $p = 0.52$), VMN (stress = $16.7 \pm 3.3 \text{ pmol} \times \text{h}^{-1} \times \text{mg protein}^{-1}$; stress+arena = $19.3 \pm 2.9 \text{ pmol} \times \text{h}^{-1} \times \text{mg protein}^{-1}$; $t = -0.60$, $df = 16$, $p = 0.56$), Tub (stress = $19.2 \pm 2.7 \text{ pmol} \times \text{h}^{-1} \times \text{mg protein}^{-1}$; stress+arena = $17.1 \pm 2.4 \text{ pmol} \times \text{h}^{-1} \times \text{mg protein}^{-1}$; $t = 0.55$, $df = 16$, $p = 0.59$). These data imply that the differences in AA observed between stressed males and stressed males paired with a female are a direct result of interacting with the female and not a result of “recovering” after stressor cessation. We did not test this idea in females because the Tub was the only nucleus that showed potential “recovery” and our earlier study demonstrated that stress-induced changes in this nucleus persist for at least 30 minutes beyond cessation of the restraint stress (10).

Effects of prior treatment—Two-way ANOVA’s to test the effects of the treatment group assigned in the first round of the behavioural tests demonstrated that there were no effects of prior treatment (stressed or non-stressed) on male AA levels in any of the nuclei studied ($F_{1,35} < 0.62$, $p > 0.45$). In addition, there were no interactions between prior treatment and subsequent treatment ($F_{1,35} < 1.14$, $p > 0.29$). Females showed an effect of prior treatment only in the mBST ($F_{1,36} = 7.8$, $p = 0.008$) with no other prior treatment effects ($F_{1,36} < 1.98$, $p < 0.17$). This effect was driven by an approximate 15% increase in AA levels in the mBST for female quail that had been previously stressed as compared to females that had not been previously stressed (regardless of subsequent treatment). The origins and potential function of this effect are difficult to interpret based on available knowledge on the regulation of aromatase and its function on the female brain. This effect may just reflect random variation in the data (type II error) or alternatively reveal an interesting long-lasting effect of stress but additional work will be needed to discriminate between these possibilities. There were no interactions between prior treatment and subsequent treatment in any nuclei in the female brain ($F_{1,36} < 1.14$, $p < 0.29$).

Changes in CORT

CORT measurements from the four paired groups were analyzed together with those of the two control groups (ns and ST) by a two way ANOVA with the sex of the subjects and the six groups (ns, ST, S+S, S+ns, ns+S, ns+ns) as independent factors. There were significant effects of sex ($F_{1,98} = 15.43$, $p = 0.0002$), group ($F_{5,98} = 15.02$, $p < 0.0001$), and a significant interaction ($F_{5,98} = 3.52$, $p = 0.006$, Fig. 4). Bonferroni post-hoc tests comparing CORT between all groups within each sex showed that in **males**, all stress groups had elevated CORT as compared to baseline concentrations. Furthermore, qualitative analysis of the male CORT data suggested that pairing with a female alone resulted in elevated CORT concentrations that did not reach stress-induced concentrations. These levels in the ns+S and ns+ns groups were, however, not significantly different from either baseline or stress-induced concentrations. **Females** had, overall, higher CORT concentrations than males and a group-related pattern that differed significantly from the male pattern as demonstrated by the interaction effect. As revealed by post-hoc tests, major differences in female CORT related to the effect of stress (ns vs. ST) but also very prominently to the status of the male partner: both stressed and non-stressed females paired with non-stressed males had significantly higher CORT than their counterpart group paired with stressed males.

Effects of prior treatment—As with AA, the treatment group assigned in the first round of the behavioural tests had no significant effects on male or female CORT concentrations in

the subsequent test ($F_{1,36}=1.01$, $p=0.22$; $F_{1,36}=0.62$, $p=0.43$, respectively). In addition, there were no significant interactions between prior treatment and subsequent treatment in males ($F_{1,36}=0.001$, $p=0.97$) or females ($F_{1,36}=1.36$, $p=0.25$). Considering that prior stress exposure did not significantly affect the CORT concentrations or AA (described above) in the second test, it is unlikely that birds had habituated to the stress stimulus or had been affected by previous stress exposure in a way that altered the neurochemical response to the second stimulation.

Effects of male behavior on female CORT concentrations—Because male behaviours that can be perceived as aggressive or injurious (such as NG and MA) have been suggested to affect female CORT release during pairing (33), we ran correlations for the number of MA versus female CORT concentrations. Combining all data, we found a correlation with a slope significantly different from zero between female CORT and male MA frequency ($R^2 = 0.19$, $F = 8.87$, $p = 0.005$, Fig. 5). Interestingly, nearly all females with CORT lower than 8 ng/mL (both stressed and non-stressed) received fewer than 10 mount attempts whereas very variable CORT measures including very high values were seen in females receiving more MA.

Thus whereas female CORT concentrations reflect both their stress status as well as their partners stress status (potentially driving behavioural changes), male CORT concentrations reflects their own status only without integrating their partner stress status.

DISCUSSION

The present study was designed to investigate the interplay between stimuli with opposing effects on neural AA and resulting behavioural output. Four main conclusions can be drawn from this study. First, the pattern of AA response to stressful and sexual stimuli shows clear regional specificity. In some regions, AA is responsive to both stress and mating such that AA is changed following acute stress but in stressed individuals AA is rapidly restored to baseline levels. This is the case in the male POM and potentially in the female Tub, two nuclei specifically involved in the control of male and female sexual behaviour respectively. By contrast, in other regions, AA seems exclusively sensitive to either mating (male VMN and Tub) or stress (female POM or VMN). Second, potentially corresponding to these neural effects, we found that stress does not dramatically affect behavioural output of either sex within a short time frame; however, fertilisation rates are decreased by stress exposure. Third, males and females appear to differ in the way they integrate their own versus their partner's stress: females physiologically respond to both their own stress exposure as well as their partner's, while male responses reflect only their own stress exposure. Finally, female CORT and fertilisation rates correlate with male sexual behaviour, suggesting that females perceive certain behaviours as less desirable or even stressful when in the breeding arena. These aspects are further discussed in the following sections.

Changes in AA and behavioural output

Males—Pairing with a female rapidly reversed the effects of stress on AA in the POM, essentially “rescuing” the region most likely driving male reproductive behaviour from the stress-induced alterations and rapidly returning AA levels to baseline. This is the first study, to our knowledge, demonstrating that inputs from one stimulus (stress) can be rapidly (5 min) negated by a second stimulus (sexual) that is known to have opposing effects on AA. Furthermore, pairing appears to decrease AA in the VMN and Tub in males regardless of stress status with the most pronounced effect observed for non-stressed males paired with non-stressed females.

Overall, the AA data are interesting when compared to those of de Bournonville et al. (12). Similar to our study, the authors found a decreased AA in Tub following copulation; here this decrease occurred in the VMN as well. Dissimilar is the observation that, in our study, AA in the male POM does not decrease below baseline levels with copulation in any condition. This may be due to a difference in experimental design between these studies since in the de Bournonville study males were pre-tested in the same arena multiple times before the test preceding brain collection while we did not pre-test our birds. If pre-testing does play a role in the decreased POM AA observed in de Bournonville et al., this would suggest that the AA decreases in the POM following copulation may be an anticipatory effect while the drop in the VMN and Tub is independent of learning and may be a pure copulatory effect.

Although the role of local oestradiol production in the male VMN and Tub is not clear and will need to be investigated further, we know that aromatisation of testosterone in the POM is critical for male reproductive behaviour (34–36). When compared to stressed non-paired controls the changes in POM AA in stressed males in our study go in the same direction (decrease) as in trained, paired males (12). Therefore, in the POM specifically, the regulation of AA appears to be an *active* mechanism, reversing the rapid effects of stress (15 min) with an even more rapid effect of sexual interaction (< 5 min); in contrast, passive “recovery” (5 min in the arena) does not alter stress-induced changes in AA.

The time scale of this effect is a potentially important novel finding. Previous studies in zebra finches have demonstrated rapid effects of behavioural context or performance on local oestradiol concentrations in the auditory telencephalon (11), but, due to technical limitations, these effects were measured on a longer time scale (30 min intervals) than in the present study. We demonstrate here that a behavioural stimulus can reverse a stress effect within 5 min, a finding that further emphasises the highly dynamic nature of the aromatisation process. Although the specific mechanisms through which these inputs affect AA remains to be investigated, data from *in vitro* studies suggest that the effect is most likely mediated by non-genomic post-translational modifications of the enzyme, affecting its kinetics, rather than genomically controlled changes in enzyme concentrations (37). The potential for rapid and reversible modulation of enzymatic output further emphasizes the capacity of this system to operate on a time scale required for membrane-initiated effects of oestrogens. Thus, the acute modulation of oestrogen synthesis (via aromatase modification) may serve as a switch, translating contextual and physiological information into neurochemical signals to rapidly improve, or even allow, reproductive behavioural output.

Since the AA data suggests a rapid “rescuing” of stress-induced AA alterations in the POM when males are paired with females, it can be expected that male quail will demonstrate normal copulatory behaviour despite stress exposure. Our behavioural data appear to confirm this prediction as we did not find reliable functionally relevant alterations in male behaviour following acute stress in the tests immediately preceding sample collection for AA. Our data further suggest that the acute stress-reproduction link is not a simple one. On the one hand, it can be argued that the quail is a domestic species “bred to breed” that may no longer reflect mechanisms, in particular some of the reactions to stress, present in wild species. On the other hand, this model may represent a domestication-induced version of a mechanism (or neurally disconnected mechanism) that is present in wild species which, in extreme circumstances, maintain reproductive functions despite stress exposure (13). Supporting the latter scenario, similar lack of reproductive behavioural suppression following acute stress has also been demonstrated in amphibians (38) and rodents (39, 40). The present study suggests a potential mechanism underlying the maintenance of sexual behaviour in stressed animals: as observed here at the level of AA, oestrogen synthesis in

brain nuclei specifically involved in the control of sexual behaviour is targeted in a way that input from sexual stimuli prevails over input from stressful stimuli.

Females—Compared to males, female AA was differentially affected by the stress of the subject and her partner and, after pairing, stress-induced changes in AA were maintained in specific nuclei. In the POM and VMN, AA remained at the level of stress-induced changes despite pairing with a male suggesting that the rescue effect that paired males demonstrate does not occur in these regions in females. In rodents, it is accepted that oestrogen action in the VMN plays a key role in lordosis behaviour (41, 42). In avian species, implants of oestradiol benzoate placed in the VMN activate female reproductive behaviour (43). Most pertinent to our study is an experiment by Meddle et al. (44) that demonstrated increased Fos-like immunoreactivity in the VMN and Tub with minimal changes in the POA following sexual interaction and correlations between Fos activation in these nuclei and proceptive behaviours. In Tub, although the directionality is different from males, the apparent pattern leads to the same conclusion even if differences are not significant: pairing seems to “rescue” AA to pre-stress levels in a way similar to what was seen in male POM. Our previous work demonstrated that contrasting with the transient stress-induced change in AA observed in the male POM the effect of stress on AA in female Tub is sustained for at least 30 min post stress. The graphical interpretation of the data provided here suggests a restoration of baseline levels after only 5 min of sexual interaction thus potentially indicating that this stress effect is an active process.

Since stressed females paired with male partners have statistically identical AA in the Tub as compared to non-stressed individuals, this restoration may be sufficient to preserve observable behaviour (we found no consistently significant effects on female behaviour). However, the sustained stress-induced effects seen in the VMN and POM may play a more subtle role in the stress-behaviour link. We plan to continue investigating the role of local oestradiol production in the female brain in order to distinguish why stress and sexual interaction might affect these nuclei differently.

Fertilisation rates

As described above, the sustained stress-induced alterations in AA may affect behaviour and/or physiology in subtle ways that are not easily measurable or observable in terms of behaviour; Although we cannot directly compare the changes in AA in the female brain with the fertilisation data from this experiment (since they were collected in two successive rounds of tests), the significant shift in fertilisation rates in favor of non-stressed females may be a downstream result of such alterations. In Japanese quail, fertilisation success from a single mating attempt is highly variable (45) and multiple studies have shown that females control to some extent whether or not a male successfully fertilises her eggs (e.g. 32, 46). Previous studies have shown that shifts in fertilisation rates correspond to female body condition (not measured in this study) and mating context (46) as well as female behaviour toward the initial male approach (running away) (45). These findings suggest that the female can assess the mate and her own condition to determine fertilisation outcomes. In addition, sustained elevation in CORT, a component of the stress response, may also result in decreased fertilisation rates (33). Overall, our results indicate that the stress status of the female plays an important role in the fertilisation rate following one sexual encounter. In addition, a male’s sexual aggressiveness towards the female (engaging in behaviour considered “potentially injurious” to the female) has been shown to correspond to lower fertilisation rates (32). In our study, the number of mount attempts correlated negatively with the number of fertilised eggs suggesting a similar conclusion: greater “sexual aggression” from the male leads to lower fertilisation rates. These findings imply that, in

some way, females are controlling fertilisation based on their physiological state and perception of the male mate.

Changes in CORT concentrations

Interestingly, females and males responded differently to stress, sexual interaction and partner status by specific modifications of CORT. First, in individuals that were not exposed to stress, it appears that both males and females had, overall, elevated CORT when paired with the opposite sex. Although, with the data collected from this experiment, we cannot exclude an effect of novelty stress related to the transfer into the test arena, we observed similar increases in CORT following sexual interaction in males pre-trained (and, therefore, habituated) in the arena (unpublished data). Previous work also evidenced an increase of CORT in male quail following exposure to a female in a familiar environment, the home cage (47). In addition, studies in rodents have shown increases in CORT following copulation (48) and other studies using quail have noted copulation-related increases in CORT that do not reach stress-induced concentrations (33); these authors suggest that such increases in CORT may be important to the act of copulation and facilitation of the breeding effort.

In **females**, status of the partner played a significant role, such that stressed females paired with a stressed male had comparably reduced CORT and non-stressed females paired with non-stressed males had comparably elevated CORT. As Correa et al. (33) demonstrate, a female's assessment of male body condition might influence her CORT concentrations. Although we did not measure male body condition, these results suggest that the female physiology is capable of responding immediately to subtle cues about the male. Various other studies have suggested that females prefer males that are less aggressive and will actively avoid males perceived to be sexually aggressive (49), even preferring a "loser" male, a male that the female observed to lose a competition, over the "winner" male (50). The female may, therefore, perceive mating tactics that may be viewed as aggressive and/or potentially injurious as stress.

When considering CORT and stressor perception, it is important to consider the mechanics of the HPA axis. CORT does not measurably increase in the blood for the first few minutes of stressor exposure (51) and while we do not know how rapidly negative feedback shuts off the HPA axis following stressor cessation, earlier data suggest that CORT concentrations in quail are very near baseline 30 min after a stressor has ceased (10). Such data suggest that females in our study made a rapid initial assessment of the male, perceiving him as a "stressor" or not. This potential stressor perception may be reflected in the females in our study as follows: stressed females either perceived the male as a "stressor" and maintained stress-induced HPA stimulation (higher CORT measured in stressed females with non-stressed males) or did not perceive the male as a stressor and allowed negative feedback to shut off the HPA stimulation (relatively reduced CORT in stressed females with stressed males); non-stressed females either perceived the male as a "stressor" and activated HPA axis (higher CORT in non-stressed females paired with non-stressed males) or did not perceive the male as a stress (relatively lower CORT in non-stressed females with stressed males).

In contrast, in **males**, there were no effects of female status (stressed or non-stressed) such that all stressed males had high CORT (as expected) and all non-stressed males had relatively low CORT irrespective of the status of their female partner. Note also that the patterns for both male and female CORT concentrations do not match those of AA (e.g. CORT in stressed males remain high when paired with females but AA levels are the same across all paired groups regardless of whether or not males were stressed). These data suggest that peripheral CORT plays either an indirect role or no role at all in stress-induced

changes in AA and this conclusion is further supported by the previously observed lack of correlation between CORT concentrations and AA (10). Other neurochemical processes that are activated by stress (e.g., secretion of CRF, AVT, or ACTH) appear to play a role in AA control (52).

To summarise, the female appears to be able to physiologically respond directly to the stress status of the male most likely via the stress system responding to the sexually aggressive behaviour expressed by males that are non-stressed versus those that are stressed. Males, however, only respond to the stress they are directly exposed to with no integration of the status of their partner.

Conclusions

This study demonstrates that stimuli derived from sexual interactions can rapidly (< 5 min) cancel out effects of an acute stress on AA in the male quail POM thus indicating that aromatase can dynamically integrate inputs from stimuli that change AA in opposite directions. Another striking result from this study is the difference between how males and females respond physiologically and behaviourally to the context of mate pairing. While the males' physiological response appears relatively simple, the females' response appears more nuanced and complex. In the male quail brain, the active and rapid change in AA in response to pairing with a mate may serve as a switch to allow normal reproductive behaviour despite stress exposure. These changes in the male AA and CORT responsiveness do not depend on the stress status of the female partner. In the female, however, not only do the physiological changes depend on her stress status but these changes and the fertilisation outcomes also depend on the stress status of her male partner and his behaviour, particularly when he is engaging in behaviour that she might perceive as aggressive or potentially injurious. These results highlight the dynamic potential of aromatase while also demonstrating that the male and female brain are not equivalent when considering responses to breeding context, mate perception, and stress.

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REFERENCES

1. Balthazart J, Cornil CA, Charlier TD, Taziaux M, Ball GF. Estradiol, a key endocrine signal in the sexual differentiation and activation of reproductive behavior in Quail. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*. 2009; 311(5):323–345.
2. Trainor BC, Marler CA. Testosterone promotes paternal behaviour in a monogamous mammal via conversion to oestrogen. *Proc R Soc Lond Ser B-Biol Sci*. 2002; 269(1493):823–829.
3. Trainor BC, Kyomen HH, Marler CA. Estrogenic encounters: How interactions between aromatase and the environment modulate aggression. *Frontiers in Neuroendocrinology*. 2006; 27(2):170–179. [PubMed: 16376420]
4. Evrard HC, Balthazart J. Aromatization of androgens into estrogens reduces response latency to a noxious thermal stimulus in male quail. *Horm Behav*. 2004; 45(3):181–189. [PubMed: 15047013]
5. Garcia-Segura LM. Aromatase in the brain: Not just for reproduction anymore. *J Neuroendocrinol*. 2008; 20(6):705–712. [PubMed: 18601693]
6. McEwen BS, Alves SE. Estrogen actions in the central nervous system. *Endocr Rev*. 1999; 20(3): 279–307. [PubMed: 10368772]
7. Woolley, CS. *Annu Rev Pharmacol Toxicol*. Palo Alto: Annual Reviews; 2007. Acute effects of estrogen on neuronal physiology; p. 657–680.

8. Balthazart J, Ball GF. Is brain estradiol a hormone or a neurotransmitter? *Trends Neurosci.* 2006; 29(5):241–249. [PubMed: 16580076]
9. Cornil CA, Dalla C, Papadopoulou-Daifoti Z, Baillien M, Dejace C, Ball GF, Balthazart J. Rapid decreases in preoptic aromatase activity and brain monoamine concentrations after engaging in male sexual behavior. *Endocrinology.* 2005; 146(9):3809–3820. [PubMed: 15932925]
10. Dickens MJ, Cornil CA, Balthazart J. Acute stress differentially affects aromatase activity in specific brain nuclei of adult male and female quail. *Endocrinology.* 2011
11. Ramage-Healey L, Maidment NT, Schlinger BA. Forebrain steroid levels fluctuate rapidly during social interactions. *Nat Neurosci.* 2008; 11(11):1327–1334. [PubMed: 18820691]
12. de Bournonville, C.; Ball, GF.; Balthazart, J.; Cornil, CA. Rapid changes of aromatase activity in discrete brain regions following social interactions. Torino, Italy: *Steroids and Nervous System*; 2011.
13. Wingfield JC, Sapolsky RM. Reproduction and resistance to stress: when and how. *J Neuroendocrinol.* 2003; 15(8):711–724. [PubMed: 12834431]
14. Bian J, Wu Y, Liu J. Breeding behavior under temporal risk of predation in male root voles (*Microtus oeconomus*). *J Mammal.* 2005; 86(5):953–960.
15. Cecconello, ALc; Raineke, C.; Sebben, V.; Lucion, AB.; Sanvito, GL. Effect of acute stress on sexual behavior in female rats: Participation of the central angiotensinergic system. *Behav Brain Res.* 2010; 207(2):429–433. [PubMed: 19879304]
16. Moore FL, Miller LJ. Stress-induced inhibition of sexual behavior: corticosterone inhibits courtship behaviors of a male amphibian. *Horm Behav.* 1984; 18:400–410. [PubMed: 6097527]
17. Lynn SE, Stampis TB, Barrington WT, Weida N, Hudak CA. Food, stress, and reproduction: Short-term fasting alters endocrine physiology and reproductive behavior in the zebra finch. *Horm Behav.* 2010; 58(2):214–222. [PubMed: 20362578]
18. Retana-Marquez S, Salazar ED, Velazquez-Moctezuma J. Effect of acute and chronic stress on masculine sexual behavior in the rat. *Psychoneuroendocrinology.* 1996; 21(1):39–50. [PubMed: 8778903]
19. Balthazart J, Ball GF. The Japanese quail as a model system for the investigation of steroid-catecholamine interactions mediating appetitive and consummatory aspects of male sexual behavior. *Annual review of sex research.* 1998; 9:96–176.
20. Adkins EK, Adler NT. Hormonal control of behavior in the Japanese quail. *Journal of Comparative and Physiological Psychology.* 1972; 81(1):27–36. [PubMed: 5074305]
21. Hutchison RE. Hormonal differentiation of sexual-behavior in Japanese quail. *Horm Behav.* 1978; 11(3):363–387. [PubMed: 753700]
22. Delville Y, Balthazart J. Hormonal control of female sexual behavior in the Japanese quail. *Horm Behav.* 1987; 21(3):288–309. [PubMed: 3666684]
23. Takada S, Ota J, Kansakuc N, Yamashita H, Izumie T, Ishikawa M, Wada T, Kaneda R, YL C, K K, Fujiwara S, Aokia H, Kisanukia H, Yamashita Y, Mano H. Nucleotide sequence and embryonic expression of quail and duck Sox9 genes. *Gen Comp Endocrinol.* 2006; 145(2):208–213. [PubMed: 16216246]
24. Clinton M, Haines L, Belloir B, McBride D. Sexing chick embryos: a rapid and simple protocol. *British Poultry Science.* 2001; 42:134–138.
25. Cornil CA, Ball GF, Balthazart J, Charlier TD. Organizing Effects of Sex Steroids on Brain Aromatase Activity in Quail. *PLoS ONE.* 2011; 6(4):e19196. [PubMed: 21559434]
26. Palkovits M. Isolated removal of hypothalamic or other brain nuclei of the rat. *Brain Res.* 1973; Vol. 59:449–450. [PubMed: 4747772]
27. Schumacher M, Balthazart J. Neuroanatomical distribution of testosterone-metabolizing enzymes in the Japanese quail. *Brain Res.* 1987; 422(1):137–148. [PubMed: 3676776]
28. Foidart A, Reid J, Absil P, Yoshimura N, Harada N, Balthazart J. Critical re-examination of the distribution of aromatase-immunoreactive cells in the quail forebrain using antibodies raised against human placental aromatase and against the recombinant quail, mouse or human enzyme. *J Chem Neuroanat.* 1995; 8(4):267–282. [PubMed: 7669272]
29. Bayle JD, Ramade F, Oliver J. Stereotaxic topography of the brain of the quail (*Coturnix coturnix japonica*). *Journal de Physiologie.* 1974; 68(2):219–241. [PubMed: 4607102]

30. Roselli, CE.; Resko, JA. In vitro assay of aromatase activity in the central nervous system. In: Greenstein, B., editor. *Neuroendocrine Research Methods*. Chur, Switzerland: Harwood Academic Publishers; 1991. p. 937-951.
31. Baillien M, Balthazart J. A direct dopaminergic control of aromatase activity in the quail preoptic area. *J Steroid Biochem Mol Biol*. 1997; 63(1-3):99-113. [PubMed: 9449211]
32. Persaud KN, Galef BG. Female Japanese quail (*Coturnix japonica*) mated with males that harassed them are unlikely to lay fertilized eggs. *Journal of Comparative Psychology*. 2005; 119(4):440-446. [PubMed: 16366777]
33. Correa SM, Horan CM, Johnson PA, Adkins-Regan E. Copulatory behaviors and body condition predict post-mating female hormone concentrations, fertilization success, and primary sex ratios in Japanese quail. *Horm Behav*. 2011; 59(4):556-564. [PubMed: 21376051]
34. Watson JT, Adkins-Regan E. Activation of sexual behavior by implantation of testosterone propionate and estradiol benzoate into the preoptic area of the male Japanese quail (*Coturnix japonica*). *Horm Behav*. 1989; 23(2):251-268. [PubMed: 2744741]
35. Watson JT, Adkins-Regan E. Testosterone implanted in the preoptic area of male Japanese quail must be aromatized to activate copulation. *Horm Behav*. 1989; 23(3):432-447. [PubMed: 2793083]
36. Balthazart J, Foidart A, Hendrick JC. The induction by testosterone of aromatase activity in the preoptic area and activation of copulatory behavior. *Physiology and Behavior*. 1990; 47(1):83-94. [PubMed: 2326347]
37. Charlier TD, Harada N, Balthazart J, Cornil CA. Human and quail aromatase activity is rapidly and reversibly inhibited by phosphorylating conditions. *Endocrinology*. 2011; 152(11):4199-4210. [PubMed: 21914772]
38. Woodley SK, Lacy EL. An acute stressor alters steroid hormone levels and activity but not sexual behavior in male and female Ocoee salamanders (*Desmognathus ocoee*). *Horm Behav*. 2010; 58(3):427-432. [PubMed: 20580723]
39. Wu Y, Bian J. Effect of acute administration of corticosterone on male sexual behavior in root voles. *Acta Theriologica Sinica*. 2006; 26(4):354-358.
40. Retana-Marquez S, Bonilla-Jaime H, Velazquez-Moctezuma J. Lack of effect of corticosterone administration on male sexual behavior of rats. *Physiology and Behavior*. 1998; 63(3):367-370. [PubMed: 9469728]
41. Pfaff, DW.; Schwartz-Giblin, S.; McCarthy, MM.; Kow, LM. Cellular and molecular mechanisms of female reproductive behaviors. In: Knobil, E.; Neill, JD., editors. *The physiology of reproduction*. 2 ed. New York: Raven Press; 1994. p. 107-220.
42. Blaustein, JD. Feminine Reproductive Behavior and Physiology in Rodents: Integration of Hormonal, Behavioral, and Environmental Influences. In: Pfaff, DW.; Arnold, AP.; Etgen, AM.; Fahrbach, SE.; Rubin, RT., editors. *Hormones, Brain and Behavior*. San Diego, CA: Academic Press; 2009. p. 68-1007.
43. Gibson MJ, Cheng MF. Neural mediation of estrogen-dependent courtship behavior in female ring doves. *JCompPhysiolPsychol*. 1979; 93:855-867.
44. Meddle SL, Foidart A, Wingfield JC, Ramenofsky M, Balthazart J. Effects of sexual interactions with a male on Fos-like immunoreactivity in the female quail brain. *J Neuroendocrinol*. 1999; 11(10):771-784. [PubMed: 10520126]
45. Adkins-Regan E. Predictors of fertilization in the Japanese quail, *Coturnix japonica*. *Anim Behav*. 1995; 50(5):1405-1415.
46. Rutkowska J, Adkins-Regan E. Learning enhances female control over reproductive investment in the Japanese quail. *Proceedings of the Royal Society B: Biological Sciences*. 2009; 276(1671):3327-3334.
47. Delville Y, Sulon J, Hendrick JC, Balthazart J. Effect of the presence of females on the pituitary-testicular activity in male Japanese quail (*Coturnix coturnix japonica*). *Gen Comp Endocrinol*. 1984; 55(2):295-305. [PubMed: 6479577]
48. Waldherr M, Nyuyki K, Maloumy R, Bosch OJ, Neumann ID. Attenuation of the neuronal stress responsiveness and corticotrophin releasing hormone synthesis after sexual activity in male rats. *Horm Behav*. 2010; 57(2):222-229. [PubMed: 19948175]

49. Ophir AG, Persaud KN, Galef BG. Avoidance of relatively aggressive male Japanese quail (*Coturnix japonica*) by sexually experienced conspecific females. *Journal of Comparative Psychology*. 2005; 119(1):3–7. [PubMed: 15740424]
50. Ophir AG, Galef BG. Female Japanese quail that 'eavesdrop' on fighting males prefer losers to winners. *Anim Behav*. 2003; 66(2):399–407.
51. Romero LM, Reed JM. Collecting baseline corticosterone samples in the field: is under three minutes good enough? *Comp Biochem Physiol Part A: Mol Integr Physiol*. 2005; 140:73–79.
52. Dickens, MJ.; Cornil, CA.; Balthazart, J. *Society for Integrative and Comparative Biology*. Salt Lake City, UT: 2011. Acute stress alters brain aromatase activity in Japanese quail (*Coturnix japonica*) in a sex-specific manner.

Figure 1. Experimental design

Individuals (either male or female) were stressed (S) or non-stressed (ns). They were then paired with an opposite sex partner that was also stressed or non-stressed immediately prior to pairing thus creating four experimental groups: Stressed individuals paired with stressed partners (S+S), Stressed individuals paired with non-stressed partners (S+ns), Non-stressed individuals paired with stressed partners (ns+S), and non-stressed individuals paired with non-stressed individuals (ns+ns). In addition, two control (CNTL) groups were tested immediately upon removal from home cage or immediately following 15 min of restraint stress (ns and S, respectively).

Figure 2. Fertilisation data

Eggs collected following the first set of behavioural experiments comparing A) number of fertilised eggs versus non-fertilised eggs laid by stressed versus non-stressed females regardless of whether her partner was stressed or not and B) number of fertilised eggs laid by a female versus the number of mount attempts (MA) she received from the male during the pairing. In (A), the number of eggs for each classification is given within the bar and the asterisk indicates that the proportions are significantly different. Eggs were collected for a total of 10 days following the behavioural tests.

Figure 3. Effects of stress and mate pairing on aromatase activity (AA in in pmol/h/mg protein) Measurements in A) the medial preoptic nucleus (POM), B) the medial portion of the bed nucleus of the stria terminalis (mBST), C) ventromedial hypothalamus (VMN) and D) tuberal hypothalamus (Tub) of male and female quail (open and hashed columns, respectively). Groups are defined in the legend as controls (non-stressed, ns or stressed, ST) and experimental groups: stressed individual with stressed partner (S+S), stressed individual with non-stressed partner (S+ns), non-stressed individual with stressed partner (ns+S), or non-stressed individual with non-stressed individual (ns+ns). Treatment groups are also labelled on the x-axis and defined as unpaired and paired groups (the latter is indicated by bars under the axis). As summarised in the insert, the darkness of the columns indicates whether subjects were acutely stressed (dark grey) or non-stressed (light grey) while the

pattern of the columns indicate whether the partner was stressed (filled bars) or non-stressed (open bars). Groups within each sex that are significantly different from each other (determined by post hoc Bonferroni tests) are represented as different letters. In females, a second two-way ANOVA to directly compare individual status versus partner status in paired groups showed a significant effect of female treatment in the POM and VMN (indicated by asterisk).

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Figure 4. Effects of stress and mate pairing on plasma corticosterone concentrations (CORT in ng/mL)

For male and female quail (open and hashed columns, respectively). Groups are defined in the legend as controls (non-stressed, ns or stressed, ST) and experimental paired groups: stressed individual with stressed partner (S+S), stressed individual with non-stressed partner (S+ns), non-stressed individual with stressed partner (ns+S), or non-stressed individual with non-stressed individual (ns+ns). Treatment groups are also labelled on the x-axis and defined as unpaired and paired groups (the latter is indicated by bars under the axis). As summarised in the insert, the darkness of the columns indicates whether subjects were acutely stressed (dark grey) or non-stressed (light grey) while the pattern of the columns indicate whether the partner was stressed (filled bars) or non-stressed (open bars). Groups within each sex that are significantly different from each other (determined by post hoc Bonferroni tests) are represented as different letters.

Figure 5. Linear regression of individual female CORT concentrations (ng/mL) versus number of mount attempts (MA) received from the male

Although the regression data shown is an analysis of all groups combined, groups for individual data points are indicated by different symbols as follows: stressed female with stressed partner (S+S), stressed female with non-stressed partner (S+ns), non-stressed female with stressed partner (ns+S), or non-stressed female with non-stressed individual (ns+ns). Square symbols indicate stressed females, circles indicate non-stressed females and filled symbols indicate stressed male partners while open symbols indicate non-stressed male partners (see insert).

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Table 1
Sexual behaviour exhibited by males during two rounds of pairing with females

Values are means ($\pm$ SE) of the either latency values (in seconds) or frequency (total number recorded during 5 min interaction). Latency to first cloacal contact movement (CCM) in Round 1 showed a significant effect of female treatment and a significant interaction but no significant effect of stress. This interaction was driven by the difference between the non-stressed males paired with non-stressed females vs. stressed females as indicated by bold text. All other statistical analyses including all measurements of mount attempts (MA) were non-significant. Male treatment prior to pairing (non-stress = ns; stress = S) is indicated by σ trt and a male treatment prior to pairing is indicated by $\varnothing$ trt.

Behaviour	σ trt	$\varnothing$ trt			
		ROUND 1		ROUND 2	
		ns	S	ns	S
MA latency (sec)	ns	8.2 $\pm$ 2.3	37.7 $\pm$ 29.3	2.3 $\pm$ 0.2	2.7 $\pm$ 0.3
	S	11.9 $\pm$ 9.8	35.2 $\pm$ 29.5	24.5 $\pm$ 19.5	41.8 $\pm$ 29.2
CCM latency (sec)	ns	18.1 $\pm$ 5.8	167.7 $\pm$ 44.5	94.9 $\pm$ 44.8	11.1 $\pm$ 3.5
	S	88.7 $\pm$ 41.1	90.0 $\pm$ 32.3	85.0 $\pm$ 40.7	94.9 $\pm$ 36.1
MA frequency	ns	13.2 $\pm$ 1.7	15.0 $\pm$ 3.9	14.2 $\pm$ 2.0	22.8 $\pm$ 3.4
	S	14.1 $\pm$ 3.3	13.0 $\pm$ 2.7	10.2 $\pm$ 2.3	10.5 $\pm$ 3.0
CCM frequency	ns	2.1 $\pm$ 0.3	1.0 $\pm$ 0.4	2.0 $\pm$ 0.6	4.3 $\pm$ 1.0
	S	2.5 $\pm$ 0.9	2.3 $\pm$ 0.7	2.0 $\pm$ 0.6	2.0 $\pm$ 0.7

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Table 2
Sexual behaviour exhibited by the females during two rounds of pairing with males

Values are means ($\pm$ SE) of the relative percentages of each behavioural display by the female relative to the total number of times the male approached her during the 5 min test. Stand up in Round 2 had a significant effect of female treatment as indicated by bold text. All other statistical analyses were non-significant. Female treatment prior to pairing (non-stress = ns; stress = S) is indicated by σ^7 trt and a male treatment prior to pairing is indicated by φ trt.

Behaviour	φ trt	σ^7 trt			
		ROUND 1		ROUND 2	
		ns	S	ns	S
% Crouch	ns	21.9 $\pm$ 9.3	28.9 $\pm$ 9.9	13.8 $\pm$ 6.5	36.7 $\pm$ 12.7
	S	14.0 $\pm$ 9.8	23.4 $\pm$ 9.9	17.1 $\pm$ 8.7	18.4 $\pm$ 3.9
% Stand up	ns	22.14 $\pm$ 7.0	18.4 $\pm$ 5.7	21.2 $\pm$ 6.3	17.2 $\pm$ 7.7
	S	39.2 $\pm$ 10.8	30.8 $\pm$ 9.8	25.2 $\pm$ 8.7	43.8 $\pm$ 4.7
% Short avoid	ns	36.6 $\pm$ 6.7	39.1 $\pm$ 7.2	52.1 $\pm$ 9.6	37.0 $\pm$ 10.4
	S	24.0 $\pm$ 8.1	34.5 $\pm$ 8.7	34.9 $\pm$ 8.4	35.8 $\pm$ 7.2
% Long avoid	ns	13.8 $\pm$ 4.3	12.9 $\pm$ 4.9	12.2 $\pm$ 5.1	7.9 $\pm$ 3.5
	S	12.7 $\pm$ 5.3	1.4 $\pm$ 1.4	10.3 $\pm$ 4.5	2.0 $\pm$ 0.9