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RESEARCH ARTICLE

Prior parental experience attenuates hormonal stress responses and alters hippocampal glucocorticoid receptors in biparental rock doves

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ABSTRACT

In the face of challenges, animals must balance investments in reproductive effort versus their own survival. Physiologically, this trade-off may be mediated by glucocorticoid release by the hypothalamic–pituitary–adrenal axis and prolactin release from the pituitary to maintain parental care. The degree to which animals react to and recover from stressors likely affects maintenance of parental behavior and, ultimately, fitness. However, less is known about how gaining parental experience may alter hormonal stress responses and their underlying neuroendocrine mechanisms. To address this gap, we measured the corticosterone (CORT) and prolactin (PRL) stress response in individuals of both sexes of the biparental rock dove (*Columba livia*) that had never raised chicks versus birds that had fledged at least one chick. We measured both CORT and PRL at baseline and after an acute stressor (30 min restraint). We also measured negative feedback ability by administering dexamethasone, a synthetic glucocorticoid that suppresses CORT release, and measured CORT and PRL after 60 min. All hormones were measured when birds were not actively nesting to assess whether effects of parental experience extend beyond the breeding bout. Experienced birds had lower stress-induced and negative-feedback CORT, and higher stress-induced PRL than inexperienced birds. In a separate experiment, we measured glucocorticoid receptor subtype expression in the hippocampus, a key site of negative feedback regulation. Experienced birds showed higher glucocorticoid receptor expression than inexperienced controls, which may mediate their ability to attenuate CORT release. Together, these results shed light on potential mechanisms by which gaining experience may improve parental performance and fitness.

KEY WORDS: Parental care, Reproduction, HPA axis, Corticosterone, Prolactin, Negative feedback

INTRODUCTION

Following life-history theory, breeding animals can maximize fitness by prioritizing resource allocation towards reproductive efforts, such as parental care of their current brood, at a cost to personal survival, self-maintenance and growth (Stearns, 1976; Williams, 1966). However, when faced with predation, food limitation, inclement weather or social challenges, animals may enter an emergency life-history stage (Wingfield et al., 1998) and abandon the current reproductive effort in order to survive (Wingfield and Sapolsky, 2003). Much research in recent decades has been conducted on the physiological mechanisms underlying these trade-offs (Ricklefs and Wikelski, 2002; Zera and Harshman, 2001), especially in the face of stressors (Romero and Wingfield, 2016).

Endocrine mechanisms, specifically glucocorticoid hormones (corticosterone or cortisol; CORT) and prolactin (PRL), have been strongly implicated in trade-offs between survival and reproduction owing to their pleiotropic effects on energetic state, metabolism and reproduction. In response to challenges, the hypothalamus releases corticotropin-releasing factor (CRF), which stimulates the anterior pituitary gland to release adrenocorticotropic hormone, which leads to CORT synthesis and release from the adrenal glands (Aguilera, 2016). This hypothalamic–pituitary–adrenal (HPA) endocrine axis is relatively conserved across vertebrates (Blas, 2015; Romero and Gormally, 2019). Increased CORT can promote survival during challenges by increasing glucose availability via gluconeogenesis, mobilizing free fatty acids as an energy source, and potentiating foraging and escape behaviors (Landys et al., 2006; Sapolsky et al., 2000; Wingfield et al., 1998; but see Taff et al., 2022). Baseline CORT can also increase to mobilize energetic resources during breeding (Bonier et al., 2011; Romero, 2002). However, elevated CORT in the face of stressors can also directly inhibit reproductive physiology and behavior, including parental behavior (Wingfield and Sapolsky, 2003). Conversely, pituitary PRL promotes resource allocation towards parental efforts in vertebrates, by facilitating lactation, offspring attendance and provisioning (as examples; Buntin, 1996; Freeman et al., 2000; Smiley, 2019). Under stress, reduced PRL may lead to less investment in parental effort and behavior in birds (the prolactin stress hypothesis; Angelier and Chastel, 2009; Chastel et al., 2005). However, acute stress often leads to increased PRL in mammals (Torner, 2016), so it is unclear whether the prolactin stress hypothesis generalizes across vertebrates. Nonetheless, the CORT and PRL stress responses can yield important insights into the trade-off between survival and energetic balance versus reproductive effort (Angelier and Chastel, 2009; Angelier et al., 2016) when measured together within individuals.

However, less is known about how previous parental experience may affect these hormonal stress responses. Young, inexperienced individuals may have constrained physiological abilities to modulate hormones in response to stress (the constraint hypothesis; Curio, 1983), or they may restrain from modulating such responses owing to higher future reproductive opportunities

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(the restraint hypothesis; Curio, 1983). Studies in long-lived seabirds suggest that age may lead to attenuated CORT and PRL stress responses, where older individuals show lower stress-induced CORT and higher stress-induced PRL (Heidinger et al., 2006, 2010). In contrast, other studies found no effects of age on stress-induced CORT, but did find that older seabirds maintained higher PRL levels at baseline or after stress (Angelier et al., 2007a,b). As gaining breeding experience necessarily requires time that ages individuals, any effects of age on stress responses seen in these studies may be modulated in part by parental experience. Indeed, previous breeding experience in these long-lived seabirds may be a better predictor of baseline CORT and PRL levels than age alone (Angelier et al., 2006, 2007a). Baseline PRL levels have also been shown to increase with subsequent breeding experiences within individuals (Smiley and Adkins-Regan, 2016). Whether parental experience itself alters hormonal stress responses when animals are relatively similar in age remains unclear.

Upstream of hormone release, neural receptor densities may also underlie differences in hormonal stress responses that may appear with breeding experience. Prior breeding experience has been shown to affect neuroendocrine systems, such as pituitary prolactin cell counts or neural prolactin receptors (Anderson et al., 2006; Christensen and Vleck, 2015; Farrar et al., 2022a), but effects on glucocorticoid-specific regulation remain unstudied. CORT exerts effects through two genomic receptor types, the high-affinity mineralocorticoid receptors (Type I; MR) and the lower-affinity glucocorticoid receptors (Type II; GR), as well as membrane-based receptors (Breuner and Orchinik, 2009). These genomic receptors are hypothesized to play distinct roles, where the high-affinity MR enacts permissive effects of CORT at baseline levels, and the lower-affinity GR enacts suppressive and adaptive actions in response to elevated CORT levels, such as those seen after stressors (Romero, 2004; Sapolsky et al., 2000). Although these receptors are found throughout the body, hippocampal MR and GR may be especially important for negative feedback of CORT after a stressor (de Kloet and Meijer, 2019). Both hippocampal MR and GR have been shown to mediate HPA axis activity and CORT release in mammals (de Kloet et al., 1998; Jacobson and Sapolsky, 1991; de Kloet and Meijer, 2019), though evidence is limited in birds (Smulders, 2017). The balance of these receptor subtypes may also play a role in maintaining homeostasis and avoiding stress pathology. For example, reduced hippocampal GR expression led to increased CORT levels after restraint stress in transgenic mice, presumably owing to reduced negative feedback inhibition, but overexpressed MR with reduced GR undid this effect (Harris et al., 2013). In birds, hippocampal GR expression can change during seasonal or breeding transitions (Krause et al., 2015; Lattin and Romero, 2013). To our knowledge, no study has evaluated the effect of prior parental experience on hippocampal GR expression while also measuring the ability of animals to negatively feed back CORT levels.

Most work on the effects of parental experience has measured hormones or neural correlates while animals were actively breeding. However, if becoming a parent alters HPA axis regulation in a long-lasting way, differences in stress responses associated with parental experience should be detectable even when animals are not engaged in care. Such persistent effects of parental experience would essentially constitute a 'carryover effect', where changes accrued during breeding affect responses in subsequent life stages (O'Connor et al., 2014).

Experiences early in development, such as parental deprivation or hormone exposures, can alter stress responses – and their underlying mechanisms – well into adulthood (Banerjee et al., 2012; Spencer et al., 2009; Wada and Coutts, 2021; Zimmer et al., 2013). It is unclear whether parental experience gained by adult animals could also lead to lasting effects on stress response regulation, even outside of breeding.

To address these questions, we first examined hormonal stress responses in CORT and PRL in non-actively nesting rock doves (*Columba livia*) that differed in prior parental experience with chicks. We used dexamethasone (DEX), a synthetic glucocorticoid, to induce maximal negative feedback when collecting stress series (an established method in avian endocrinology; Lattin and Kelly, 2020), allowing us to compare baseline, stress-induced and negative feedback levels of each hormone. In a second experiment, we extended our analysis into the brain, where we measured hippocampal gene expression of *MR* and *GR* using quantitative PCR in non-actively nesting birds. By capitalizing on a captive breeding population of biparental rock doves, we were able to collect data from individuals of both sexes with known breeding histories and ages.

We tested a variation of the constraint and restraint hypotheses (Curio, 1983), modified for the effects of parental experience (instead of age). We hypothesized that prior parental experience would lead to reduced constraint on hormonal modulation, thus improving the ability to attenuate stress responses and invest in reproduction. Accordingly, we predicted that birds with prior experience with chicks would have lower CORT and higher PRL after an acute stressor and after negative feedback than birds that had never previously raised chicks. Further, we hypothesized that parental experience alters hippocampal GR, enabling more flexible hormonal stress responses. We then predicted that birds who had raised chicks would have higher hippocampal *GR* and/or *MR* expression than inexperienced birds. As both sexes of rock doves incubate eggs, care for chicks and pseudo-'lactate' via crop milk production (Abs, 1983; Horseman and Buntin, 1995; Johnston, 1992), we hypothesized that shared parental behavior and physiology would lead to similar effects, if any, of parental experience. We thus predicted no sex differences in hormone stress responses or GR subtypes.

MATERIALS AND METHODS

Experiment 1: Hormonal responses to stress

Subjects and study design

We collected stress series (consisting of three blood samples) from 35 adult rock doves (*Columba livia* Gmelin 1789) of both sexes between March and June 2021. All subjects were born in captivity and housed in a semi-natural, social aviary environment. Each outdoor flight aviary (1.5×1.2×2.1 m) was exposed to ambient temperatures and natural daylight, which was supplemented with artificial lighting on a 12 h:12 h light:dark photoperiod. Birds were provided *ad libitum* food (whole corn and turkey/game bird protein starter, 30% protein; Modesto Milling, CA, USA), grit and water. Each aviary housed 10–12 breeding pairs of rock doves and included wooden nest boxes and nesting material (straw). Birds were allowed to naturally form breeding pairs and select and defend nest sites. Nest boxes were checked daily and the identity of the attending parent, presence and number of eggs or chicks was recorded. This daily data collection yielded a full breeding history for each individual bird.

To examine the effect of prior parental experience on hormonal stress responses in a non-parental state, we also collected blood samples from birds that had ('experienced') and had not ('inexperienced') raised chicks in previous nests. Experienced

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birds had raised at least one chick in a prior nest. Sample sizes were 16 experienced birds (8 females, 8 males) and 19 inexperienced birds (10 females, 9 males). Samples were collected when birds had no active nest. Birds with no active nest were considered to be in a non-parental, 'baseline' state, as they were not caring for eggs or chicks (including no fledged juveniles). However, birds were likely still in a 'reproductively active' state when not nesting, as sex steroid hormone and gonad data from other studies in our population suggest (Austin et al., 2021b; Farrar et al., 2022a,b). Of the inexperienced birds, 68% (n=13) had never previously laid an egg, while the remaining six birds had laid eggs previously. There was no significant difference in either CORT or PRL concentrations at any time point between inexperienced birds that had laid eggs previously and those that had not (two-sample t-tests, all P>0.1; Table S1).

The average time since the last nest effort was completed did not significantly differ between experienced and inexperienced birds (8.4 days versus 21.5 days on average; t=1.03, P=0.317). Experienced birds were older than inexperienced birds at sampling time (1.84 years versus 1.38 years on average; t=3.31, P=0.002). We continued to collect breeding data on these birds after blood samples were collected, and experienced birds initiated a new nest effort (defined as the first day an egg was laid) significantly sooner than inexperienced birds (8.6 days versus 24.9 days on average; t=−1.03, P=0.032).

All methods and procedures were approved by the University of California Davis IACUC (protocol no. 22407). Sample sizes were determined to maximize statistical power while minimizing the number of animals used.

Dexamethasone dosage validation

To test the maximal negative feedback ability of birds after a stressor, we used DEX, a synthetic glucocorticoid that selectively binds glucocorticoid receptors to initiate negative feedback and downregulate CORT release (Lattin and Kelly, 2020), including in rock doves (Westerhof et al., 1994). To ensure DEX reduced CORT levels significantly below stress-induced levels and to levels similar to baseline, we conducted a validation experiment with multiple dosages. We captured non-breeding rock doves (total n=19) and placed them in an opaque cloth bag for 30 min to simulate an acute stressor. This capture–restraint method is a classic handling stress paradigm that has been used to reliably increase CORT levels in birds (Romero and Wingfield, 2016; Wingfield et al., 1982), including in our rock dove population (Calisi et al., 2018). After 30 min, we removed birds from bags and took an ∼100 µl blood sample from the alar wing vein. We then immediately injected birds intramuscularly with DEX (catalog no. D1756, Sigma Aldrich, Milwaukee, WI, USA) at either 1 mg kg^{-1} (n=3), 2 mg kg^{-1} (n=5) or 4 mg kg^{-1} (n=5), or with 0.9% physiological saline as a vehicle control (n=6). Birds were returned to their home cage to recover, then recaptured and bled after an additional 60 and 90 min post-DEX (∼100 µl each sample). All blood samples were taken between 08:00 and 11:00 h in February 2020.

Plasma CORT levels in birds treated with saline vehicle did not change after 60 or 90 min of recovery post-stressor (one-way ANOVA: $F_{2,16}$=0.4, P=0.957; Fig. 2). Despite being effective in other bird species (Dickens et al., 2009a; Lattin et al., 2012), 1 mg kg^{-1} DEX also did not significantly reduce CORT levels 60 or 90 min after stress ($F_{2,5}$=1.1, P=0.396). DEX dosages of 2 mg kg^{-1} ($F_{2,12}$=7.4, P=0.008) and 4 mg kg^{-1} ($F_{2,12}$=7.7, P=0.007) significantly decreased CORT after 60 and 90 min recovery. Additionally, both 2 mg kg^{-1} and 4 mg kg^{-1} DEX doses significantly differed from vehicle after 60 and 90 min ($F_{3,16}$=4.1, P=0.023). Thus, we chose to use the lowest effective DEX dose, 2 mg kg^{-1}, measuring post-DEX CORT levels after 60 min of recovery.

Stress-series blood collection

For experimental stress series, we collected three blood samples from each bird under the classic capture–restraint protocol (Fig. 1). First, we collected a sample of blood from the alar wing vein using a 26 G needle within 3 min of entering the aviary cage (109±36 s). Samples collected within 3 min of capture are considered representative of baseline levels for both circulating CORT and PRL (Chastel et al., 2005; Romero and Reed, 2005), and we found no effect of time to sample on either baseline concentration of either hormone (CORT: $F_{1,28}$=0.57, P=0.458; PRL: $F_{1,27}$=0.31, P=0.583). We then placed each bird in an opaque

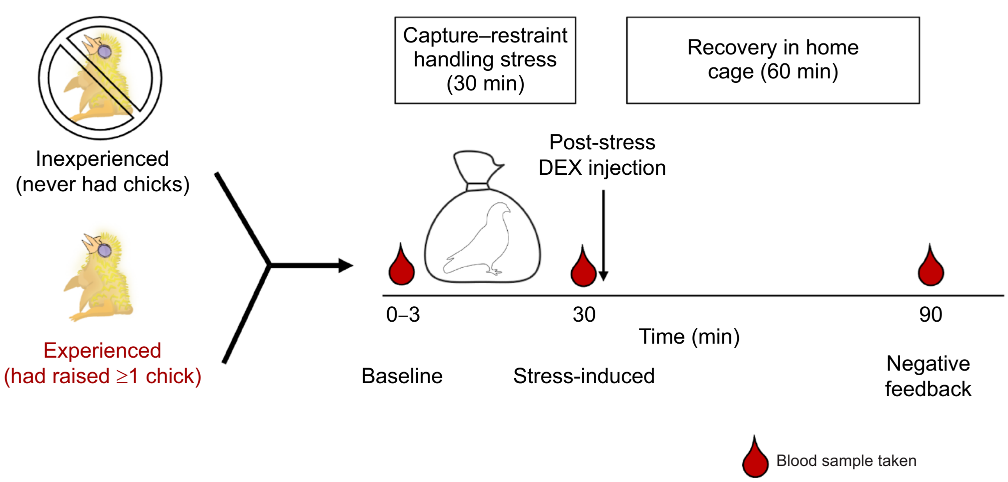

Fig. 1. Sampling paradigm for experiment 1. We collected stress series from inexperienced (never raised chicks, had laid eggs) and experienced birds (had raised at least one chick) that currently had no active nest to understand the influence of prior parental experience on plasma hormone levels after a stressor. Three blood samples were taken from birds to assess hormonal responses to stress: (1) baseline (<3 min from capture), (2) stress-induced (after 30 min in an opaque cloth bag, representing a classic capture–restraint stressor) and (3) negative feedback [60 min after injection with dexamethasone (DEX) and recovery in home cage]. DEX was injected immediately after the stress-induced blood sample was taken. Plasma from blood samples were used to measure corticosterone (CORT) and prolactin. Sample sizes for each group can be found in the Materials and Methods.

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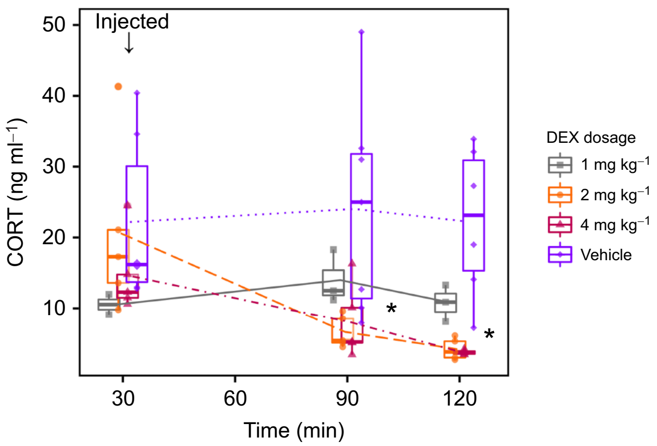

Fig. 2. Plasma CORT response to injections of various doses of DEX or vehicle after 30 min of restraint. Birds were either injected with a dose of DEX (1 mg kg^{-1}, *n*=3, gray; 2 mg kg^{-1}, *n*=5, orange; 4 mg kg^{-1}, *n*=5, red) or saline vehicle (*n*=6, purple) after being exposed to an acute capture–restraint stressor for 30 min (injection time indicated with an arrow). Boxplots show first quartile, median and third quartile. **P*<0.05 for 2 and 4 mg kg^{-1} doses compared with the 1 mg kg^{-1} and vehicle groups at the same time point in one-way ANOVA.

cloth bag to simulate an acute handling stressor and collected a second blood sample 30 min later to measure stress-induced hormone levels. After this blood sample was taken, we injected each bird intramuscularly with 2 mg kg^{-1} DEX (dosage validated as described above) and then returned birds to their home cage to recover. We collected the last blood sample 60 min after DEX injection to measure negative feedback hormone levels. All blood samples were approximately 100 µl each and were collected between 08:00 and 12:00 h in March–June 2021. We found no effect of time of day on either CORT ($F_{1,100}$=0.01, P=0.939) or PRL concentration ($F_{1,97}$=0.19, P=0.660). We found no significant effect of date of sample on CORT concentration ($F_{1,100}$=2.75, P=0.101), but PRL concentrations were significantly higher later in the season ($F_{1,97}$=6.34, P=0.017). Thus, we added date of sampling as a random effect to general linear models (see Statistical analysis, below).

We centrifuged blood samples for 10 min at 10,000 rpm (∼12,300 ***g***) to separate plasma. Plasma aliquots were then stored at −80°C until further analysis. All samples were brought up to 4°C before being run in immunoassays.

Corticosterone radioimmunoassays

Circulating CORT concentrations were measured from plasma at the UC Davis Metabolomics core using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals, Orangeburg, NY, USA). A serial dilution was performed prior to the assay, and plasma samples from 0, 30 and 90 min time points were run at 1:11, 1:26 and 1:11 dilutions, respectively. Cross-reactivity with *C. livia* CORT was validated previously for this assay (Austin et al., 2021a; Calisi et al., 2018), and the assay had a limit of detection of 0.0385 ng ml^{-1}. Samples were run in duplicate, and mean intra-assay and inter-assay coefficients of variation (CV) were 5.0% and 6.5%, respectively. All samples from the DEX dosage validation were run in a single assay.

Avian prolactin enzyme-linked immunoassay

We measured circulating PRL using a heterologous competitive enzyme-linked immunoassay (ELISA) using the methods described in detail in Smiley and Adkins-Regan (2016) and developed by ADS Biosystems (San Diego, CA, USA). This assay has previously been used with rock dove plasma. The full protocol can be accessed at protocols.io: dx.doi.org/10.17504/protocols.io.36wgq7zoovk5/v1.

Briefly, biotinylated recombinat chicken PRL (ADS Biosystems) is added to samples and standards and competes for binding sites on the bound rabbit anti-chicken PRL antibodies (1:20,000 dilution; A. F. Parlow, National Hormone and Peptide Program, Los Angeles, CA, USA). Visualization occurs through an enzymatic reaction using streptavidin horseradish peroxidase (HRP) (catalog no. 21130, Thermo Fisher Scientific, Waltham, MA, USA). Chicken PRL antibodies have been successfully used in ELISA to measure prolactin from other avian species, including zebra finches and brown-headed cowbirds (Lynch et al., 2020; Smiley and Adkins-Regan, 2016). We confirmed parallelism of serially diluted *C. livia* plasma against the chicken PRL standard curve (research and development by ADS Biosystems), and spike-recovery of chicken PRL spiked into a *C. livia* plasma sample. We used two pooled validation samples, one from non-breeding birds (low pool) and one from birds at incubation day 17 (high pool) to calculate intra-assay CV. Mean intra-assay CV was 5.79%. We ran all plasma series (0, 30 and 90 min samples) for an individual bird on the same ELISA plate. All samples were run in duplicate, along with a standard curve on each 96-well plate. Plates were read on an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm with background subtracted from 595 nm. Concentrations were interpolated from the standard curve using a four-parameter fit (iMark software v.1.04, Bio-Rad Laboratories). One individual (inexperienced, no active nest) was not run owing to hemolysis that contaminated the plasma samples.

Statistical analysis

All statistical analyses were performed in the R statistical language (v.4.0.3). For each hormone (CORT and PRL), we created a linear mixed-effects model using the lme4 package (v.1.1.27) (Bates et al., 2015). In this model, we tested how the independent variables of experience level (experienced with chicks or inexperienced), stress-series time point, sex and their interactions affected the dependent variable of hormone concentration. We also included random effects of (1) date of sample collection and (2) individual bird to account for the repeated-measures design of our stress series. To improve distribution of the data, all hormone concentrations were $\log_{10}$-transformed. Individual bird and date of sampling were included as random effects. Normality of residuals and homogeneity of variance were checked for each model using the performance package (v.0.9.2) (Lüdecke et al., 2021). As we found non-homoscedasticity of residuals, we used robust estimation of our mixed-effects model using the robustlmm package in R (v.3-0.2) (Koller, 2016). Robust linear mixed modeling differentially weights residuals from outliers to reduce their effects on model estimates and to address deviations from model assumptions. We report model estimates and standard errors for robust linear mixed models. We also report the results of *post hoc* comparisons performed with estimated marginal means, corrected with Benjamini–Hochberg false discovery rate (FDR) corrections in the emmeans package (v.1.5.2) (https://CRAN.R-project.org/package=emmeans). All code for statistical analysis and figures can be found at: https://github.com/vsfarrar/experience-stress-hippocampus.

Experiment 2: Hippocampal and pituitary gene expression

In a second experiment, we extended results from experiment 1 that showed an effect of parental experience on CORT and PRL release

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after an acute stressor. Here, we examined gene expression in brain and pituitary tissues collected from birds with and without prior experience with chicks, to determine if genes involved in stress response regulation were differentially expressed in experienced birds versus inexperienced ones. Specifically, we examined GR (also known as NR3C1) and MR (or NR3C2) in the hippocampus, as these two receptors are known to regulate negative feedback of the glucocorticoid stress response and HPA axis regulation (Herman et al., 2012; Phillips et al., 2006).

Tissue collection

Whole brains were collected from 30 reproductively mature rock doves (age range: 1–2 years old) that were not actively nesting. Of these, 16 (n=8 males, 8 females) birds had previously raised at least one chick (average chicks raised: 2.5±1.46) and 14 (n=7 males, 7 females) had never raised chicks. The mean time since birds last had an active nest (at time of collection) did not significantly differ with experience (6.6 days for experienced versus 10.0 days in inexperienced birds: t=0.85, P=0.419). As in above, experienced birds were older than inexperienced birds, but only by a matter of days; all birds were between 1 and 2 years old (average age: 1.41 years for inexperienced versus 1.66 years for experienced individuals, t=−2.16, P=0.041).

We euthanized birds using methods previously used in rock doves (Calisi et al., 2018; MacManes et al., 2017). Within 3 min of capture, birds were euthanized with an overdose of isoflurane and then rapidly decapitated. Whole brains and pituitary glands were removed and flash-frozen on dry ice, then stored at −80°C until further analysis. All tissues were collected between 08:00 and 11:00 h in March 2020. As these methods are terminal, different individual animals were used in this experiment than those in experiment 1.

Hippocampi microdissection

To analyze gene expression specific to the hippocampus, we microdissected the hippocampus from whole brains using a Leica CM1950 cryostat. We collected hippocampus tissue using a 3 mm diameter punch from 100 µmol l^{-1} slices. We used landmarks from the Karten and Hodos (1966) pigeon brain atlas to locate the hippocampus, starting with when the commissura anterior visibly crossed the coronal section and ending when the cerebellum was visible (Fig. 5A, plates A 7.75–A 4.25 in Karten and Hodos, 1966; average of 27–30 punches at 100 µmol l^{-1}). Hippocampus tissue punches were stored in 200 µl TriSure Reagent (BioLine, Meridian Bioscience, Cincinnati, OH, USA) at −80°C until RNA extraction.

Quantitative PCR

To extract total RNA from hippocampal tissue, we used a modified protocol of the Direct-zol RNA extraction kit (catalog no. R2501, Zymo Scientific, Irvine, CA, USA) along with TriSure reagent (catalog no. 38032, BioLine, Meridian Life Science, Memphis, TN, USA). A full RNA extraction protocol can be accessed online at protocols.io: dx.doi.org/10.17504/protocols.io.5qpvob6p9l4o/v1. Investigators were blind to sex and experience level during all molecular analyses.

RNA concentration and quality was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). All samples passed quality assurance and had 260/280 ratios and 260/230 ratios >1.80. We removed any remaining genomic DNA from RNA samples using Quanta Perfecta DNase I (catalog no. 95150-01K, Quanta Biosciences, Gaithersburg, MD, USA). We then converted RNA to single-stranded complementary DNA (cDNA) via reverse transcription using qScript cDNA Supermix (catalog no. 95048-100, Quanta Biosciences). We diluted total cDNA 5-fold in preparation for qPCR.

Using quantitative PCR (qPCR), we measured relative gene expression for GR and MR in the hippocampus. We also measured expression of two reference genes, *HPRT1* and *RPL4*, to account for variation in total transcription between samples. All primers were designed using the *C. livia* transcriptome v2.10 (NCBI accession no. GCA_000337935.2) as a template. We also validated each primer for ideal replication efficiencies and singular melt curves using a standard curve consisting of five 10-fold dilutions of pooled tissue cDNA. Primer details can be found in Table 1.

We ran each sample in triplicate on a 384-well plate using the following qPCR reaction mix: 1 µl diluted cDNA template, 5 µl 2X SSOAdvanced SYBR Green PCR mix (Bio-Rad Laboratories) and 10 µmol l^{-1} each of primer (total volume: 10 µl; Invitrogen, Waltham, MA, USA). We ran plates on a CFX384 Touch Real-Time PCR detection system (Bio-Rad Laboratories) under the following thermocycling protocol: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 30 s. Plates also included no-template controls. We ran all samples of each tissue–gene combination on a single plate.

To calculate relative gene expression from raw qPCR data, we used the Livak and Schmittgen (2001) $\Delta\Delta C_t$ method. To do this, we first normalized the expression (cycle threshold, C_t) of each gene of interest to the geometric mean of reference gene expression for that sample. We used *HPRT1* and *RPL4* as reference genes, as recommended for avian neural tissue (Zinzow-Kramer et al., 2014). We verified that expression of these reference genes did not differ with parental experience ($F_{1,27}$=2.14, P=0.155) or sex ($F_{1,27}$=0.09, P=0.766) in our samples using two-way ANOVA. Then, we relativized normalized expression (ΔC_t) for each sample to the average normalized expression for the control group ($\Delta\Delta C_t$), which in this case was inexperienced birds. Lastly, we calculated fold change, or $2^{-\Delta\Delta C_t}$. Fold change was $\log_{10}$-transformed for statistical analyses.

Statistical analysis

For each gene of interest (*GR* or *MR*), we ran a linear model to test how the dependent variable, log fold change, may be affected by the independent variables of experience with chicks, sex and their interaction. We also calculated the ratio of *MR* to *GR* expression (MR:GR ratio) and examined whether this ratio was also affected by experience with chicks, sex or their interaction using a linear model. We report ANOVA based upon these linear models.

We ran each gene in a separate model because (1) different transcription factors and promoters are known to underlie expression of these receptors (Biddie and Hager, 2009; Herman and Spencer, 1998) and (2) direct comparisons are not recommended owing to the relative expression calculations used in the Livak and Schmittgen (2001) method.

RESULTS

Experiment 1: Hormonal responses to acute stress

When compared between birds that were not actively nesting (i.e. not actively caring for eggs or chicks in nests), previous parental experience with chicks significantly altered the CORT and PRL stress responses. We found a significant interaction between experience and stress-series time point on CORT levels (Table 2). Robust linear mixed model estimates show this effect is driven by experienced birds having lower CORT after stress (Table 2). However, as models indicated no significant effect of sex, nor

Table 1. Primers used in quantitative PCR

Gene (abbreviation)	NCBI accession number	Amplicon length (bp)	Efficiency (%)		Primer sequence
Hypoxanthine phospho-ribosyl-transferase 1 (*HPRT1*)	XM_005500563.2	150	95.0	F	GCCCCATCGTCATACGCTTT
				R	GGGGCAGCAATAGTCGGTAG
Ribosomal protein L4 (*RPL4*)	XM_005511196.1	78	105.4	F	GCCGGAAAGGGCAAAATGAG
				R	GCCGTTGTCCTCGTTGTAGA
Glucocorticoid receptor (*GR*)[a]	XM_021301096.1	77	90.5	F	TGCTTAACTCGTCGGATCAA
				R	AAAGTCCATCACGATCCCTC
Mineralocorticoid receptor (*MR*)[b]	XM_021296726.1	158	103.8	F	AGAACATGGCTTCCTCGGTG
				R	CTAGAAAGCGGAGACCCGAC

[a]Also referred to as nuclear receptor subfamily 3 group C member 1 (*NR3C1*).
[b]Also referred to as nuclear receptor subfamily 3 group C member 2 (*NR3C2*).

interaction of sex with other variables (Table 2), we also compared the effect of experience averaged over levels of sex. When averaged across sexes, experienced birds showed lower CORT levels after stress (t=−2.18, P=0.033) and after DEX-induced negative feedback (t=−2.63, P=0.011), but not at baseline (t=−0.19, P=0.853; Fig. 3A and 4A). Time point as a main effect was significant (Table 2), as expected. When controlling for experience and sex, CORT levels increased in response to 30 min of acute restraint stress compared with baseline, and subsequently decreased after 60 min of DEX-induced negative feedback. Negative feedback CORT levels were also significantly higher than baseline levels.

In PRL levels, we found a significant three-way interaction between experience, sex and time point (Table 2). This interaction implies that previous parental experience affects the stress response sequence differently between the sexes. As with CORT, we found a significant interaction between experience and time point when controlling for sex (Table 2). Averaged across the sexes, experienced birds showed higher levels of PRL at both 30 (t=2.62, P=0.038) and 90 min (t=2.69, P=0.035) after a stressor. However, this relationship differed significantly between the sexes. The difference between experienced and inexperienced females was larger at 30 min than the difference between experience levels in males at this time point (Figs 3B and 4B). Females also had overall higher PRL levels at baseline and after 30 min than males. Overall, the shape of the PRL stress response differed with experience when birds were not actively nesting, with experienced birds showing a slight, but not significant, PRL increase post-stressor, and inexperienced birds showing the typical, significant decrease after an acute stressor (Figs 3B and 4B).

Experiment 2: Hippocampal and pituitary gene expression

In the hippocampus, prior experience with chicks significantly increased *GR* gene expression ($F_{1,26}$=11.1, P=0.002; Fig. 5B) but did not affect *MR* gene expression ($F_{1,26}$=2.7, P=0.113; Fig. 5C). There was no significant effect of sex ($F_{1,26}$=0.5, P=0.530) nor was there a significant interaction between sex and parental experience ($F_{1,26}$=2.0, P=0.164) on *GR* expression. However, *MR* expression did show a significant interaction between sex and experience with chicks ($F_{1,26}$=6.7, P=0.015). This effect appears to be due to inexperienced females having significantly lower *MR* expression than inexperienced males (t=−2.86, P=0.007), but this sex difference in *MR* expression was not present in experienced birds (t=0.71, P=0.486). However, we found no significant effect of experience ($F_{1,26}$=0.9, P=0.350), sex ($F_{1,26}$=2.5, P=0.127) or their interaction ($F_{1,26}$=2.6, P=0.120) on the MR:GR expression ratio.

DISCUSSION

We found that previous parental experience with chicks decreased stress-induced and DEX-induced negative-feedback CORT levels and led to increased stress-induced PRL in rock doves without

Table 2. Robust model estimates and standard errors for effects of parental experience, stress-series time point, sex and their interactions on $\log_{10}$-transformed concentrations of corticosterone and prolactin

	Corticosterone			Prolactin		
Variable	β	SE	*P*	β	SE	*P*
Experience (reference level: never raised chicks)						
Had raised chicks	0.177	0.084	**0.036**	−0.544	0.298	0.068#
Sex (reference level: male)						
Female	−0.018	0.084	0.831	0.158	0.054	**0.003**
Time point (reference level: 0 min, baseline)						
30 min (stress-induced)	−1.241	0.072	**<0.001**	0.184	0.069	**0.007**
90 min (negative feedback post-DEX)	1.454	0.071	**<0.001**	0.028	0.068	0.677
Experience×Sex						
Experienced×Female	0.068	0.084	0.418	−0.004	0.054	0.943
Experience×Time point						
Experienced×30 min	−0.160	0.072	**0.026**	0.380	0.069	**<0.001**
Experienced×90 min	0.046	0.071	0.512	−0.202	0.068	**0.003**
Sex×Time point						
Female×30 min	0.065	0.072	0.361	0.189	0.069	**0.006**
Female×90 min	−0.028	0.071	0.695	−0.120	0.068	0.078#
Experience×Sex×Time point						
Experienced×Female×30 min	0.007	0.072	0.917	0.205	0.069	**0.003**
Experienced×Female×90 min	0.073	0.071	0.301	−0.061	0.068	0.371

Significant effects at the α=0.05 level are indicated in bold, # indicates a trend (0.05<α<0.10).

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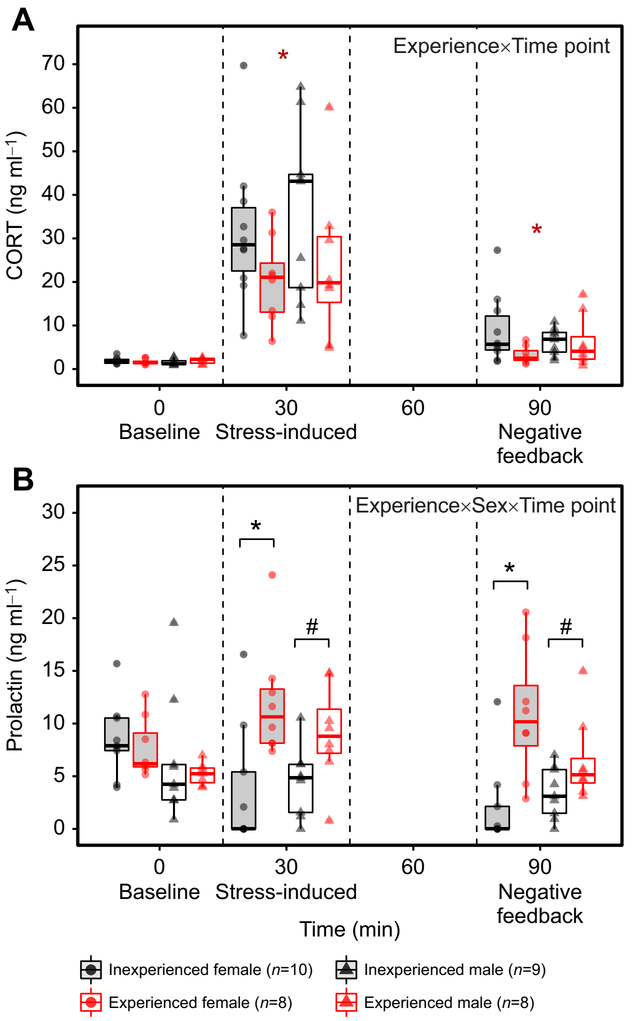

Fig. 3. Circulating hormones vary with parental experience, stress-series time point and sex. (A) Plasma CORT and (B) plasma prolactin were measured in birds without active nests that varied in previous parental experience with chicks (coded by line color; black and red represent inexperienced and experienced birds, respectively). Hormones were measured at baseline (0–3 min after capture), after capture–restraint stress (30 min post capture) and after DEX-induced negative feedback (60 min of recovery post stressor, 90 min after capture). Sampling time points are separated visually with dashed lines. Points represent individual birds, and boxplots represent the first quartile, median and third quartile for each sex and stage. These stress series were collected for both females (circles; boxplots shaded in gray) and males (triangles, boxplots unshaded). The highest-level, significant predictors from the linear mixed model that included experience, time point, sex and their interactions are shown in the upper right corner (see Table 2). In A, red asterisks indicate a significant effect (P<0.05) of experience averaged over levels of sex in *post hoc* analyses. In B, brackets represent significant differences in experience between the sexes across time points in *post hoc* contrasts (two-tailed t-tests, $^{\#}P$<0.10, $^{*}P$<0.05).

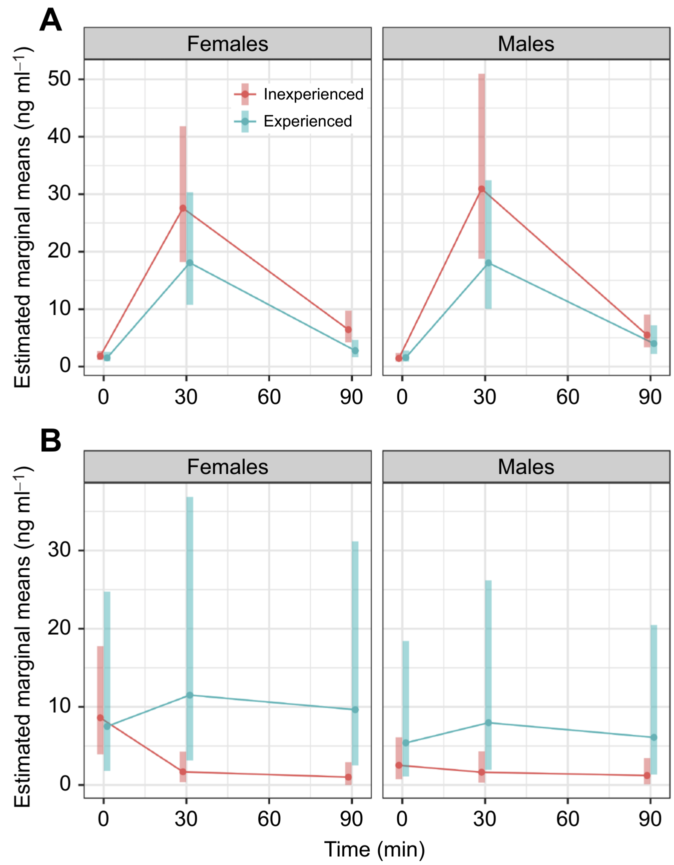

Fig. 4. Interaction plot for estimated marginal means of hormone concentrations across parental experience, sex and time point in non-actively nesting birds. Predicted responses (estimated marginal means) from mixed linear models that predict include the effects of experience, sex and sampling time point on (A) CORT and (B) prolactin are shown. Experienced (blue) and inexperienced (red) birds are across the stress-series time points and sexes. Dots represent estimated marginal means and shaded areas represent 95% confidence intervals around these means. Units are hormone concentration (ng ml^{-1}). Plot produced using the emmeans package in R statistical language (https://CRAN.R-project.org/package=emmeans). See Materials and Methods for model details. Sample size: 16 experienced birds (8 females, 8 males) and 19 inexperienced birds (10 females, 9 males).

active nests (i.e. in a pre-parental state). Further, in a separate experiment, we found that experienced birds of both sexes also had higher hippocampal *GR* expression than inexperienced birds. Increased *GR* expression may lower the threshold for negative feedback and suppressive effects on the HPA axis in experienced birds (Sapolsky et al., 2000), potentially mediating the observed changes in the CORT stress response. Together, these results suggest that inexperienced birds may be constrained by their HPA axis physiology and may not be able to attenuate their stress responses to prioritize future reproduction (support for the constraint hypothesis; Curio, 1983).

Parental experience and hormonal stress responses

Prior parental experience with chicks led to lower CORT and higher PRL levels, both after an acute stressor and after DEX-induced negative feedback. Previous studies that examined effects of prior breeding experience on CORT and PRL only measured baseline hormone levels (Angelier et al., 2006, 2007a), and found that experienced albatross had higher baseline CORT and PRL during brooding than birds breeding for the first time. Higher baseline PRL has also been found in experienced zebra finches (Smiley and Adkins-Regan, 2016) and cotton-top tamarin monkeys (Ziegler et al., 1996) during breeding. However, we did not find any significant effects of experience on baseline CORT or PRL levels in pre-parental birds with no active nest. Experience only significantly altered hormone levels after a stressor or during negative feedback in our study, highlighting the importance of measuring hormone

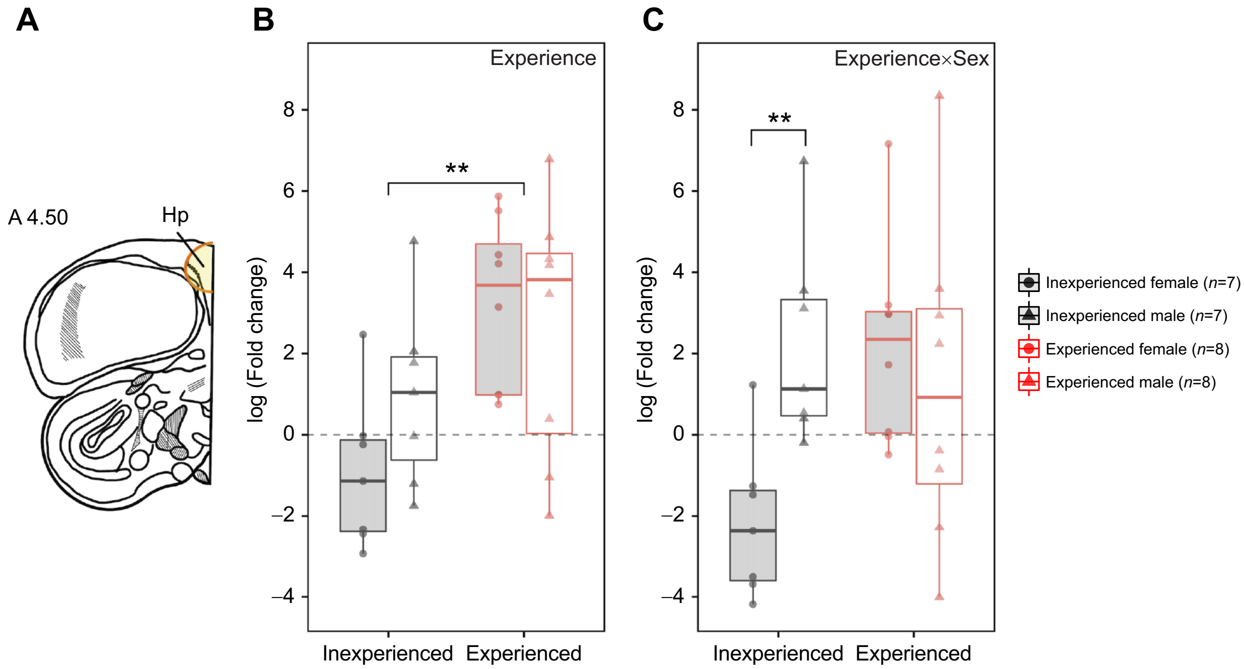

Fig. 5. Relative expression of glucocorticoid receptor types in the hippocampus of birds with and without prior parental experience with chicks. (A) Representative hippocampal (Hp) section, in which (B) glucocorticoid (*GR*) and (C) mineralocorticoid receptor (*MR*) expression was measured using quantitative PCR and compared across birds that had never previously raised chicks (inexperienced, gray) and birds that had previously raised at least one chick (experienced, red). Hippocampal section drawings modeled after Karten and Hodos (1966) (plate A4.50). Points represent individual birds, and boxplots represent the first quartile, median and third quartile for each sex and experience level. Sex is denoted by boxplot fill and point shape (females with shaded boxplots, circles; males with unshaded boxplots, triangles). Significant predictors from the linear model including experience, sex and their interactions are shown in the upper right corner (see Results). Brackets indicate specific significant *post hoc* comparisons (two-sample *t*-tests) after FDR correction (***P*<0.01).

responses beyond baseline levels to understand HPA axis plasticity. Although our findings did not align with previous work on breeding experience, they did mirror patterns seen with increasing age. In common terns, a long-lived seabird, CORT and PRL were lower and higher, respectively, after acute restraint stress in older parents compared with younger ones during incubation (Heidinger et al., 2006, 2010). Similarly, younger snow petrel females had lower stress-induced PRL than older females (Angelier et al., 2007b), and senescent albatross had lower CORT, but not PRL, levels than younger birds (Angelier et al., 2006). In our study, the age range was small (0.5–3 years, with 80% between 1 and 2 years old), making age less likely to drive our observed effect of experience. Instead, the effect of age on stress responses seen in prior studies may be mediated in part by accumulated parental experience. Indeed, when both were measured, breeding experience appeared to better statistically predict hormone levels than age (Angelier et al., 2006, 2007a).

Our observation that inexperienced birds exhibit more reactive hormonal stress responses than experienced birds lends support for the constraint hypothesis about why reproduction may improve over the lifespan (Curio, 1983). Under the constraint hypothesis, inexperienced birds may be limited (constrained) in their ability to invest in reproduction in the face of stressors. That is, inexperienced birds may be less able to attenuate HPA axis activity or maintain PRL secretion during stress. This hypothesis implies that mechanisms of HPA regulation may differ between inexperienced and experienced birds. We found evidence for such differences in the hippocampus, where experience was associated with higher *GR* expression. Alternatively, inexperienced birds may limit (restrain) their parental investment owing to relatively larger opportunities for future reproduction compared with older, more experienced breeders (restraint hypothesis; Curio, 1983). We found less support for this interpretation, however, as the age difference between inexperienced and experienced birds in our study (mean difference: 0.46 years), while statistically significant, was likely negligible given the lifespan of a rock dove (3–5 years for feral birds, 15 years in captivity) (Johnston, 1992).

Another interpretation is that experienced birds were closer to a parental state than inexperienced birds, driving stress response differences. Although birds did not have active nests when sampled, experienced birds did initiate new nests sooner after sampling than inexperienced birds on average (8.6 versus 24.9 days), though the time since last nest effort did not differ significantly (9.9 versus 14.9 days). Thus, we cannot rule out that the effects of experience may be due to differences in reproductive state or engagement in pre-parental behaviors. Even under this interpretation, however, our results would still be consistent with the parental care hypothesis (Wingfield et al., 1995). This hypothesis states that birds more involved in parental effort show more attenuated stress responses than those not engaged in care. Comparing stress responses in birds of varying experience during the parental period (i.e. during incubation or brooding) would clarify whether our results are due to differential reproductive states or truly represent a persistent effect of experience.

Parental experience and hippocampal glucocorticoid receptors

When we examined hippocampal GR, we found that, when not actively nesting, birds of both sexes that had previously had chicks had higher *GR* expression than inexperienced birds. Combined with our hormonal stress response results, this suggests that increased hippocampal *GR* may allow experienced birds to enact negative feedback on their HPA axis more rapidly and/or at a lower threshold level of circulating CORT, leading to overall lower stress-induced and negative-feedback CORT compared with inexperienced birds. Thus, hippocampal receptors provide a potential molecular mechanism for the constraint hypothesis, where young, inexperienced birds may be limited (constrained) in their ability to attenuate stress responses and prioritize current reproductive efforts (Curio, 1983). Although modified hippocampal *GR* expression has been a target of interest regarding the effects of stress in early development (Harris and Seckl, 2011; Lupien et al., 2009), our finding opens further investigation into plasticity of this mechanism in adults. However, it remains unclear whether the differences with experience we observed persist throughout the parental care period, which would be important to establish in future studies.

Our results contrast with previous work in avian species, which suggests that the hippocampal MR may be more important in modulating the glucocorticoid stress response than GR. For example, hippocampal *MR*, but not *GR*, expression was altered in zebra finch lines selected for highly responsive HPA axes (i.e. high stress-induced CORT) (Hodgson et al., 2007). Developmental stress, such as egg CORT injections or postnatal food restriction, affected hippocampal *MR*, but not *GR*, expression in Japanese quail (Soleimani et al., 2011; Zimmer and Spencer, 2014). Similarly, neither chronic stress nor translocation to captivity affected hippocampal GR in starlings or chukar (Dickens et al., 2009a,b, 2011). Hypothalamic GR may be more important for HPA axis regulation than hippocampal GR in other species, as chronic and prenatal stress reduced GR in the hypothalamus of European starlings and Japanese quail, respectively (Dickens et al., 2009a,b; Zimmer and Spencer, 2014). However, no differences in hypothalamic or hippocampal *GR* expression were found during breeding stages, where stress responses are typically attenuated (Gambel's white-crowned sparrows, Krause et al., 2015; house sparrows, Lattin and Romero, 2013). Given this, our results align more closely with mammalian studies, where changes in hippocampal GR affected stress-induced CORT release (Harris et al., 2013; Ratka et al., 1989; van Haarst et al., 1996).

We did not find an overall effect of experience on hippocampal *MR* expression, in contrast with other studies that found altered hippocampal *MR* expression in birds. In these studies, artificial selection for highly reactive stress profiles, chronic stress, developmental stressors and breeding transitions all altered hippocampal *MR* expression (Dickens et al., 2009b; Hodgson et al., 2007; Krause et al., 2015; Zimmer and Spencer, 2014), with all associating decreased *MR* expression with reduced stress-induced CORT release. We did not find support for this relationship, but did observe an apparent sex difference present in inexperienced birds, with females having lower *MR* expression than males, that was not present in experienced birds. Although this result suggests that inexperienced females may have lower MR densities, allowing GR to be bound more rapidly, potentiating faster negative feedback, this was not borne out in the plasma CORT data. Most previous studies only measured these receptors in one sex, though those that included both sexes found no significant differences in both stress response and hippocampal *MR* expression (Dickens et al., 2009a,b; Hodgson et al., 2007). These results emphasize the importance of studying these mechanisms in both sexes to understand where sex differences in HPA axis regulation may arise.

The discrepancies we found in hippocampal gene expression patterns compared with other avian studies may be due to differences in the context of our study (parental experience) and/or species differences. It is possible that prior reproductive cycles, and the many endocrine changes involved (Austin et al., 2021b), may alter hippocampal gene regulation in different ways than stress contexts or the annual cycle transitions examined in other studies. Additionally, we measured *GR* and *MR* gene expression, not proteins, so functional receptor counts in the hippocampus may show a different pattern (Maier et al., 2009). Finally, we cannot rule out the role of species differences, as the continuously breeding rock dove may differ in stress regulation from seasonal breeders. For instance, rock doves may not downregulate CORT responses during molt as other species do (Romero and Wingfield, 2001), suggesting possible differences in HPA axis regulation.

Conclusions

Overall, we found evidence in support of the constraint hypothesis for why inexperienced birds may be poorer breeders than experienced individuals; these groups may differ in their ability to attenuate the CORT and PRL stress responses. In turn, the ability of experienced birds to attenuate hormonal stress responses, specifically CORT release, may be mediated by increased hippocampal GR involved in HPA axis regulation.

Our finding that effects of experience are detectable in stress responses and the brain even after the parental effort suggests long-lasting changes to gene regulation. Raising chicks may thus constitute an experience such as those seen early in development, where HPA axis responsiveness is altered in subsequent life stages (e.g. Wada and Coutts, 2021). If the effects of experience uncovered here continue during the next breeding effort and beyond, acting as a carryover effect (O'Connor et al., 2014), future work should examine (a) what elements of the parental experience cause lasting changes in HPA axis gene expression, and (b) how such gene expression changes are 'programmed' at the molecular level. Potential causal mechanisms may include exposure to hormones involved in parental care, such as PRL or mesotocin (Rogers and Bales, 2019; Smiley, 2019). At the level of the gene, epigenetic mechanisms such as changes in DNA methylation (Bentz et al., 2016; Siller and Rubenstein, 2019) or histone modifications (Stolzenberg and Champagne, 2016) may influence GR transcription. Thus, our results provide a foundation upon which to explore how experience-dependent mechanisms can alter parental stress responses and, ultimately, fitness.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: V.S.F.; Methodology: V.S.F.; Validation: V.S.F.; Formal analysis: V.S.F.; Investigation: V.S.F., J.M.G.; Resources: R.M.C.; Writing - original draft: V.S.F.; Writing - review & editing: R.M.C., V.S.F.; Supervision: R.M.C.; Funding acquisition: R.M.C.

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Data availability
Data for this paper can be found on the Dryad data repository (Farrar et al., 2022): https://doi.org/10.25338/B8KK91. Analysis code can be found at: https://github.com/vsfarrar/experience-stress-hippocampus.

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