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THE NEUROENDOCRINE ROLE OF PROLACTIN IN LIFE-HISTORY TRADEOFFS AND
TRANSITIONS IN A BIPARENTAL BIRD

By

VICTORIA SOPHIA FARRAR

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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ABSTRACT

During parental care, animals must allocate resources towards offspring care that may come at a cost to personal survival, growth, and future reproductive efforts. Hormones physiologically mediate many of these tradeoffs. Prolactin, a pituitary peptide best known for its role in mammalian lactation, also promotes parental behaviors across vertebrates. However, less is known how prolactin affects reproductive behavior and physiology, or the endocrine stress response, during parental care. Further, how prolactin enacts these effects at the neuroendocrine level remains less studied. To address these gaps, I examined the effects of prolactin on behavior, hormones and gene expression in a biparental bird, the rock dove (*Columba livia*). Rock doves exhibit nearly-egalitarian biparental care and prolactin-driven pseudolactation, making them an ideal system to study prolactin's effects on physiology in both sexes. First, I characterized prolactin and prolactin receptor gene expression across the endocrine axis controlling reproduction: the hypothalamic-pituitary-gonadal (HPG) axis in both sexes, as well as in the crop sac, the site of pseudolactation. Next, I used hormone manipulations to determine the causal role of prolactin in transitions from mating to parental behavior. I found that treatment with prolactin promoted parental behaviors, even after nest removal, but did not significantly alter expression of courtship or copulation behaviors as compared with vehicle-treated controls. I found that prolactin-treated birds showed increased sensitivity to hypothalamic hormones in the pituitary and sex-specific changes in the gonads, where males showed increased sensitivity to gonadotropins, but females did not. These results suggest the HPG axis may compensate for elevated prolactin (within the physiological range) in order to maintain reproductive behavior and function.

Lastly, I examined how prolactin system regulation compares across birds with a range of parental experience and ages. I found that neural expression of prolactin receptors nor vasoactive intestinal peptide (a peptide involved in prolactin release) changed with increasing parental experience. This result suggesting that increased fitness with parental experience may be mediated through other mechanisms or elsewhere in the brain. However, when I examined the effects of parental experience on

the hormonal stress response, I found that experienced parents released lower levels of glucocorticoids and maintained higher plasma prolactin levels after an acute stressor than inexperienced individuals. This hormone pattern appears to be mediated by increased glucocorticoid receptor density in the hippocampus. Taken together, this dissertation integrates across neurobiology, endocrinology and animal behavior to provide novel insights into reproduction and stress in vertebrates.

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CHAPTER 1

Prolactin and prolactin receptor expression in the HPG axis and crop during parental care in both sexes of a biparental bird (*Columba livia*)

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1.1. ABSTRACT

During breeding, multiple circulating hormones, including prolactin, facilitate reproductive transitions in species that exhibit parental care. Prolactin underlies parental behaviors and related physiological changes across many vertebrates, including birds and mammals. While circulating prolactin levels often fluctuate across breeding, less is known about how relevant target tissues vary in their prolactin responsiveness via prolactin receptor (*PRLR*) expression. Recent studies have also investigated prolactin (*PRL*) gene expression outside of the pituitary (i.e., extra-pituitary *PRL*), but how *PRL* gene expression varies during parental care in non-pituitary tissue (e.g., hypothalamus, gonads) remains largely unknown. Further, it is unclear if and how tissue-specific *PRL* and *PRLR* vary between the sexes during biparental care. To address this, we measured *PRL* and *PRLR* gene expression in tissues relevant to parental care, the endocrine reproductive hypothalamic-pituitary- gonadal (HPG) axis and the crop (a tissue with a similar function as the mammalian mammary gland), across various reproductive stages in both sexes of a biparental bird, the rock dove (*Columba livia*). We also assessed how these genes responded to changes in offspring presence by adding chicks mid-incubation, simulating an early hatch when prolactin levels were still moderately low. We found that pituitary *PRL* expression showed similar increases as plasma prolactin levels, and detected extra-pituitary *PRL* in the hypothalamus, gonads and crop. Hypothalamic and gonadal *PRLR* expression also changed as birds began incubation. Crop *PRLR*

expression correlated with plasma prolactin, peaking when chicks hatched. In response to replacing eggs with a novel chick mid-incubation, hypothalamic and gonadal *PRL* and *PRLR* gene expression differed significantly compared to mid-incubation controls, even when plasma prolactin levels did not differ. We also found sex differences in *PRL* and *PRLR* that suggest gene expression may allow males to compensate for lower levels in prolactin by upregulating *PRLR* in all tissues. Overall, this study advances our understanding of how tissue-specific changes in responsiveness to parental hormones may differ across key reproductive transitions, in response to offspring cues, and between the sexes.

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1.2. INTRODUCTION

In animals that exhibit offspring care, an array of physiological changes must occur to facilitate parental behaviors. This transition requires synchronized changes at many physiological levels, from the brain (Bridges, 2015; Champagne and Curley, 2012) to the reproductive organs (Stiver and Alonzo, 2009). Hormones facilitate those changes, including those produced by the reproductive or hypothalamic-pituitary-gonadal (HPG) axis, through their pleiotropic effects on multiple behavioral and physiological traits (Ketterson et al., 2009; Zera and Harshman, 2001). Similarly, tissue responsiveness to hormones, via hormone receptor expression, must also change to produce a synchronized parental phenotype across the brain and periphery (Ball and Balthazart, 2008).

One such hormone, prolactin, plays an important role in parental behavior across vertebrates, but is particularly important in birds (Angelier and Chastel, 2009). Best known for promoting lactation in mammals, prolactin also underlies the onset and maintenance of parental behaviors in birds such as incubation onset, offspring defense and provisioning (Angelier and Chastel, 2009; Buntin, 1996; Smiley, 2019). Circulating prolactin is released by the anterior pituitary gland, and acts upon specific receptors to trigger signaling pathways in target cells. Once in circulation, prolactin acts upon its receptor (PRLR) to activate secondary messenger cascades in target cells, such as the signal transducer and activator of transcription 5 (STAT5) pathway (Austin and Word, 2018; Freeman et al., 2000). Prolactin receptors have been identified in nearly every tissue type in both mammalian and avian species, reinforcing its role in multiple physiological and behavioral processes including reproduction, immune function, and homeostasis (Nagano and Kelly, 1999; Zhou et al., 1996). Additionally, evidence for local prolactin expression beyond the pituitary gland (i.e. extra-pituitary prolactin) has been identified in tissues ranging from the gonads to the mammary glands and the brain (Ben-Jonathan et al., 1996; Marano and Ben-Jonathan, 2014).

While circulating prolactin often increases during parenthood, less is known about how concordant responsiveness to prolactin changes in the brain. In female rats, *PRLR* mRNA increases in some hypothalamic nuclei, and hypothalamic responsiveness to prolactin (measured via STAT5 phosphorylation downstream of the PRLR) increases with reproductive experience (Anderson et al., 2006; Sjoeholm et al., 2011). In birds, brain responsiveness to prolactin increases during breeding compared to non-breeding individuals of multiple species (Buntin and Buntin, 2014; Smiley et al., 2021), and prolactin binding varies seasonally, including during breeding (Smiley et al., 2020). However, how hypothalamic responsiveness to prolactin varies during transitions *within* the breeding cycle remains less studied. Understanding these subtle changes in *PRLR* expression is important, as changing neural responsiveness to prolactin may prepare behavioral and endocrine systems for the onset of offspring care. For instance, in mammals, prolactin and placental lactogen secretion increases during pregnancy to facilitate lactation and

maternal adaptations for postnatal care (Bridges, 2015). In birds, prolactin increases after egg laying to promote incubation behavior with a subsequent increase around hatching to facilitate chick brooding and provisioning in species that exhibit these behaviors (Angelier et al., 2016b; Buntin, 1996; Smiley, 2019). Thus, changes in *PRLR* expression with offspring cues and fluctuating plasma prolactin levels may play a role in prolactin's facilitation of parental behaviors.

Beyond the brain, peripheral endocrine systems can also respond to prolactin and may influence behavior through altered hormone regulation. *PRLR* gene and protein expression has been documented in the pituitary gland, gonads and other tissues across vertebrates (Aoki et al., 2019; Nagano and Kelly, 1999; Zhou et al., 1996). Prolactin can have an "anti-gonadal" effect in some species, where high circulating levels inhibit sex steroid release and gonadal function (Grattan, 2018; Meier, 1969; Moult and Besser, 1981), which may serve to maintain parental efforts on the current brood rather than continuing breeding or starting a new clutch (Angelier et al., 2016b). These effects may be modulated in part by prolactin's effects on pituitary gonadotroph cells in the release of luteinizing hormone (LH) or follicle-stimulating hormone (FSH), or by direct action on sex steroid production in the gonads (Bachelot and Binart, 2007). Any of these diverse effects on the HPG axis, and ultimately, reproductive behaviors, would depend upon a tissue's function and ability to respond to prolactin. Thus, measuring how *PRLR* varies across the HPG axis during parental care is key to understanding how prolactin may exert pleiotropic effects during breeding.

Further, local prolactin expression in the brain and other tissues may also vary during parental care and play an autocrine/paracrine role in hormone regulation (Ben-Jonathan et al., 1996; Marano and Ben-Jonathan, 2014). Extra-pituitary prolactin (*ePRL*) gene expression has been measured in various tissues, including the brain, gonads and mammary glands, though its specific role and function remains unclear (Ben-Jonathan et al., 1996; Marano and Ben-Jonathan, 2014). While there is some debate whether *ePRL* becomes a functional protein (Grattan and Le Tissier, 2015), hypophysectomized rats have been shown to have immunoreactive prolactin protein in their brains (DeVito, 1988), giving evidence that

bioactive prolactin can be locally translated beyond the pituitary. Characterizing if, and how, *ePRL* expression changes in the HPG axis and responds to offspring cues will lay the groundwork to explore any potential role this gene may play in reproductive physiology or behavior.

Rock doves (*Columba livia*) provide a powerful model to explore the dynamics of prolactin and its receptor across parental care and between the sexes. These birds form monogamous bonds and exhibit biparental care, with both sexes incubating eggs and provisioning offspring. Additionally, rock doves produce "crop milk" to feed their offspring, which is regulated by circulating prolactin (Abs, 1983; Horseman and Buntin, 1995). Unlike mammals, both male and female rock doves pseudo-lactate, allowing the comparison of sex differences without the confounds of pregnancy and female-only lactation. In doves, prolactin maintains incubation behaviors and facilitates the onset of chick provisioning, rising mid-incubation and peaking around hatch in both sexes (Austin et al., *in review*; Cheng and Burke, 1983; Horseman and Buntin, 1995; Ramsey et al., 1985). Prolactin then remains elevated post-hatching to facilitate both crop milk production and chick brooding/provisioning, a pattern typical of avian species with altricial young (Angelier and Chastel, 2009; Smiley, 2019). Additionally, we detected *PRLR* and *PRL* gene transcripts across the HPG axis in previous RNAseq studies (Austin et al., 2021a; Calisi et al., 2018; MacManes et al., 2017), setting a foundation to examine patterns of expression in these genes during parental care.

In this study, we examined how reproductive tissues vary in prolactin responsiveness and local prolactin expression across breeding and in response to offspring presence. Our goal was to understand how regulation at the tissue level may facilitate and coordinate reproductive transitions beyond circulating hormones alone. First, we characterized the expression of prolactin (*PRL*) and its receptor (*PRLR*) across multiple stages of parental care in the hypothalamus, pituitary, and gonads of both sexes. We also characterized these genes in the crop sac ("crop"), which is where crop milk is produced in doves. Then, we tested the influence of offspring cues on *PRL* and *PRLR* by introducing chicks at mid-incubation, before plasma prolactin is elevated and crops are fully functional for chick provisioning and lactation

(Dong et al., 2012; Horseman and Buntin, 1995). We compared this "early hatch" manipulation to the equivalent stage at mid-incubation as a control group. Through this manipulation, we assessed to what degree offspring presence influences prolactin gene dynamics separate from the rise in circulating prolactin normally seen before hatch (Austin et al., 2021b). We hypothesized that offspring presence drives prolactin and prolactin responsiveness in key tissues. Therefore, we predicted plasma prolactin levels and *PRLR* expression would increase when chicks were added mid-incubation to compensate for normally low circulating prolactin levels at this stage. Alternatively, the priming effect of circulating prolactin before hatch may drive tissue responsiveness to prolactin. In this case, we predicted that chick presence alone would not increase *PRLR* expression, as hormonal priming was not yet completed. These hypotheses are not mutually exclusive and may be supported in some tissues under examination, but not others. Lastly, because both male and female rock doves exhibit the same suite of parental behaviors, we hypothesized that prolactin gene dynamics would be similar between the sexes.

1.3. METHODS

This project was conducted in conjunction with a larger RNAseq study of the HPG axis during reproduction and parental care in rock doves (*Columba livia*). However, the focus of this study is prolactin-related gene dynamics in key tissues, including the crop. Thus, in addition to the HPG tissues (*n* ≅ 10/sex/sampling point, see Table 1.2 for exact sample sizes), we also collected crop tissue from a randomly-selected subset (n = 73) of these male-female pairs of breeding rock doves at focal stages of reproduction. We also collected crop tissues from an additional 20 individuals who were not part of the RNAseq study to increase sample sizes per stage (total n = 93, see Table 1.2*)*. We focused on the following stages of reproduction: nest building (building), clutch completion/early incubation (incubation day 3: incubation begins when the first egg is laid in this species) (Abs, 1983), mid-incubation (incubation day 9), and the day the first chick hatched (hatch) (see Austin et al., 2021b, for more details). Additionally, to understand the influence of external cues on candidate gene expression, we also included a manipulation group (early hatch), where we experimentally reduced the length of the incubation period

by replacing eggs with one young chick at mid-incubation (on incubation day 8) and then collected the pair ~24 hours later. Circulating hormone data for these same individual birds across multiple stages of parental care were reported previously in Austin et al. (2021b). Here, we extend that study with the first gene expression data from these individuals, reporting *PRL* and *PRLR* gene counts across the hypothalamus, pituitary, and gonads and crop. F

Study Animals

Rock doves *(Columba livia)* were socially housed in outdoor flight aviaries (1.5 x 1.2 x 2.1 m), each containing 8-10 breeding pairs, and were provided with nesting material (straw) and nest sites (wooden nest boxes, 16 per aviary). These outdoor aviaries exposed the birds to natural photoperiod for the area (Davis, California, USA), and photoperiod was supplemented with 14L:10D artificial lighting year-round. Birds were fed whole corn, turkey/game bird starter (30% protein; Modesto Milling, CA) and grit *ad libitum.* We used birds that were reproductively experienced and < 2 years old in this study. Further details can be found in Austin et al. (2021b).

Tissue Collection

Brain, pituitary, gonads, crop and trunk blood (for circulating hormones) were collected from birds at each timepoint following approved IACUC protocols (UC Davis protocol #20618). Tissues were flash frozen (brain, crop) or immediately placed in RNALater (Thermo Fisher) then flash frozen (pituitary and gonads) and stored at -80°C until use in downstream analyses. An additional 20 birds were collected in the same manner for crop tissues. For detailed collection methods and handling of HPG tissues (see Austin et al. 2021a; Calisi et al. 2018; MacManes et al. 2017), and for experimental design see Austin et al. (2021b). All of the subjects in this study, with the exception of the additional 20 birds collected for crop tissues alone, are included in Austin et al. (2021b).

RNA-sequencing for total gene expression

Before RNA processing, the hypothalamus and lateral septum were isolated using punch biopsy on a Leica CM 1860 cryostat and stored in RNALater at - 80 C before analysis (see Calisi et al 2018; MacManes et al, 2017 Austin et al. 2021a for details). For gonadal tissue, we sequenced tissue from whole homogenized testes in males and a homogenate consisting of both ovaries and oviduct tissue in females. We took this approach to allow direct comparisons with, and to be consistent with previous transcriptomic studies in rock doves (Austin et al., 2021a; Calisi et al., 2018; Lang et al., 2020; MacManes et al., 2017). Although gonadal tissue typically refers to the testes in males and the ovaries in females, and does not typically include the oviduct, we included the oviduct as our study is one of the first to examine transcriptomic responses to transitions in parental care beyond the brain, and we wished to broadly capture transcriptomic changes that could occur across female reproductive tissues. Hereafter, we refer to the testes and ovaries/oviducts as "gonadal" tissue, as gonadal gene expression is encompassed in these samples and to align with the hypothalamic-pituitary-gonadal (HPG) reproductive axis framework.

Tissue processing for RNA sequencing is described in detail in Austin et al., 2021a and Lang et al., 2020. Briefly, RNA from the hypothalamus, pituitary, testes and ovaries/oviducts was prepared for Illumina sequencing using the NEB Next Ultra Directional RNA Library Prep Kit, and sequenced on an Illumina HiSeq 400 via 125 base pair paired-end sequencing (Novogene). Reads were pseudomapped (*kallisto:* Bray et al., 2016) to the Rock Dove transcriptome v1.1.0 whose transcripts were annotated with genes from *Gallus gallus* genome v5 using BLAST. Transcriptomic data were then imported into the R statistical language using tximport (Soneson et al., 2016) and gene counts were variance-stabilized using the DEseq2 package (Love et al., 2014). Variance-stabilized gene counts for each sample were used in statistical analysis.

Quantitative PCR

To measure gene expression in the crop, we ran quantitative PCR (qPCR) on a subset of crops from each of the reproductive timepoints. For crop sample sizes by stage and sex, see Table 1.2.

To extract total RNA from crops, we first homogenized an approximately 10 mg sample from each crop tissue using the OmniTip Tissue Homogenizer (Omni International), followed by RNA extraction using the Direct-zol RNA Miniprep kit (Zymo) with modifications recommended for lipid-rich tissues. We verified RNA purity and concentration using a NanoDrop 2000c (Thermo Scientific). For each sample, we treated 500 ng of RNA with DNase (Perfecta; QuantaBio) then performed cDNA reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen). We then ran real-time qPCR reactions with SYBR Green detection chemistry using the following reaction mix: 10 µL total reaction volume containing 1 µL cDNA template (diluted 1:5), 5 µL 2X SSOAdvanced SYBR Green PCR mix (BioRad), and 10 µM each of primer. We ran each reaction under the following conditions: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 sec and 60°C for 30 sec. We ran samples in duplicate for each gene on the same 384-well plate using a CFX384 Touch Real-time PCR detection system (BioRad). We validated all primers for this study by running a 10-fold serial dilution to determine amplification efficiencies (average: 97.2% $\pm$ 5.63) and checked melt curves for a single product. Primer sequences, efficiencies, and amplicon lengths can be found in Table 1.3.

We then quantified the relative expression of each gene of interest (*PRL* and *PRLR*) relative to the geometric mean of the reference genes, beta-actin (*ACTB*) and ribosomal protein L4 (*rpL4*) (Zinzow-Kramer et al., 2014) using the ddCt method (Livak and Schmittgen, 2001). We found no significant effect of reproductive stage ($F_{4,83} = 1.6$, $p = 0.17$), sex ($F_{1,83} = 0.9$, $p = 0.36$) or their interaction ($F_{4,83} = 1.2$, $p = 0.33$) on mean reference gene expression, indicating stable reference genes for crop tissue. Samples that did not cross the cycle threshold within 40 cycles had Ct values set to 40. Normalized expression (dCt) was calculated as the average Cq value between technical replicates of each gene minus the geometric mean of the reference genes for each sample. We calculated relative expression (ddCt) as the normalized value (dCt) minus the average normalized expression for the nest-building stage. Nest-building was used as a reference as it was the first reproductive stage included in the study, and birds were not yet caring for

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eggs or chicks. Fold change equals $2^{(-ddCt)}$. We then log-transformed ($\log_e$ or ln) fold change values for statistical analysis to improve model fit and visualization.

Hormone measurements

Plasma hormones, including prolactin, were measured and described in rock doves across multiple stages of parental care in Austin et al., (2021b). Here, we used circulating prolactin data from Austin et al. (2021b) for our stages of interest (nest building, incubation day 3, incubation day 9, hatch and the manipulation on incubation day 8) to correlate plasma prolactin with *PRL* and *PRLR* gene expression, newly reported here. Briefly, plasma prolactin levels were measured from trunk blood using a heterologous radioimmunoassay (RIA) run at the Center for Biological Studies at Chizé, France (CEBC-CNRS) as detailed in (Angelier et al., 2007). This RIA had previously been validated in rock doves by creating a dose-response curve with pooled rock dove plasma and determining parallelism with standard curves consisting of chicken prolactin (Angelier et al., 2016a). Samples for this project were run in two separate assays with intra- and inter-assay coefficients of variation (CVs) of 9.58 and 11.83%, respectively. The minimal detectable prolactin level was 0.45 ng/ml.

Statistical analysis

All statistical analyses were performed in R (v.4.0.3, R Core Team, 2020). We compared gene expression in each gene-by-tissue combination using general linear models (glm), where gene expression (either variance-stabilized gene counts for RNAseq data or log-transformed fold change for qPCR) was predicted by stage, sex, and their interaction. We analyzed each gene-by-tissue combination in a separate model for three main reasons. First, we used two different methods for estimating gene expression, RNAseq and qPCR, and thus the expression data are not directly comparable across tissues. Second, we were interested in how each gene expression in tissue changed over time, responded to external manipulation, and varied by sex. Third, evidence shows that in different tissues genes for prolactin and its

receptor are regulated by different promoters and transcription factors (Aoki et al., 2019; Featherstone et al., 2012), and therefore their expression should be considered independently.

For each glm, we ensured that our data met the model assumptions. If main effects were significant (alpha = 0.05), we compared group differences using pairwise comparisons of our *a-priori* hypotheses. The interaction between stage and sex was not significant for *PRL* and *PRLR* in any tissue, which suggests that males and females responded similarly across stage and to external manipulation. Because sex interactions were not significant, we did not include this term in future models (gene expression ~ stage + sex). We also present estimates,standard errors, and *p*-values of *a priori* contrasts of biological interest. Following Austin et al. (2021b), we compared each reproductive stage to the adjacent or subsequent stage in the normal course of parental care: nest building vs. clutch completion, clutch completion vs. mid-incubation, and mid-incubation vs. hatch. This approach allowed us to compare gene expression changes during key reproductive transitions. We also compared how external manipulation affected gene expression, by comparing the early hatch group to its equivalent control stage, i.e., early hatch vs. mid-incubation. To determine if adding chicks mid-incubation had a similar effect to that seen when chicks naturally hatch after 18 days of incubation, we also compared early hatch vs. hatch. A list of pairwise contrasts can be found in Table 1.1. Finally, we examined relationships between plasma prolactin levels and gene expression within each tissue by calculating Spearman's correlation coefficients (ρ).

1.4. RESULTS

We examined the effect of reproductive stage and sex on plasma prolactin, and gene expression of *PRL* and *PRLR* in HPG and crop tissues. Results from *a priori* pairwise comparisons for all tissues and circulating prolactin can be found in Table 1.1.1.

Plasma prolactin levels

As in our larger analysis of circulating prolactin (Austin et al., 2021b), we found that plasma prolactin levels varied significantly across the stages examined in this study (stage: $F_{4,106} = 83.6$, p < 0.01) and with sex ($F_{1,106} = 10.4$, p < 0.01). Prolactin significantly increased from nest building to clutch completion, and from mid-incubation to hatch, but did not differ from clutch completion to mid-incubation (Fig.1.1.2). When chicks were added mid-incubation (early hatch), circulating prolactin did not significantly differ from the equivalent stage at mid-incubation, and was significantly lower than the level seen at typical hatching. Across all stages, females had significantly higher prolactin levels than males.

Hypothalamic PRL and PRLR expression

While there was no significant difference in hypothalamic *PRL* expression across stage in our models ($F_{4,94} = 0.7$, $p = 0.569$), this effect was largely driven by earlier time points. When we investigated a priori hypotheses of gene expression difference across stage, we found that birds at hatch had higher *PRL* expression compared with those at mid-incubation. When we investigated how external manipulation influenced gene expression, we found that the addition of chicks (early hatch) at mid-incubation did not significantly affect hypothalamic *PRL* levels above those seen at its control at mid-incubation. We found that *PRL* at early hatch was significantly lower than at a typical hatch (Fig.1. 3A, Table 1.1). We found a suggestive trend ($0.05 < p < 0.10$) of sex on hypothalamic *PRL* in our models ($F_{1,94} = 2.9$, $p = 0.092$), suggesting that males may express hypothalamic *PRL* at slightly higher levels than females. Hypothalamic *PRL* and plasma prolactin levels were not significantly correlated (Fig.1.4A; $\rho_{99} = 0.12$, $p = 0.200$).

Hypothalamic *PRLR* expression significantly differed by stage ($F_{4,94} = 7.7$, $p < 0.01$) and sex ($F_{1,94} = 10.8$, $p < 0.01$). Specifically, *PRLR* counts increased at clutch completion compared with nest building (Fig.1. 3B; Table 1.1.1). When we compared the early hatch manipulation to its equivalent control at mid-incubation, *PRLR* levels significantly increased (Fig.1. 3B, Table 1.1.1). Further, *PRLR* expression at the

early hatch manipulation was also significantly higher compared to hatch. We found no significant correlation between hypothalamic *PRLR* and plasma prolactin (Fig.1.4E; $\rho_{99} = -0.09$, $p = 0.355$).

Pituitary PRL and PRLR expression

Like plasma prolactin, pituitary *PRL* gene expression varied significantly with stage ($F_{4,98} = 47.9$, $p < 0.001$) and sex ($F_{1,98} = 6.0$, $p = 0.016$). Pituitary *PRL* expression also increased from mid-incubation to hatching (Fig.1. 3C; Table 1.1.1). Unlike plasma prolactin levels, however, pituitary *PRL* significantly increased from clutch completion to mid-incubation but did not significantly change from nest building to clutch completion (Table 1.1). Pituitary *PRL* gene counts were significantly higher in females than males, as seen in plasma levels. As expected, pituitary PRL expression and plasma prolactin were significantly positively correlated (Fig.1.4B; $\rho_{101} = 0.78$, $p < 0.001$).

Pituitary *PRLR*, in contrast, did not significantly differ across stages (Fig.1. 3D; $F_{4,98} = 0.7$, $p = 0.616$). However, we found a significant effect of sex ($F_{1,98} = 29.0$, $p < 0.001$), where males expressed higher levels of pituitary *PRLR* than females. Unlike pituitary *PRL*, *PRLR* expression did not correlate with plasma prolactin levels (Fig 4F; $\rho_{101} = -0.14$, $p = 0.146$).

Gonadal PRL and PRLR expression

PRL expression in the testes and ovaries/oviducts did not significantly differ with stage, though there was a suggestive trend ($F_{4,98} = 2.2$, $p = 0.079$). This trend appears to be driven by the early hatch manipulation, which significantly increased *PRL* in both the testes and ovaries/oviduct compared to the mid-incubation control and hatching stages (Fig.1. 3E, Table 1.1). *PRL* in these tissues also differed significantly by sex ($F_{1,98} = 5.7$, $p = 0.019$), where testes expressed *PRL* at higher levels than ovaries and oviducts. We found no correlation between gonadal *PRL* expression and plasma prolactin (Fig.1. 4C; $\rho_{101} = -0.02$, $p = 0.851$).

Gonadal *PRLR* expression significantly differed with stage ($F_{4,98} = 3.0$, $p = 0.023$). *PRLR* in the testes and ovaries/oviducts decreased at clutch completion compared with nest building, but did not differ from clutch completion to mid-incubation or mid-incubation to hatch (Fig.1. 3F, Table 1.1). At early hatch, gonadal *PRLR* expression did not significantly change compared to mid-incubation levels, though early hatch levels were significantly lower than at hatch. Further, there was a significant sex effect ($F_{1,98} = 154.4$, p < 0.001), where testes expressed more *PRLR* than ovaries/oviducts at all stages. Gonadal *PRLR* expression was significantly negatively correlated with plasma prolactin (Fig.1.4G; $\rho_{101} = -0.28$, $p = 0.005$).

Crop PRL and PRLR expression

In the crop, *PRL* expression remained relatively constant, with no significant stage effect detected (Fig.1. 3G; $F_{4,83} = 0.21$, $p = 0.930$). However, we found crop *PRL* expression differed by sex ($F_{1,83} = 4.50$, $p = 0.037$) with males having higher *PRL* than females. Crop *PRL* was not correlated with plasma prolactin (Fig.1.4D; $\rho_{80} = -0.03$, $p = 0.827$).

Unlike *PRL*, crop *PRLR* expression differed significantly by stage ($F_{4,87} = 4.30$, $p = 0.003$). This effect was likely driven by increased expression at hatch, which was higher than every other stage in contrasts (Fig.1. 3H, Table 1.1). However, crop *PRLR* levels did not significantly differ after the early hatch manipulation compared to mid-incubation controls. We did not find a significant effect of sex on crop *PRLR* expression ($F_{1,87} = 0.19$, $p = 0.665$). We found a suggestive positive correlation between crop *PRLR* and plasma prolactin ($\rho_{80} = 0.25$, $p = 0.060$), which is likely driven by levels at hatch (Fig.1.4H).
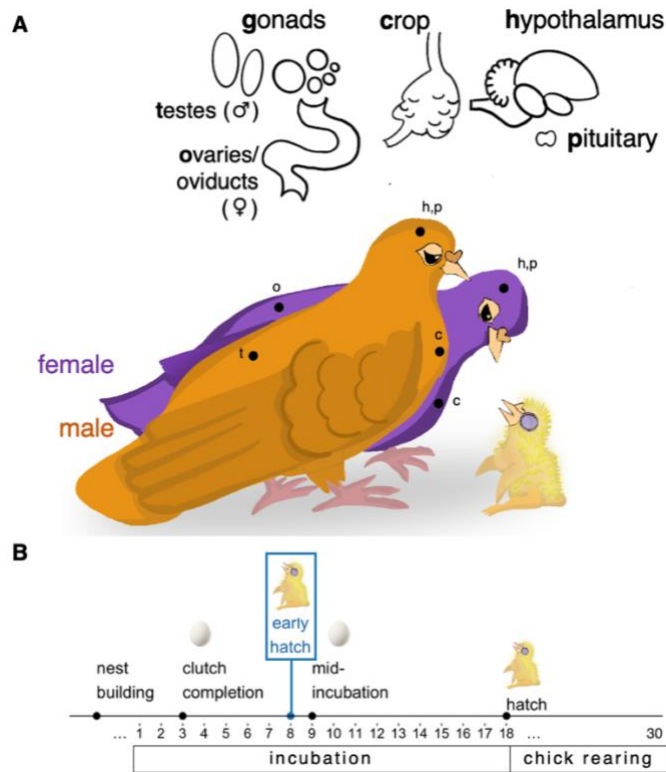
Figure 1.1. Schematic diagram of experimental design. (**A**) Tissues sampled in both males and females include the hypothalamus, pituitary, gonads (testes in males, ovaries and oviduct in females), and crop. Relative locations of each tissue are shown on the diagram with lowercase letters representing each tissue. (**B**) These tissues were collected from breeding pairs at the following stages of the rock dove reproduction: nest building, where pairs were engaged in nest building behaviors but had not yet laid an egg; clutch completion (incubation day 3), three days after the first egg was laid and the onset of incubation, when the second egg is laid (completing the two-egg clutch; this population of rock doves had a one day gap between laying the 1st and 2nd eggs); mid-incubation (incubation day 9), nine days after the first egg was laid and the onset of incubation; hatch, the day of the first chick hatching; and early hatch, a manipulation group where eggs were removed on the eighth day of incubation and replaced with a young chick(s) to test the impact of external cues (offspring presence) on gene expression and circulating prolactin concentration.
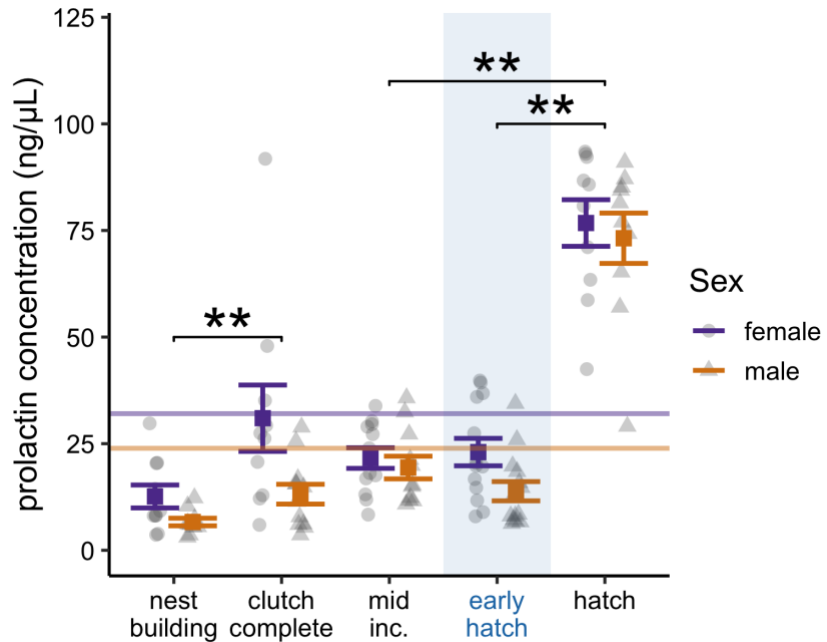
Figure 1.2. Plasma prolactin across reproductive stages. Prolactin plasma concentrations (ng/mL) of each stage for females (purple, triangles) and males (orange, circles). Means and standard errors are shown for each stage and sex. The mean value for each sex is shown as a horizontal line. Significant pairwise comparisons between stages are indicated (** $p < 0.01$; for a full list of *a priori* defined comparisons, see Table 1.1). Plasma prolactin data were originally presented in Austin et al (2021).
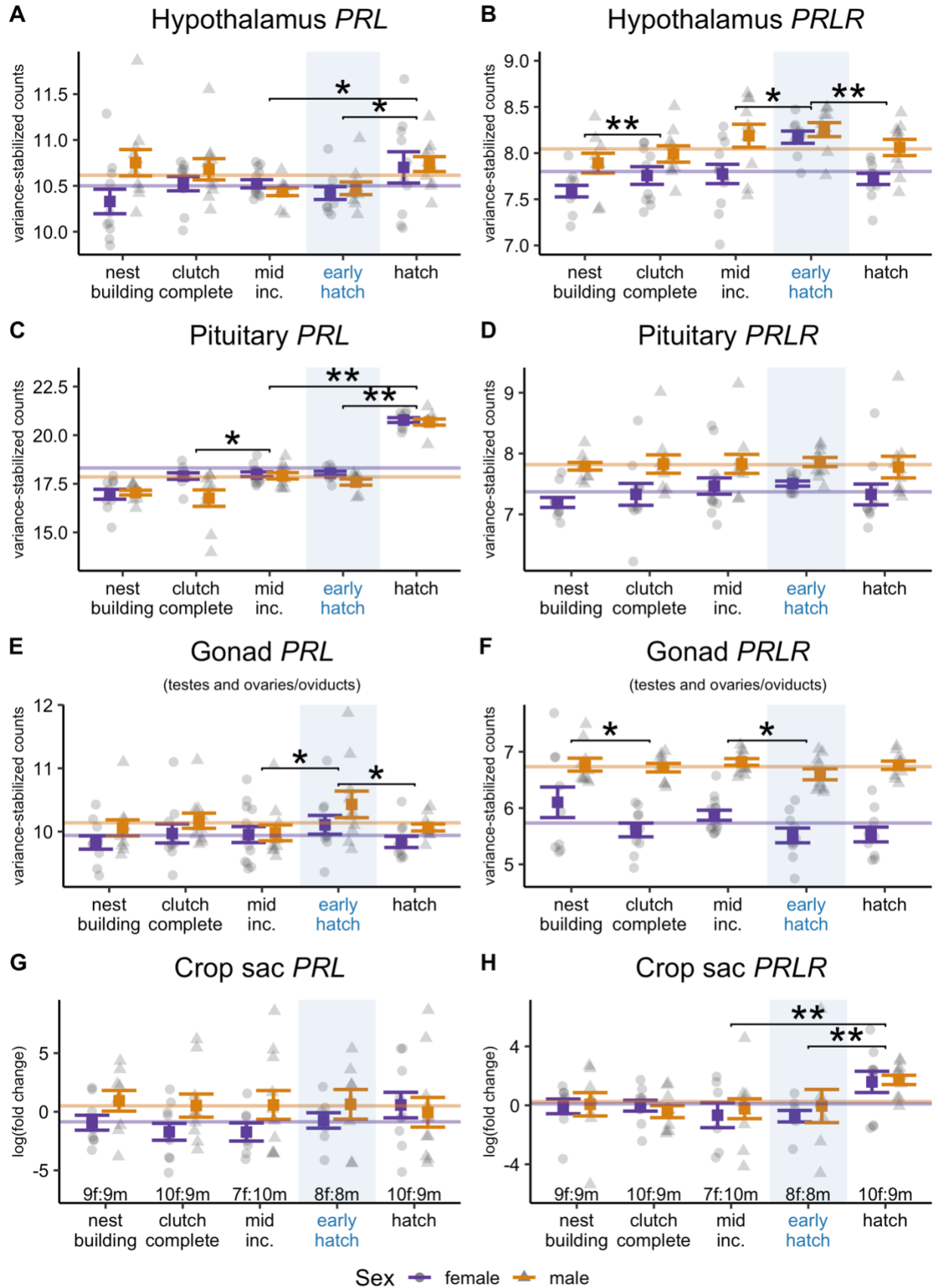
Figure 1.3. ***PRL and PRLR*** **gene expression across tissues.** *PRL* (left) and *PRLR (*right) expression, respectively, in the (**A-B**) hypothalamus, (**C-D**) pituitary, (**E-F**) gonads (testes and ovaries /oviducts), and (**G-H**) crop across reproductive stages. Early hatch, a manipulation group where we added chick(s) at mid-incubation, is highlighted in blue. Male (orange, triangles) and female (purple, circles) means and standard errors of the gene count mean (SEM) for each stage. The mean value for each sex is shown as a horizontal line. Significant pairwise comparisons between stages are indicated (* $p < 0.05$, ** $p < 0.01$; for a full list of *a priori* contrasts, see Table 1.1).
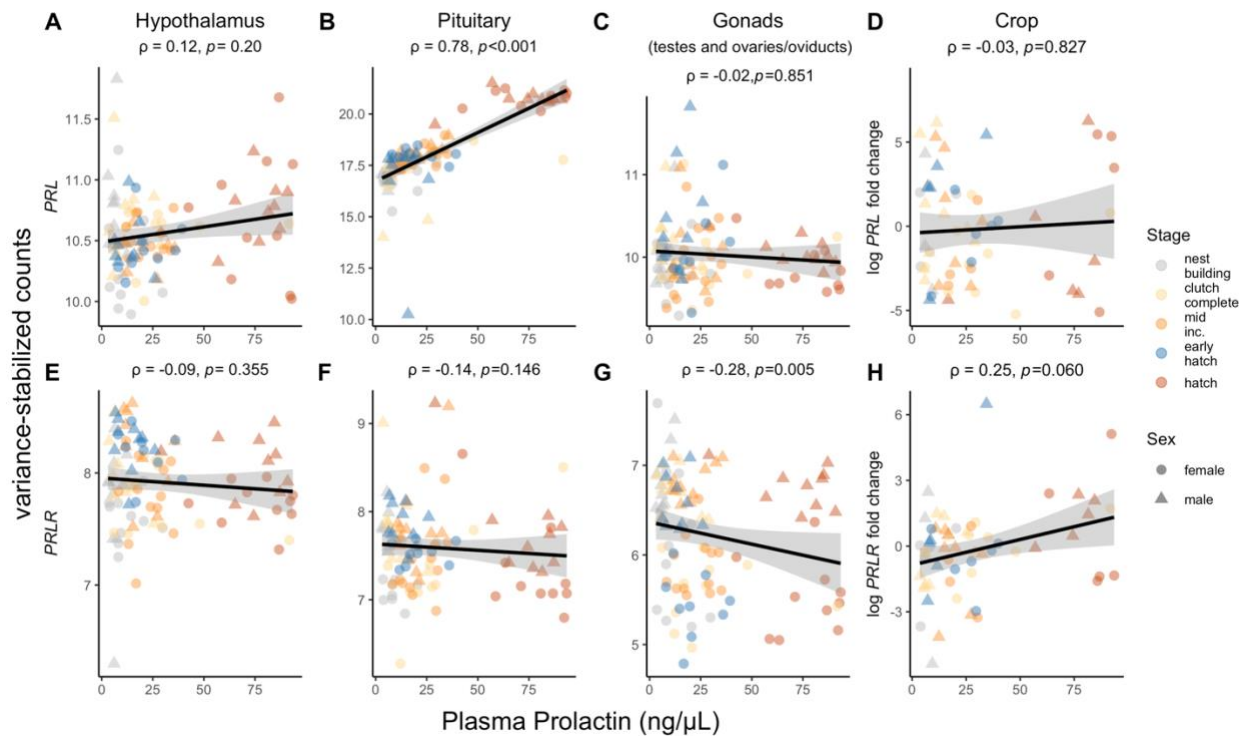
Figure 1.4. **Correlations between plasma prolactin concentrations and gene expression across tissues**. Correlations between plasma prolactin (as measured by RIA) and gene expression of (**A**) hypothalamic *PRL*, (**B**) pituitary *PRL*, (**C**) gonadal *PRL* (testes and ovaries/oviducts), (**D**) crop *PRL*, (**E**) hypothalamic *PRLR*, (**F**) pituitary *PRLR*, (**G**) gonad *PRLR* (testes and ovaries/oviducts), and (**H**) crop *PRLR* for each individual bird. Spearman's correlation coefficient (ρ) and *p*-values are shown for each gene-tissue combination. Gray shading around the line of best fit represents the 95% confidence interval. Point color corresponds to reproductive stage, and males and females are indicated with circles and triangles, respectively.

Contrasts	Circulating Prolactin	PRL Hypothalamus	Pituitary	Gonads (testes and ovaries/oviducts)	Crop	PRLR Hypothalamus	Pituitary	Gonads (testes and ovaries/oviducts)	Crop
COMPARISONS BY STAGE									
Reproductive stages compared with adjacent stage									
Clutch completion – nest building	11.94 ± 3.99, $p = 0.003$	n.s.	n.s.	n.s.	n.s.	0.21 ± 0.10, $p = 0.040$	n.s.	-0.27 ± 0.13, $p = 0.040$	n.s.
Mid-incubation – clutch completion	n.s.	n.s.	0.61 ± 0.30, $p = 0.043$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Hatch – mid-incubation	54.57 ± 3.95, $p < 0.001$	0.23 ± 0.10, $p = 0.026$	2.79 ± 0.30, $p < 0.001$	n.s.	n.s.	n.s.	n.s.	n.s.	2.08 ± 0.61, $p < 0.001$
Manipulation group compared with controls									
Early hatch – mid-incubation	n.s.	n.s.	n.s.	0.29 ± 0.13, $p = 0.022$	n.s.	0.24 ± 0.10, $p = 0.019$	n.s.	-0.29 ± 0.12, $p = 0.02$	n.s.
Early hatch – hatch	-56.55 ± 3.81, $p < 0.001$	-0.27 ± 0.10, $p = 0.011$	-3.28 ± 0.31, $p < 0.001$	0.32 ± 0.13, $p = 0.018$	n.s.	0.32 ± 0.10, $p = 0.002$	n.s.	n.s.	-2.05 ± 0.63, $p = 0.002$
COMPARISONS BY SEX									
Males - females	-7.86 ± 2.44, $p = 0.002$	n.s.	-0.47 ± 0.19, $p = 0.016$	0.20 ± 0.08, $p = 0.019$	1.38 ± 0.65, $p = 0.036$	0.22 ± 0.07, $p = 0.001$	0.44 ± 0.09, $p < 0.001$	1.00 ± 0.08, $p < 0.001$	n.s.

Table 1.1.1. Pairwise contrasts for circulating prolactin, and PRL and PRLR within each tissue.

Using a priori hypotheses, we developed contrasts to compare relevant transitions during parental care. We compared adjacent stages of reproduction and then compared the early hatch manipulation group with its equivalent control at mid-incubation and with concentration (circulating proalctin) /gene counts (PRL and PRLR) typically seen at natural hatch after 18 days of incubation. We also compared values between the sexes. Estimates ± standard errors are presented as A - B, where the estimate is group A minus group B. Only contrasts with p-values < 0.05 are shown. Comparisons where values increased in A relative to B are highlighted in yellow, where those where values decreased are in blue. For sex differences, purple indicates when values are higher in females and orange when values are higher in males.

Stage	Sex	HPG RNAseq data (n)	Crop (total) (n)	Crops without associated RNAseq data (n)
Nest building	F	10	10	4
	M	10	8	4
Clutch completion (incubation day 3)	F	10	10	1
	M	10	9	0
Mid-incubation (incubation day 9)	F	12	9	0
	M	10	10	2
Early Hatch manipulation (manipulation on incubation day 8)	F	10	8	0
	M	10	8	0
Hatching	F	10	10	4

	M	10	11	5

Table 1.1.2. **Sample sizes for tissues by stage and sex**. Total sample sizes (n) are shown for the HPG

RNAseq data (all individuals included had gene count data for all three tissues, the hypothalamus,

pituitary and gonads). Total crop sample sizes are shown by stage and sex. The majority of crops came

from individuals who also had HPG RNAseq data, except for 20 additional individuals that were included

to increase crop sample size. The number of additional crops that were collected separately from the

RNAseq study are shown in the right column.

Gene	NCBI Accession Number	Amplicon length (base pairs)	Efficiency (%)	Primer sequence (Forward and Reverse primers)
Prolactin receptor (*PRLR*)	NM_001282822.1	158	95.2	**F** - TCTTCCTTGCACACATGAAACC **R**- TCCAGGGTATGATTGACCAGT
Prolactin (*PRL*)	XM_005506024.2	181	92.6	**F** - GGCGGGTTCATACTGGTGAG **R** - TGGATTAGGCGGCACTTCAG
Beta actin (*ACTB*)	AB980793.1	147	95.5	**F** - TTAACCAACACCCACACCTT **R** - GACACCTTCACCGTTCCAGTT
Ribosomal	XM_005511196.1	78	105.4	**F** -

protein L4				GCCGGAAAGGGCAAAATGAG
(*rpL4*)				**R** - GCCGTTGTCCTCGTTGTAGA

Table 1.1.3. **Primers used in quantitative PCR**. All primers were designed using the NCBI Primer-BLAST tool using gene sequences specific to *Columba livia* (NCBI Accession numbers show the specific gene sequence from which the primers were designed). Replication efficiencies are based upon a standard curve of five 10-fold dilutions of purified gene product.

1.5. DISCUSSION

We characterized how circulating prolactin, *PRL* and *PRLR* gene expression in the HPG axis and crop varied across four reproductive stages (nest building, clutch completion, mid-incubation, and hatch) in both male and female rock doves. We then tested how externally manipulating the development period by adding offspring halfway through incubation influenced prolactin and HPG and crop tissue *PRL* and *PRLR* gene expression levels ˜24 hours later. This study thus provides a finer resolution into how prolactin gene expression changes across specific reproductive stages and within specific tissues important for parental care.

We found that circulating prolactin was lowest at nest building and highest after chicks hatch. Pituitary *PRL* gene expression mirrored this pattern, as expected. Hypothalamic *PRL* also increased at hatching. We did not observe significant differences in gonad or crop *PRL* expression across the reproductive stages measured. *PRLR* expression also did not differ across reproductive stages in the HPG or crop. However, some tissues showed significant increases in *PRLR* across specific transitions during parental care (such as from nest building to early incubation), though the overall effect size of these increases was relatively small, and the biological significance of these changes remains to be tested. We

also found significant sex differences in prolactin and *PRL/PRLR* gene expression. In response to offspring presence, we found no significant difference in circulating prolactin levels as compared to the mid-incubation control. However, chick presence significantly increased hypothalamic *PRLR* and decreased gonadal *PRL*. The early hatch manipulation did not affect pituitary or crop gene expression.

Characterization of PRL and PRLR expression across the HPG and crop

In the hypothalamus, a key regulatory center for reproductive and parental behavior, we found that *PRL* gene expression increased when chicks hatched. Prolactin can act upon hypothalamic nuclei, such as the preoptic area (POA), to regulate key parental behaviors in birds and mammals (Brown et al., 2017; Dobolyi et al., 2014; Slawski and Buntin, 1995). Prolactin can also physiologically coordinate parental care through actions on the hypothalamus, such as affecting overall HPG axis regulation via hypothalamic gonadotropin releasing hormone (GnRH) neurons (Grattan et al., 2007; Rozenboim et al., 1993), or regulating energy balance and hyperphagia through hypothalamic neuropeptide Y (Buntin et al., 1991; Lopez-Vicchi et al., 2020; Slawski and Buntin, 1995). In birds, both prolactin protein and gene expression, as well as prolactin binding and receptors have been identified in the hypothalamus (Buntin and Ruzycki, 1987; Buntin and Walsh, 1988; Chaiseha et al., 2012; Ramesh et al., 2000; Smiley et al., 2021). We found that *PRL* expression significantly changed from mid-incubation to hatching in the brain. This is consistent with rodent studies, where hypothalamic *PRL* mRNA also increased from pregnancy to lactation in female rats (Torner et al., 2004, 2002). Extra-pituitary *PRL* may play a role in regulating the stress hyporesponsiveness seen during maternal care (Torner et al., 2004), though it remains unclear whether hypothalamic *PRL* is actually translated into a functional protein. We thus extend previous studies characterizing hypothalamic *PRL* expression in the avian brain by showing that its expression changes during parental care.

We also found that hypothalamic *PRLR* increased from nest building to clutch completion. This increase in hypothalamic responsiveness to prolactin may facilitate incubation behavior (Buntin 1996).

25

Studies in birds have linked incubation behavior with increases in circulating prolactin (Angelier and

Chastel, 2009; Hope et al., 2020; Ramsey et al., 1985; Sockman et al., 2000). We found that plasma

prolactin increased from nest building to clutch completion (the third day of incubation in this species),

and that hypothalamic *PRLR* also increased during this transition. This increase suggests that behavioral

centers in the brain become more responsive to prolactin levels to facilitate incubation behavior. Previous

studies show intracerebroventricular injections of prolactin increased incubation in turkey hens

(Youngren et al 1991), but did not induce incubation in ring doves (Buntin and Tesch, 1985). In light of

our findings, it is possible that the isolated, non-breeding doves in Buntin and Tesch (1985) may have not

upregulated *PRLR* levels sufficiently to behaviorally respond to the injections of prolactin. Progesterone

and estradiol may also upregulate hypothalamic *PRLR* during this transition, as these hormones facilitate

incubation in doves (Michel, 1977; Silver, 1978). However, prolactin itself does not appear to upregulate

its receptor in the hypothalamus, as we found no significant correlation between hypothalamic *PRLR* and

plasma prolactin. This result differs from turkey hypothalamic *PRLR*, which was negatively correlated

with plasma prolactin (Zhou et al., 1996). Causal studies which manipulate prolactin or other hormones

involved in incubation behavior are needed to further understand drivers of hypothalamic *PRLR* across

this transition.

In the pituitary, we found that *PRL* gene expression mirrored plasma prolactin patterns, while

PRLR did not. Nearly all circulating prolactin originates from lactotroph cells in the anterior pituitary

(Freeman et al., 2000), thus, a correlation between pituitary *PRL* and plasma prolactin was expected. Like

plasma levels, pituitary *PRL* was lowest at nest building, rose at clutch completion/early incubation, and

peaked at hatch, consistent with other studies in doves and pigeons (Cheng and Burke, 1983; Dong et al.,

2012; Horseman and Buntin, 1995). Slight differences in pituitary *PRL* in comparison to plasma levels

may be due to different drivers for prolactin peptide secretion versus gene transcription. For instance, we

observed a significant increase in plasma prolactin at clutch completion, but no concordant significant

change in pituitary *PRL*. Stored prolactin peptide may have been released to facilitate the onset of

incubation, as prolactin has been shown to increase after the first egg is laid (when incubation begins in doves;(Cheng and Burke, 1983; Lea et al., 1986). Vasoactive intestinal peptide (VIP), a neuropeptide that stimulates the release of prolactin from the pituitary in birds (Macnamee et al., 1986; Vleck and Patrick, 1999) typically increases around incubation as well (Cloues et al., 1990) which may have caused prolactin release but not upregulation of *PRL*. Later, we find that pituitary *PRL* mRNA significantly increased from clutch completion to mid-incubation, but observed no change in plasma levels. This difference may be due to increased lactotroph recruitment in the pituitary (Pitts et al., 1994), which would lead to higher overall *PRL* transcription that may be stored for release later in incubation. Lastly, we found that *PRLR* did not change across the reproductive stages measured. While the role of the *PRLR* in the pituitary remains unclear, it may play a role in autocrine negative feedback (as seen in mammals; Ferraris et al., 2012). However, this potential role remains untested in birds.

In contrast, *PRL* and *PRLR* in the testes and ovaries/oviducts did not differ across the reproductive stages we measured. We found no significant changes in gonadal *PRL* in either sex, though *PRLR* increased in both the testes and ovaries/oviducts at clutch completion compared to nest building. Prolactin treatment has been shown to have an anti-gonadal effect in birds, leading to reduced gonad size (Meier, 1969; Meier et al., 1971) and sex steroid secretion (Camper and Burke, 1977; Reddy et al., 2002). In chickens, FSH, but not LH, increased ovarian *PRLR* (Hu et al., 2017). However, FSH has been shown to increase during nest building and decrease around ovulation / laying in doves (Cheng and Balthazart, 1982), which does not support that FSH may drive gonadal *PRLR*. This relationship, however, may differ across sexes and species. In male rats, for instance, FSH treatment decreased testicular *PRLR* expression in the Sertoli cells (Guillaumot and Benahmed, 1999). *PRLR* may play a role in spermatogenesis, as hyperprolactinemia reduces sperm count in mammals (Gill-Sharma, 2009), though such a relationship remains unstudied in birds. The increased gonadal *PRLR* in this study could indicate that *PRLR* regulates sex steroids, which are often higher before laying than during parental care in doves (Austin et al., 2021b; Dong et al., 2012; Feder et al., 1977). We did not find significant changes in estradiol or testosterone

between nest building and clutch completion (where gonadal *PRLR* increased) (Austin et al., 2021b), though progesterone levels fluctuate significantly as birds began incubation (Austin et al., 2021b). Increased prolactin responsiveness in the testes and ovaries/oviducts may possibly alter steroidogenic pathways and progesterone release, though this has not been tested. Clearly, more comparative research into the effects of prolactin on gametogenesis and steroidogenesis in the gonads is needed.

In the crop, *PRLR* expression patterns more closely mirrored plasma prolactin levels, though we found no variation in crop *PRL*. Like circulating prolactin and pituitary *PRL*, crop *PRLR* significantly increased at hatching, but did not differ across nest building and incubation. This pattern is consistent with crop weight changes across the dove breeding cycle, where crop thickness and weight peaks around hatching in conjunction with crop milk production (Cheng and Burke, 1983). As the crop is highly responsive to prolactin (Horseman and Buntin, 1995; Riddle and Braucher, 1931) and prolactin regulates its own binding in this tissue (Shani et al., 1981), our results reiterate that prolactin levels likely drive crop *PRLR* gene expression. Crop *PRLR* dynamics are consistent with mammalian mammary gland cells, where prolactin also upregulates *PRLR* expression (Bera et al., 1994; Swaminathan et al., 2008). While low, relative expression of *PRL* was detectable in both sexes. In mammals, autocrine ePRL plays a role in mammary gland differentiation and initiation of lactation (Chen et al., 2012), as well as in milk protein expression (Hennighausen et al., 1997). Unlike the mammary gland, the crop epithelium proliferates but does not differentiate (Gillespie et al., 2013); whether autocrine *PRL* plays a role in crop development remains unknown. Our results show that prolactin gene dynamics may be similar across convergently evolved organs for lactation, which opens the door for exciting comparative studies of "milk" production across species.

Effects of offspring presence on PRL and PRLR gene expression

In response to the early hatch manipulation, where chicks were added mid-incubation to examine response to offspring presence, neither circulating/plasma prolactin levels nor pituitary *PRL* expression

significantly changed compared to mid-incubation. Exposure to chicks increased plasma prolactin in previous studies (Buntin, 1979; Hansen, 1966; Lea and Vowles, 1985). In doves, chick exposure for four days in early or mid-incubation led to significant increases in crop weight, suggesting increased prolactin (as the crop is known to be highly prolactin-responsive) (Hansen, 1966). In parental doves deprived of their own young for 24 hours, pituitary reserves of prolactin decreased after just one hour of chick exposure, indicating prolactin was released into circulation from the pituitary (Buntin, 1979). However, we did not see an increase in plasma prolactin or pituitary PRL transcription after 24 hours of chick exposure. This lack of response may have occurred because our sampling time course ($\cong$24 hours after chicks were added) may have missed the window of any significant changes in prolactin. We may have missed an initial spike in prolactin release or transcription, as Buntin (1979) observed after one hour with chicks. Alternatively, 24 hours may have been not enough time to reliably upregulate *PRL* transcription or release. Secondly, it is possible that sufficient priming, either by hormonal secretion or internal rhythms during incubation, had not occurred. Indeed, 5 hours of offspring presence only stimulated prolactin release in non-breeding female ring doves that had been primed through estradiol and progesterone treatments (Lea and Vowles, 1985). In previous studies, doves were already in a chick-rearing state (deprived of their own chicks; Buntin, 1979) or had been given sufficient time to respond (i.e., more than one day; Hansen, 1966). Thus, if the manipulation had occurred later in incubation and closer to a natural hatch date, birds may have been more flexible in their ability to elevate prolactin in response to chick cues. Comparisons of our findings with a manipulation later in incubation could test this hypothesis.

Although plasma prolactin remained unchanged, hypothalamic *PRLR* increased when chicks were added, to levels significantly above those of mid-incubation or typical hatch. This increase suggests that neural responsiveness to prolactin may have increased to compensate for the typically low circulating prolactin at this stage and to facilitate a parental response to chicks. Indeed, parental behaviors can spontaneously occur without subsequent increases in prolactin (Wang & Buntin 1999), and we observed parents brooding and attempting to feed chicks during this manipulation (Austin et al., 2021b). This

behavior may have been facilitated by the increasing responsiveness to prolactin in hypothalamic nuclei like the POA, where prolactin is critical for chick feeding in doves (Buntin et al., 1991; Slawski and Buntin, 1995). Our findings also suggest that the hypothalamus may be able to respond more quickly to offspring cues than prolactin release from the pituitary, as plasma prolactin remained unchanged after the same period of chick exposure. Although we examined the hypothalamus as a whole, future examination of specific nuclei or cell-types could clarify where this *PRLR* response occurs.

We also observed significant upregulation of *PRL* and downregulation *PRLR* in both the testes and ovaries/oviducts. Like hypothalamic *PRLR*, gonadal expression of these genes differed from both the mid-incubation control and typical hatch. Studies show that sex steroids like estradiol or progesterone are required to exhibit parental behaviors in birds (El Halawani et al., 1986; Hutchison, 1975), including response to chicks (Lea and Vowles, 1985). As previously suggested, increased local *PRL* transcription could shift steroidogenic pathways to increase necessary sex steroid production and facilitate a parental response. However, this hypothesis is not supported by the fact that estradiol significantly decreased in females in this study when chicks were added mid-incubation, and testosterone remained unaffected (Austin et al., 2021b). Alternatively, altered prolactin regulation could play a role in a gonadal stress response, as this manipulation increased circulating corticosterone in this study compared to mid-incubation (Austin et al., 2021b). This hypothesis is not supported because gonadal *PRL* or *PRLR* transcription did not change in non-breeding rock doves after an acute stressor (Calisi et al., 2018), though this response may differ when animals are in a parental state. Lastly, it is unclear why *PRLR* would be downregulated, ostensibly reducing prolactin responsiveness in the testes and ovaries/oviducts. The gonadal response in *PRL* and *PRLR* could diverge because the two genes respond to different factors beyond prolactin, such as changes in other hormones or transcription factors that were affected during manipulation. These two genes do exhibit differential regulation in mammalian cells (Aoki et al., 2019; Featherstone et al., 2012), which if true in birds, could partially explain their opposing responses to chick

presence. Overall, the transcriptional response in reproductive tissues to offspring cues merits further study to understand its importance in parental behavior.

Sex differences in PRL and PRLR gene expression

In almost all tissues examined, we uncovered consistent patterns of sex differences in *PRL* and *PRLR* gene expression. We found that females had higher levels of plasma prolactin and pituitary *PRL* expression than males, but males expressed higher levels of *PRLR* than females in all tissues. These sex differences are consistent with other studies in biparental birds, where females also had higher plasma prolactin than males (Hector & Goldsmith, 1985, Vleck 1998). In mammals, higher prolactin levels in females are explained by estrogen-responsive elements in the *PRL* gene promoter (Maurer and Notides, 1987), though this mechanism remains unconfirmed in birds (Kurima et al., 1995). Interestingly, we found no significant sex differences in hypothalamic *PRL*, and gonadal *PRL* was more expressed in males than females. This result is consistent with the idea that gene regulation differs for extra-pituitary *PRL* compared to "dogmatic" pituitary *PRL*, which has been found in mammalian cell lines (Marano and Ben-Jonathan, 2014). Further studies are needed to determine whether autocrine extra-pituitary prolactin could compensate locally for sex differences in circulating prolactin of pituitary origin. Our finding that males had higher *PRLR* across all tissues differs from rodent studies, where *PRLR* expression or prolactin-binding is often lower in males than females in the brain (Cabrera-Reyes et al., 2015; Pi and Voogt, 2002; Salais-López et al., 2018). The mechanism by which male doves may upregulate *PRLR* remains unclear, though testosterone may play a possible role, as castration significantly reduces prolactin binding in the rat brain (Salais-López et al., 2018). Our findings highlight the need to study prolactin dynamics in both sexes, as most studies of *PRL/R* expression in birds to date only included one sex or did not compare sex differences (Buntin and Buntin, 2014; Chaiseha et al., 2012; Ramesh et al., 2000; Smiley et al., 2021, 2020)**.**

Together, our results support the hypothesis that different gene expression pathways can allow sexes to converge on a behavioral phenotype, preventing behavioral differences rather than promoting them (De Vries, 2004). A compensatory mechanism appears to be at play in our study, where females produced more hormonal signal (prolactin), but males increased downstream tissue responsiveness to that signal (via *PRLR*). This compensation may allow the sexes to exhibit a similar suite of parental behaviors despite sex differences in circulating prolactin levels. Indeed, several other bird species also exhibit higher prolactin levels in females, but both sexes show similar parental behaviors (Angelier et al., 2007; Angelier and Chastel, 2009). Sex differences in brain and peripheral *PRLR* may explain how similar parental behaviors can be maintained despite sex-biased differences in circulating prolactin. While much focus is on sex differences in behaviors or hormone levels, our results highlight the need to examine underlying mechanisms that may allow the sexes to converge to a similar phenotype (McCarthy and Konkle, 2005). Examining hormone and receptor dynamics in both sexes will be important to determine if this pattern occurs in other biparental species.

1.6. CONCLUSIONS

In summary, we report dynamic expression of prolactin and its receptor in various tissues important for reproduction and parental care, including the HPG endocrine axis and the crop. By examining specific stages of reproduction and parental care, we show that subtle changes in tissue-specific gene expression may help coordinate the overall response to prolactin and transitions between parental phenotypes. We show that *PRL* and *PRLR* gene expression in key tissues like the hypothalamus and gonads can respond to offspring cues even when plasma prolactin levels remain unaffected. Our results emphasize the need to examine how target tissues and endocrine axes transcriptomically respond to changing offspring stimuli, even in the absence of hormonal changes. Lastly, we uncovered consistent sex differences in prolactin regulation across the HPG axis, suggesting a compensatory mechanism by which the sexes may converge on similar parental behaviors in a biparental system. Future studies will be required to determine how regulation of these genes differs across tissues and the sexes, including

manipulations of hormones that may drive gene expression. Overall, this study shows that tissue- and sex-specific changes in local production or responsiveness to a hormone can occur across an endocrine axis to coordinate physiological and behavioral breeding transitions.

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CHAPTER 2

Prolactin maintains parental responses and alters reproductive axis gene expression, but not courtship behaviors, in both sexes of a biparental bird

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2.1. ABSTRACT

Prolactin, a hormone involved in vertebrate parental care, is hypothesized to inhibit reproductive hypothalamic-pituitary-gonadal (HPG) axis activity during parenting, thus maintaining investment in the current brood as opposed to new reproductive efforts. While prolactin underlies many parental behaviors in birds, its effects on other reproductive behaviors, such as courtship, remain unstudied. How prolactin affects neuropeptide and hormone receptor expression across the avian HPG axis also remains unknown. To address these questions, we administered ovine prolactin (oPRL) or a vehicle control to both sexes in experienced pairs of the biparental rock dove (*Columba livia*), after nest removal at the end of incubation. We found that oPRL promoted parental responses to novel chicks and stimulated crop growth compared to controls, consistent with other studies. However, we found that neither courtship behaviors, copulation rates nor pair maintenance differed with oPRL treatment. Across the HPG, we found oPRL had little effect on gene expression in hypothalamic nuclei, but increased expression of *FSHB* and hypothalamic hormone receptor genes in the pituitary. In the gonads, oPRL increased testes size and gonadotropin receptor expression, but did not affect ovarian state or small white follicle gene expression. However, the oviducts of oPRL-treated females were smaller and had lower estrogen receptor expression compared with controls. Our results highlight that some species, especially those that show multiple brooding, may be able to maintain mating behavior despite elevated prolactin. Thus, mechanisms may exist for prolactin to promote investment in parental care without concurrent inhibition of reproductive function or HPG axis activity.

Keywords: prolactin, parental care, mating, courtship, copulation, hypothalamus, pituitary, gonads, GnIH, birds, gene expression

2.2. INTRODUCTION

Animals that exhibit parental care must balance current reproductive efforts that prioritize care and provisioning of the current brood versus future reproductive opportunities, such as mating (Stearns, 1976). These reproductive transitions require physiological mediators (Ricklefs and Wikelski, 2002; Zera and Harshman, 2001), including endocrine mechanisms that are well-known to facilitate investment and resources in line with such life-history tradeoffs (Hau and Wingfield, 2011; Williams, 2012).

Prolactin, a hormone involved in parental care across vertebrates (Bachelot and Binart, 2007; Freeman et al., 2000), can mediate key transitions in reproductive investment and behavior. Prolactin (PRL) is a 23 kD peptide protein produced in the anterior pituitary with receptors expressed in nearly every tissue across the body, including the brain (Grattan and Bridges, 2009) and gonads (Harris et al., 2004). Best known for facilitating mammalian lactation, PRL also promotes parental motivation and care behaviors in both males and females of many vertebrates (Brown et al., 2017; Hashemian et al., 2016; Smiley, 2019). In order to facilitate reproductive transitions, PRL must interact with the hypothalamus-pituitary-gonadal (HPG) endocrine axis, which coordinates reproductive behaviors and physiology. In the HPG axis, kisspeptin in the hypothalamus stimulates gonadotropin-releasing hormone (GnRH) release onto the anteriory pituitary gland, which then releases luteinizing hormone (LH) and follicle-stimulating

hormone (FSH), stimulating the gonads to release sex steroids (e.g. progesterone, testosterone, estradiol). Additionally, gonadotropin-inhibitory hormone (GnIH) from the hypothalamus can inhibit the release of GnRH, LH and FSH in some vertebrates (Ubuka et al., 2013).

PRL is hypothesized to exert an "anti-gondal" effect on the HPG axis in multiple species. Lactational amenorrhea presents a classic example of this effect, where PRL inhibits ovulation during the energy-intensive period of milk production after pregnancy in mammals (Fourman and Fazeli, 2015). This anovulatory effect is mediated through the inhibition of kisspeptin in rodents (Brown et al 2019, Grattan 2018), which consequently reduces GnRH, gonadotropin and sex steroid release. In seasonally-breeding birds, PRL has been implicated in gonadal regression as birds transition into photorefractoriness (Dawson and Sharp, 1998; Sharp et al., 1998; Small et al., 2008), and a rich body of classic studies show exogenous PRL treatment can induce gonadal regression in birds (Bates et al., 1937; Meier, 1969; Tewary et al., 1983). Further, experiments in doves show that systemic and intracerebroventricular (icv) PRL injections, at levels akin to those circulating during parental care, maintain parental responses and reduce gonad size and LH plasma levels (Buntin et al., 1991, 1988; Buntin and Tesch, 1985; Janik and Buntin, 1985). The evidence for an inhibitory effect of PRL on the HPG axis has been connected to its possible role in photorefractoriness (Dawson and Sharp, 1998), clutch size regulation (Ryan et al., 2015; Sockman et al., 2000) and parental care (Angelier et al., 2016; Buntin, 1996).

In contrast, many other studies have not found evidence for such an anti-gonadal effect of PRL. For instance, classic studies in multiple bird species did not find any effect of PRL treatment on gonadal regression (house finches- Hamner 1968; quail - Jones 1969; sparrow *spp.* - Meier and Dusseau 1968, Laws and Farner 1960). Although an anti-gonadal effect seems clear in male doves, not all studies found changes in testes weight or LH with icv PRL treatment (Foreman et al., 1990). In domestic fowl, immunization against PRL, which reduces functional peptide levels, did not affect LH levels (Crisóstomo et al., 1998), and reduced laying rates (Li et al.,2011). Further, a pro-gonadal function appears in some seasonally-breeding mammals, such as hamsters and sheep, where PRL administration can actually

stimulate testes growth in periods of gonadal recrudescence (Bartke et al., 1980, 1975; Howell-Skalla et al., 2000; Sanford and Baker, 2010). This mixed literature indicates that PRL's effects on the HPG axis may be species and breeding context- specific.

Despite this mixed evidence, few studies have examined how non-parental reproductive behaviors respond to PRL, and how multiple HPG signals and receptors may mediate this relationship. Many studies have established PRL's role in parental behaviors (Brown et al., 2017; Buntin et al., 1991; Horseman and Buntin, 1995), but its influence, if any, on other reproductive behaviors, like courtship and mating, remains understudied. Most of these studies measured gonad size, and/or plasma hormone levels, such as LH or sex steroids. However, hormone receptor expression, which modulates tissue responsiveness to circulating signals, may also underlie any effect PRL has on the HPG axis. Further, no study to our knowledge has examined if there is a causal relationship between gonadotropin inhibitory hormone (GnIH, also known as RFRP-3) and PRL. GnIH has been shown to increase during transitions in parental care in starlings and rodents (Calisi et al., 2016), including at timepoints where PRL levels also rise in birds (Austin et al., 2021; Dawson and Sharp, 1998). Thus, GnIH and its receptor may be an important, but unexplored, HPG target of PRL.

To address these questions, we manipulated PRL levels and examined the effects on HPG axis gene expression and non-parental reproductive behaviors in a monogamous, biparental bird, the rock dove (*Columba livia*). Both male and female rock doves participate in all stages of offspring care, including nest building, egg incubation and chick provisioning (Abs, 1983). Both sexes also produce crop milk, a nutrient-rich substance which offspring depend exclusively on for the first few days of life (Davies, 1939). Like mammalian milk, crop milk production is also driven by PRL (Horseman and Buntin 1995). This avian model thus removes the confounding effects of female-only pregnancy and lactation seen in mammals, allowing us to compare sex-typical biases in HPG regulation and behavior. Lastly, we capitalized upon detailed ethograms of courtship behaviors developed for doves to quantify species-specific reproductive behaviors (Cheng, 1973; Goodwin, 1983, 1956; Lehrman, 1964).

Our study had three main aims: to examine the effect of PRL on 1) parental responsiveness, 2) non-parental reproductive behaviors, like courtship and copulation, and 3) gene expression of hormones and their receptors across the entire HPG axis, with a specific eye towards effects on GnIH. We administered exogenous ovine prolactin (oPRL) or a vehicle control to experienced breeding pairs of rock doves and then removed their nests, forcing birds from a parental state back to a courtship/mating state as they restart their nest efforts. We then recorded parental behaviors in response to a novel chick and observed naturally-occurring courtship behaviors. We also collected brain, pituitary and gonadal tissues to measure HPG gene expression. We hypothesized that oPRL would maintain the HPG in a parental state, favoring current reproductive effort and depressing gonadal activity that may promote future reproductive efforts. Thus, we predicted that oPRL-treated birds would exhibit more parental behaviors in response to a novel chick, would be more delayed in the progression of the dove courtship cycle, and would copulate less often than vehicle-treated pairs. We also predicted that oPRL treatment would reduce GnRH, LH and FSH expression in the hypothalamus and pituitary, increase GnIH expression, and alter gonadotropin receptor expression in the gonads.

2.3. METHODS

Hormone manipulation

To determine how PRL may causally drive transitions between parental and other reproductive behaviors, we treated breeding pairs of rock doves (*Columba livia*) with exogenous PRL during a transition where birds are switching between parental and mating behavior. On day 16 of the 17 day incubation period, we surgically implanted osmotic pumps (Model 2001, release rate: 1.0 µl/hr, Alzet, DURECT Corp.) containing either ovine PRL in 0.87% physiological saline (dose of 3.33 µg/hr, 80 µg/day; A.F. Parlow, National Hormone and Peptide Program) or vehicle (0.9% physiological saline;1.0 µl/hr, 24 µl/day) in both the male and female of the breeding pair. We did not measure circulating PRL because oPRL is not reliably detected by chicken PRL antibodies, and endogenous PRL would likely be

lower in oPRL-treated birds due to negative feedback (Z.Wang, F. Angelier *pers. comm.*). However, using the plasma concentration estimation described in Sockman et al., (2000), we estimate that this dose will lead to a plasma concentration of approximately 46.25 ng/mL in the average rock dove in our population. This concentration is on par with PRL levels observed in late incubation and during nestling rearing in previous studies of rock doves (Austin et al., 2021). These osmotic pumps reliably release 1.0 µl/hr for seven days (Alzet, DURECT Corp.), thus the experimental period lasted seven days (Figure 1). oPRL delivered through osmotic pumps has been shown to activate PRL receptors and signalling pathways in the brain of a closely-related species, the ring dove (Buntin and Buntin, 2014), and we replicated the osmotic pump model and dose used in that study.

Sixteen breeding pairs received pumps with oPRL (32 birds), while 12 received vehicle (24 birds), for a total of 56 birds. All breeding pairs used in this study were reproductively experienced and had raised at least one chick to fledging before the experiment. Birds were socially housed in a semi-natural environment, where they selected their own mate and nest box within a colonial aviary, which houses 10 - 12 other breeding pairs (see MacManes et al., 2017 for details).

To induce a shift from parental behaviors to re-mating, we removed eggs and hay from the nest box on incubation day 17, the day after surgical pump implantation and the last day of incubation before expected hatching (hatching typically occurs after 18 days of incubation). Eggs and hay were removed between 09:00-11:00 hours on incubation day 17. As rock doves breed nearly continuously in captivity (Johnston, 1992), egg removal ends the current nest effort and birds will court and re-mate to start a new clutch.

Behavior: Response to novel chick

As a proof of principle that PRL promotes parental behaviors and responses, we tested birds in both groups on their response to a novel chick after nest loss. On the fifth day of the seven-day

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experimental period (Fig. 2.1), we placed a young chick (average age: 6.6 days old) from another nest into the focal pair's nest box for approximately 120 minutes between 08:00 - 12:00, and video-recorded the behavioral response. To ensure that chicks elicited parental responses from subjects, we removed chicks from their home nests at least twelve hours before the behavioral trial to restrict them from feedings and induce begging behavior when placed in the subject's nest. Unlike previous studies in ring doves (Buntin et al., 1991; Wang and Buntin, 1999), we did not remove either sex during the behavioral trial, as to avoid introducing a stressor (due to loss of the mate or separation from the mate, or the stress of being isolated while the mate was tested). Instead, we recorded the response of both birds in the pair simultaneously during these trials. At the end of the live behavioral trial, we measured the change in weight of the chick compared to at the start of the trial (post-trial weight minus pre-trial weight / pre-trial weight), as an additional measure of feeding attempts by the focal subjects.

Video recordings were scored on BORIS v.7.9 (Friard and Gamba, 2016) by trained observers who were blind to treatment. We recorded the occurrence and duration of the following chick-oriented behaviors: a) entering and standing in the nest box while the chick is present, b) brooding the chick, by standing or squatting over the chick, with body-to-body contact, and c) feeding the chick, where a bird engaged in mouth-to-mouth regurgitation behaviors with the chick. We also recorded the duration of time spent aggressing the chick (vigorously, offensive pecking) if it occurred. For each pair, we averaged the time each bird spent expressing each behavior during the trial, and present this data as a percent of the total trial time recorded. Two trials were excluded from behavioral scoring because the video recording equipment failed to record or save the recording. Both of these excluded trials came from oPRL-treated pairs, but we still had chick weight data as a proxy for feeding attempts by these pairs. In total, we scored videos for 26 pairs (n = 14 oPRL, n = 12 vehicle).

To analyse these behavioral responses, we first compared the percent of pairs that expressed each of the four behaviors across treatment groups (i.e. oPRL vs vehicle) using pairwise chi-square tests. Then,

we compared whether the percent of time pairs expressed each behavior differed with treatment using pairwise t-tests.

Behavior: Courtship and reproductive behaviors

To examine how PRL may affect non-parental reproductive behaviors, we recorded occurrences of various courtship behaviors and copulations for a pre-selected subset of pairs. On day six of the seven day experiment (Fig. 2.1), we recorded video from two cameras, one placed facing the focal pair's nest box and one recording activity on the entire aviary cage floor. We recorded reproductive behaviors on day six of the seven day experimental period for two reasons. First, this timepoint was furthest in time from nest removal (and thus closest to any possible ovulation/egg laying event, if it occurs), and second, these behaviors would be the most temporally connected to gene expression as tissues were collected the morning of day seven. We recorded videos for eight oPRL-treated pairs (n = 16 birds) and seven vehicle-treated pairs (n = 14 birds) in total.

Nest box videos were used to measure species-specific courtship behaviors observed in doves, such as bow-coos, nest-coos, and nest building through hay manipulation (Cheng, 1973). Nest videos were recorded from 07:30 to 18:30 for all pairs, and 30-minute videos for scoring were sampled every 90 minutes, resulting in four hours (240 minutes) of total scored nest video per pair. Trained observers, blinded to treatment, scored videos in BORIS v.7.9 (Friard and Gamba, 2016) for the following dove courtship behaviors: a) bow-cooing in the nest, where the bird struts and turns in the nest box while cooing and stamping feet up and down (Goodwin, 1956)**, b)** nest-cooing or "nodding", where the bird takes a posture with head down and tail up and inflates the crop to coo (Cheng, 1973; Goodwin, 1956), c) wing-flipping, where the bird's head is low, tail is up and it gently flips or flicks the tips of its wings while nest-cooing (note: this behavior always occurs during nest cooing, but nest cooing can occur without wing-flipping Cheng, 1973; Miller and Miller, 1958), and d) nest-building, via hay manipulation, where a bird picks up a single hay stick into its bill, with or without bringing the hay to the nest (Cheng

and Balthazart, 1982; Miller and Miller, 1958). These courtship behaviors have been well-described in the stereotypical courtship progression of doves, including *Columba livia* (Cheng, 1992; Goodwin, 1983).

We also scored the following pair-maintenance behaviors: a) allopreening or "hetero-preening", where the bird preens its mate, typically around the head and neck (Miller and Miller, 1958)**,** b) allofeeding or billing, where a bird, typically the male, opens their bill towards the mate and feeds the mate similarly to how chicks would be fed (this usually occurs after a bout of allopreening) (Miller and Miller, 1958). These pair maintenance behaviors usually precede copulations, for which we scored the following: c) soliciting copulations, where the bird (typically female) squats down, slightly spreads wings and shoulders to facilitate a mount (also called the "sex crouch" - Miller and Miller 1958), and d) mounting, where a bird mounts another in a copulatory position (typically the male) (Goodwin, 1956; Miller and Miller, 1958)**.** All behavioral descriptions are consistent with Miller and Miller 1958, and were aligned to the representative courtship stage as described in Cheng 1992. All behaviors were scored as state events, with a start, end, and duration, except: nest building (hay manipulation), soliciting copulation and mounting, which were scored as point behaviors with only occurrences counted.

As most copulations occur outside the nest box (*pers.obs.*), we also scored aviary floor videos for copulation attempts. Aviary floor videos, which had a lower resolution, were recorded from 07:30 to 18:30 for all pairs, and all 11 hours of the floor video was scored to capture the daily copulation rate. Copulation attempts were scored by counting the occurrence of a) soliciting copulations, and b) mounting, as described above. Interestingly, reciprocal mountings, where males appear to "solicit" mountings by females, or females mount their mate, have been observed in rock doves (Goodwin, 1956)**,** and we observed both males and females mounting and soliciting. However, we only compared male mounts and female soliciting in our data analyses.

To analyse courtship and other reproductive behaviors, we sorted behaviors by the sex that performs them typically during the stereotyped dove courtship cycle (Cheng, 1992). We compared the

following behavior*sex combinations: male bow-coo, male nest-coo and wing-flip, female nest-coo and wing-flip, male billing, male mounting,and female soliciting copulations. We compared allopreening and nest-building in both sexes. For each behavior*sex combination, we then compared if the proportion of time spent exhibiting the behavior or occurrence (for state and point behaviors, respectively) differed between oPRL and vehicle-treated birds using pairwise t-tests.

Tissue Collection

Seven days after surgery (six days after nest removal), we euthanized birds using an overdose of isoflurane anesthetic followed by swift decapitation. Brains were removed and flash frozen on dry ice within 3 minutes of capture from the cage (as described in MacManes et al 2017). Trunk blood, pituitary gland, gonadal tissue, and crop sac tissue were flash-frozen and stored at -80°C for future gene expression analyses. All methods were approved under UC Davis IACUC protocol #20618.

During collection, the entire crop sac was removed from each bird, fat and crop milk removed, patted dry and weighed for wet weight before flash freezing. We also measured the weight of the ovaries and oviduct combined in females and both testes in males, the diameter and state of the largest ovarian follicle in females, and the length of the testes in males. Crop and gonad weights were normalized by dividing by overall bird body weight. We assessed normality of distributions using Shapiro-Wilks tests, and since all but testes length were significantly non-normal ($p < 0.05$), we compared crop and gonad sizes across treatment groups using the non-parametric Mann-Whitney U tests instead of t-tests.

Hypothalamic nuclei microdissection

To examine expression in specific nuclei of the hypothalamus, we microdissected the paraventricular nucleus (PVN) and preoptic area (POA) with 2 mm punches using a Leica CM1950 cryostat. We used nuclei landmarks described in Karstens & Hodos 1966 to isolate nuclei. Briefly, we started collecting the POA when the tractus septomesencephalicus (TSM) terminates at the bottom of the

brain, stopping when the TSM is no longer visible, and began collecting the PVN when the quintofrontal tracts appear until the optic tecta are visible (see Table 2.5 for details and Figure 4 for representative punches). Hypothalamic nuclei punches were weighed and then stored in 200 µL of TriSure reagent (BioLine) at -80°C until RNA extraction.

Quantitative PCR

We extracted RNA from the brain nuclei punches, whole pituitary glands, and gonad samples using TriSure (BioLine) along with a modified protocol of the Zymo Direct-zol RNA miniprep spin-column extraction kit (Zymo Scientific). For gonad analysis, we took a 10 mg sample from the midsection of the left testis, a 10 mg sample of small white follicles (pre-yolk deposition) from the ovary, and a 10 mg sample of the magnum region of the oviduct (Apperson et al., 2017). We measured total RNA quality using a Nanodrop ND-1000 spectrophotometer (ThermoScientific) and RNAs with 260/280 and 260/230 ratios > 1.8 were used in downstream analyses.

We then treated total RNA with a second round of DNase treatment using the Quanta Perfecta DNase kit (QuantaBio), and then converted 8 uL of DNase-treated RNA to complementary DNA (cDNA) using qScript cDNA Supermix (QuantaBio). We diluted cDNA 1:5 for quantitative PCR analyses.

We ran qPCR reactions for the following genes of interest: *GNRH1, GNIHR* in the POA, *GNIH* in the PVN, *AR, ESR1, ESR2, PRLR* in the POA and PVN, *CGA, FSH, GNIHR, GNRHR* in the pituitary, *AROM, FSHR, LHCGR and GNIH* in the testes and ovarian follicles, and *ESR1* and *ESR2* in the oviduct (see Table 2.6 for full gene names and primers). For each tissue, we measured the expression of HPG reproductive hormones or enzymes expressed in that tissue, as well as the relevant receptors to upstream hormones. We measured *GNIH* in the PVN as this is the main location of GnIH-expressing cell bodies in birds (Ubuka et al., 2013). GnIH neurons have been shown to project to GnRH-1 neurons in the POA and the pituitary gland via the median eminence (Ogawa and Parhar, 2014), thus we measured *GNIHR* in the

POA and the pituitary gland. As GnIH gene expression has also been measured in avian gonads (Bentley et al., 2017), including the rock dove (MacManes et al., 2017), we also measured GNIH in the testes and follicles. We also included *ACTB, GAPDH, HPRT1, RPL4* as reference genes, which have been shown to be reliable reference genes in avian tissues (Zinzow-Kramer et al., 2014).

We designed all primers used in this study to be specific to *C.livia* using the assembled Rock Dove transcriptome v2.10 (NCBI accession no. GCA_000337935.2). We validated primers by running a 10-fold serial dilution to calculate replication efficiencies (95.6 ± 1.12 %) and confirmed a single product through melt curve analysis (see Table 2.6 for accession numbers and primer efficiencies). We ran qPCR reactions in triplicate for each sample using the following mix: 1 µL cDNA template (diluted 1:5), 5 µL 2X SSOAdvanced SYBR Green PCR mix (BioRad), and 10 µM each of primer (total volume: 10 µL). 384-well qPCR plates were run on a CFX384 Touch Real-Time PCR detection system (BioRad) in the following thermocycling conditions: 50℃ for 2 min, 95℃ for 10 min, and then 40 cycles of 95℃ for 15 sec and 60°C for 30 sec. All samples for each tissue-gene combination were run on a single plate.

We calculated relative gene expression using the delta-delta-Ct method ($2^{-\Delta\Delta C}$;[t] Livak and Schmittgen, 2001). In this method, gene expression is first normalized to the geometric mean of two reference genes run for each sample (dCt). We used *HPRT1* and *GAPDH* as reference genes for hypothalamic nuclei, *HPRT1* and *RPL4* for pituitary glands,*ACTB* and *GAPDH* for ovarian follicles, and *ACTB* and *RPL4* for oviducts and testes. We confirmed each of these reference gene combinations to be stably expressed in each tissue (no effect of treatment, sex, or their interaction on reference gene Ct, Table 2.7). Normalized expression (dCt) was then expressed relative to average value for a "control" sample group, in this case vehicle-treated females (ddCt). Fold change equals $2^{(- ddCt)}$. We log-transformed fold changes for analysis.

For each tissue-gene combination, we ran independent general linear models (glms) comparing the effect of treatment (oPRL or vehicle), sex (for hypothalamic nuclei and the pituitary), and their

interaction on gene expression. For each gonad type (testes or ovaries), which are unique to one sex, glms only included treatment as an independent variable. We present ANOVA run on these glms. We ran each tissue-gene combination as a separate model because a) the ddCt method does not allow for direct comparisons between genes (Livak and Schmittgen, 2001), and b) each of these genes are regulated by different promoters and transcription factors, and are subject to different tissue-specific regulation, thus we considered their expression independently. Due to the number of linear models run, we adjusted p-values for ANOVA using Benjamini-Hochberg false discovery rate (FDR) correction for multiple comparisons (Benjamini and Hochberg, 1995). All statistical analyses were completed in R (v.4.0.3, R Core Team, 2020).

2.4. RESULTS

Parental behavior and crop weights

As a confirmation of PRL's effect on parental behavior, we found that oPRL treatment significantly increased the likelihood of exhibiting parental behaviors towards a novel chick after nest removal (Table 2. 1; Fig. 2.2A). Almost all oPRL-treated pairs brooded or fed the novel chick (Table 2. 1; 92.9% of pairs), while only one pair of vehicle-treated birds (8.3% of pairs) exhibited these responses to the chick. Further, oPRL significantly increased the average proportion of time birds spent brooding, and also led to a suggestive trend towards increased time spent feeding (Table 2. 1; as only one pair of vehicle-treated birds showed feeding, the feeding showed by this pair was lower than the average oPRL treated pair, 1.2% of the trial versus an average of 4.4% of the trial for oPRL pairs). Although we did not separate birds from their mates and pairs were tested together, we also examined the likelihiood of each sex to respond in the trial. Most of the response in oPRL-treated pairs was driven by females, as 12 oPRL females (85.7%) fed the chick at least once, compared to one vehicle female (8.3%) ($\chi^2 = 13.3, p < 0.01$). In males, five (35.7%) oPRL-treated males fed the chick at least once, compared with no vehicle-treated

males ($\chi^2 = 5.30$, $p = 0.021$). Both treatment groups were equally likely to enter the nest box, presumably to investigate the chick, though oPRL led to significantly more time spent in the nest box. Both groups were equally likely to exhibit aggressive behaviors towards the chick, and the average proportion of time spent showing these behaviors did not significantly differ (Table 2. 1).

As further evidence that PRL promoted chick feeding, we found that chicks placed in oPRL nests significantly gained more weight during the trial (Fig. 2.2B; average 9.79% weight increase in oPRL nests vs. 0.08% increase in vehicle nests, Cohen's $d = 1.24$, $t = 3.11$, $p = 0.005$). When crops were weighed at collection, oPRL-treated birds had significantly larger crops than vehicle-treated birds (Fig. 2.3A; 2.00 % of body weight in oPRL vs 0.68% in vehicle, Cohen's $d = 2.81$, $t = 12.20$, $p < 0.001$). All oPRL crops had clearly visible crop milk and evidence of thick cornification of the crop (Gillespie et al., 2013) compared to the thinner, less-vascularized crops of the vehicle-treated birds.

Courtship and reproductive behaviors

We found no significant effect of treatment on any of the stereotypical dove courtship behaviors (Figure 2C, Table 2. 2; male bow-coo, male nest-coo and wing-flip, female nest-coo and wing-flip, or nest building of either sex, all $p > 0.09$). Further, we found no significant effects of treatment on male or female allopreening bouts, or male-initiated allofeeding/billing, and all birds were observed allopreening their mate at least once (Table 2. 2). We observed male mounting in all pairs. There was no significant effect of treatment on copulation rates, measured either through male mounting or female solicitations (all $p > 0.1$, Table 2. 2).

Gonad morphology

We found a significant effect of treatment on both male and female gonad weights. Normalized testes weights were significantly higher in oPRL-treated birds than vehicle birds ($U = 117$, $p = 0.010$, Cohen's $d = 0.76$). Female normalized ovaries and oviduct weights were significantly lower in oPRL

birds than vehicle birds ($U = 30$, $p = 0.017$, Cohen's $d = -1.08$). However, the diameter of the largest ovarian follicle did not differ significantly with treatment ($U = 81.5$, $p = 0.516$, Cohen's $d = -0.08$); all females had large yolky follicles at collection. Similarly, there was no significant difference in testes length between treatment groups in males ($t = 0.11$, $p = 0.909$, Cohen's $d = 0.04$). No birds laid eggs during the experimental period, and no females were near laying at collection.

Hypothalamic gene expression

In the POA, we saw no significant effect of treatment or sex on *GNRH* or *GNIHR* expression (Table 2. 3; Fig. 2.4). Similarly, we saw no significant effect of treatment or sex on *GNIH* expression in the PVN (Table 2. 3; Fig. 2.4C). As for sex steroid receptors measured in both the POA and the PVN, there was no effect of treatment, sex, or nuclei on *AR* expression. *ESR1* tended to be expressed at lower levels in oPRL-treated birds compared to vehicle ($p = 0.04$), but after Benjamini-Hochberg corrections, this effect was no longer significant ($p_{adj} = 0.16$; Table 2.8) There was no effect of sex or nuclei on *ESR1* expression. *ESR2* did not significantly differ with treatment or sex, but the POA appeared to have marginally lower expression than the PVN ($p = 0.05$). However, this effect of nuclei did not persist past Benjamin-Hochberg correction ($p_{adj} = 0.21$; Table 2.8). We did not find any effects of treatment, sex or nuclei on *PRLR* expression (Table 2.8).

Pituitary gene expression

In the pituitary, we found a significant effect of treatment on *FSHB* ($p_{adj} < 0.01$, Cohen's $d = 0.99$) but not *CGA* ($p_{adj} > 0.05$; Table 2. 3; Fig. 2.5). In terms of receptors, treatment significantly affected *GNRHR* and *GNIHR* expression (Table 2. 3; Fig. 2.5), with oPRL-treated birds expressing higher levels of both receptors than vehicle birds (*GNRHR*, Cohen's $d = 0.77$; *GNIHR*, Cohen's $d = 0.85$).

Gonad gene expression

In the testes, we found a significant effect of treatment on *FSHR* and *LHCGR* expression, where oPRL-treated birds expressed higher levels of these receptors than those given vehicle (Table 2. 4; Fig. 2.6A; Cohen's *d* = 1.49 and 1.41, for *FSHR* and *LHCGR*,respectively). However, treatment had no significant effect on *FSHR* or *LHCGR* in the small white preovulatory follicles in the female ovary(Table 2. 4; Fig. 2.6B). There was no significant effect of treatment on *AROM* or *GNIH* expression in either the testes or the ovaries (Table 2. 4; Fig. 2.6).

In the oviducts, we found a significant effect of treatment on both estrogen receptors, *ESR1* and *ESR2*, in that oPRL treatment reduced expression of both genes compared to vehicle-treated birds (Table 2. 4; Fig. 2.6C; Cohen's d = -2.02 and -0.84 for *ESR1* and *ESR2*, respectively).
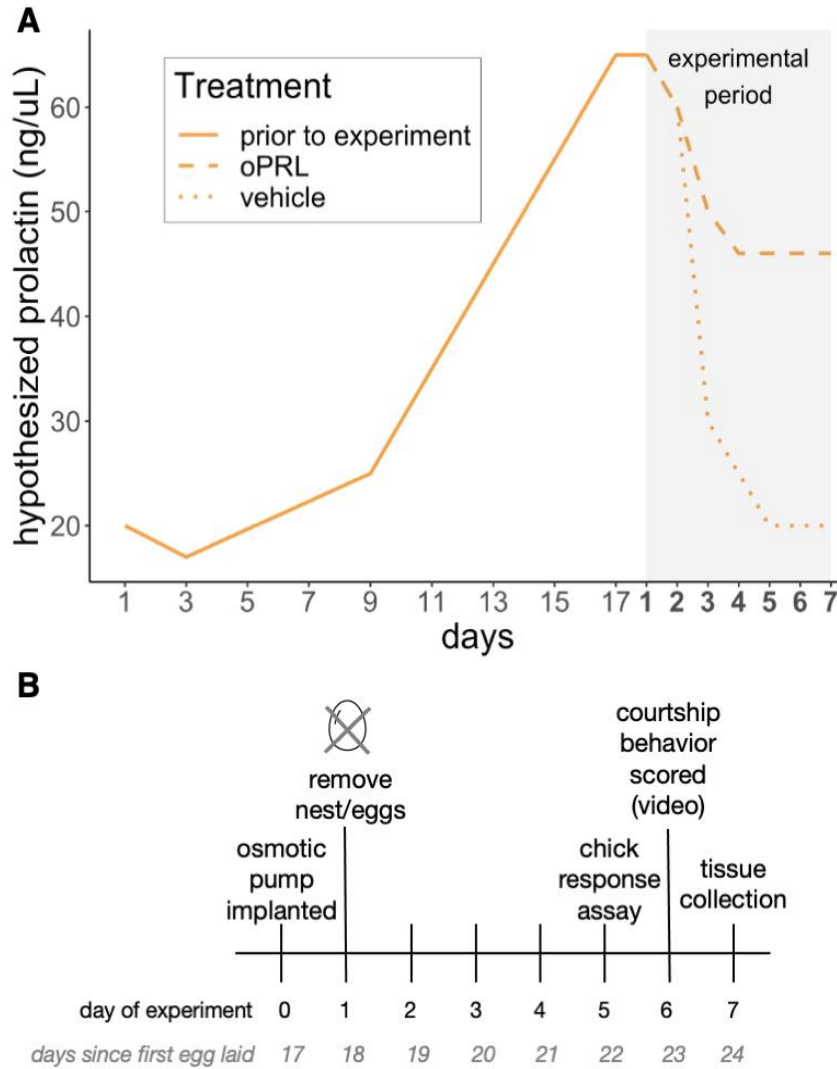
Figure 2.1. Experimental timeline. (A) Hypothesized concentrations of prolactin during the typical dove incubation period and as expected during the experimental period, both in the experimental treatment (oPRL, dashed) and control (vehicle, dotted) groups. Hormonal data during incubation based on data from Austin et al., 2021; Dong et al., 2012 and expected experimental values are drawn from patterns seen in ring doves (Lea & Sharp, 1991; Ramsey et al., 1985). The experimental period is shaded in gray. **(B)** Both male and female birds in a pair had osmotic pumps containing either ovine prolactin or vehicle implanted surgically on day 17 of incubation. The experiment began on the expected hatch date, incubation day 18, when eggs and nests were removed, and ended seven days later when tissues were collected. For full details, see Methods.
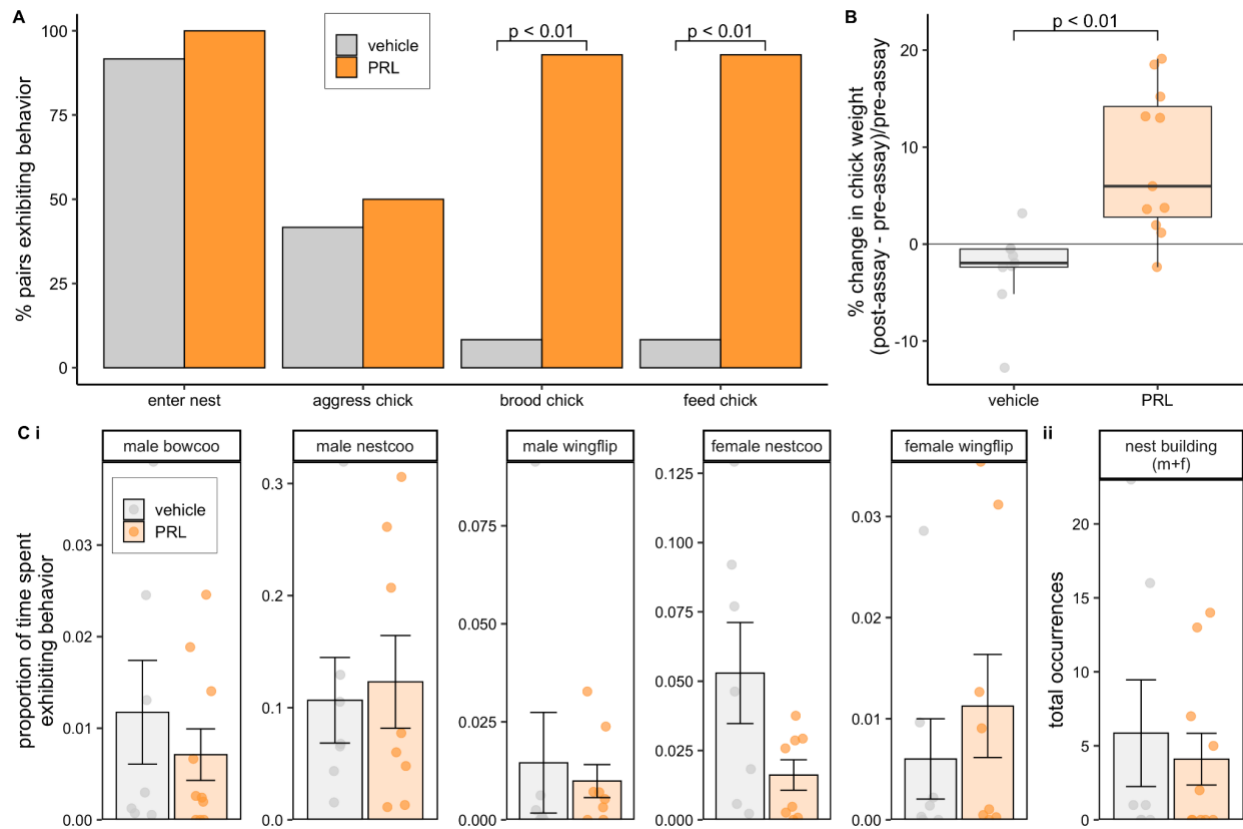
Figure 2.2. **Behavioral responses to a novel chick and observed reproductive behaviors.** After five days of experimental treatment (vehicle or oPRL), a novel chick was placed in each pair's home nest box. (**A**) The percent of pairs tested where at least one bird showed a behavioral response during this chick response assay is shown, as well as (**B**) the percent change in body weight of the stimulus chick, which is measured as post-assay body weight minus pre-assay body weight, divided by the pre-assay body weight. (**C**) After 6 days of treatment, courtship behaviors were observed, with the proportion of the observed time a bird exhibited the behavior (out of 1.0 max, or 100% of the observed time) shown for (**i**) state behaviors and (**ii**) the total number of occurrences shown for point behaviors. Means ± SEM are shown, and points represent pairs (A) or individual birds (B-C).
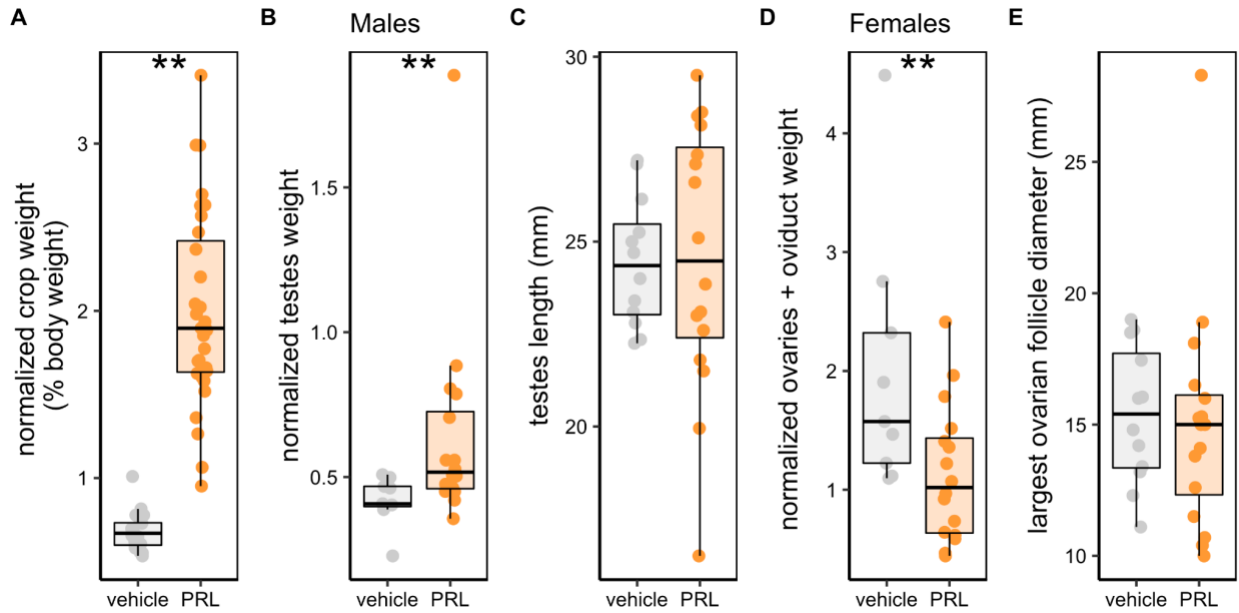
Figure 2.3. Measurements of crop and gonads in birds treated with oPRL or vehicle. (A) Crop sac weights, normalized to body weight (% of overall body weight), (**B**) male testes weight (normalized to body weight), (**C**) vertical length of testes (in millimeters), (**D**), female ovary and oviduct weights, weighed together and normalized to body weight, and **(E)** the diameter (mm) of the largest ovarian follicle (mm) were measured at tissue collection. ** signifies $p < 0.01$ on a Mann-Whitney non-parametric U test. Vehicle-treated birds are shown in gray, oPRL-treated birds in orange. Boxplots show median and first and third quartiles, and dots represent individual samples.
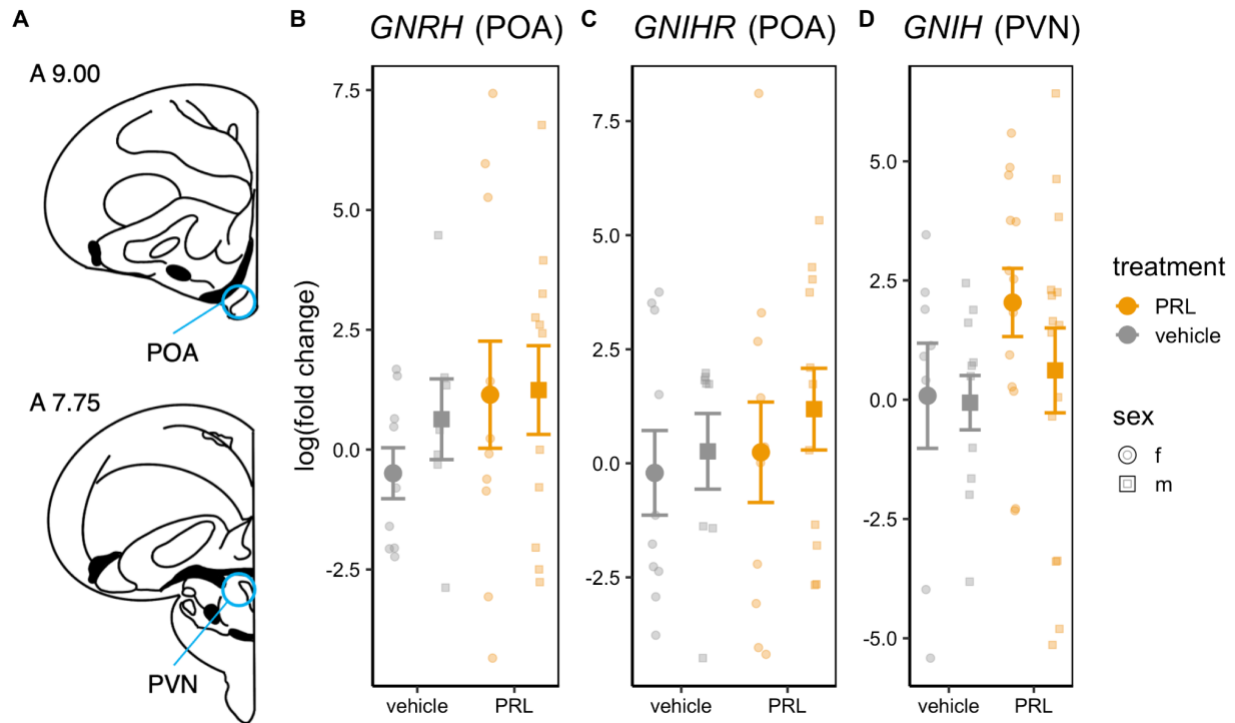
Figure 2.4. HPG gene expression in hypothalamic nuclei. (**A**) Genes of interest were measured in microdissected punches of the preoptic area (POA) and paraventricular nucleus (PVN) using the Karstens & Hodos (1966) atlas (representative coronal slices with atlas plate numbers are shown). (**B**) Gonadotropin releasing hormone (GNRH) expression and (**C**) gonadotropin inhibitory-hormone receptor (GnIH-R) expression were measured in the POA, and (**D**) GnIH itself was measured in the PVN, where GnIH-neuron cell bodies are found. Circles represent females and squares represent males. Mean and SEM are shown for each sex and treatment.
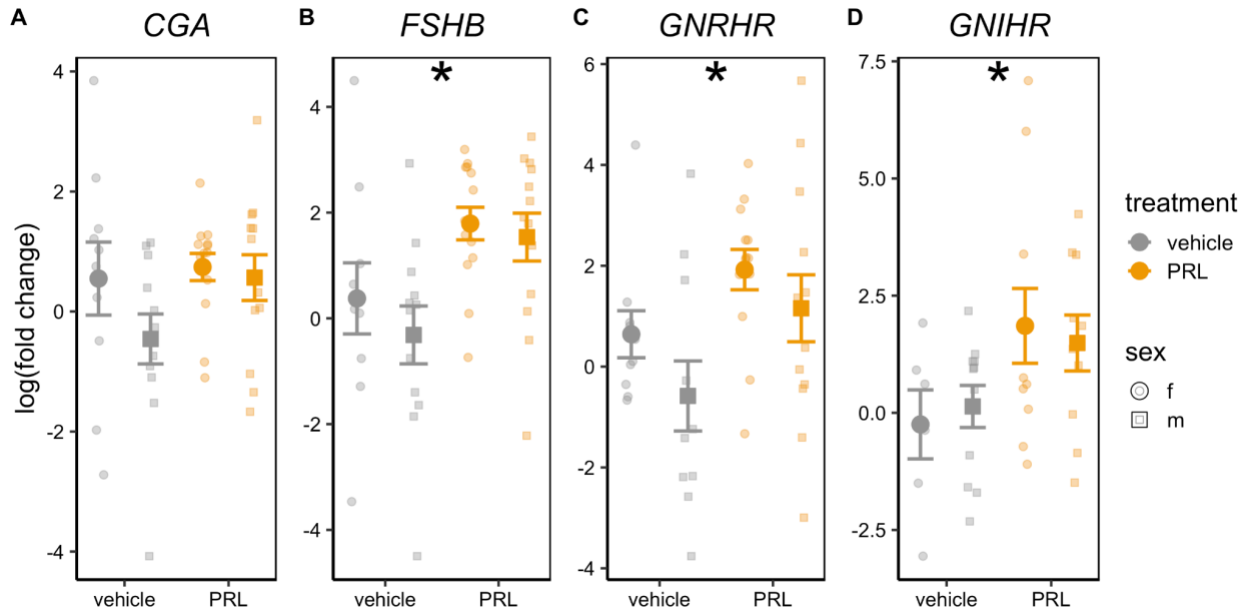
Figure 2.5. Pituitary gene expression. (**A**) Luteinizing hormone (or CGA), (**B**) follicle stimulating hormone b (FSH), (**C**) GnRH receptor (GnRH-R) and (**D**) GnIH receptor (GnIH-R) gene expression in the pituitary gland of males and females treated with vehicle or ovine PRL. Mean and SEM are shown for females and males in each treatment group, using circles and squares respectively. Small points represent individual birds. Asterisks (*) represent a significant main effect of treatment ($p_{adj} < 0.05$).
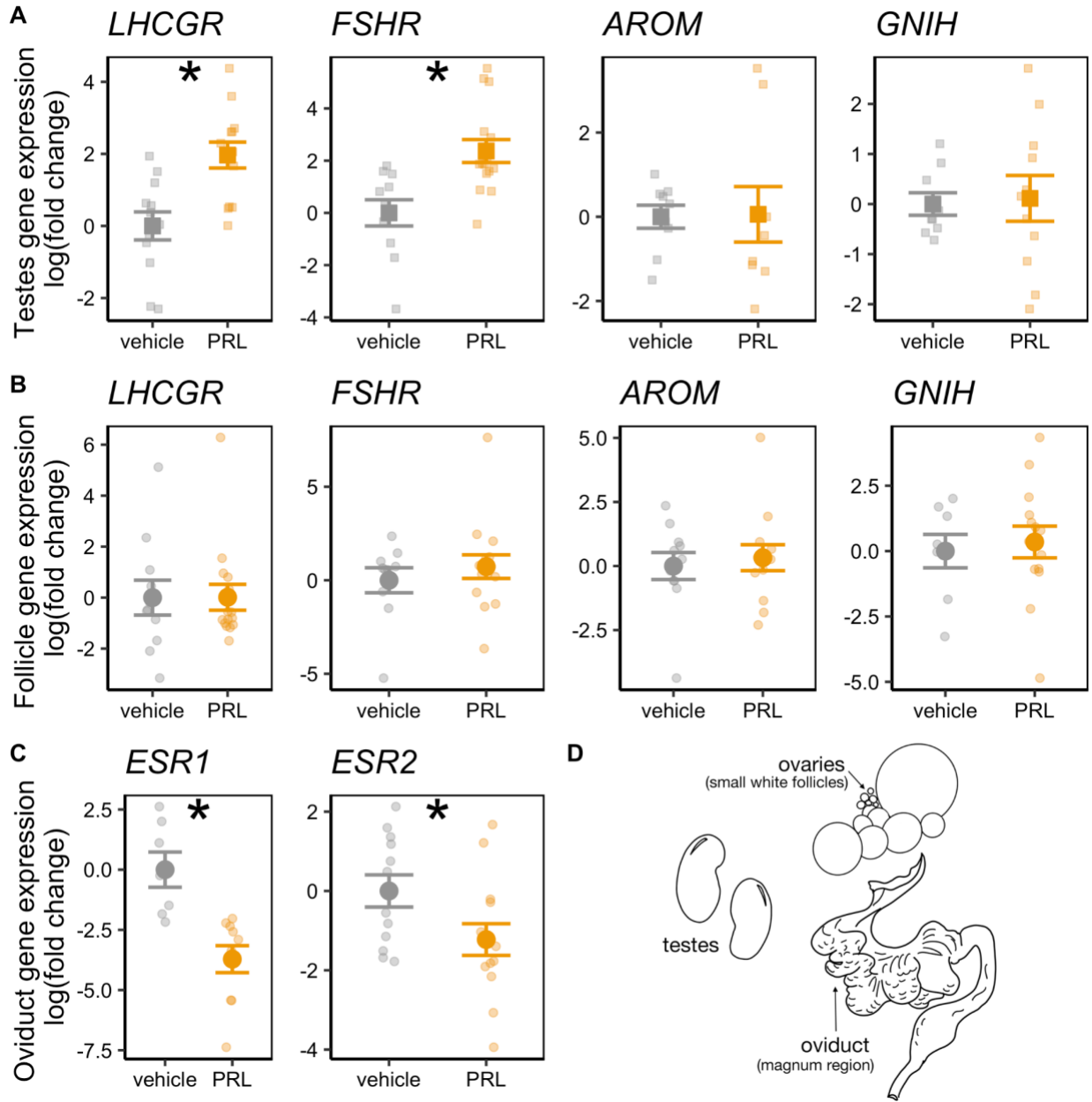
Figure 2.6. Gonadal gene expression. Relative expression of luteinizing hormone receptor (*LHCGR*), follicle stimulating hormone receptor (*FSHR*), aromatase (*AROM)* and gonadotropin-inhibitory hormone (*GNIH*) relative expression in (**A**) male testes and (**B**)small white ovarian follicles of females , as well as of (**C**) estrogen receptor 1 (*ESR1*) and 2 (*ESR2)* in female oviducts in birds treated with oPRL (orange) or

vehicle control (gray). Means and SEM are shown. Small points represent individual birds. Asterisks (*)

represent a significant main effect of treatment ($p_{adj} < 0.05$). Male tissues are shown with squares and

females with circles.

	Percent (number) of pairs exhibiting behavior					Proportion of time spent exhibiting behavior				
Behavior	Vehicle	PRL	χ^2	p	p_{adj}	Vehicle	PRL	t	p	p_{adj}
Enter / in nest box	91.67% (11)	100% (14)	0.006	0.937	0.976	0.155 ± 0.075	0.354 ± 0.051	2.198	0.040	0.073[†]
Aggress chick	41.67% (5)	50% (7)	0.001	0.976	0.976	0.017 ± 0.014	0.002 ± 0.001	-1.120	0.286	0.286
Brood chick	8.33% (1)	92.86% (13)	15.330	**<0.001**	**<0.001**	0.017*	0.196± 0.04	3.953	0.001	**0.004**
Feed chick	8.33% (1)	92.86% (13)	15.330	**<0.001**	**<0.001**	0.012*	0.044 ± 0.01	2.028	0.055	0.073[†]

Table 2.1. Behavioral responses to novel chick after five days of experimental treatment. The

percentage and number of pairs that exhibited the behavior at least once, as well as the average proportion

of the assay time (2 hours) those pairs spent exhibiting the behavior are listed. Proportions are listed out

of 1, and mean ± SEM are shown. Significant differences between treatment groups (indicated by chi-

squared tests for percentage and t-tests for proportions of time) are indicated in bold.

*As only one pair exhibited these behaviors, no standard error was calculated.

[†] represents a suggestive trend towards significance ($0.05 < p < 0.10$).

State behavior	Vehicle		Prolactin			
	Percent (number) of birds exhibiting behavior	*Proportion of time spent*	*Percent (number) of birds exhibiting behavior*	*Proportion of time spent*	*t*	*p*
Courtship behaviors						
Male bow-coo	100% (7)	0.012 ± 0.006	87.5% (7)	0.007 ± 0.003	-0.46	0.652
Male nestcoo	100% (7)	0.107 ± 0.038	100% (8)	0.123 ± 0.041	0.30	0.769
Male wingflip	71.4% (5)	0.015 ± 0.013	75% (6)	0.01 ± 0.004	-0.34	0.745
Female nestcoo	100% (7)	0.053 ± 0.018	87.5% (7)	0.016 ± 0.005	-1.93	0.095
Female wingflip	71.4% (5)	0.006 ± 0.004	87.5% (7)	0.011 ± 0.005	0.79	0.445
Pair-bond maintenance behaviors						
Allopreen (male-initiated)	100% (7)	0.018 ± 0.006	100% (8)	0.036 ± 0.01	1.64	0.125
Allopreen (female-initiated)	100% (7)	0.03 ± 0.011	100% (8)	0.031 ± 0.012	0.07	0.949
Male allofeed ("billing")	42.9% (3)	0 ± 0	62.5% (5)	0.002 ± 0.001	1.40	0.195

Point behavior	*Percent exhibiting behavior*	*Occurrences*	*Percent exhibiting behavior*	*Occurrences*	*t*	*p*
Female nest building	42.9% (3)	1.714 ± 1.392	50% (4)	1.333 ± 0.678	0.25	0.811
Male nest building	28.6% (2)	4.143 ± 2.694	37.5% (3)	2.083 ± 1.282	0.69	0.508
Copulation behaviors						
Mount (male)	100% (7)	2.933 ± 0.7	100% (8)	2.579 ± 0.953	0.30	0.766
Solicit copulations (female)	28.6% (2)	0.714 ± 0.474	50% (4)	1 ± 0.423	-0.45	0.660

Table 2.2. Reproductive behaviors expressed after six days of experimental treatment. Courtship and pair-bond behaviors were scored over four randomly sampled hours of nest video, and copulation behaviors scored from 11 hours of aviary floor video. Percentage and number of birds that exhibited the behavior at least once is shown for all behaviors. For state behaviors (behaviors with a clear beginning and end), average duration ($\pm$ SEM) is shown, and for point behaviors, average occurrences ($\pm$ SEM) are shown. Raw p values and p values adjusted using the Benjamini-Hochberg false-discovery rate method for t-tests comparing treatment groups are shown.

Tissue	Gene	df	Effect	β ±SE	F	p	p_{adj}
POA	*GNRH*	1,34	Treatment	1.64 ± 1.29	1.60	0.22	0.59
			Sex	1.13 ± 1.45	0.32	0.58	0.59
			Treatment*Sex	-1.03 ±1.90	0.29	0.59	0.59
	GNIHR	1,36	Treatment	0.452 ±1.34	0.54	0.47	0.70
			Sex	0.473 ±1.45	0.57	0.46	0.70
			Treatment*Sex	0.472 ±1.95	0.06	0.81	0.81
PVN	*GNIH*	1,43	Treatment	1.96 ±1.28	2.27	0.14	0.42
			Sex	-0.14 ±1.33	1.18	0.28	0.42
			Treatment*Sex	-1.28 ±1.71	0.56	0.46	0.46
Pituitary	*CGA*	1,45	Treatment	0.19 ±0.58	2.66	0.11	0.27
			Sex	-1.01 ±0.60	1.87	0.18	0.27
			Treatment*Sex	0.83 ±0.81	1.06	0.31	0.31
	FSHB	1,45	Treatment	1.42 ±0.70	11.72	**0.00**	**0.00**
			Sex	-0.69 ±0.73	0.86	0.36	0.54
			Treatment*Sex	0.43 ±0.98	0.20	0.66	0.66
	GNIHR	1,34	Treatment	2.10 ±1.03	6.41	**0.02**	**0.05**
			Sex	0.38 ±1.03	0.00	0.94	0.94
			Treatment*Sex	-0.75 ±1.36	0.30	0.59	0.88
	GNRHR	1,43	Treatment	1.28 ±0.83	7.13	**0.01**	**0.03**
			Sex	-1.22 ±0.86	2.87	0.10	0.15
			Treatment*Sex	0.46 ±1.15	0.16	0.69	0.69

Table 2.3. Linear models testing differences in relative gene expression as an effect of treatment, sex, and the interaction for hypothalamic nuclei and the pituitary. Estimates (β) and standard errors are shown in log(fold change), calculated using the Livak & Schmittgen (2001) method. Females treated

with vehicle were used as the reference group. Degrees of freedom for each component of the gene linear

model. *P*-values are adjusted using the Benjamin-Hochberg false discovery rate correction, significant

values ($p < 0.05$) are in bold.

Tissue	Gene	df	Independent Variable	β ±SE	F	p	p_{adj}
Testes	*AROM*	1,16	Treatment	0.06 ±0.71	0.01	0.94	0.94
	FSHR	1,23	Treatment	1.97 ±0.53	13.8	**0.001**	**0.003**
	GNIH	1,18	Treatment	0.11 ±0.55	0.04	0.84	0.94
	LHCGR	1,24	Treatment	2.37 ±0.67	12.5	**0.002**	**0.003**
Follicles	*AROM*	1,22	Treatment	0.32 ±0.74	0.20	0.66	0.95
	FSHR	1,24	Treatment	0.01 ±0.83	0.00	0.99	0.99
	GNIH	1,20	Treatment	0.35 ±0.94	0.14	0.72	0.95
	LHCGR	1,23	Treatment	0.73 ±0.95	0.59	0.45	0.95
Oviducts	*ESR1*	1,15	Treatment	-3.72 ±0.91	16.8	**0.001**	**0.002**
	ESR2	1,24	Treatment	-1.23 ±0.58	4.59	**0.04**	**0.04**

Table 2.4. Linear models testing differences in relative gene expression between treatment group in

gonadal tissues. Gene expression was measured in testes in males, and small white ovarian follicles and

oviducts in females. Estimates (β) and standard errors are shown in log(fold change), calculated using the

Livak & Schmittgen (2001) method. Vehicle-treated birds are used as a reference group. Degrees of

freedom for each component of the gene linear model. *P*-values are adjusted using the Benjamin-

Hochberg false discovery rate correction, significant values ($p < 0.05$) are in bold.

Nuclei	Start Plate	Start Landmarks	End Plate	End Landmarks	Punch diameter (mm)
Preoptic area (POA)	A 9.00	Tractus septomesencephalicus (TSM) extends to bottom of brain.	A 8.50	TSM no longer visible, cloudy Tractus quintofrontalis (QF) appears.	2
Paraventricular nucleus (PVN)*	A 8.25	QF apparent.	A 6.75	Tractus opticus (TrO) appears.	2

Table 2.6. Atlas plates and landmarks used to delineate hypothalamic nuclei. Plate numbers are referenced from the Karten & Hodos (1966) brain atlas. Landmark names are carried over from the terminology of that atlas. * The PVN is noted as Nucleus paraventricularis magnocellularis (PVM) in Karten & Hodos (1966). For full details on hypothalamic microdissection, see Methods.

Gene (Abbreviation)	NCBI Accession number	Amplicon length (base pairs)	Efficiency (%)	Primer sequence	Notes
Androgen receptor (*AR*)	XM_005509361.1	156		F GCTCTTCTTCAGCATCATTCC R ACCTTGGTGAGCTGGTAAAA	
Aromatase (*AROM*)	XM_021297872.1	125	95.6	F TGATGATTGCTGCTCCCGAC R ATCTCTGTCACCCATAACAGTCTC	
Chorionic gonadotropin alpha (*CGA*)	XM_005499841.2	169	99.9	F ACAAGGGAGACAGATCATGGA R CGCTCCTGGCTTGGAAAAGA	Equivalent to luteinizing hormone (LH)
Estrogen receptor 1 (*ESR1*)	XM_013368702.2	82		F CCTGTGTCATGTGATCCCTCC R TGGCAGTCCACATTGATCCC	Also known as ER-alpha
Estrogen receptor 2 (*ESR2*)	NM_001282841.1	139	100.6	F GGGAATGATGAAATGTGGCTC R GATCTCTTTTACGCGGGTTG	Also known as ER-beta
Follicle stimulating hormone (*FSH*)	FJ913876.1	140	91.2	F AGTGAAGATCCCTGGTTGCG R TGAAGGAACAGTAGGACGGC	
Follicle stimulating hormone receptor (*FSHR*)	XM_005498409.3	150	97.1	F TGCGTGTTTGCTGATGTTCC R AGGACAAATCTCAGTTCGGTGG	
Gonadotropin inhibitory hormone (*GNIH*)	XM_005513478.1	144	96.3	F AAGGTATCACACACAGGCTTGG R TAGTCTTCATTTCCCTGGTTCA	Gene name in NCBI: Neuropeptide VF precursor (*NPVF*)
Gonadotropin releasing hormone 1 (*GNRH1*)	XM_005513520.3	105	94.1	F GAAGTGCAGAAGAGCGAATG R AATCTTCCGTCTGGCTTCTC	
Gonadotropin-releasing hormone receptor (*GNRHR*)	XM_013369955.2	133	96.1	F GGCACGAGACCCTCTACAAC R TGTGAGGAGAAGAGGCTGGA	
Gonadotropin-inhibitory hormone receptor, or RFamide-related peptide receptor (*GNIHR*)	AB193127.1	119	99.4	F CTGGACACTGACGCTGCTGA R GGTTGGCACTGCTGTTGAAG	Also known as RFamide-related peptide receptor (*RFRPR*) or neuropeptide FF receptor 1 (*NPFFR1*)
Luteinizing hormone choriogonadotropic hormone receptor (*LHCGR*)	XM_021287698.1	167	90	F CCAGATGTCCTGGATGTTTCTT R CAGTGGCTGGGATACGTTAGA	Also known as Lutropin-choriogonadotropic hormone receptor
Reference genes					
Beta actin (*ACTB*)	XM_005504502.2	107	95.8	F ATGTGGATCAGCAAGCAGGAG R CATTTCATCACAAGGGTGTGGG	
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	NM_001282835.1	99		F AGCAATGCTTCCTGCACTAC R CTGTCTTCTGTGTGGCTGTG	
Hypoxanthine phosphoribosyltransferase 1 (*HPRT1*)	XM_005500563.2	150	94.72	F GCCCCATCGTCATACGCTTT R GGGGCAGCAATAGTCGGTAG	
Ribosomal protein L4 (*RPL4*)	XM_005511196.1	78	105.4	F GCCGGAAAGGGCAAAATGAG R GCCGTTGTCCTCGTTGTAGA	

Table 2.7. Primers used in quantitative PCR.

	POA	PVN	Pituitary	Testes	Ovarian Follicles	Oviducts
Treatment	1.14 (0.292)	1.57 (0.216)	0.04 (0.851)	1.01 (0.323)	1.21 (0.282)	0.10 (0.944)
Sex	0.24 (0.627)	1.76 (0.191)	0.40 (0.530)			
Treatment*Sex	0.13 (0.718)	0.184 (0.670)	0.58 (0.449)			

Table 2.8. **Reference gene stability across treatment, sex, and their interaction for each tissue**. For each tissue, we ran ANOVA upon general linear models of the form: *mean reference gene Ct ~ treatment*sex*, except for gonadal tissues and oviducts, where only treatment was included as an independent variable. *F* statistics from these ANOVA for each factor are shown, along with *p* values in parentheses. No reference gene combination showed significant differences across treatment, sex, or their interaction for any tissue, illustrating that reference genes were indeed stable in these tissues.

Tissue	Gene	Independent Variable	β ± SE	F	p	p_{adj}
Hypothalamus (POA & PVN)	*AR*	Treatment	-0.89 ± 0.81	1.41	0.24	0.61
		Sex	-0.55 ± 0.81	0.27	0.61	0.61
		Nuclei	-0.50 ± 0.62	0.70	0.41	0.61
		Treatment*Sex	0.56 ± 1.07	0.27	0.61	0.61
	ESR1	Treatment	-0.79 ± 0.83	4.49	0.04	0.16
		Sex	0.37 ± 0.84	0.00	0.99	0.99
		Nuclei	-0.52 ± 0.66	0.60	0.44	0.76
		Treatment*Sex	-0.65 ± 1.13	0.33	0.57	0.76
	ESR2	Treatment	-0.39 ± 0.65	0.76	0.38	0.77
		Sex	-0.001 ± 0.68	0.001	0.98	0.98
		Nuclei	-0.86 ± 0.44	3.87	0.05	0.21
		Treatment*Sex	0.05 ± 0.89	0.003	0.95	0.98
	PRLR	Treatment	-1.21 ± 0.51	1.46	0.23	0.76
		Sex	0.88 ± 0.52	0.79	0.38	0.76
		Nuclei	0.08 ± 0.40	0.01	0.93	0.93
		Treatment*Sex	-0.08 ± 0.69	0.01	0.93	0.93

Table 2.9. Linear models testing differences in hypothalamic sex steroid and prolactin receptors (measured in both hypothalamic nuclei). Estimates (β) and standard errors are shown in log(fold change), calculated using the Livak & Schmittgen (2001) method. Vehicle-treated birds are used as a reference group. Degrees of freedom for each component of the gene linear model. P-values are adjusted using the Benjamin-Hochberg false discovery rate correction

2.5. DISCUSSION

We found that rock dove pairs given ovine prolactin (oPRL) maintained parental responses after nest loss, but courtship behaviors and copulation rates were not affected compared to vehicle controls. Hypothalamic gene expression was relatively unaffected by oPRL treatment, but we found increased *FSHB* and hypothalamic hormone receptor expression in the pituitary. The gonads showed sex specific responses, where testes increased in size and gonadotropin receptor expression, while ovaries remained unaffected and oviduct size and estrogen receptor expression decreased. To our knowledge, our study is the first to measure the effect of PRL on courtship and copulation and integrate these behaviors with HPG-wide gene expression.

Effects on parental and reproductive behaviors

We found that oPRL maintained a parental phenotype, as it increased the likelihood birds would show parental responses towards a novel chick (i.e. feeding, brooding) after nest loss. Supporting this finding, chicks given to oPRL-treated pairs gained weight during the trial (presumably due to regurgitation of crop milk, water or food), whereas chicks in vehicle-treated nests did not. Further, oPRL treatment led to increased crop sac weights, with visible crop milk production, a classic indicator of PRL bioactivity (Lebovic and Nicoll, 1992; Riddle and Braucher, 1931). Together, these results confirm that systemic oPRL administration through the osmotic pumps did bind the avian PRL receptor, leading to crop sac growth and milk production, as well as affected expression of parental behaviors. Our results are consistent with previous studies in ring doves, where systemic PRL administration increased regurgitation feeding rate and chick weight gain in reproductively-experienced doves (Buntin et al., 1991; Wang and Buntin, 1999).

Unlike parental responses, however, courtship and copulation behaviors remained unaffected by PRL treatment. The typical courtship cycle in doves consists of male bow coo to attract their mates' attention, male nest cooing in potential nest sites, female nest cooing if she accepts the nest site, then both

males and females participate in nest building before oviposition (Cheng, 1992; Goodwin, 1983). Pairs cycle through these behaviors in a reliable sequence over a few days (Lehrman, 1964). We found no significant differences between the treatment groups in any of these behaviors, though there was a weak trend suggesting oPRL-treated females may express lower nest cooing. All birds, regardless of treatment, appeared to express courtship behaviors and be at similar courtship stages, with male nest cooing being the dominant behavior for most pairs. We similarly observed no differences in copulation rates. Copulation rates in our study align with those observed in established ring dove pairs allowed to copulate for short periods before ovulation (Cheng et al., 1981). Females in both groups solicited copulations, a behavior coordinated by both GnRH and estrogen actions (Cheng et al., 1981; Gibson and Cheng, 1979). However, female ring doves can exhibit copulatory behaviors even when ovariectomized, showing that ovarian hormones are not necessarily required for this behavior (Cheng, 1973).

The similar courtship and copulation patterns between groups suggests that PRL manipulation did not affect these reproductive behaviors in established pairs. There are several possible explanations. First, our oPRL dose may not have been high enough to inhibit or abolish non-parental reproductive behaviors. Our expected oPRL dose, using calculations described in Sockman et al.(2000), approximates levels seen during mid-chick-rearing in our population (Austin et al., 2021). The mid- to late- chick rearing period can include clutch overlap in rock doves, especially in captive conditions and with experienced pairs (Burley, 1980). Thus, oPRL levels administered may have been similar to a period where mating and courtship co-occur with parental care. Second, stimulatory environmental cues, such as a mate presence, nestbox and nesting material, may have overridden any inhibitory effects of PRL, especially if levels were not supraphysiological. Indeed, mate access and photostimulation lead to nest building and courtship behaviors, as well as increase in gonadotropin release in many bird species, including doves (Cheng and Balthazart, 1982; Lehrman et al., 1961; Shields et al., 1989). Previous studies showing anti-gonadal effects of PRL in doves were conducted in isolation, with birds removed from mates and conspecifics (Buntin et al., 1991, 1988; Foreman et al., 1990). Lastly, courtship and copulation may be relatively

unaffected by manipulation of a single hormone in intact animals. Indeed, estradiol implants did not affect courtship and breeding cycle in wild lapland longspurs (Hunt and Wingfield, 2004), and testosterone implants did not affect courtship in captive ring doves (Fusani and Hutchison, 2003). Future studies that manipulate PRL in different doses or in conjunction with other hormones could tease apart these possibilities.

Effects on hypothalamic gene expression

In the hypothalamus, we found oPRL did not significantly alter GnRH, GnIH, GnRH-R or sex steroid receptor expression. Although not in support of our initial hypothesis, this result is consistent with previous studies in our rock dove population, where hypothalamic gene expression shows fewer significant changes compared to other tissues during parental care (Harris, 2020; Harris et al., 2020). The absence of differences in reproductive behaviors may offer one explanation; mate presence and participation in courtship may have normalized hypothalamic gene expression. For instance, just one to two hours of courtship can increase POA GnRH activity in male doves (Mantei et al., 2008). Our birds interacted continuously for seven days, which may have normalized GnRH expression despite any effects of oPRL. Alternatively, oPRL treatment may have downregulated prolactin responsiveness via *PRLR*, thus reducing the mechanism by which oPRL would alter reproductive gene expression. However, we did not find support for this hypothesis, as *PRLR* did not differ with treatment in either nuclei.

Another possible explanation is that oPRL did not reach the brain or cross the blood brain barrier in our birds given the peripheral placement of osmotic pumps. However, various lines of evidence support that oPRL likely reached the brain. We replicated methods used in the ring dove that showed oPRL treatment led to higher phosphorylated STAT5, a secondary-signalling protein downstream of the PRL receptor, in the PVN and POA (Buntin & Buntin 2014). PRL binding sites have also been well-characterized in the dove brain (Buntin and Ruzycki, 1987; Fechner and Buntin, 1989), and autoradiography experiments show that radiolabeled PRL can cross the blood-brain barrier via the

choroid plexus in doves (Buntin and Walsh, 1988) as it does in rodents (Walsh et al., 1987). We also found behavioral differences in parental response that are most likely mediated in part by PRL actions on the brain, though perhaps not through the HPG axis (Grattan and Bridges, 2009).

Despite this evidence, it remains possible that only a portion of the systemic dose reached the brian. Previous studies showing anti-gonadal effects in doves used intracerebroventricular injections to separate out causal neural mechanisms (Buntin et al., 1991, 1988; Buntin and Tesch, 1985). Buntin and Tesch (1985) found that i.c.v. oPRL injections had similar effects on the gonads as the higher doses in previous peripheral administrations (Janik and Buntin, 1985). Even so, not all i.c.v. injections led to gonadal changes or altered gonadotropin release in doves (Foreman et al., 1990). Nonetheless, our methods mimic the physiological release of PRL into the periphery from the pituitary, and we found clear evidence for peripheral effects in our study. At the most conservatively, we can thus interpret PRL actions on the pituitary, gonads and behavior even if only low doses ultimately affected neural gene expression. Direct manipulations of neural PRL are needed to ultimately clarify if the changes in HPG gene expression we observed are mediated through the hypothalamus.

Effects on pituitary gene expression

In the pituitary, we found significantly increased expression of hypothalamic hormone receptors (*GnRH-R* and *GnIH-R*) and *FSHB* with oPRL treatment. The fact that *FSHB* expression increased, rather than was inhibited, by oPRL was surprising and did not support our original hypothesis. Gonadotropins can be stimulated by salient breeding stimuli, such as presence of nest boxes or a reproductively-receptive mate (Cheng and Balthazart, 1982; Cheng, 1977; Cheng and Follett, 1976; Shields et al., 1989; Silver et al., 1980). In birds, as in mammals, FSH serves to stimulate steroidogenesis and hierarchical development in ovarian follicles (Johnson, 2015) and testes (Deviche et al., 2011). In our study, courtship behaviors were maintained and gonadal size was either maintained or increased with oPRL treatment. Increased *FSHB* expression, combined with increased GnRH receptors, may act as an underlying mechanism to

compensate for any potentially inhibitory effects of PRL and maintain reproductive function. Lastly, lack of changes in LH (via *CGA* expression) is consistent with some previous studies, where immunization against PRL or VIP did not affect LH levels (Crisóstomo et al., 1998; Li et al., 2011). LH levels may thus be less sensitive than FSH to PRL manipulation. However, these hypotheses require further testing, as we did not directly measure gonadotropin release.

Effects on GnIH and GnIH-R expression

A specific aim of this study was to examine the potential relationship between PRL and expression of GnIH and its receptor across the HPG axis. Previous studies show GnIH can increase during transitions in parental care where PRL also rises, such as after hatch or birth in starlings and rodents, respectively (Calisi et al., 2016). While one study shows that GnIH administration does not affect ovine pituitary *PRL* gene expression *in vitro* (Sari et al., 2009)*,* no studies have examined the causal relationship between PRL and GnIH expression to our knowledge. As GnIH inhibits the HPG axis during acute stress and seasonal transitions (Calisi et al., 2011, 2008; Kirby et al., 2009), we hypothesized GnIH may also play a role in any inhibitory effect of PRL on avian reproductive function.

We found that hypothalamic and gonadal *GNIH* expression were unaffected by oPRL treatment, but pituitary *GNIHR* increased in both sexes given oPRL. Classically, hypothalamic GnIH inhibits gonadotropin release by inhibiting GnRH synthesis and release from PVN neurons (Tsutsui and Bentley, 2009; Ubuka et al., 2013), but GnIH can also directly inhibit gonadotropin release by acting on pituitary receptors (Ciccone et al., 2004; Clarke et al., 2008). Locally-expressed gonadal GnIH may also affect reproductive function (Bentley et al., 2017). Our results suggest that oPRL does not affect the HPG axis via hypothalamic or gonadal *GNIH* expression when PRL levels are equivalent to those at mid-chick rearing. Increased *GNIHR* in the pituitary may affect HPG axis regulation (Maddineni et al., 2008), but we did not observe any concordant decrease in gonadotropin expression. It remains possible that PRL manipulation generally dysregulated HPG axis gene expression in a way that would be detrimental to

fitness (Bonier and Cox, 2020). However, GnIH neuropeptide translation or release could also have been possibly affected by oPRL treatment, or higher oPRL levels than our dose are required to affect GnIH expression. Further studies examining neural protein levels and/or administering oPRL at doses at the higher end of the physiological range could more definitively determine if the GnIH system is indeed independent of PRL.

Effects on gonads and gonadal gene expression

In the gonads, we found sex-specific differences in morphology as well as gene expression. We observed slightly *increased* testes size, which stands against the purported "anti-gonadal" role of PRL and contrasts with previous studies in doves (Janik and Buntin, 1985). Design differences may explain this discrepancy; males in our study stayed with their mates in a colonial aviary setting, while birds were isolated in previous studies. Indeed, access to females promotes gonad growth and maintenance in male cowbirds (Dufty and Wingfield, 1986), and spermatogenesis and testosterone release in male starlings (Pinxten et al., 2003; Schwab and Lott, 1969). The presence of mates and the maintenance of courtship behaviors may have led to increased testes size in some males to compensate for any effects of PRL on their own or their mates' reproductive axis. These differences highlight the influence of environmental stimuli, and the importance of studying hormone regulation in a semi-natural environment (Calisi and Bentley, 2009) in addition to highly-controlled conditions.

Further, gene expression in the testes was consistent with increased testes size. We found oPRL increased *FSHR* and *LHCGR* expression in the testes, suggesting increased responsiveness to gonadotropins in males. LH acts upon Leydig cells to promote androgen synthesis, and FSH stimulates spermatogenesis (Deviche et al., 2011). However, treatments with FSH have been shown to stimulate larger testes growth and development than treatments of LH alone (though both led to growth) (Brown et al., 1975; Deviche et al., 2011). FSH treatment also reduced the inhibitory effect of pharmacological doses of PRL on the testes, and when combined with a lower dose of PRL, actually led to testes growth in

non-breeding pigeons (Bates et al., 1937). Taken together with increased pituitary *FSHB* expression, increased *LHCGR* and *FSHR* in oPRL-treated testes is consistent with testes stimulation and the hypothesis that birds may be compensating for any "anti-gonadal effects" of PRL treatment to maintain reproductive behaviors.

In females, oPRL-treatment did not affect ovarian state, but significantly decreased oviduct weight. We found no differences in the largest ovarian follicle diameter between groups, as all birds had a distinct follicular hierarchy with large yolky follicles. PRL has been associated with the end of lay and ovulation in birds (Ryan et al., 2015; Sockman et al., 2000), but other studies show that PRL manipulation does not inhibit or delay laying rates or ovulation (Li et al., 2011; Opel and Proudman, 1984). In ovaries, FSH leads to the development of the follicular hierarchy and release of sex steroids, while LH stimulates ovulation and steroidogenesis (Johnson, 2015; Mishra et al., 2020). The increased pituitary *FSHB* expression we observed may thus have allowed ovarian state to be maintained in oPRL-treated females. Further, ovarian state may have also been maintained by environmental cues in the social aviary setting. As in males, exposure to a mate, courtship and nesting material can also stimulate gonadotropin release and ovarian development in females (Barfield, 1971; Cheng, 1974; Lehrman et al., 1961). Male presence alone can stimulate female doves, as even castrated males, who do not typically show courtship behaviors,can still induce ovulation if prior ovarian development has occurred (Cheng, 1974). Female nest coos themselves can stimulate ovarian activity (Cheng, 1992). As we observed male courtship behaviors and female nest coos in both treatment groups, the interaction of these behaviors and stimuli may have helped maintain ovarian state in the face of oPRL.

Concordantly, we found no differences in gonadotropin receptor or aromatase expression in the small white follicles. PRL can inhibit ovarian steroidogenesis in birds (Camper and Burke, 1977) and in ovaries *in vitro* (Zadworny et al., 1989; but see Hammond et al., 1982; Hrabia et al., 2004). PRL-inhibited steroidogenesis may occur through reduced aromatase expression, specifically in small white follicles (Tabibzadeh et al., 1995); we did not observe this. However, reduced estradiol release and aromatase

expression only occurred after 14 days of oPRL treatment in Tabibzadeh et al. 1995, and was not observed after eight days of oPRL (the timeline that aligns more with our study). Additionally, oPRL doses used in these studies may have been higher than ours. Physiological doses of oPRL do not always affect ovarian estradiol and progesterone release *in vitro* (Hammond et al., 1982; Hrabia et al., 2004), suggesting that high doses may be required to inhibit steroidogenesis and affect ovarian gene expression. Although we did not directly measure estradiol levels here, we expect estradiol likely was unaffected given the similar ovarian state and gene expression between treatment groups. However, oPRL may have altered steroidogenesis through other enzymatic pathways and steroid hormone measurements would be required to address this hypothesis.

In contrast, oPRL treatment reduced oviduct size and oviduct estrogen receptor gene expression. Typically, oviduct weight and ovarian follicle state are strongly correlated in birds (Barfield, 1971; Hutchison, 1974). The developed follicular hierarchy releases estradiol, which stimulates the magnum region of the oviduct to grow, differentiate, distend and produce albumin in preparation for laying (Johnson, 2015). Estrogen is more important for this process than gonadotropins, which regulate more of ovarian development (Mishra et al., 2020). Thus, reduced estrogen responsiveness (via reduced *ESR1* and *ESR2* expression) in oPRL-treated females corresponds with observed reductions in oviduct weight. One possibility for the apparent disconnect between ovarian state and oviduct weight may be PRL responsiveness. The oviduct may differ in PRL responsiveness across breeding in a way that the ovaries do not.

Despite recent studies of the oviduct transcriptome (Jeong et al., 2012; Yin et al., 2020), however, links between oviduct gene expression and PRL remain unexplored. Lastly, we cannot rule out the possibility that hormone manipulation disconnected oviduct development from ovarian state, dysregulating the HPG axis in a way that would reduce fitness (Bonier and Cox, 2020).

Conclusions: Maintaining reproductive state in the face of elevated prolactin

Overall, we found support that PRL manipulation can maintain parental care, but found only mixed support for the hypothesis that PRL acts "anti-gonadally" and inhibits HPG axis regulation. Our results contribute to a mixed literature, where some field and laboratory studies found PRL treatment inhibited gonadal function (Bates et al., 1937; Buntin et al., 1991, 1988, p. 19; Buntin and Tesch, 1985; Janik and Buntin, 1985; Meier, 1969), where others did not (Foreman et al., 1990; Hamner, 1968; Jones, 1969; Li et al., 2011; Meier and Dusseau, 1968; Opel and Proudman, 1984).

One hypothesized reason we did not observe a strong anti-gonadal effect of PRL may be due to the multiple brooding strategy seen in doves and other species. Many birds engage in clutch overlap when resources are available during the breeding season (Burley, 1980; Westmoreland et al., 1986), including seasonal breeders (Grüebler and Naef-Daenzer, 2010; Morton, 2002; Stępniewski and Halupka, 2018; Walsh and Bock, 1997). This reproductive strategy results in co-occurrence of mating (to start a new clutch) and parental care of the still-dependent chicks. A similar phenomenon occurs in some rodents, where females enter a postpartum estrus shortly after birth and lactate pups while pregnant (Connor and Davis, 1980; Roy and Wynne-Edwards, 1995). During multiple brooding, PRL may continue to promote parental behaviors while also occurring at levels low enough to leave gonadal function uninhibited. HPG activity may also naturally increase as PRL declines. In fact, the oPRL dose we used approximates levels observed at nine-days post-hatch in rock doves (Austin et al.,2021), a period where clutch overlap often occurs in experienced pairs in our population (*pers. obs.*). These PRL levels, lower than those seen around late-incubation/hatch, may have allowed both courtship and parental responses to be maintained. Thus, PRL's effects on the HPG axis appear to depend on the breeding cycle context, even within a species, and this context may be represented by circulating levels. As PRL has often been studied as animals transition out of breeding, such as into seasonal photorefractoriness or molt (e.g., Sharp et al., 1998), subtle shifts in PRL responsiveness *during* breeding may change how this hormone mediates reproductive transitions, especially in species where multiple-brooding occurs.

Taken together, our results support the idea that mating effort and parental care are not mutually-exclusive (Stiver and Alonzo, 2009), and that proximate mechanisms involved in parental care, like prolactin, may not always inhibit other reproductive functions. When we consider contexts where parental and mating behaviors co-occur, such as during multiple brooding, we may find subtle shifts in HPG axis regulation not seen at other breeding stages or in hormone profiles alone. With this in mind, our study highlights the importance of studying hormonal effects on signal production and responsiveness across the entire HPG axis to understand the complex picture of reproductive regulation during breeding transitions.

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CHAPTER 3

Effects of parental experience and age on expression of prolactin, vasoactive intestinal peptide and their receptors in a biparental bird (*Columba livia*)

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3.1. ABSTRACT

As animals gain parental experience, they often show more rapid and efficient parental care responses that likely lead to increased offspring survival and fitness. Changes in circulating hormones that underlie reproductive behaviors, including prolactin, have been found to correlate with parental experience in birds and mammals. Altered responsiveness to prolactin in key behavioral centers of the brain may also underlie the effects of experience on parental behaviors. Further, experience may also affect responsiveness to prolactin stimulatory hormones, such as hypothalamic vasoactive intestinal peptide (VIP). While experience has been shown to upregulate neural prolactin receptors and responsiveness in rodents, its effects on prolactin receptor gene expression remain unstudied in birds. To address this, we examined gene expression of pituitary prolactin, hypothalamic prolactin receptors in the preoptic area, hypothalamic VIP, and pituitary VIP receptors in both sexes of the biparental rock dove (Columba livia) when birds were not actively nesting. As age and parental experience are often confounded (experienced parents also tend to be older than their inexperienced counterparts), we measured gene expression in birds of varying combinations of age (0.5 - 3 years) and prior reproductive experience (0-12 chicks raised). We found that increasing experience with chicks led to decreased *PRLR* expression in the preoptic area, and age decreased *VIP* expression in birds of both sexes. Pituitary *PRL* and *VIPR* expression appeared unaffected by parental experience or age. These results suggest there may be long-lasting effects of experience and age on neural responsiveness to, and regulation of, prolactin in birds.

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3.2. INTRODUCTION

In animals that show parental care, prior parental or reproductive experience often leads to higher likelihood of offspring survival, and ultimately, higher fitness (Fowler, 1995). Behavioral differences between experienced and first-time parents appear to mediate this effect. For instance, female rodents with prior parental experience (hereafter, "experienced") show higher responsiveness to pups and motivation to care than those without parental experience ("inexperienced") (reviewed in Bridges, 2016). Similar effects have been found in ring doves, where experienced female birds were more likely to show spontaneous care of novel chicks, even in the absence of hormonal treatment, than inexperienced females (Wang & Buntin, 1999). In birds, chicks raised by inexperienced parents also tend to grow at slower rates than those raised by experienced parents (Coulson & Porter, 2008; Pugesek, 1995; Skagen, 1988), suggesting differences in incubation or feeding behaviors. These effects of parental experience extend beyond typical offspring care behaviors to also improve performance in memory tasks and reduce anxiety-like behaviors, which may facilitate more efficient care (Pawluski et al., 2006).

Beyond parental experience, age can also affect reproductive success. Life history theory posits that since the opportunities for future reproduction decrease with age, the value of each successive brood increases (Forslund & Pärt, 1995; Williams, 1966), leading to changes in behavioral priorities. Experimental evidence supports this, as parental care effort increases with age in some animals (Paitz et al., 2007). For example, some bird species show greater investment in chicks as the parents age (Geslin et al., 2004; Pugesek, 1995; Robertson & Rendell, 2001), though these effects can differ by sex (McGraw et al., 2001). However, in most cases, age and reproductive experience are confounded, as gaining parental experience requires time and accrues age. Studies that have controlled for experience show that age itself

may have an effect on behavior; for instance, younger inexperienced Japanese quail took longer to spontaneously express maternal behaviors towards novel chicks than older, inexperienced birds (Pittet et al., 2012). Thus, controlling for age is important to dissociate the separate effect of experience on behavior and physiological mediators.

Lasting changes in circulating hormones may mediate the effects of both experience and age on parental care behaviors. One candidate hormone that may facilitate these effects is prolactin, a peptide hormone that promotes lactation in mammals and parental care behaviors across vertebrates of both sexes (Austin & Word, 2018; Freeman et al., 2000). Prolactin release from the pituitary gland is controlled by hypothalamic neuropeptides, including dopamine in mammals and vasoactive intestinal peptide (VIP) in birds (Fitzgerald & Dinan, 2008; Lea & Vowles, 1986; Kulick et al., 2005). In female rodents, prolactin levels tend to decrease during pregnancy in experienced/multiparous individuals versus those less experienced (Bridges & Byrnes, 2006; Bridges & Hammer, 1992 ; reviewed in Bridges, 2016), but in male primates, circulating prolactin has been shown to increase during offspring care with experience (Almond et al., 2008; Ziegler et al., 1996).

In birds, parental experience also alters prolactin release. For example, circulating prolactin increases with parental experience in various bird species (black-browed albatross- Angelier et al., 2007; common terns - Riechert et al., 2012; zebra finches - Smiley & Adkins-Regan, 2016). Experience also led to increased numbers of prolactin-immunoreactive cells in the pituitary gland even when zebra finches were not breeding (i.e. not actively caring for chicks or eggs) (Christensen & Vleck, 2015). Further, experienced ring dove females showed increased behavioral responses towards chicks when administered prolactin (Wang & Buntin, 1999), but had lower VIP levels in the hypothalamus later in breeding (Cloues et al., 1990). This suggests that experienced birds may have increased sensitivity to prolactin and VIP, which may better facilitate the onset and maintenance of key parental behaviors.

While such hormonal changes may persist in experienced individuals, neural sensitivity to circulating prolactin may also differ with age and experience. Prolactin receptors may change correspondingly in key brain regions for parental care, such as the preoptic area (POA; Dobolyi et al., 2014; Dulac et al., 2014), as experience is gained. Differential responsiveness to stimulatory signals, such as via increased VIP receptors in the pituitary gland, may also lead to differences in baseline prolactin release (Kulick et al., 2005). Such altered sensitivity to circulating prolactin could lead to a greater physiological or behavioral response to offspring cues, facilitating the more efficient care seen in experienced parents. While reproductive experience has been shown to increase prolactin receptor expression and downstream signaling cascades in the medial POA of female rats (Anderson et al., 2006; Sjoeholm et al., 2011), the effects of experience and age on prolactin receptor expression remains unexplored in birds.

To address this gap, we examined how both experience and age affect gene expression of prolactin, VIP and their respective receptors in the brain and pituitary of a biparental bird, the rock dove (*Columba livia*). Rock doves are a species well-suited for evaluating the effects of parental experience on the prolactin system. The two sexes exhibit nearly-egalitarian parental care (e.g. both males and females nest-build, incubate eggs and feed chicks), allowing direct comparisons between the sexes (Abs, 1983). Further, both sexes pseudo-lactate via the production of a nutrient rich "crop milk" used to provision chicks (Horseman & Buntin, 1995). Like mammalian milk, crop milk production is driven by prolactin, and prolactin also has been shown to causally enhance expression of parental behaviors in doves (Horseman & Buntin, 1995). Capitalizing upon a captive breeding population of rock doves with known age and reproductive history, we examined the effects of experience on prolactin and VIP systems while controlling for age. We hypothesized that variation in prolactin responsiveness and regulation mediates differences in parental behavior observed in experienced parents. We thus predicted that increasing parental experience will alter prolactin receptor expression, VIP and VIP receptor expression, and will do so similarly in both sexes of this biparental species.

3.3. METHODS

Study animals

We collected brains and pituitaries from 62 rock doves (*Columba livia*) of varying ages and degrees of parental experience. All birds used in this study were born and housed in a semi-natural environment where they are exposed to natural ambient temperatures and daylight. Daylight is supplemented with artificial lighting set to 14L:10D and animals are protected from the elements. We provided *ad libitum* food (whole corn and game bird protein starter), grit and water. Each social aviary (1.5 x 1.2 x 2.1m) houses mixed-sex flocks and includes 16 nest boxes and nesting material (straw). Breeding birds naturally select their mates and compete for nest sites in the aviary. All methods used in this study were approved by the University of California Davis IACUC (protocol no. 22407).

To compare the effects of experience with chicks ("parental experience") controlling for age, we collected birds that ranged in levels of experience and age. Birds either were inexperienced (never had chicks, n = 31) or experienced (had reared one or more chicks to fledging, n = 31) (Fig.3.1A), and ranged in age from 0.62 to 3.3 years old, with an average of 1.52 years (Fig.3.1B). All birds were collected when they were not actively caring for a nest (i.e., did not have any eggs or chicks) to control for the effects of parental care stage, as prolactin and VIP gene expression have been shown to vary across incubation and chick care in rock doves (Farrar et al., 2022; Harris, 2020). Note that all inexperienced birds had not reared any chicks nor laid any eggs. As nests are checked daily to record eggs laid, chicks hatched, and identity of attending parents, we had exact hatch dates as well as breeding histories for each bird. Age was calculated as hatch date subtracted from date of tissue collection. We also totaled the number of chicks hatched for each bird (see distribution in Fig.3.1C). Age at first reproduction in our population is around four months, as we have observed multiple first nests initiated at this age (*pers. obs.*).

Tissue collection and brain microdissection

We collected brains and pituitaries from birds using methods previously described (Calisi et al., 2018; MacManes et al., 2017). All birds lacked an active nest when they were collected (i.e. birds were not incubating eggs or rearing chicks, but may have been participating in courtship or nest initiation behaviors). Briefly, birds were euthanized using an overdose of isoflurane and then swiftly decapitated within three minutes of removal from their cage. The brain and pituitary were removed, flash frozen on dry ice, then stored at -80°C. All tissues were collected between 8:00 and 11:00 AM during March 2020.

To evaluate gene expression in specific hypothalamic nuclei, we microdissected nuclei of interest using a Leica CM1950 cryostat. We used a species-specific brain atlas to identify landmarks for the preoptic area (POA) and the infundibulum (INF) (Karten & Hodos, 1966). We focused on the POA as it is key to regulation of parental behaviors in doves (Slawski & Buntin, 1995) and other vertebrates (Dobolyi et al., 2014; Dulac et al., 2014), and is rich in prolactin receptors (Buntin et al., 1993; Buntin & Buntin, 2014; Smiley et al., 2021). The infundibulum is the main site of VIP release into the median eminence in birds (Kosonsiriluk et al., 2008), and VIP-immunoreactive neuron counts there correlate with prolactin levels (Cloues et al., 1990; Péczely & Kiss, 1988). For the POA, we punched 100 µM coronal slices beginning when the tractus septomesencephalicus (TSM) extended to the bottom of the brain, and ending when the TSM was no longer visible and the cloudy tractus quintofrontalis (QF) appeared (plates A 9.00 - 8.50 in Karten & Hodos, 1966). We punched the INF when the optic tecta appeared and the tractus occipitomesencephalicus (OM) was no longer visible, until the cerebellum appeared (plates A 5.00 - 4.25 in Karten & Hodos, 1966; approximately 4-5 punches). We dissected both nuclei using 2 mm punches (see Fig.3.2A for representative slices). Hypothalamic nuclei punches were weighed and then stored in 200 µL of TriSure reagent (BioLine) at -80°C until RNA extraction.

Quantitative PCR

We extracted RNA from POA and INF punches, as well as whole pituitaries, using TriSure reagent (BioLine) and a modified protocol of the Zymo Direct-zol RNA extraction kit (Catalog No.

R2501, Zymo Scientific). We measured the quantity and integrity of extracted RNA using a Nanodrop

ND-1000 spectrophotometer (Thermo Scientific). We then treated total RNA with DNase to remove any

remaining genomic DNA using Quanta Perfecta DNase I (Catalog No. 95150-01K, Quanta Biosciences),

then reverse transcribed RNA to single-stranded complementary DNA (cDNA) using qScript cDNA

Supermix (Catalog No. 95048-100, Quanta Biosciences). Before qPCR, we diluted cDNA 1:2 for

hypothalamic nuclei and 1:5 for pituitary samples. Due to tissue storage issues, we were only able to

extract RNA from 36 INF samples (n = 15 inexperienced, < 1 year old ; 5 inexperienced, 1-2 years; 9

experienced, 1-2 years; 7 experienced > 2 years). We also excluded nine POA punches and five

pituitaries from analysis due to low RNA quality (260/280 and 260/230 ratios < 1.80), leading to final

sample sizes of n= 54 POA and n = 58 pituitaries.

Using qPCR, we measured relative gene expression for prolactin receptor (*PRLR*),*VIP*, and VIP

receptor (*VIPR*) in the POA, INF, and pituitary respectively. We also measured three reference genes:

HPRT1,*RPL4*, and *ACTB* to account for variation in overall mRNA levels between samples. Primers for

these genes were designed on the *Columba livia* transcriptome v2.10 (NCBI accession no.

GCA_000337935.2) using NCBI PrimerBlast. We validated each primer for ideal efficiencies by running a

standard curve consisting of five 10-fold dilutions of standard tissue cDNA and ensured single amplicons

by assessing melt curves. Gene accession numbers, amplicon lengths and efficiencies for each primer can

be found in Table 2.

We used the following qPCR reagent mix: 1 µL diluted cDNA template, 5 µL 2X SSOAdvanced

SYBR Green PCR mix (BioRad), and 10 µM each of primer (total volume: 10 µL, Invitrogen). All

samples, as well as no-template controls, were run in triplicate. We ran each 384 well plate under the

following thermocycling protocol: 50℃ for 2 min, 95℃ for 10 min, and then 40 cycles of 95℃ for 15

sec and 60℃ for 30 sec. Plates were run on a CFX384 Touch Real-Time PCR detection system

(BioRad). All samples for each tissue/gene were run on a single plate, eliminating the need to account for

interplate variation.

To calculate relative gene expression, we used the Livak and Schmittgen (2001) delta-delta Ct method. Here, gene of interest expression (Ct) for each sample is normalized first to the geometric mean of reference gene expression for that sample (dCt). For reference genes, we used *HPRT1* and *RPL4* for brain tissue (both POA and INF) and *HPRT1* and *ACTB* for pituitary. We validated that expression of these genes did not significantly differ with experience (total chicks raised),age or sex in any tissue, thus making them suitable as reference genes (see Table 3.2 for analysis). Then, normalized expression is calculated relative to the average normalized expression for the "control" group (ddCt). As the control group for relative expression, we used birds that were inexperienced with chicks and less than one year old. We analyzed data as fold change, or $2^{(-ddCt)}$.

Statistical analysis

We analyzed all data in R v. 4.0.3 (R Statistical Team, 2021). For each gene, we ran a general linear model with fold change in gene expression as the independent variable and total chicks, age in years, and sex as the explanatory variables. We $\log_{10}$-transformed fold change data to meet assumptions of normality. The interaction between age and total chicks was not significant for any gene, suggesting that experience has a similar effect across a range of ages; we therefore excluded the interaction term from our models. We ran separate models for each gene as 1) direct comparisons between genes is not recommended using the ddCt method (Livak & Schmittgen, 2001), especially when different reference genes are used to calculate relative expression, and 2) *PRLR, VIPR* and *VIP* are all regulated by different gene promoters and transcription factors (Chaiseha et al., 1998, 2004; Schennink et al., 2015), and their expression should be considered independent. As age and experience are inevitably related (i.e., animals must age in time as they gain parental experience), we assessed the linear model for each gene for collinearity between the predictors, age and experience, by calculating variance inflation factors (VIF) using the car package(v 3.0.10, Fox & Weisberg, 2019). VIF was < 5 in all models (Table 3.3), suggesting that most of the variation in age is not explained by experience and vice versa (James et al., 2013; Zuur et al., 2007)**.** Thus, we kept all predictors in our models.

We present ANOVA run on the linear models (calculated using the car package, v.3.0.10, Fox & Weisberg, 2018). However, as our hypotheses revolved around the categorical effect of experience with chicks, we also visualized predicted values across levels of experience for those genes where experience had a significant effect. These "predictor effect displays" hold the effects of age and sex constant (Fox & Weisberg, 2018) to examine the effect of experience with other variables accounted for. Predictor effect plots were created using the jtools package (v.2.1.0, Long, 2020).

3.4. RESULTS

Prolactin receptor (PRLR)

In the POA, we found a significant effect of number of chicks raised ($F_{1,50} = 6.62$, $p = 0.013$) on *PRLR* expression when controlling for age in years and sex (Fig.3.2B). This effect of experience led to decreased *PRLR* gene expression as total chicks raised increased ($\beta = -0.32 \pm 0.12$). This experience effect appears to be due to lower predicted *PRLR* expression in birds with high levels of experience (greater than five nests) when age and sex are held constant (see predictor effect plot, Fig.3.3). Age ($F_{1,50} = 0.85$, $p = 0.350$) and sex ($F_{1,50} = 0.32$, $p = 0.778$) themselves did not significantly affect *PRLR* expression when other variables were accounted for in the model.

Vasoactive intestinal peptide (VIP)

In the infundibulum, we found a significant effect of age ($F_{1,32} = 48.89$, $p = 0.018$), but not total chicks raised ($F_{1,32} = 7.68$, $p = 0.329$) or sex ($F_{1,32} = 0.11$, $p = 0.741$), on *VIP* expression (Fig.3.2C). Age appeared to negatively affect infundibular VIP, as expression decreased in older birds ($\beta = -2.46 \pm 0.98$).

Pituitary prolactin (PRL)

In the pituitary gland, we measured *PRL* gene expression as a proxy for pituitary gland prolactin content and plasma prolactin levels. Previous studies in rock doves have shown that plasma prolactin and

pituitary *PRL* expression are highly correlated, but may not always match one-to-one (Farrar et al., 2021). We found no significant effects of experience (number of chicks raised) ($F_{1,53} = 0.06$, $p = 0.801$) , age ($F_{1,53} = 0.86$, $p = 0.358$) , or sex ($F_{1,50} = 0.10$, $p = 0.758$) on pituitary *PRL* expression in birds that were not actively breeding (Fig.3.2D).

VIP receptor (VIPR)

Lastly, in the pituitary, we found no significant effect of age ($F_{1,53} = 0.51$, $p = 0.477$) or total chicks raised ($F_{1,53} = 0.07$, $p = 0.787$) on *VIPR* expression (Fig.3.2E). However, there was a suggestive trend towards a sex difference in pituitary *VIPR* ($F_{1,53} = 3.09$, $p = 0.085$), with males appearing to express *VIPR* at higher levels than females ($\beta = 1.25 \pm 0.71$) when controlling for age and experience.
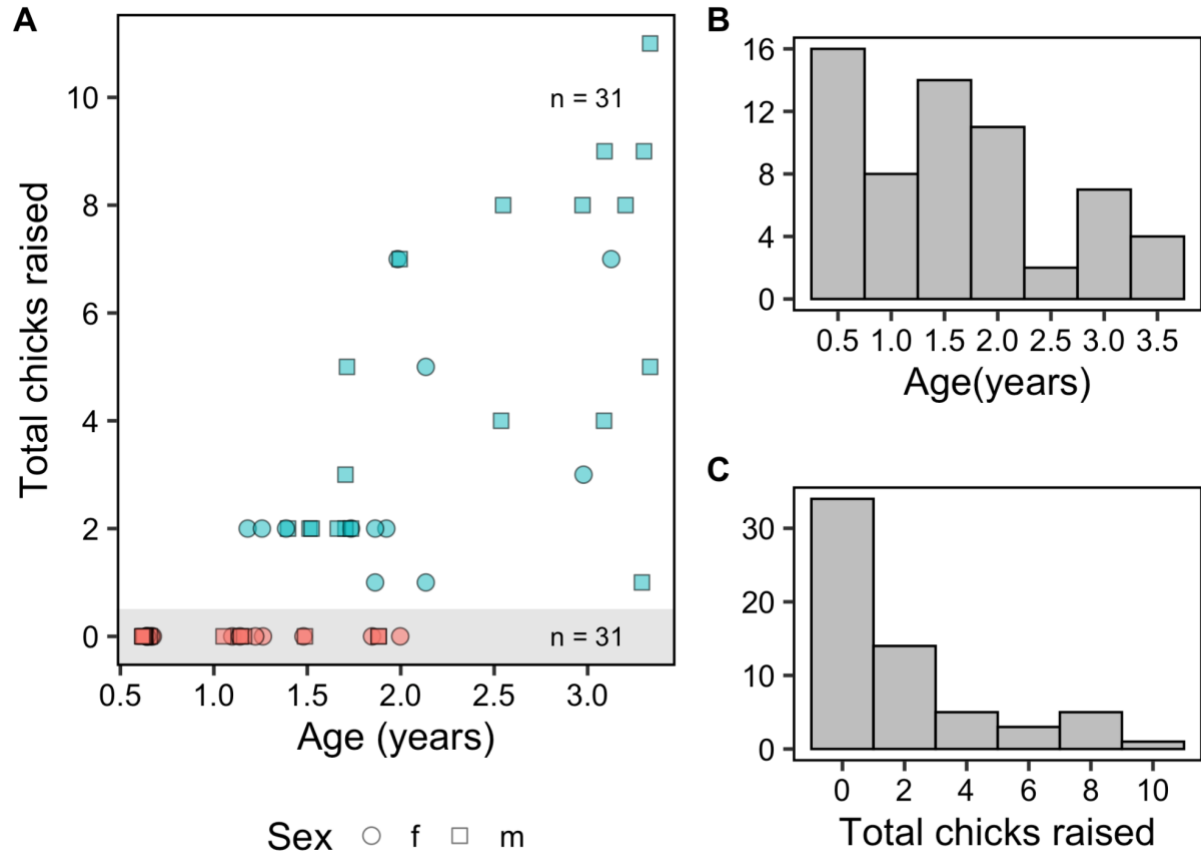
Figure 3.1. **Sample size spread across age and degree of parental experience**. (**A**) Age (in years) at collection versus total chicks raised for each individual sample. Total sample size is 62, with 31 individuals inexperienced (never raised chicks, total chicks = 0, shaded in gray) and 31 individuals experienced with chicks (total chicks ≥ 1). Shape depicts sex, with circles as females (n = 27) and males as squares (n = 35). Histograms show distribution of (**B**) age and (**C**) experience, measured by total chicks raised across the sample size.
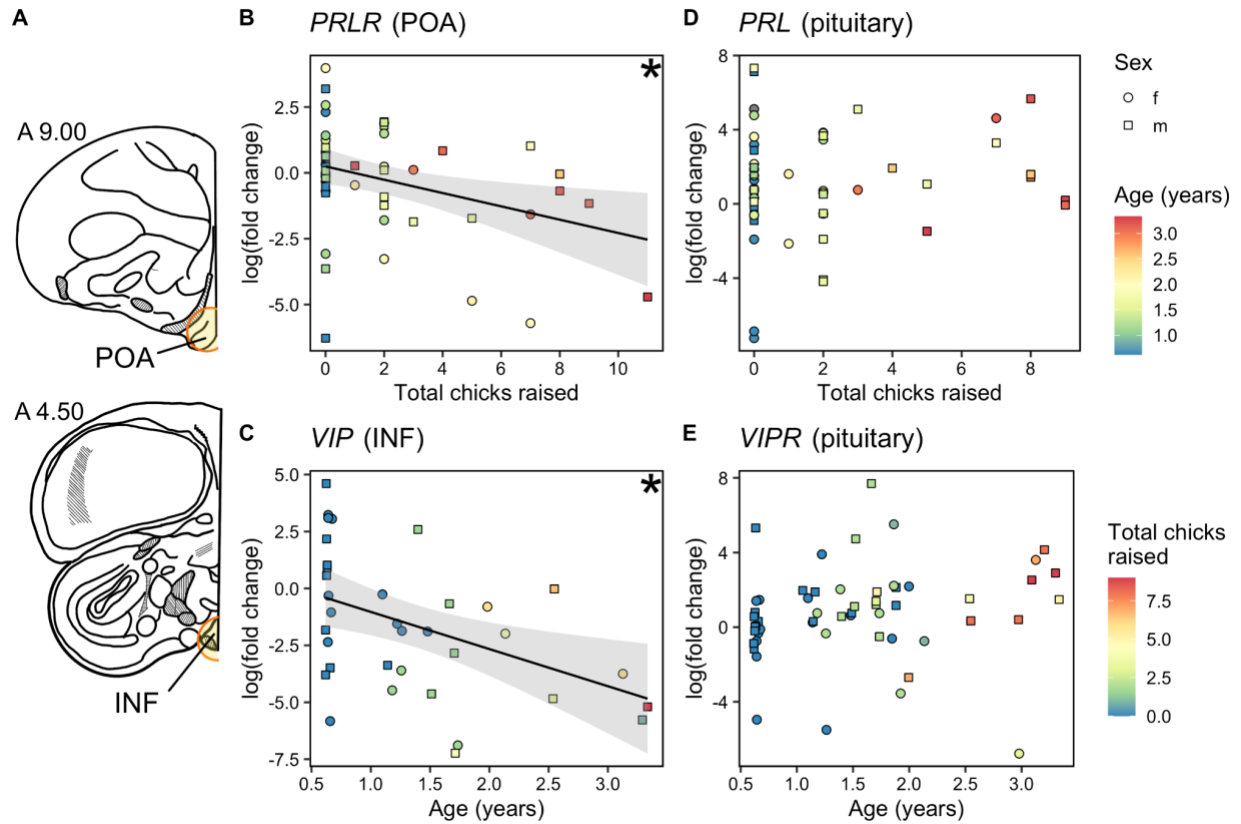
Figure 3.2. **Gene expression in hypothalamic nuclei and the pituitary as a function of total chicks raised and age in years.** (**A**) Representative coronal slices showing where hypothalamic nuclei punches (top, preoptic area - POA; bottom, infundibulum - INF). Images recreated from Karten and Hodos (1966). Relative gene expression of (**B**) prolactin receptor (*PRLR*) in the POA, (**C**) vasoactive intestinal peptide (*VIP*) in the INF, (**D**) pituitary prolactin (*PRL*) and (**E**) pituitary vasoactive intestinal peptide receptors (*VIPR*). Sex is represented by shape, with circles and squares for males and females, respectively. For panels B and D, gene expression is shown versus total chicks raised, with color representing age, and vice versa for panels C and E. Genes with significant relationships between x and y plotted variables (as determined by ANOVA) are shown with an asterisk, and regression lines with 95% confidence intervals shaded in gray are shown.

Gene (Abbreviation)	NCBI Accession number	Amplicon length (base pairs)	Efficiency (%)		Primer sequence	Notes
Reference Genes						
Beta actin (*ACTB*)	XM_005504502.2	107	95.8	F	ATGTGGATCAGCAAGCAGGAG	Suggested reference genes for avian tissue by Zinzow-Kramer et al. (2014)
				R	CATTTCATCACAAGGGTGTGGG	
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	
Candidate Genes						
Prolactin (*PRL*)	XM_005506024.2	181	92.6	F	GGCGGGTTCATACTGGTGAG	Previously used in Farrar et al. (2021)
				R	TGGATTAGGCGGCACTTCAG	
Prolactin receptor (*PRLR*)	NM_001282822.1	158	95.2	F	TCTTCCTTGCACACATGAAACC	
				R	TCCAGGGTATGATTGACCAGT	
Vasoactive intestinal peptide (*VIP*)	XM_005507654.3	115	103.9	F	TGCTCCTCTCTGGACATACCA	
				R	GGCATTCTGTTTCCCAATCCCA	
Vasoactive intestinal peptide receptor (*VIPR*)	XM_013369762.2	135	100.9	F	AGGGATTTGTGGTGGCTGTT	
				R	TGCCTAAGGAAGGGTGGTGA	

Table 3.1. Primers used in quantitative PCR. Primers were designed using the NCBI Primer-BLAST tool on species-specific gene templates for *Columba livia* (accession numbers show the template upon which each primer was designed). Primer efficiencies were calculated by running a standard curve of five 10-fold dilutions of purified PCR product, and primers were evaluated for single products via melt-curve analysis during validation.

Tissue	Gene	Predictor	df	F value	p value
Infundibulum (INF)	HPRT1	total_chicks	1	0.10	0.75
		age_years	1	0.12	0.73
		sex	1	0.98	0.33
		Residuals	31		
	RPL4	total_chicks	1	0.02	0.89
		age_years	1	0.19	0.67
		sex	1	0.58	0.45
		Residuals	32		
Preoptic area (POA)	HPRT1	total_chicks	1	2.47	0.12
		age_years	1	2.09	0.15
		sex	1	1.38	0.25
		Residuals	50		
	RPL4	total_chicks	1	0.44	0.51
		age_years	1	1.74	0.19
		sex	1	0.17	0.68
		Residuals	50		
Pituitary	ACTB	total_chicks	1	0.11	0.74
		age_years	1	0.04	0.84
		sex	1	2.66	0.11
		Residuals	53		
	RPL4	total_chicks	1	0.56	0.46
		age_years	1	0.02	0.89
		sex	1	2.60	0.11
		Residuals	53		

Table 3.2. Reference genes are stably expressed across all model predictor variables and all tissues. For each tissue and reference gene used, we show the results of an ANOVA on a linear model of reference gene expression ~ total_chicks + age_years + sex. As none of these predictor variables were significant for any gene:tissue combination at the level of $\alpha = 0.05$, we determined that these genes were stably expressed across all variables of interest and thus suitable for use as reference genes.

Gene	Variance Inflation Factor (VIF)		
	Age (years)	Experience (total chicks raised)	Sex
PRL	2.80	2.75	1.03
PRLR	2.70	2.63	1.04
VIP	3.01	3.02	1.03
VIPR	2.80	2.74	1.03

Table 3.3. Variance inflation factors for each linear model for each gene. To assess collinearity of predictor variables, specifically age and experience, we calculated VIF for each model and each gene. VIF of 1 represents the absence of collinearity, where VIF > 5 presents a problematic amount of collinearity which may reduce the ability of the model to detect effects (James et al., 2013).

3.5. DISCUSSION

This is the first study to examine the effects of parental experience with chicks and age on gene expression of prolactin, VIP and their respective receptors in an avian species. We found that neural *PRLR* expression tends to decrease as the number of chicks reared increases and neural *VIP* expression decreases with age in both sexes of reproductively mature rock doves in a non-nesting state. Pituitary *PRL* and *VIPR* expression were not significantly predicted by parental experience or age in non-nesting birds. These gene expression results suggest that there may be changes in regulation of the prolactin system associated with experience and age in birds that may persist even when birds are not actively engaging in parental care.

Effects of experience on PRL and PRLR

In the pituitary, we found no significant effects of age nor experience in *PRL* gene expression. The lack of an effect of age on baseline pituitary PRL is consistent with results in zebra finches(Christensen & Vleck, 2008).   In contrast, previous research on the effects of reproductive experience on circulating prolactin in birds is mixed. In wild, long-lived seabirds, baseline prolactin concentrations increased with experience, where black-browed albatross that had had 2-10 nests had significantly higher prolactin levels than birds on their first nesting attempt (Angelier et al., 2007), and prolactin concentrations increased within individual common terns across the first three years of breeding (Riechert et al., 2012). Second-year male juncos, entering their first breeding season, showed an earlier decline in plasma prolactin during breeding than after-second-year males that had bred at least once before (Deviche et al., 2000). However, all of these studies measured plasma prolactin *during* breeding, when birds were actively incubating eggs or brooding chicks. Here, we measured prolactin gene expression in birds that did not have active nests, and while likely in a reproductive state, were not expressing parental behaviors. Indeed, in captive zebra finches, prior breeding experience only led to significant increases in plasma prolactin from late incubation onwards (Smiley & Adkins-Regan, 2016),

and there were no significant differences between first and second breeding cycles earlier in incubation. This study also did not measure effects of breeding experience outside of the nesting period (Smiley & Adkins-Regan, 2016). Therefore, parental experience may exert its effects on prolactin levels during the breeding cycle, but these differences may not be observed when birds are not actively nesting. Previously, we showed that plasma prolactin and pituitary PRL (which were highly correlated), do fluctuate throughout the rock dove breeding cycle (S. H. Austin et al., 2021; Farrar et al., 2021), but we did not control for experience or age in those studies. Future work should examine whether pituitary *PRL* gene expression also differs with experience *during* breeding and parental care.

In contrast, we found that *PRLR* expression in the preoptic area (POA), a hypothalamic nucleus important for expression of parental care behaviors (Dulac et al., 2014), significantly decreased with increasing experience. While not studied previously in birds, reproductive experience led to upregulated *PRLR* mRNA in the POA of primiparous rats when not actively parenting, compared to virgins (Anderson et al., 2006). In ring doves, experienced birds may have different behavioral responses to prolactin treatment than inexperienced parents (Wang & Buntin, 1999), suggesting different prolactin responsiveness via a possible neural mechanism.

However, we found decreased *PRLR* in the brain in non-nesting birds, contrary to what one might expect if experienced birds show increased prolactin responsiveness. We consider three possible explanations for decreased *PRLR* expression with increasing experience. First, *PRLR* dynamics may be higher in experienced birds *during* the parental care period or in response to chick cues, but these patterns may not appear when birds are not actively breeding. As suggested above, experience may lead to different prolactin responsiveness when parents are actively incubating eggs or responding to chicks. This explanation, however, does not consider that we still found significantly decreased *PRLR* expression in birds without active nests as the total chicks reared increased. Alternatively, gaining parental experience may reduce reliance on hormonal drivers of parental care, and thus lead to reduced *PRLR*. In rodents, maternal responsiveness to pups in experienced females is less dependent on hormonal cues, whereas this

response in primiparous females appears to be more reliant on hormones associated with pregnancy and lactation (Fleming et al., 1996). Without hormonal stimulation, virgin mice were slower to show spontaneous pup care than non-breeding experienced females (Stolzenberg & Rissman, 2011). However, even ovariectomized virgin mice, without hormonal stimulation, could be induced to show care through experience and exposure to pups (Stolzenberg & Rissman, 2011). Similar results were found in ring doves. Experienced ring doves without hormonal stimulation showed more spontaneous chick care behaviors than inexperienced doves (Wang & Buntin, 1999). Together, this evidence suggests that experiential effects on parental response may not always be linked to lasting changes in neural hormone responsiveness. Similarly, prolactin levels could change with experience, and consequently, prolactin responsiveness could be less affected by experience. If experience leads to higher prolactin during breeding, as has been shown in other birds (Angelier et al., 2007; Riechert et al., 2012; Smiley & Adkins-Regan, 2016), experienced birds may be able to rely on increased circulating prolactin instead of increased neural responsiveness to prolactin to maintain parental behaviors. Evidence suggests female rodents tend to show decreased circulating prolactin, but increased neural prolactin responsiveness, with experience (reviewed in Bridges, 2016). Birds may show the opposite signal-receptor dynamic, favoring higher hormonal signal and lower receptor expression with experience. To test this hypothesis, future studies should extend our work and compare prolactin levels and *PRLR* expression in inexperienced and experienced birds during parental care and/or in response to chicks. Lastly, we cannot rule out the possibility that the transcription of *PRLR* differs from functional protein levels in the brain (Vogel & Marcotte, 2012). Experienced parents could possibly already have higher existent PRLR protein levels and thus require lower baseline transcription of this gene. Without protein measurements at non-nesting and during parental care, however, this possibility remains untested.

Effects of experience on VIP and VIPR

In the *VIP* system, we found no effects of parental experience on expression of *VIP* and its receptor, but did see an effect of age on infundibular *VIP* expression. In birds, VIP is a main prolactin-

releasing factor that stimulates prolactin secretion from the pituitary (Kosonsiriluk et al., 2008; Lea & Vowles, 1986; Macnamee et al., 1986). Again, any possible effects of parental experience on VIP and its receptor may not appear outside of the breeding cycle, as previous studies in ring doves show experiential effects on neural VIP levels during chick rearing (Cloues et al., 1990). As with prolactin, *VIP* and *VIPR* expression has been shown to fluctuate during breeding in turkeys (Chaiseha et al., 1998, 2004), and the parental period may be where any experiential effects appear in our species. We also found that age decreases *VIP* expression. However, age did not predict pituitary *PRL* levels in our study, apparently inconsistent with age leading to lower *VIP*. Reduced neural *VIP* transcription, if correlated with stored peptide levels, could suggest reduced ability of older birds to upregulate prolactin secretion when needed. Reduced onset or release of prolactin could lead to delayed responses to offspring cues, such as chick demands (as tested in Farrar et al., 2021). Experiments that measure prolactin upregulation in response to chick stimuli or VIP injections as birds age would be needed to test this hypothesis.

Methodological considerations

Some methodological considerations should also be taken into account when interpreting our results. As discussed, our birds were collected outside of the parental care period, when they did not have an active nest (but likely had active reproductive axes and may have been showing courtship and mating behaviors). We used this sampling paradigm in order to control for the effects of incubation or chick rearing stage on prolactin system gene expression, which we found in previous studies (Farrar et al., 2022; Harris, 2020). However, nearly all previous studies examining the effect of experience, and age, on parental hormones such as prolactin found effects while birds were actively incubating eggs or rearing chicks (Angelier et al., 2007; Cloues et al., 1990; Riechert et al., 2012; Smiley & Adkins-Regan, 2016), or were stimulated by the presence of chicks (Wang & Buntin, 1999) . The apparent presence or absence of experiential effects in non-nesting birds may not preclude differences that may appear during parental care or in response to chick cues. While we aimed to address the effect of experience while controlling for age, biological constraints in bird breeding limited the full ability to control for age and experience. For

instance, we did not sample any experienced birds less than one year old. This limitation is due to the semi-natural aviary setup, where young, first-time breeders often are not able to compete for a nest box with more established breeding pairs, and therefore tend not to fledge chicks until they are older than one year. On the other hand, we were not able to sample inexperienced birds older than two years old, as most birds have established nest boxes and have bred by that age, unless they were isolated or kept from breeding. While our semi-natural rearing environment is a strength and we captured a range of ages and experience levels, future studies could use different designs or isolate breeding pairs in order to more precisely control these variables.

Further, reproductive "experience" may have an effect in forms beyond exposure to chicks. Indeed, Michel (1977) separated out the effects of courtship, nest building, incubation and full chick experience on the ability of progesterone to facilitate incubation onset in doves, and found that experience at the nest building stage or further into incubation was enough to potentiate progesterone responsiveness. Pair bond duration could also be considered, as pair bond strength affects reproductive success and hormonal synchronization between individuals (Fowler, 1995; Ouyang et al., 2014). Future studies could define reproductive experience at a more precise resolution than we did here, allowing for a better delineation of the effects of experience versus age.

Additionally, the range of ages in our study is relatively small (up to three years) compared to studies in long-lived sea birds with lifespans upwards of 20 years. Rock doves can have long lives in captivity (record 31 years), but the lifespans of feral rock doves average 2.4 years due to predation (Johnston, 1992) . Considering we measured these variables in captive birds, the short age range measured may not be ecologically significant, and should not be interpreted as indicating "senescence." Despite the short age range, we still found significant age effects independent of experience and sex. The biological relevance of these small effect sizes in gene expression remains to be determined. Future

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research should compare our study with expression of these genes in wild birds, during parental care, and in response to hormonal manipulations such as VIP injection or prolactin administration.

3.6. CONCLUSIONS

In sum, we examined how parental experience (number of chicks raised) and age affect expression of pituitary *PRL*, neural *PRLR*, *VIP* and its receptor in the pituitary. This initial study is the first to our knowledge to examine prolactin and VIP receptor dynamics in the context of experience and/or age in birds. In non-nesting birds of both sexes, we found mixed effects of age and experience, where *PRLR* decreased with total chicks raised and *VIP* decreased with age. We did not observe any significant changes in pituitary *PRL* or *VIPR* expression in birds with no active nest. These effects may be more or less prominent if measured during the breeding cycle or in response to chick stimuli. Our results offer a foundation for future research to test the physiological and behavioral relevance of such changes in gene expression seen with experience and age, as well as compare if these trends are present across wild and seasonally-breeding avian species. Lastly, if the gene expression differences observed here do indeed persist even outside of the parental care period (as our results suggest), this study lays the groundwork to explore if epigenetic mechanisms underlie the effects of experience on prolactin receptor regulation in birds.

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CHAPTER 4

Prior parental experience, but not incubation stage, alters hormonal stress responses and hippocampal glucocorticoid receptors in the biparental rock dove, *Columba livia*

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4.1. ABSTRACT

In the face of challenges, animals must balance investments in reproductive effort versus their own survival. Physiologically, this tradeoff may be mediated by glucocorticoid release by the hypothalamic-pituitary-adrenal (HPA) axis and prolactin release from the pituitary to maintain parental care. The degree to which animals react to, and recover from, stressors likely affects their ability to maintain parental behavior and ultimately, fitness. However, less is known about how the stress response changes when animals gain parental experience, and what mechanisms may underlie any effect of experience on hormonal stress responses. While studies have shown hormonal stress responses can change across the annual cycle, less is known about if and how the stress response changes *within* a breeding stage. To address these questions, we measured the corticosterone (CORT) and prolactin (PRL) stress response in both sexes of the biparental rock dove (*Columba livia*) across three stages of incubation as investment in eggs increased. To understand the effect of parental experience, we also compared the stress response in non-actively-nesting birds 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 minutes restraint). We also measured negative feedback ability by administering dexamethasone, a synthetic glucocorticoid that suppresses CORT release, measuring CORT and PRL after 60 minutes. While the stress response did not change significantly across incubation, birds with parental experience 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. We found that experienced birds expressed higher glucocorticoid

receptors than inexperienced controls, which may mediate their ability to attenuate the hormonal stress response. Together, these results shed light on potential mechanisms by which gaining experience may improve parental performance and fitness.

4.2. INTRODUCTION

Following life-history theory, animals will maximize fitness by modulating their physiology and behavior across their life cycles, including during the energetically-costly breeding period (Stearns, 1976). Breeding animals often prioritize resource allocation towards current reproductive efforts, such as parental care of their current brood, at a cost to personal survival, self-maintenance, and growth (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 on the physiological mechanisms underlying these tradeoffs (Ricklefs and Wikelski, 2002; Zera and Harshman, 2001), especially in the face of stressors (Romero and Wingfield, 2016).

Endocrine mechanisms, specifically the glucocorticoid hormones (corticosterone or cortisol; CORT) and prolactin (PRL), have been strongly implicated in tradeoffs between survival and reproduction due to their pleiotropic effects on energetic state, metabolism, and reproduction. 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 naturally during energetically-costly stages, like 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). In contrast, 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). In a stress context,

reduced PRL may reduce investment away from 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 the prolactin stress hypothesis may not generalize across vertebrates. Nonetheless, the CORT and PRL stress responses can yield important insights into the tradeoff between survival and energetic balance versus reproductive effort (Angelier et al., 2016; Angelier and Chastel, 2009) when measured together within individuals.

Multiple hypotheses have been proposed to connect these hormonal stress responses with the reproductive value of and investment in an animal's current brood (Harris, 2020). For instance, the "parental care hypothesis" posits that individuals, sexes, or species that invest more in parental efforts will be less hormonally-responsive to stressors (i.e. show attenuated changes in hormone levels) than those that invest relatively less (Wingfield et al., 1995). Multiple studies have found support for the parental care hypothesis with regard to CORT levels, especially in bird species where the sexes differ in parental investment across breeding (Holberton and Wingfield, 2003; Jesse S. Krause et al., 2015; Meddle et al., 2003; O'Reilly and Wingfield, 2001; Reneerkens et al., 2002). Many studies compare hormonal stress responses across gross reproductive stages defined broadly (i.e., "pre-parental" vs "parental"). It remains unclear if these hormonal stress responses change *within* breeding stages, such as egg incubation, as time invested in the brood increases.

Similarly, the reproductive value of offspring, relative to an individuals' future reproductive opportunities, could also alter their response to stressors. The "brood value" hypothesis (Bókony et al., 2009; Heidinger et al., 2006) predicts that if the fitness value of the current brood is high (i.e., if it is larger, occurs later in life, or if breeding opportunities are limited), individuals should modulate their stress response to maintain parental effort and ensure brood success. The brood value hypothesis has been supported in the context of age, where maximum stress-induced CORT levels declined with age, while PRL levels decreased less rapidly in older individuals of a long-lived seabird (Heidinger et al., 2010, 2006). However, other studies found no effects of age on CORT stress responses, but did find older birds

maintained higher PRL levels at baseline or after stress (Angelier et al., 2007; Angelier et al., 2007). Alternatively to the brood value hypothesis, these effects of age on CORT and PRL stress responses could be due to constrained physiological ability of young individuals to modulate these hormones, or they restrain from modulating these responses due to relatively higher future reproductive opportunities ("constraint" and "restraint" hypotheses) (Curio, 1983).

Beyond the effects of age, less is known about how previous parental experience may affect hormonal stress responses. A few studies in long-lived seabirds show that previous breeding experience may be a better predictor of baseline CORT and PRL levels than age alone (Angelier et al., 2007b, 2006). Baseline PRL levels have also been shown to increase with subsequent breeding experiences (Smiley and Adkins-Regan, 2016). Prior breeding experience may alter endocrine systems in ways that also change the hormonal stress response, such as through pituitary prolactin cell counts or neural prolactin receptors (Anderson et al., 2006; Christensen and Vleck, 2015) (Farrar et al., *submitted*). However, the effects of prior breeding experiences on CORT and PRL stress responses, beyond just baseline levels, remains understudied. As gaining breeding experience necessarily requires time that ages individuals, some of the effects of age on stress responses seen in other studies may be modulated by experience, and requires exploration. Further, no study to our knowledge has examined how experience affects the negative feedback ability after stressors, which would further test the brood value, constraint and restraint hypotheses.

Upstream of hormone release, neural receptors densities may also underlie differences in hormonal stress responses that may appear across parental care and with breeding experience. CORT exerts effects through two genomic receptor types, the high-affinity mineralocorticoid receptors (Type I; *MR*) 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). While these receptors are found throughout the body, hippocampal *MR* and *GR* may be especially important for negative feedback of CORT after a stressor. Both hippocampal *MR* and *GR* have been shown to mediate hypothalamus-pituitary-adrenal axis activity and CORT release in mammals (de Kloet et al., 1998; Jacobson and Sapolsky, 1991; R. de Kloet and C. Meijer, 2019), though evidence is limited in birds (Smulders, 2017). The balance of these receptor subtypes has also been hypothesized to 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 due to reduced negative feedback inhibition, but overexpressed *MR* with reduced *GR* undid this effect (Harris et al., 2013). In birds, hippocampal glucocorticoid receptor expression can change during seasonal or breeding transitions (J. S. Krause et al., 2015; Lattin and Romero, 2013), and thus may mediate observed changes in the CORT stress response across these transitions (Lattin et al., 2016). However, no study to our knowledge has evaluated how prior parental experience may alter hippocampal glucocorticoid receptor expression, nor connected receptor densities with ability to negatively feedback on CORT levels.

To address these questions, we first examined hormonal stress responses in CORT and PRL a) across the incubation period and b) in non-actively-nesting rock doves (*Columba livia*) that differed in prior parental experience with chicks. We used dexamethasone, 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 then extended our results into the brain, where we measured hippocampal gene expression of *MR* and *GR* using quantitative PCR. By capitalizing on a captive breeding population of biparental rock doves, we were able to collect data on both sexes at precise timepoints during incubation and in individuals with known breeding histories and ages. Using captive doves also allowed for neural analyses of gene expression, a challenge in wild populations.

In this study, we aimed to test a variation of the "constraint" and "restraint" hypotheses (Curio 1983) and hypothesized that prior parental experience would lead to reduced constraint and improved ability to attenuate stress responses to promote investment in reproduction. We thus 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 glucocorticoid receptors, thus reducing constraints on hormonal stress responses. We then predicted that birds who had raised chicks would have higher hippocampal *GR* and/or *MR* expression than inexperienced birds. We also tested a variation of the "parental care hypothesis" (Wingfield et al. 1995), hypothesizing that increased parental investment, here via time spent incubating eggs, will lead to reduced hormonal responses to stressors in order to maintain the parental effort. We test this hypothesis at a finer resolution - specific stages of egg incubation - rather than broadly-defined reproductive stages seen in previous studies (i.e., "pre-parental" versus "parental"). We predicted that as incubation duration increased, CORT would be lower and PRL would be higher after an acute stressor and after negative feedback. As both sexes of rock doves participate nearly equally in incubation (Abs, 1983; Johnston, 1992), we predicted we would observe no sex differences in hormone stress responses.

4.3. METHODS

Experiment 1: Hormonal responses to stress

Subjects and experimental design

We collected stress series (three blood samples; see "*Stress series blood collection*" below for timepoints) from 96 total adult, captive rock doves (*Columba livia*) of both sexes between March and June 2021 (see Table 4.1 for sample size). All subjects were born in captivity and housed in a semi-natural, social aviary environment. Each outdoor flight aviary (1.5 x 1.2 x 2.1m) is exposed to ambient temperatures and natural daylight, which is supplemented with artificial lighting on a 14L:10D

photoperiod. Birds are provided *ad libitum* food (whole corn and turkey/game bird protein starter, 30% protein; Modesto Milling, CA), grit and water. Each aviary houses 10-12 breeding pairs of rock doves and includes wooden nest boxes (16 total) and nesting material (straw). Birds are allowed to naturally form breeding pairs and select and defend nest sites. Nest boxes are checked daily and the identity of the attending parent, presence and number of eggs or chicks is recorded. This daily data collection allowed us to collect samples at precise stages of incubation and yielded a full breeding history for each individual bird.

To understand if and how increasing circulating PRL and duration of incubation may affect hormonal responses to stress, we collected blood samples from both sexes of breeding pairs at the following reproductive stages: 1) the third day of incubation, when clutch completion typically occurs (clutches in this species are typically 2 eggs), 2) the ninth day of incubation, (about halfway through the 18 day incubation period for this population), and 3) day 17 of incubation, the day before expected hatching (which typically occurs after 18 days of incubation). All timepoints were compared to a control group, birds with no active nest (i.e. not currently incubating eggs or rearing chicks, but could be participating in courtship or nest building behaviors). Sample sizes for each stage are shown in Table 4.1. We selected these stages as circulating prolactin has been shown to increase at clutch completion as compared to nest building, and increases from mid- through late-incubation (Austin et al., 2021b). We also limited stages to incubation only to control for offspring cue type (eggs), as presence of chicks has been shown to alter prolactin and prolactin responsiveness in rock doves (Austin et al., 2021b; Farrar et al., 2021). All birds measured during incubation had never raised chicks or had a chick hatch previously ("inexperienced"), though they may have had previous nests with eggs.

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, and had not, had raised chicks in previous nests. For this comparison, blood samples were taken when birds had no active nest. Birds with no active nest can be considered in a non-parental, "baseline" state, as they have no eggs nor chicks to attend,

though they are likely reproductively active and may be engaged in courtship or nest building. "Inexperienced" birds for this comparison were the same birds used as controls described above. "Experienced" birds had raised at least one chick in a prior nest. The average time since the last nest effort ended did not significantly differ between experienced and inexperienced birds (9.9 days vs. 14.9 days on average; $t_{36} = -0.91$, $p = 0.372$). Experienced birds were older than inexperienced birds at sampling time (1.84 years vs. 1.38 years on average; $t_{36} = 3.55$, $p = 0.001$). 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 vs. 24.9 days on average; $t_{36} = -2.47$, $p = 0.032$).

All methods and procedures were approved by the University of California Davis IACUC (protocol #22407).

Breeding stage	Female (n)	Male (n)
No active nest (Experienced)	8	8
No active nest (Inexperienced)	10	9
Incubation day 3	11	10
Incubation day 9	10	10
Incubation day 17	10	10

Table 4.1. Stress series collected by breeding stage. Full stress series (three blood samples each bird) were collected from 96 birds total. "Inexperienced" birds had never had chicks in a previous nest (though may have previously had nests with eggs), while "experienced" birds had raised at least one chick in a previous nest. All birds sampled during incubation stages were inexperienced with chicks, and we sampled both the male and female of nesting pairs.

Dexamethasone dosage validation

To test birds' maximal negative feedback ability after a stressor, we used dexamethasone (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 minutes 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 minutes, we removed birds from bags, took a ~100 µL blood sample from the alar wing vein. We then immediately injected birds intramuscularly with DEX (Cat No. D1756, Sigma Aldrich, Milwaukee, WI, USA) at either 1 mg/kg ($n = 3$), 2 mg/kg ($n = 5$), or 4 mg/kg ($n = 5$) 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 minutes post-DEX (~ 100 µL each sample). All blood samples were taken between 0800 - 1100 (PST) in February 2020.

Plasma CORT levels in birds treated with saline vehicle did not change after 60 or 90 minutes of recovery post-stressor (Fig.4.2; one-way ANOVA: $F_{2,16} = 0.4$, $p = 0.957$). Despite being effective in other bird species (M. J. Dickens et al., 2009; Lattin et al., 2012), 1 mg/kg DEX also did not significantly reduce CORT levels 60 or 90 minutes after stress ($F_{2,5} = 1.1$, $p = 0.396$). DEX dosages of 2 mg/kg ($F_{2,12} = 7.4$, $p = 0.008$) and 4 mg/kg ($F_{2,12} = 7.7$, $p = 0.007$) significantly decreased after 60 and 90 minutes recovery. Additionally, both 2 mg/kg and 4 mg/kg DEX doses significantly differed from vehicle after 60 and 90 minutes ($F_{3,16} = 4.1$, $p = 0.023$). Thus, we chose to use the lowest effective DEX dose, 2 mg/kg, measuring post-DEX CORT levels after 60 minutes 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.4.1A). First, we collected a sample of blood from the alar wing vein using a 26G needle within three minutes of capture (106 ± 30 seconds) from the bird's home cage. Samples collected within three minutes 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: $F_{1,245} = 0.002$, $p = 0.96$; PRL: $F_{1,243} = 0.27$, $p = 0.607$). We then placed each bird in an opaque cloth bag to simulate an acute handling stressor and collected a second blood sample 30 minutes later to measure stress-induced hormone levels. After this blood sample was taken, we injected each bird intramuscularly with 2 mg/kg DEX (dosage validated as described above) and then returned birds to their home cage to recover. We collected the last blood sample 60 minutes after DEX injection to measure negative feedback hormone levels. All blood samples were approximately 100 µL each and were collected between 0800 - 1100 PST in April - June 2021. We found no effect of time of day on either CORT ($F_{1,286} = 0.21$, $p = 0.649$) or PRL concentration ($F_{1,275} = 3.3$, $p = 0.069$). For birds collected during incubation stages, we collected blood samples from both the male and female of the pair on the same morning.

We centrifuged blood samples for 10 minutes at 10,000 rpm 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 (CORT) radioimmunoassays

Circulating corticosterone (CORT) concentrations were measured from plasma at the UC Davis Metabolomics core using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals, Orangeburg, NY). A serial dilution was performed prior to the assay, and plasma samples from 0 min, 30 min and 90 min timepoints 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. 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 (ELISA)

We measured circulating prolactin (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, Inc. (San Diego, CA). This assay has previously been used with rock dove plasma (Booth et al., *submitted*). Briefly, biotinylated recombinant chicken PRL (ADS Biosystems, Inc., San Diego, CA) is added to samples and standards and competes for binding sites on the bound rabbit anti-chicken PRL antibodies (A.F. Parlow, National Hormone and Peptide Program, Los Angeles, CA). Visualization occurs through an enzymatic reaction using streptavidin horseradish peroxidase (HRP) (Cat. No. 21130, Thermo Fisher, Waltham, MA). 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 with a chicken PRL standard curve, 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 coefficients of variation (CV). Mean intra-assay %CV was 5.79%. We ran all plasma series (0 min, 30 min, 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) at 450 nm with background subtracted from 595 nm. Concentrations were interpolated from the standard curve using a 4-parameter fit (iMark software v.1.04, Bio-Rad Laboratories). Two samples (one in incubation day 17, one inexperienced no-active-nest) were not run due 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 mixed effects linear model, where a random effect of individual bird was included to account for the repeated-measures design of our stress series. Models were created using the lme4 package (v.1.1.27)(Bates et al., 2015). In these models, we tested how the independent variables of incubation stage (or experience level, experienced with chicks or inexperienced), stress series timepoint, sex, and their interactions affected the dependent variable of hormone concentration. To improve distribution of the data, all hormone concentrations were $\log_{10}$-transformed. We report results from ANOVA (Type III) run on these mixed effects models using the car package (v.3.0.1)(Fox and Weisberg, 2019). 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)(Lenth, 2020).

Experiment 2 : Hippocampal and pituitary gene expression

In this second experiment, we followed up upon results from Experiment 1 that showed an effect of parental experience on CORT and PRL release 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 glucocorticoid receptor (GR; also known as NR3C1) and mineralocorticoid receptor (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). We also measured corticotropin releasing hormone receptor 1 (CRHR1) in the pituitary, as this receptor mediates responsiveness to hypothalamic CRH signals of HPA activation (Bonfiglio et al., 2011).

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 (nor had nests with eggs). As described above, doves that are not actively nesting are not currently incubating eggs or attending to chicks, but may be engaging in other reproductive behaviors, such as courtship, pair-bond maintenance and nest building. 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 vs. 10.0 days in inexperienced birds; t_{28} = 0.85, p = 0.419). We euthanized birds using methods previously used in rock doves (e.g., Calisi et al., 2018; MacManes et al., 2017). Within three minutes of capture, birds were euthanized with an overdose of isoflurane 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 0800-1100 PST 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 µM slices. We used landmarks from the Karten and Hodos (1966) pigeon brain atlas to locate the hippocampus, starting with when the commissura anterior (CA) visibly crossed the coronal section and ending when the cerebellum was visible (Fig.4.5A, plates A 7.75 - A 4.25 in Karten and Hodos 1966; average of 27-30 punches at 100 µM). 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 Bioscience). RNA concentration and quality was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 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 glucocorticoid receptors (*GR*) and mineralocorticoid receptors (*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 *Columba 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 4.2.

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 (BioRad), and 10 µM each of primer (total volume: 10 µL, Invitrogen). We ran plates on a CFX384 Touch Real-Time PCR detection system (BioRad) under the following thermocycling protocol: 50℃ for 2 min, 95℃ for 10 min, and then 40 cycles of 95℃ for 15 sec and 60℃ for 30 sec. Plates also included no-template controls. To reduce the need to account for interplate variation, 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 Ct method. To do this, we first normalized the expression (cycle threshold, Ct) 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 ($F_{1,26} = 2.14$, $p = 0.155$) or sex ($F_{1,26} = 0.09$, $p = 0.766$) in our samples using two-way ANOVA. Then, we relativized normalized expression (delta-Ct) for each sample to the average normalized expression for the control group (delta-delta-Ct), which in this case was inexperienced birds. Lastly, we calculated fold change, or $2^{(- ddCt)}$. 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 due to the relative expression calculations used in the Livak and Schmitgen (2001) method.

4.4. RESULTS

Experiment 1 : Hormonal responses to acute stress

Effects of parental experience on birds without active nests

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 timepoint on CORT levels (Table 4. 3). Post-hoc analyses show that this interaction is driven by experienced birds having lower CORT post-stressor (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.4.3A). Further, timepoint as a main effect was significant (Table 4. 3), as expected. Averaged over levels of experience and sex, CORT levels increased in response to 30 minutes of acute-restraint stress compared to baseline (t = -23.5, p < 0.001) and subsequently decreased after 60 minutes of DEX-induced negative feedback (t = 14.6, p < 0.001). Negative feedback CORT levels were also significantly higher than baseline levels (t = -9.14, p < 0.001). There was no significant main effect of sex, nor a significant interaction between sex and experience or sex and timepoint in CORT levels (Table 4. 3).

In PRL levels, we found a significant three-way interaction between experience, sex, and timepoint (Table 4. 3). This suggests that how previous parental experience affects the stress response sequence differs between the sexes. As with CORT, we found that experience altered levels across timepoints when averaged across sexes (Table 4. 3), with experienced birds having higher levels of PRL both 30 (t = 4.85, p < 0.001) and 90 minutes (t = 4.99, p < 0.001) after a stressor. However, this relationship differed significantly between the sexes. The difference between experienced and inexperienced females was larger at both 30 and 90 minutes than the difference between experience levels in males at these timepoints (Fig.4.3B; Figure 4.6). At 30 minutes, experienced females had 4.9 ± 1.6 (SE) times higher PRL than inexperienced females (t = 4.84, p < 0.001), while experienced males only had 1.9 ± 0.6 times higher PRL than inexperienced males (t = 2.02, p = 0.048). Similarly, after 90 minutes, experienced females had 5.8 ± 1.8 times higher PRL levels compared to their inexperienced counterparts (t = 5.24, p <0.001), compared to only 1.8 ± 0.6 times for males (t = 1.82, p = 0.073) (Fig.4.3B). 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 (Fig.4.3B; Fig. 4.6).

Effects of incubation stage on birds inexperienced with chicks

Across incubation in birds inexperienced with chicks, we found a significant main effect of stress-series timepoint, along with significant interaction between incubation stage and timepoint (Table 4. 4). As expected, CORT increased after 30 minutes of restraint stress compared to baseline (t = -24.3, p < 0.001) and post-DEX negative feedback levels (t = -13.4, p < 0.001) when averaged across all incubation stages and sexes. Negative feedback CORT levels were also significantly higher than baseline levels (t = -10.8, p < 0.001) on average. The significant interaction between incubation stage and timepoint appears to be due to the large effect of timepoint. However, in post-hoc comparisons, negative feedback CORT levels appeared to decrease significantly from incubation day 3 to day 9 (t = 2.99, p =0.019; Fig.4. 4A). Trends also show stress-induced CORT (30 min) decreases slightly from incubation day 3 to day 9 (t = 2.12, p =0.070), and then subsequently increases in birds at incubation day 17 compared to day 9 (t = 2.16, p =0.070). However, these trends did not reach significance at α = 0.05 after FDR correction. Lastly, we found a significant main effect of sex, where females had higher CORT overall than males on average (t = 2.60, p =0.012).

In PRL levels, we found a significant interaction between both incubation stage and timepoint, and between incubation stage and sex (Table 4. 4). The main effect of incubation stage, as well as the interaction with timepoint, is largely driven by significantly higher PRL at incubation day 17 compared to all other stages (all post-hoc comparisons p < 0.001) (Fig.4.4B). The PRL stress response pattern also differed across stages. Inexperienced birds with no active nest, PRL significantly declined after 30 minutes of acute stress (t = 4.26, p =0.001) and stayed significantly lower after negative feedback (t = 5.57, p <0.001) when averaged across sex. Birds at incubation day 3 showed a significant decline after the

stressor (t = 2.60, p =0.012), but this difference was no longer significant after negative feedback and recovery. At incubation day 9, birds showed no significant changes in PRL across the stress series. Finally, on incubation day 17, there was a trend towards decreasing PRL 90 minutes post-stressor (t = 2.35, p =0.056), but not after 30 minutes (t = 1.53, p =0.276). These stress series responses by stage are shown in an interaction plot of estimated marginal means in Figure 4.7. Lastly, while there was a significant interaction between stage and sex, no post-hoc comparisons remained signfiicant after FDR correction. We also detected no significant main effect of sex on PRL levels.

Experiment 2 : Hippocampal and pituitary gene expression

In the hippocampus, prior experience with chicks significantly increased GR gene expression (Fig.4.3A; $F_{1,26}$ = 11.1, p = 0.002) but did not affect MR gene expression (Fig.4.3B; $F_{1,26}$ = 2.7, p = 0.113). There was no significant effect of sex ($F_{1,26}$ = 0.5, p = 0.530) nor 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 signifcant interaction between sex and experience with chicks (Fig.4.3B; $F_{1,26}$ = 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 is 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), nor their interaction ($F_{1,26}$ = 2.6, p = 0.120) on MR:GR expression ratio.
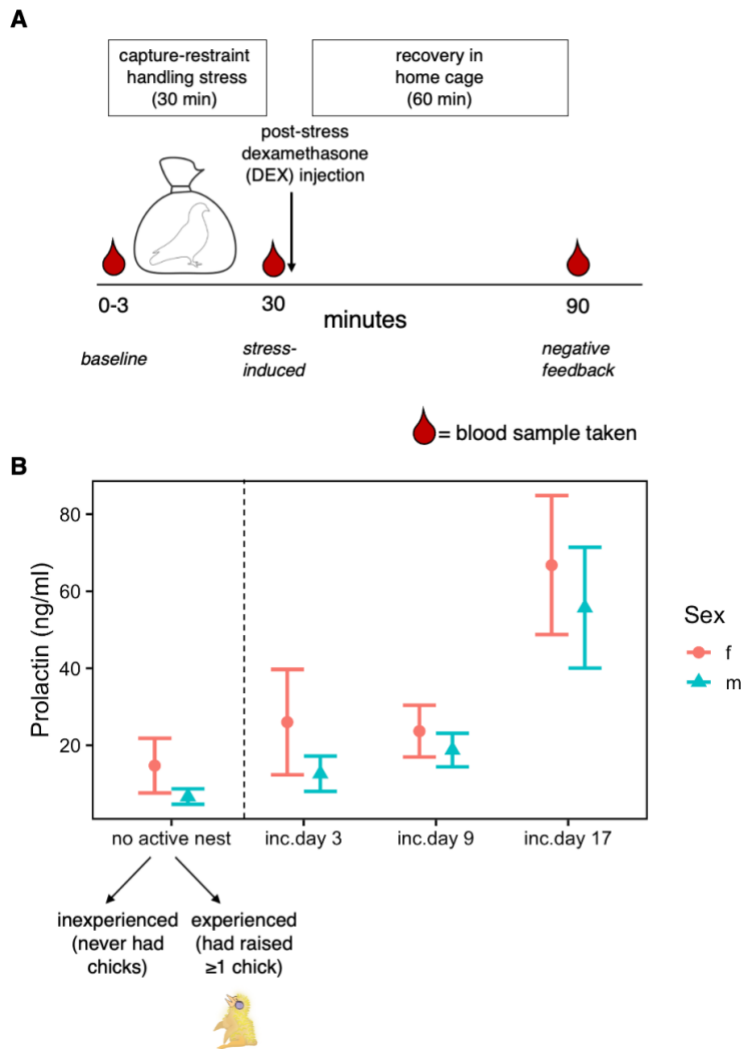
Figure 4.1. Sampling paradigm for Experiment 1. (A) Three blood samples were taken from birds to

assess hormonal responses to stress: 1) baseline (< 3 minutes from capture), 2) stress-induced (after 30

minutes in an opaque cloth bag, representing a classic capture-restraint stressor), and 3) negative feedback

(60 minutes after injection with dexamethasone and recovery in home cage). Dexamethasone was injected

immediately after the stress-induced blood sample was taken. Plasma from blood samples were used to

measure corticosterone (CORT) and prolactin. **(B)** Expected baseline circulating prolactin concentrations

(means ± 95% confidence intervals) at the reproductive stages sampled. We sampled from four

reproductive stages: 1) no active nest, when birds may be courting or nest building, 2) incubation day 3,

the day of clutch completion in this population, 3) incubation day 9, approximately halfway through the

incubation period, and 4) incubation day 17, the day before expected hatching. Additionally, we collected

blood samples 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. Sample sizes for each stage can be found in Table 4. 1. Circulating prolactin data is

reproduced from previous radioimmunoassay data published in our rock dove population (Austin et al.,

2021b) (nest building was used as equivalent to "no active nest" in this study).
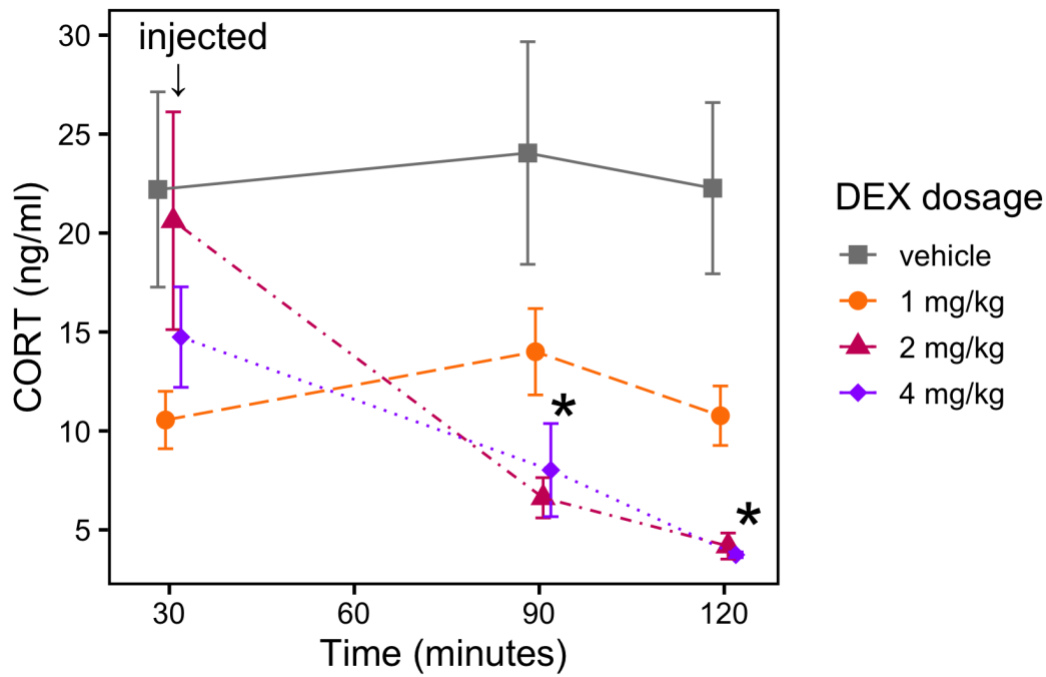

Figure 4.2. Plasma corticosterone (CORT) response to injections of various doses of dexamethasone

(DEX) or vehicle after 30 minutes of restraint. Birds were either injected with a dose of DEX (1 mg/kg,

$n = 3$; 2 mg/kg, $n = 5$, 4 mg/kg, $n = 5$) or saline vehicle ($n = 6$) after being exposed to an acute capture-

restraint stressor for 30 minutes (injection time indicated with an arrow). Mean ± SEM are shown. * = p

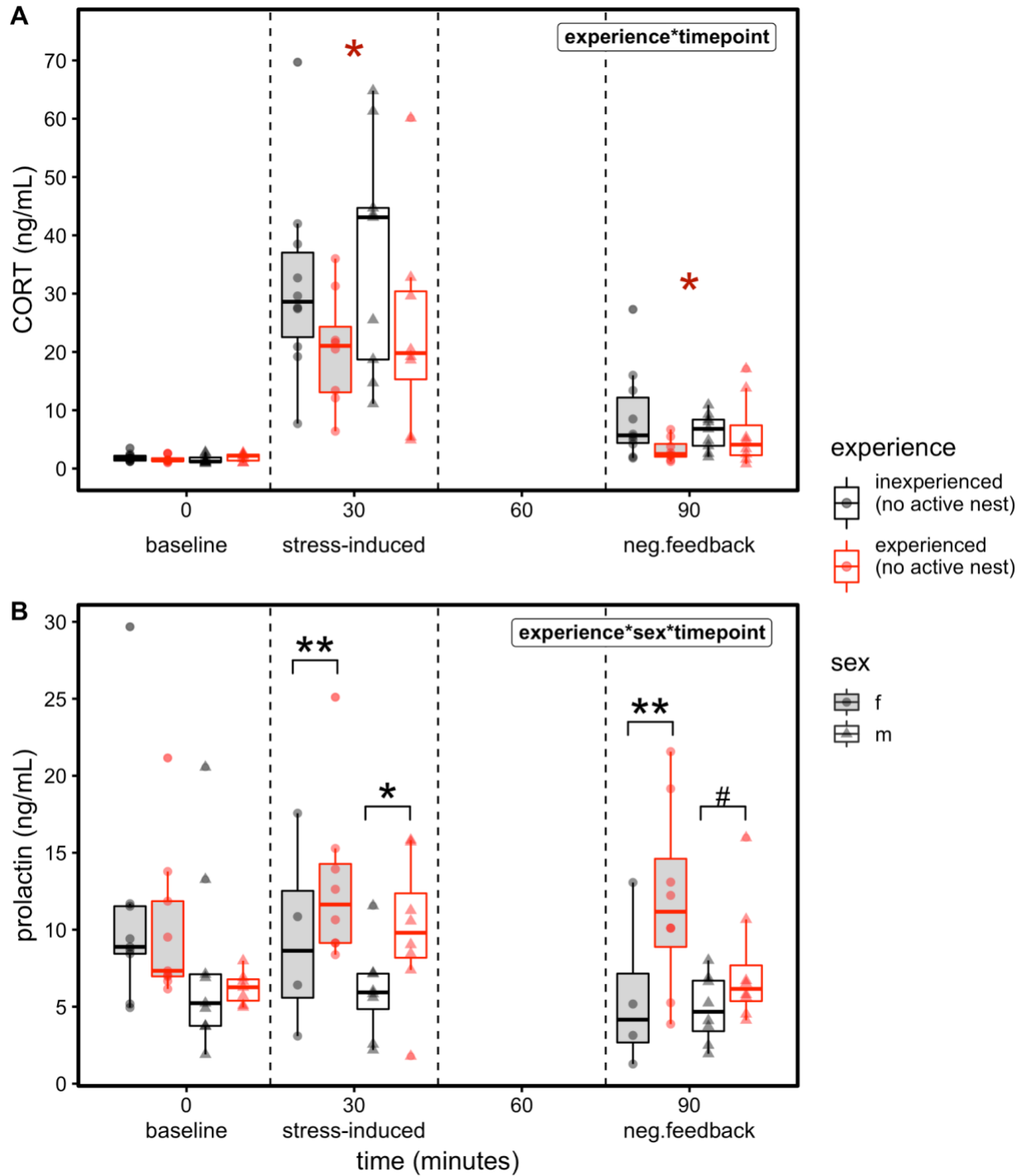< 0.05 for 2 mg/kg and 4 mg/kg doses compared to the 1 mg/kg and vehicle groups at the same timepoint.

Figure 4.3. Circulating hormones vary with parental experience, stress-series timepoint, and sex.

(**A**) Plasma corticosterone (CORT) and (**B**) plasma prolactin was 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 minutes after capture), after capture-restraint stress (30 min post capture) and after dexamethasone (DEX) induced negative feedback (60 minutes of recovery post stressor, 90 min after capture). Sampling timepoints are separated visually with dashed lines. Points represent individual birds, and boxplots represent the where 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, timepoint, sex and their interactions are shown in bold in the upper right corner (see Table 4. 3). In plot A, red asterisks (*) indicate a significant effect ($p < 0.05$) of experience averaged over levels of sex in post-hoc analyses. In plot B, brackets represent significant differences in experience between the sexes across timepoints in post-hoc contrasts, where # = $p < 0.1$, * = $p < 0.05$, and * * = $p < 0.01$.
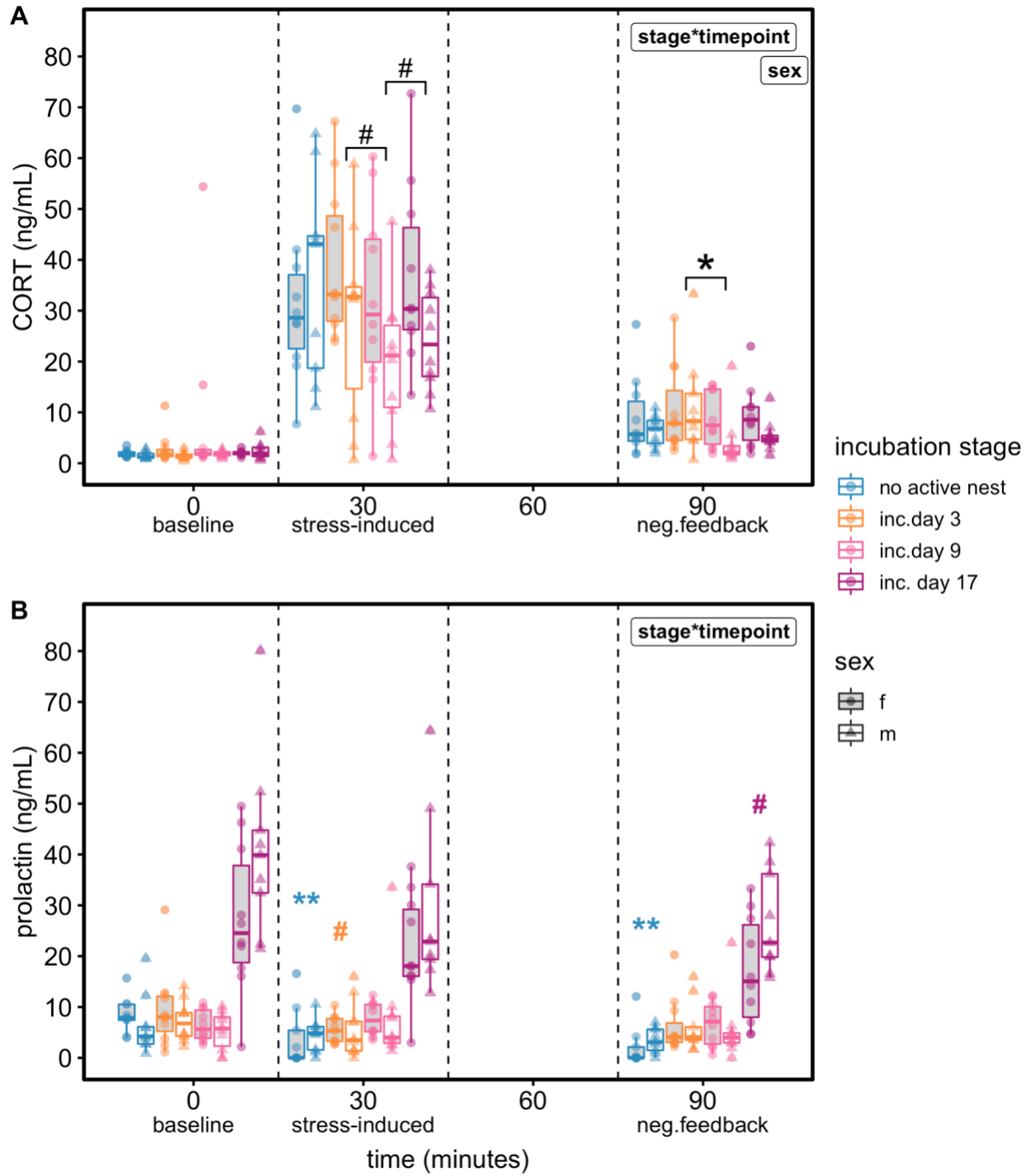
Figure 4.4. Circulating hormones across stress-series timepoints, incubation stage and sex. (**A**)

Plasma corticosterone (CORT) and (**B**) plasma prolactin was measured for each incubation stage (coded

by color) at baseline (0-3 minutes after capture), after capture-restraint stress (30 min post capture) and

after dexamethasone (DEX) induced negative feedback (60 minutes of recovery post stressor, 90 min after capture). Sampling timepoints are separated visually with dashed lines. Points represent individual birds, and boxplots represent the where 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) in a breeding pair. The highest level significant predictors from the linear mixed model including stage, timepoint, sex and their interactions are shown in bold in the upper right corner (see Results; Table 4. 4). In plot A, significant post-hoc analyses showing effects of stage across timepoint are shown with brackets comparing different incubation stages. In plot B, significance markers denote where the 30 minute or 90 minute timepoints significantly differed from baseline in post-hoc contrasts, and are colored by stage. $\# = p < 0.1$, $^* = p < 0.05$, and $^{**} = p < 0.01$.
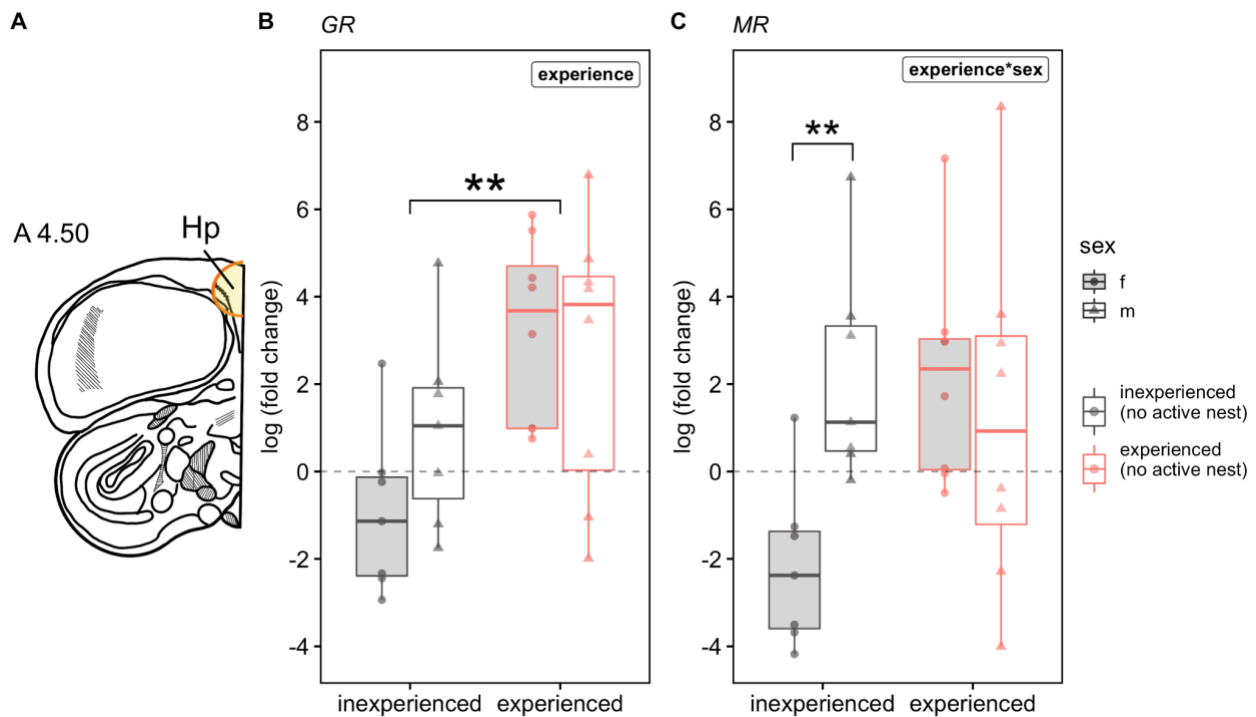
Figure 4.5. Relative expression of glucocorticoid receptor types in the hippocampus of birds with and without prior parental experience with chicks. (A) Representative hippocampal sections, in which (B) glucocorticoid and (C) mineralocorticoid receptor expression was measured using quantitative PCR and compared across birds who had never previously raised chicks ("inexperienced", gray) and birds who had previously raised at least one chick ("experienced", red). Points represent individual birds, and boxplots represent the where 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, and males with unshaded boxplots, triangles). Significant predictors from the linear model including experience,sex and their interactions are shown in bold in the upper right corner (see Results). Brackets indicate specific significant post-hoc comparisons after FDR correction, with $**= p < 0.01$.

Gene (Abbreviation)	NCBI Accession number	Amplicon length (base pairs)	Efficiency (%)		Primer sequence	Notes
Reference genes						
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	
Genes of interest						
Glucocorticoid receptor *(GR)*	XM_021301096.1	77	90.5	F	TGCTTAACTCGTCGGATCAA	Also referred to as nuclear receptor subfamily 3 group C member 1 (*NR3C1*)
				R	AAAGTCCATCACGATCCCTC	
Mineralocorticoid receptor (*MR*)	XM_021296726.1	158	103.8	F	AGAACATGGCTTCCTCGGTG	Also referred to as nuclear receptor subfamily 3 group C member 2 (*NR3C2*)
				R	CTAGAAAGCGGAGACCCGAC	

Table 4. 2. Primers used in quantitative PCR. Primers were designed using the NCBI Primer-BLAST tool on gene templates for *Columba livia* (indicated by NCBI accession numbers). Primer efficiencies were calculated by running a standard curve of five 10-fold dilutions of purified PCR product, and primers were evaluated for single products via melt-curve analysis during validation.

	Corticosterone			Prolactin		
Variable	*df*	*F*	*p*	*df*	*F*	*p*
Experience	1	4.24	0.048	1	16.49	**<0.001**
Sex	1	0.01	0.913	1	0.04	0.845
Timepoint	1	283.92	**<0.001**	1	6.14	**0.004**
Experience x Sex	2	0.39	0.537	2	3.01	**0.093[#]**
Experience x Timepoint	1	3.15	**0.050**	1	14.93	**<0.001**
Sex x Timepoint	2	0.46	0.637	2	2.52	**0.089**
Experience x Sex x Timepoint	2	1.03	0.363	2	3.65	**0.032**
Residuals	92			89		

Table 4. 3. ANOVA from mixed effect model for effects of parental experience, stress series timepoint, sex and their interactions on $\log_{10}$-transformed concentrations of corticosterone and prolactin. This model included a random effect of individual bird to account for the repeated measures design of the stress series. All birds included in this dataset were not actively nesting (i.e., not actively incubating eggs or caring for chicks). Significant effects at the $\alpha = 0.05$ level are indicated in bold, and # indicates a trend at the $\alpha = 0.10$ level.

	Corticosterone			Prolactin		
Variable	*df*	*F*	*p*	*df*	*F*	*p*
Incubation Stage	3	1.74	0.161	3	65.02	**<0.001**
Sex	1	6.74	**0.012**	1	0.25	0.621
Timepoint	2	298.15	**<0.001**	2	13.19	**<0.001**
Stage x Sex	3	1.22	0.305	3	3.55	**0.016**
Stage x Timepoint	6	2.38	**0.032**	6	3.89	**0.001**
Sex x Timepoint	2	0.09	0.912	2	1.59	0.206
Stage x Sex x Timepoint	6	1.03	0.410	6	1.50	0.182
Residuals	214			209		

Table 4. 4. ANOVA from mixed effect model for effects of incubation stage, stress series timepoint, sex and their interactions on $\log_{10}$-transformed concentrations of corticosterone and prolactin. This model included a random effect of individual bird to account for the repeated measures design of the stress series. Significant effects at the $\alpha = 0.05$ level are indicated in bold.
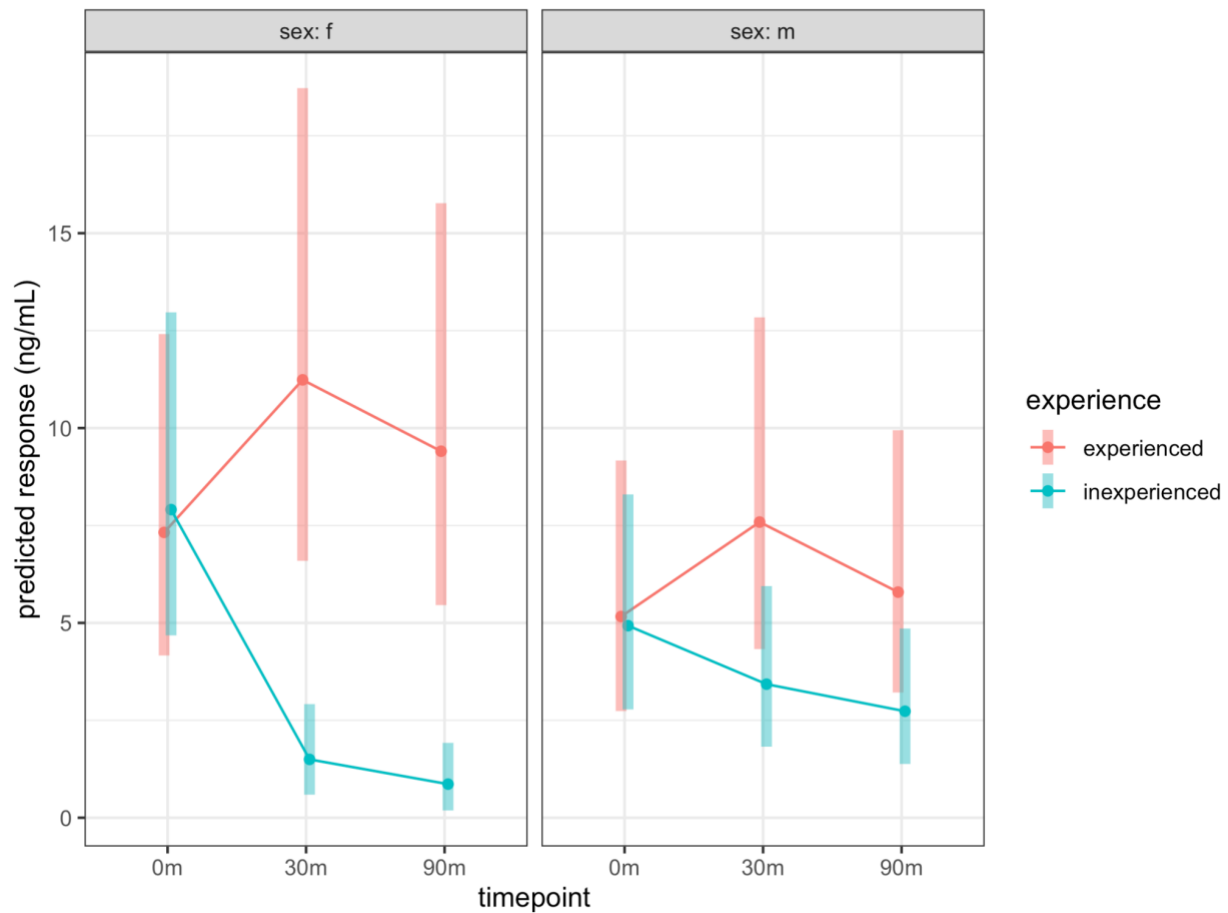
Figure 4.6. Interaction plot for estimated marginal mean PRL across parental experience, sex, and timepoint in non-actively nesting birds. Predicted responses from the mixed linear model, shown in concentration (ng/mL), for experienced (red) and inexperienced (blue) birds are across the stress series timepoints and sexes. The left plot shows trends in females and the right in males. Dots represent estimated marginal means and shaded areas represent 95% confidence intervals around these means. Plot produced using the emmeans package in R statistical language (Lenth, 2020).
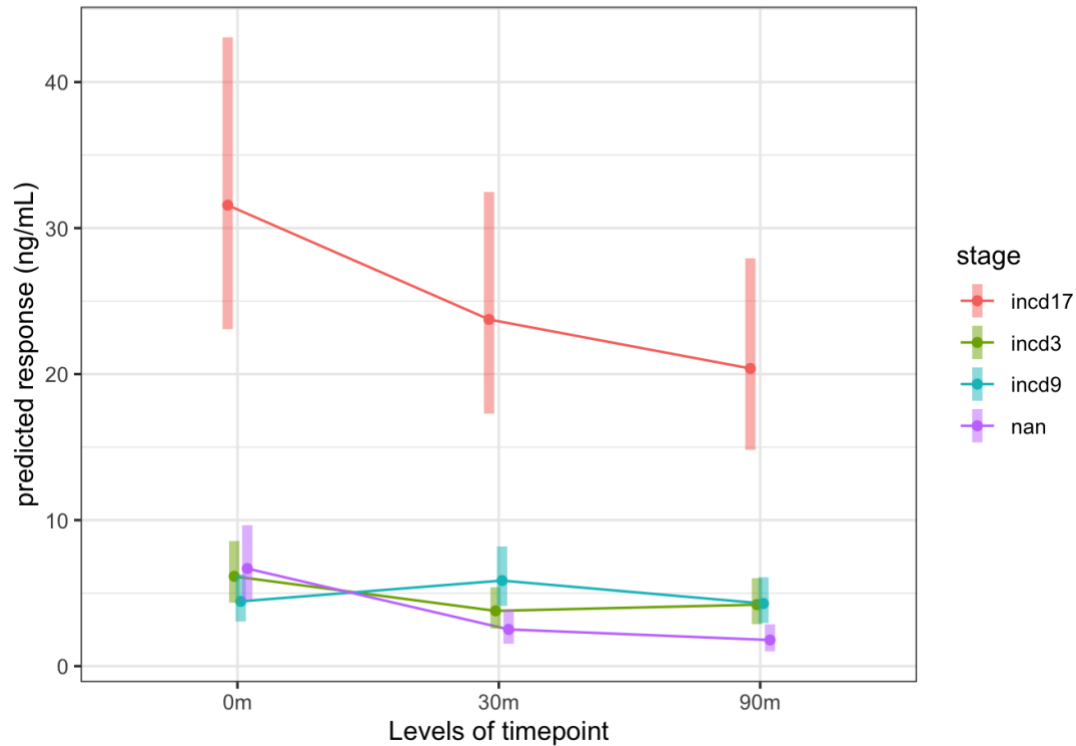
Figure 4.7. Interaction plot for estimated marginal mean PRL across incubation stage and timepoint, averaged over levels of sex. Predicted responses from the mixed linear model, shown in concentration (ng/mL), for each incubation stage across the stress series timepoints. Dots represent estimated marginal means and shaded areas represent 95% confidence intervals around these means. Color corresponds to the incubation stage. Plot produced using the emmeans package in R statistical language (Lenth, 2020).

4.5. DISCUSSION

We found that previous parental experience with chicks decreased stress-induced and dexamethasone-induced negative-feedback CORT levels, and led to increased stress-induced PRL in rock doves without active nests (i.e. in a pre-parental state) Further, in a separate experiment, we found that birds of both sexes with previous experience with chicks also had higher hippocampal *GR* than inexperienced birds. Increased *GR* may lower the threshold for negative feedback and suppressive effects on the HPA axis in experienced birds (Sapolsky et al., 2000), thus potentially mediating the changes in the CORT stress response we observed. 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). We also evaluated the parental care hypothesis (Wingfield et al., 1995) within a breeding phase (egg incubation). While we found CORT increased after stress and decreased with dexamethasone-induced negative feedback, consistent with other avian species, we did not find meaningful variation in the stress response across the incubation period in rock doves. We also only found mixed evidence of PRL stress responses across the incubation period, where baseline PRL appears most variable.

Effects of parental experience on hormonal stress responses

Prior parental experience with chicks led to lower CORT, and higher PRL levels, both after an acute stressor and after dexamethasone-induced negative feedback. Previous studies that examined effects of prior breeding experience on CORT and PRL only measured baseline hormone levels(Angelier et al., 2007b, 2006), 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 led to significant effects after a stressor or

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during negative feedback in our study, highlighting the importance of measuring hormone 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 to younger ones during incubation (Heidinger et al., 2010, 2006). Similarly, younger snow petrel females had lower stress-induced PRL than older females (Angelier et al., 2007a), and senescent albatross had lower CORT levels, but not PRL, levels than younger birds (Angelier et al., 2006). In our study, the range of ages was small (0.5 - 3 years, with 80% between 1-2 years old), making age less likely to drive our observed effect of experience. Instead, increasing age may lead to increasing breeding experience (which would be correlated in most populations), so the effects of age on hormonal stress responses seen in prior work may be mediated in part by effects of parental experience. Indeed, when both were measured, breeding experience appeared to better statistically predict hormone levels than age (Angelier et al., 2007b, 2006).

Our observation that birds without prior parental experience exhibit a more reactive stress response in both CORT and PRL than experienced birds lends support for both the "constraint" and "restraint" hypotheses about why reproduction may improve with age (Curio 1983). Under the constraint hypothesis, inexperienced birds may be limited (constrained) in their ability to invest in reproductive efforts over personal survival in the face of stressors. That is, these inexperienced birds may not be able to modulate down and attenuate the HPA axis or maintain PRL secretion under stress. This interpretation implies that there may be mechanistic differences in HPA regulation between inexperienced and experienced birds, which we found evidence for in the hippocampus (see below). Alternatively, as the inexperienced birds we sampled were slightly, but significantly, younger than experienced birds (mean 1.84 vs 1.38 years), the "restraint" hypothesis may also be supported (Curio 1983). In this case, inexperienced, younger birds may limit (restrain) their parental investment due to their relatively larger

opportunities for future reproduction compared to older, more experienced breeders (lower residual reproductive value; Stearns, 1976). This interpretation is also consistent with the "brood value hypothesis" (Heidinger et al., 2010; Lendvai et al., 2007), where older, experienced birds may modulate their stress response because their current / next brood has relatively higher reproductive value.

Another interpretation is that experienced birds were actually closer to parental care (either had more recently ended a chick care bout, or were closer to restarting another nest) than inexperienced birds, and this drove 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 vs 24.9 days), though the time since last nest effort did not differ significantly (9.9 vs 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. If this was the case, our results would align with the "parental care hypothesis" (Wingfield et al., 1995) where birds more involved in parental effort show attenuated stress responses than those not engaged in care. Examining the effects of experience on birds during the parental period (i.e. during incubation or brooding) would make it clear if our results are due to differential reproductive effort or actually persistent effects of experience.

Hormonal stress responses across incubation

Across the incubation period, we did not find significant changes in baseline, stress-induced, or negative-feedback CORT in breeding birds that had not raised chicks in previous nests. We therefore did not find support for the parental care hypothesis in CORT (Wingfield et al.,1995), as we did not observe

more attenuated stress responses as the time invested in the parental effort increased. A substantial body of work has found support for the parental care hypothesis in birds (Bókony et al., 2009). As examples, red knots that started incubation (i.e., had brood patches) had lower stress-induced CORT than those that had not yet begun incubation (Reneerkens et al., 2002). Often, the differences in CORT stress responses across breeding are sex-dependent, as the sexes participate in parental care to differing degrees in many species. In multiple sparrow species, males had higher stress-induced CORT than females in the "pre-parental" incubation stages when males do not participate extensively in care, but the sexes converged to a lower CORT profile during chick rearing (Holberton and Wingfield, 2003). Similar findings were found in Smith longspurs, with females having overall more attenuated stress responses than males, but males decreased theirs during the chick-rearing period (Meddle et al., 2003). Across specific breeding stages, male Gambel's white-crowned sparrows decreased stress-induced CORT levels from territory establishment to breeding, while females decreased from incubation to chick rearing, where both sexes converged on an attenuated parental stress response profile (Krause et al., 2015). While we found females had overall higher CORT than males (similar to previous studies), we did not find a sex difference in response to stressors. The lack of sex differences in stress responses is consistent with the predictions of the parental care hypothesis, as both males and female rock doves participate nearly equally in incubation (Abs, 1983).

An alternative interpretation may be that our findings are consistent with the parental care hypothesis, if parental investment and effort was similar across incubation. If birds' parental investment does not meaningfully differ from clutch completion to late incubation (before hatch), then we might not expect to see differences in the stress response across these stages. During incubation, both male and female rock doves incubate the eggs nearly constantly, with the sexes taking time-specific shifts that do not differ across incubation (Abs, 1983; *personal observation*). Indeed, (Holberton and Wingfield, 2003) found that female sparrows, who provide extensive care during both incubation and chick-rearing, did not

show differences in stress-induced CORT between these stages, compared with males who are more involved in chick care. Thus, the timescale of comparisons (incubation stages) may not be as functionally different as breeding stage comparisons seen in other studies (i.e., pre-lay, incubation, versus chicks), and we may have observed differences when parental demands meaningfully differed, such as incubation versus chick rearing. We also cannot rule out that our captive population, with *ad libitum* food and protection from the elements, may not express the stress attenuation necessary for successful reproduction in wild animals living in extreme and unpredicTable 4. environments (Krause et al., 2016; Lendvai et al., 2014; Wingfield, 2002). These effects may also be taxa-specific, as a similar study in wild mourning doves did find differences in stress-induced CORT across the nestling period (Miller et al., 2009).

In PRL, baseline levels differed with stage, but stress-induced or negative-feedback levels did not meaningfully change across incubation. Baseline PRL levels have been shown previously to differ during incubation in rock doves (Austin et al., 2021b; Farrar et al., 2022), as it facilitates egg brooding and the production of nutrient-rich crop milk for chick provisioning (Buntin et al., 1996; Horseman and Buntin, 1995)**.** PRL stress responses, where PRL significantly decreased after an acute stressor, appeared in inexperienced birds with no active nests and at incubation day 3, but overall were not present in birds later in incubation. These results are somewhat consistent with the prolactin stress response hypothesis (Angelier and Chastel, 2009; Chastel et al., 2005), which relates to the parental care hypothesis and posits that parental birds will attenuate the typical reduction in PRL when the value of the brood is high. While PRL stress responses have been found during breeding in some birds, others did not find any effect of 30 minutes of acute stress on wild birds' PRL levels (Jesse S. Krause et al., 2015), or that the PRL stress response did not change during breeding (Miller et al., 2009).

Lastly, we found the PRL stress responses may be detecTable 4. at late incubation, but only 90 minutes after a stressor, when maximal negative feedback has been induced. To our knowledge, this is the first

study to examine how PRL, in addition to CORT, responds to maximal glucocorticoid levels. While this was not a main goal of our study and we did not compare the natural recovery without dexamethasone, future PRL stress series should be extended past 30 minutes of an acute stressor.

Effects of parental experience on hippocampal glucocorticoid receptors

When we examined hippocampal glucocorticoid receptors, we found that, when not actively nesting, birds of both sexes that had previously had chicks had higher *GR* expression than birds inexperienced with chicks. 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 to 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). 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 expression, but not GR, 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*, in Japanese quail (Soleimani et al., 2011; Zimmer and Spencer, 2014)**.** Similarly, chronic stress nor translocation to captivity, which both led to attenuated HPA axis responses, affected hippocampal GR in starlings or chukar (M. Dickens et al., 2009; Dickens et al., 2011). Alternatively, GR in the hypothalamus, another potential site of negative feedback (Smulders, 2021), may be more important for HPA axis regulation in

other species, as chronic and prenatal stress reduced GR in the hypothalamus of European starlings and Japanese quail, respectively (Dickens et al., 2009; Zimmer and Spencer, 2014). Other studies of seasonal transitions, however, found no differences in hippocampal or hypothalamic GR across breeding stages when stress responses had been shown to attenuate (Gambel's white-crowned sparrows (Krause et al., 2015) ; house sparrows (Lattin and Romero, 2013)). However, 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).

Similarly, we did not find an overall effect of experience on hippocampal *MR* expression, in contrast with other studies that found altered hippocampal *MR* in birds. In the aforementioned studies, selection for highly-reactive stress profiles, chronic stress, developmental stressors, and breeding transitions all altered hippocampal *MR* expression (Dickens et al., 2009; Hodgson et al., 2007; Krause et al., 2015; Zimmer and Spencer, 2014), with all associating decreased *MR* expression with reduced stress-induced CORT release. Again, we did not find this effect. However, we found an apparent sex difference present in inexperienced birds, with females expressing lower *MR* in males, that was not present in experienced birds. While 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 signfiicant differences in both stress response and hippocampal *MR* (Dickens et al., 2009; Hodgson et al., 2007). These results emphasize the importance of studying these mechanisms in both sexes, as to further understand what contexts may lead to presence, and absence, of sex differences in HPA axis regulation.

The discrepancies we found with other avian studies may be due to differences in the context of our study (parental experience) and/or species differences. To our knowledge, our study is the first to

investigate how prior parental experiences may affect hippocampal *GR* and *MR* expression in birds. It is possible that prior reproductive cycles, and the many endocrine changes involved (Austin et al., 2021b), may alter hippocampal gene regulation in ways that differ from those observed in stress contexts or other annual cycle transitions. Indeed, female rats show attenuated stress response and reduced hippocampal *GR* expression in late pregnancy(Johnstone et al., 2001). The experience of the changing hormonal milieu during gestation and preparation for lactation may be responsible for some of these changes (as suggested by (Torner and Neumann, 2002). In our study, it remains to be seen if the effects of experience continue beyond the pre-parental stage, when birds enter their next nesting attempt. If these effects truly persist into future breeding efforts, manipulations of hormones involved in the gaining of parental experience, such as fluctuating prolactin or oxytocin/mesotocin, would help to uncover the causes of HPA axis regulation in the hippocampus. Additionally, negative feedback may be mediated through other mechanisms,, such as steroid-metabolizing enzymes in target cells or corticosterone-binding globulins in plasma (Wingfield et al., 2015). Finally, we cannot rule out the role of species differences, as the continuously-breeding rock dove may differ in stress regulation from the seasonal breeders previously mentioned. Indeed, rock doves do appear to regulate CORT differently from other birds in some ways, such as not downregulating HPA activity during molt (Romero and Wingfield, 2001).

4.6. CONCLUSIONS

Overall, we found evidence in support of the "constraint" and "restraint" hypotheses for why younger, inexperienced birds may be poorer breeders than older, experienced individuals, that this effect may be related to the 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 glucocorticoid receptors involved in HPA axis regulation. We found mixed evidence for the parental care hypothesis and prolactin stress hypothesis across incubation in the rock dove, but future work comparing parental care stages that are distinct in behavior, energetic demands, and offspring cues may further test these hypotheses. Overall, investigations of the effect of parental

experience on hormonal stress responses and neural HPA axis regulation are few, and the results here may provide potential mechanisms for further exploration. These results set the stage for future studies examining how experience may enact lasting changes in HPA axis regulation, such as epigenetic mechanisms (Rubenstein et al., 2016; Siller and Rubenstein, 2019), as well as link these mechanisms to behavioral and fitness consequences of gaining parental experience.

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