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Prolactin promotes parental responses and alters reproductive axis gene expression, but not sexual behaviors, in a biparental bird

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ABSTRACT

Prolactin, a hormone involved in parental care, is hypothesized to inhibit reproduction by the hypothalamic-pituitary-gonadal (HPG) axis during parenting, maintaining investment in current broods as opposed to new reproductive efforts. While prolactin is known to act on parental behaviors, its effects on other reproductive behaviors, such as courtship, is understudied. How prolactin affects peptide hormone receptor expression across the HPG axis also remains unknown. To address these questions, we administered ovine prolactin (oPRL) or vehicle control to both sexes of experienced parental rock doves (Columba livia) after nest removal at the end of incubation. We found that oPRL promoted parental responses to chicks and stimulated crop growth compared to controls, consistent with other studies. However, we found that courtship behaviors and copulation were maintained after oPRL treatment. Across the HPG, we found PRL altered gene expression primarily in the pituitary, increased expression of FSHB and hypothalamic hormone receptor genes in the pituitary, and PRL increased testes gonadotropin receptor expression, as well as ovarian small white follicle gene expression. However, the oviducts of PRL-treated females were smaller and had lower estrogen receptor expression compared to controls. Our results highlight that in species that show multiple brooding, continued expression of mating behaviors may persist despite elevated prolactin. This mechanism may allow prolactin to promote parental care without the continued inhibition of reproductive function or HPG axis activity.

1. Introduction

Animals that exhibit parental care must balance current reproductive effort with the future reproductive opportunities (Stearns, 1976). These trade-offs require coordination of reproductive physiology and behavior (Ricklefs and Wikelski, 2002; Zera and Harshman, 2001), including endocrine mechanisms that are well-known to mediate life-history trade-offs (Hau and Wingfield, 2011; Williams, 2012).

Prolactin, a hormone involved in parental care across vertebrates (Bachelot and Binart, 2010; Freeman et al., 2000), is a key mediator of the transition into parental care. Prolactin (PRL) is a 23 kDa peptide hormone produced primarily in the anterior pituitary and acts via its receptor, PRLR, expressed widely across the body, including in the brain, multiple tissues (Grattan and Bridges, 2009) and gonads (Freitas et al., 2004). Best known for its crucial role in mammalian lactation, PRL also promotes parental motivation and behavior in males and females of many vertebrates (Brown et al., 2017; Hashemian et al., 2016; Smiley, 2019). In addition to promoting parental care, PRL is thought to inhibit the reproductive hypothalamic-pituitary-gonadal (HPG) axis. Within the HPG axis, the hypothalamus releases gonadotropin-releasing hormone (GnRH), which stimulates the pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH), stimulating gonadal release of sex steroids (e.g., testosterone, progesterone). Additionally, gonadotropin-inhibitory hormone (GnIH) in the hypothalamus can inhibit the release of GnRH, LH and FSH in some vertebrates (Ubuka et al., 2013). PRL is hypothesized to exert "anti-gonadal" effects on the HPG axis at multiple sites, as for example. Best of the few PRL individuals, during energy-inten

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period of milk production after pregnancy in mammals (Fourman and Fazeli, 2015). This anovulatory effect is mediated through the inhibition of kisspeptin production (Brown et al., 2019; Grattan, 2018), which consequently reduces GnRH, gonadotropin, and sex steroid release. In seasonally-breeding birds, PRL has also been implicated in ending breeding seasons and birds' transitions into photorefractoriness (Dawson and Sharp, 1998; Sharp et al., 1998; Small et al., 2008), and a body of classic studies on exogenous PRL treatment found gonadal regression in birds (Bates et al., 1937; Meier, 1969; Tewary et al., 1983). Further, experiments show that systemic or intracerebroventricular (icv) PRL injections lower circulating gonadotropins, impair gonads, and decrease 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 to the many studies that show evidence for such anti-gonadal effects of PRL, other studies fail to find these effects. For instance, studies in multiple species did not find any effect of PRL treatment on gonadal regression (house finch: Hamner, 1968; quail: Jones, 1969; sparrows: Meier and Dusseau, 1968; Laws and Farner, 1960). Although anti-gonadal effects seem consistent in male doves, studies also found no changes in testes weight with icv PRL treatment (Freeman et al., 1990). In domestic fowl, immunization against PRL, which reduces functional PRL peptide, led to elevated LH levels (Crisóstomo et al., 1998) and reduced laying (Li et al., 2011). Further, pro-gonadal functions appear in some seasonally breeding mammals, such as hamsters, where PRL administration actually stimulates regrowth in the period of gonadal recrudescence (Bartke et al., 1980, 1987; Howell-Skalla et al., 2000; Sanford and Dakin, 2010). This mixed literature indicates that PRL's effects on the HPG axis may be species and breeding context-specific.

Despite this mixed evidence, studies examining how non-parental reproductive behaviors respond to PRL, and how multiple HPG signaling components mediate this relationship, are limited. Many studies have established PRL's role in parental behavior (Brown et al., 2017; Buntin et al., 1991; Horseman and Buntin, 1995), but its influence, if any, on other reproductive behaviors, such as courtship and mating, remains understudied. Most of these studies used gonad size and/or plasma levels of LH or sex steroids. However, hormone receptor expression, which modulates responses to circulating signals, may underlie the effects of PRL on the HPG axis. Further, to our knowledge, no study has examined the causal relationship between gonadotropin-inhibitory hormone (GnIH, also known as RFRP-3) and PRL. GnIH has been shown to increase during transitions into parental stages (Calisi et al., 2016), including when PRL levels also rise (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 and examined its effect 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, incubation, and chick provisioning (Abs, 1983). Both sexes produce crop milk, a nutrient-rich substance which is fed to offspring exclusively for the first few days of life (Davies, 1939). Like mammalian milk production, crop milk production is also driven by PRL (Horseman and Buntin, 1995). This animal model thus removes the confound of female-only lactation in mammals, allowing comparisons of sex-typical and atypical HPG regulation and behavior. Lastly, we capitalized on a detailed ethogram of courtship behaviors developed to quantify species-specific reproductive behavior (Cheng, 1979; Goodwin, 1983, 1955; Lehrman, 1964).

Our study had three main aims: to examine the effect of PRL on 1) parental responses, 2) non-parental reproductive behavior, like courtship and copulation, and 3) gene expression of hormones and their receptors across the HPG axis, to specify where effects occur. We administered exogenous ovine prolactin (oPRL) or a vehicle control to experienced pairs of rock doves and then removed their nests, forcing birds from a parental state back to a courtship/mating state. We further recorded parental behavior responses to a novel chick, and observed naturally-occurring courtship behavior. We also collected brain, pituitary, and gonadal tissues to measure HPG gene expression. We hypothesized that PRL would hold the HPG in a parental state, favoring current reproductive effort and depressing gonadal activity promoting future reproductive effort. Thus, we predicted that PRL-treated birds would exhibit more parental behavior response to a novel chick, but would be more delayed in the progression of courtship, and would copulate less than vehicle-treated pairs. We also predicted that PRL treatment would reduce GnRH, LH, and FSH expression in the hypothalamus and pituitary, increase GnIH expression, and reduce gonadotropin receptor expression in the gonads.

2. Methods

2.1. 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 when birds were switching from parental to mating behavior. On day 16 of the 17 day incubation period, we surgically implanted osmotic pumps (Model 2001, release rate 1.0 µl/h, ALZET, DURECT Corp.) containing either PRL in 0.87% physiological saline (dose: 1.25 µg/h, 30 µg/day; A.F. Parlow, National Hormone and Peptide Program) or vehicle (0.9% physiological saline; 1 µl/h, 24 µl/day) to both male and female members of breeding pairs. Osmotic pumps were placed subcutaneously between the scapulae, using local anesthetic (topical lidocaine, 2%) at the insertion site. Both vehicle and PRL pumps were filled to full volume (200 µl).

We did not measure circulating PRL because oPRL is not reliably detected by chicken PRL antibodies, and endogenous PRL would likely be below detection in oPRL-treated birds due to negative feedback (Z. Wang & F. Angelier, pers. comm.). However, using plasma concentration estimates described in Sockman et al. (2000), we estimate that the dose led to a plasma concentration of approximately 46.25 ng/ml in the average rock dove population. This concentration is within PRL levels observed during incubation and chick rearing in previous studies of rock doves (Austin et al., 2021). The osmotic pumps reliably release 1.0 µl/hour for seven days (ALZET, DURECT Corp.), thus the experiment lasted seven days (Fig. 1). oPRL delivered through osmotic pumps has been shown to activate PRL receptor signaling in a closely-related species, ring doves (Buntin and Buntin, 2014), and we replicated the osmotic pump model and doses used in that study. Further, radiolabeled PRL crosses the blood-brain barrier into the hypothalamus of doves (Buntin and Walsh, 1988) as it does in rodents (Walsh et al., 1987).

Sixteen breeding pairs received PRL (32 birds), while 12 received vehicle (24 birds), total 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 semi-natural aviaries, where they selected their own mates and bred within a colonial aviary, with about 10–12 other breeding pairs (see MacManes et al., 2017 for details).

To induce a shift from parental behavior towards mating, we removed eggs from the nest box on day 17, the day after surgical pump implantation and the last day of incubation before expected chick hatching (hatching typically occurs after 18 days of incubation

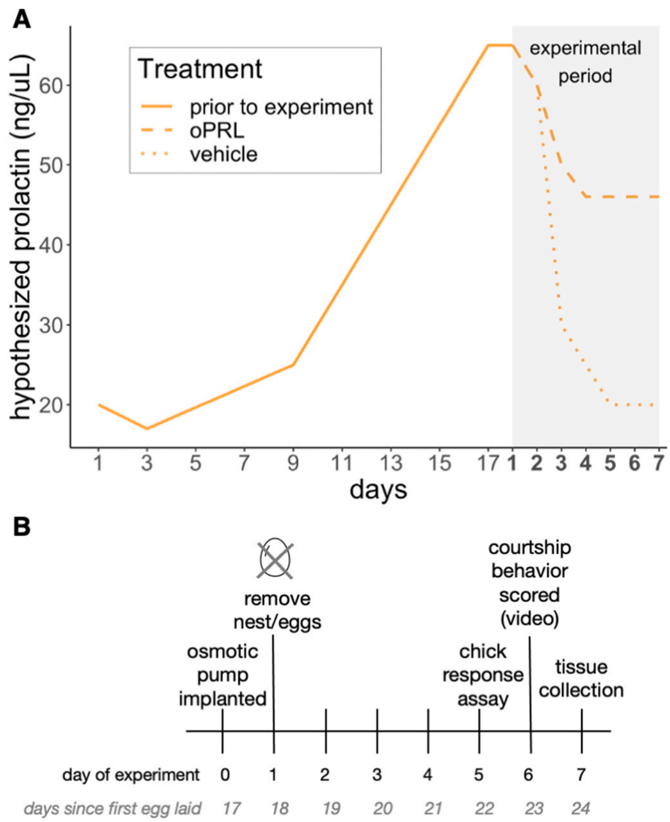

Fig. 1. Experimental timeline. (A) Hypothesized circulating prolactin concentrations during typical incubation period and during the experimental period for both the experimental treatment (oPRL, dashed) and control (vehicle, dotted) groups. Hypothesized curve during incubation drawn from Austin et al., 2021; Dong et al., 2012 and expected experimental values are drawn from pattern seen in doves (Lea and Sharp, 1989; Ramsey et al., 1985). The experimental paradigm (B). Both male and female birds in a pair had osmotic pumps containing either prolactin or vehicle implanted subcutaneously at day 17 of incubation. The next day of the experiment (day 18 of incubation), eggs and nest were removed and behavioral assays were done daily, after which tissues were collected. For full details, see Methods.

Egg removal at mid-incubation has been shown to significantly decrease plasma prolactin in male and female rock doves 24 h later (Austin et al., 2021) and delay the rise in plasma prolactin in ring doves (Ramsey et al., 1985). Eggs and nest were removed between 09:00–11:00 h on incubation day 17. As rock doves are continuously in captivity (Johnston, 1992), egg removal and nest removal counteract the remaining effects of the eggs. The egg removal paradigm allows us to control the target of parental care and minimize incidental effects independent of our treatments.

2.2. Behavior: response to novel chick

As a proxy for increased PRL promoting parental behaviors in response to chicks, we presented novel chicks to pairs. On the fifth day of the seven-day experiment (Fig. 1), we placed a novel chick (aged 2–6 days old) from another nest into the focal pair's nest box for approximately 20 min between 08:00–10:00 h (video-recorded). To ensure the chick would solicit, chicks were fasted before the trial. Chicks were placed in the subject's cage when the behavior was tested, following previous studies (Buntin et al., 1991; Wang and Buntin, 1999), which did not remove the

during the behavioral trials, to avoid introducing cross-fostered chicks of the same age as the treatment, the test chicks were isolated while the treatment was set (test chicks were reared, corresponding to the birds' simultaneous incubating eggs). Because of the nature of the behavior, only one parent could brood or feed the chick at the same time (i.e., male and female did not overlap in expression of the behavior). At the end of the behavioral trial, we measured the change in weight of the chick compared to the start of the trial (post-trial weight – pre-trial weight/pre-trial weight) as an additional measure of feeding by the subjects.

Videos were scored using BORIS v. 7.9 (Friard and Gamba, 2016) by trained observers blind to treatment. We recorded the occurrence and duration of the following chick-oriented behaviors: a) sitting and standing next to the chick (chick present), brooding the chick, and nuzzling the chick (body-to-body contact), feeding the chick (when bird engaged in mouth-to-mouth regurgitation with the chick). We also recorded the duration of time spent aggressively pecking (vigorously pecking the chick) if it occurred. For each behavior, we averaged each bird's expressed behavior during the trial, presenting this data as a percent of the total time recorded. Two birds were excluded from behavioral scoring due to video recording equipment malfunction. Both birds excluded also came from oPRL-treated pairs, and did not have chick weight data as a proxy for feeding by these pairs. In total, we recorded videos for 26 pairs (n = 14 oPRL, n = 12 vehicle).

2.3. Behavior: courtship and reproductive behaviors

To examine how PRL may affect non-parental behaviors, we conducted courtship behavior assays on a subset of pairs on day 7 of the seven-day experiment (Fig. 1). We recorded two video samples placed facing the entire aviary for the courtship behaviors on day 7 of the seven-day experiment. First, this time point was selected as the furthest in time from treatment onset, and the most possible ovulation/egg laying would occur, so courtship behaviors would be the most temporally close to the expression of these behaviors. Tissues were collected on day 7. We recorded for eight oPRL-treated pairs (n = 16 birds) and seven vehicle pairs (n = 14 birds) total.

Nest box videos were used to measure species-specific courtship behaviors, such as bow-coos, nest-coos, and nest building (including nest manipulation) (Cheng, 1973). Nest videos were recorded from 07:30–08:30 and 12:30–13:30 and 30-min video samples were scored per time point by trained observers blind to treatment using BORIS v. 7.9 (Friard and Gamba, 2016) for the following courtship behaviors: a) bow-coo (male bows to the female while cooing, head stretched and tail down) (Goodwin, 1956), b) nest-coo (bird crouches in the nest, tail up and head down) (Cheng, 1973; Goodwin, 1956), c) wing-flip (bird flips its wings while nest-cooing) (Cheng, 1973; Miller and Miller, 1958), and d) nest-building (bird manipulates nest material, picks it up or arranges it with the bill) (Cheng and Balthazart, 1982; Miller and Miller, 1958). These behaviors are well-described stereotypical courtship behaviors in pigeons and doves (Cheng, 1992; Goodwin, 1983).

We also scored the following mating behaviors: a) preening, the bird preens the other, typically around the head and neck (Miller and Miller, 1958), b) allofeeding

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pair was tested. The number of trials in which the male approached the female within a 2 cm radius of the female's cage was recorded as a proximity event. The duration of time spent in proximity was also recorded. Behavioral data were collected using JWatcher software (Blumstein et al., 2000). Two observers scored the behavior of the male in each pair. Inter-observer reliability was assessed using Cohen's kappa (κ) and was found to be high for both proximity events (κ = 0.96) and proximity duration (κ = 0.98). Data from the observer with the higher reliability score were used for analysis.

2.3. Statistical analysis

Statistical analyses were performed using R (version 3.6.1; R Core Team, 2019). Generalized linear models (GLMs) were used to test for differences between treatment groups in the number of proximity events and the duration of proximity. Because the number of proximity events was a count variable, we used a GLM with a Poisson error distribution and a log link function. Because the duration of proximity was a continuous variable, we used a GLM with a Gaussian error distribution and an identity link function. Model assumptions were checked by visual inspection of residual plots. Post-hoc pairwise comparisons between treatment groups were conducted using Tukey's honestly significant difference (HSD) test, implemented with the multcomp package (Hothorn et al., 2008). The significance level was set at $\alpha = 0.05$.

3. Results

3.1. Effects of PRL on proximity behavior

Males exposed to PRL in their drinking water spent more time in proximity to females than males exposed to control water ($\chi^2 = 4.21$, $df = 1$, $p = 0.040$; Fig. 1). The number of proximity events did not differ significantly between the two groups ($\chi^2 = 2.87$, $df = 1$, $p = 0.090$). Males exposed to PRL had a mean proximity duration of 142.6 ± 18.3 s (mean ± SEM), while control males had a mean proximity duration of 89.4 ± 14.7 s.

3.2. Effects of PRL on aggression

Males exposed to PRL in their drinking water showed a significantly lower rate of aggressive behavior than control males ($\chi^2 = 5.63$, $df = 1$, $p = 0.018$; Fig. 2). The mean rate of aggressive behavior was 0.31 ± 0.07 events per minute for PRL-exposed males and 0.58 ± 0.09 events per minute for control males. The duration of aggressive behavior did not differ significantly between the two groups ($\chi^2 = 3.12$, $df = 1$, $p = 0.077$).

3.3. Effects of PRL on courtship behavior

Males exposed to PRL in their drinking water showed a significantly higher rate of courtship behavior than control males ($\chi^2 = 6.94$, $df = 1$, $p = 0.008$; Fig. 3). The mean rate of courtship behavior was 0.74 ± 0.11 events per minute for PRL-exposed males and 0.39 ± 0.08 events per minute for control males. The duration of courtship behavior did not differ significantly between the two groups ($\chi^2 = 2.45$, $df = 1$, $p = 0.118$).

3.4. Effects of PRL on body mass

Males exposed to PRL in their drinking water had a significantly greater body mass than control males ($\chi^2 = 4.87$, $df = 1$, $p = 0.027$; Fig. 4). The mean body mass was 24.6 ± 0.4 g for PRL-exposed males and 23.7 ± 0.3 g for control males. The difference in body mass between the two groups was 0.9 g, which corresponds to a 3.8% increase in body mass for PRL-exposed males relative to control males.

4. Discussion

The present study found that exposure to prolactin (PRL) in drinking water affected several behaviors in male zebra finches. Males exposed to PRL spent more time in proximity to females, showed a lower rate of aggressive behavior, showed a higher rate of courtship behavior, and had a greater body mass than control males. These findings are consistent with the hypothesis that PRL promotes behaviors associated with parental care and reduces behaviors that may interfere with parental care.

The finding that PRL-exposed males spent more time in proximity to females is consistent with the hypothesis that PRL promotes affiliative behavior toward females. This result is also consistent with previous studies showing that PRL administration increases the duration of time spent in proximity to females in male zebra finches (Goodson et al., 2009). The finding that PRL-exposed males showed a lower rate of aggressive behavior is consistent with the hypothesis that PRL reduces aggression toward females. This result is also consistent with previous studies showing that PRL administration reduces aggression toward females in male zebra finches (Goodson et al., 2009).

The finding that PRL-exposed males showed a higher rate of courtship behavior is consistent with the hypothesis that PRL promotes courtship behavior toward females. This result is also consistent with previous studies showing that PRL administration increases the rate of courtship behavior in male zebra finches (Goodson et al., 2009). The finding that PRL-exposed males had a greater body mass than control males is consistent with the hypothesis that PRL promotes body mass gain. This result is also consistent with previous studies showing that PRL administration increases body mass in male zebra finches (Goodson et al., 2009).

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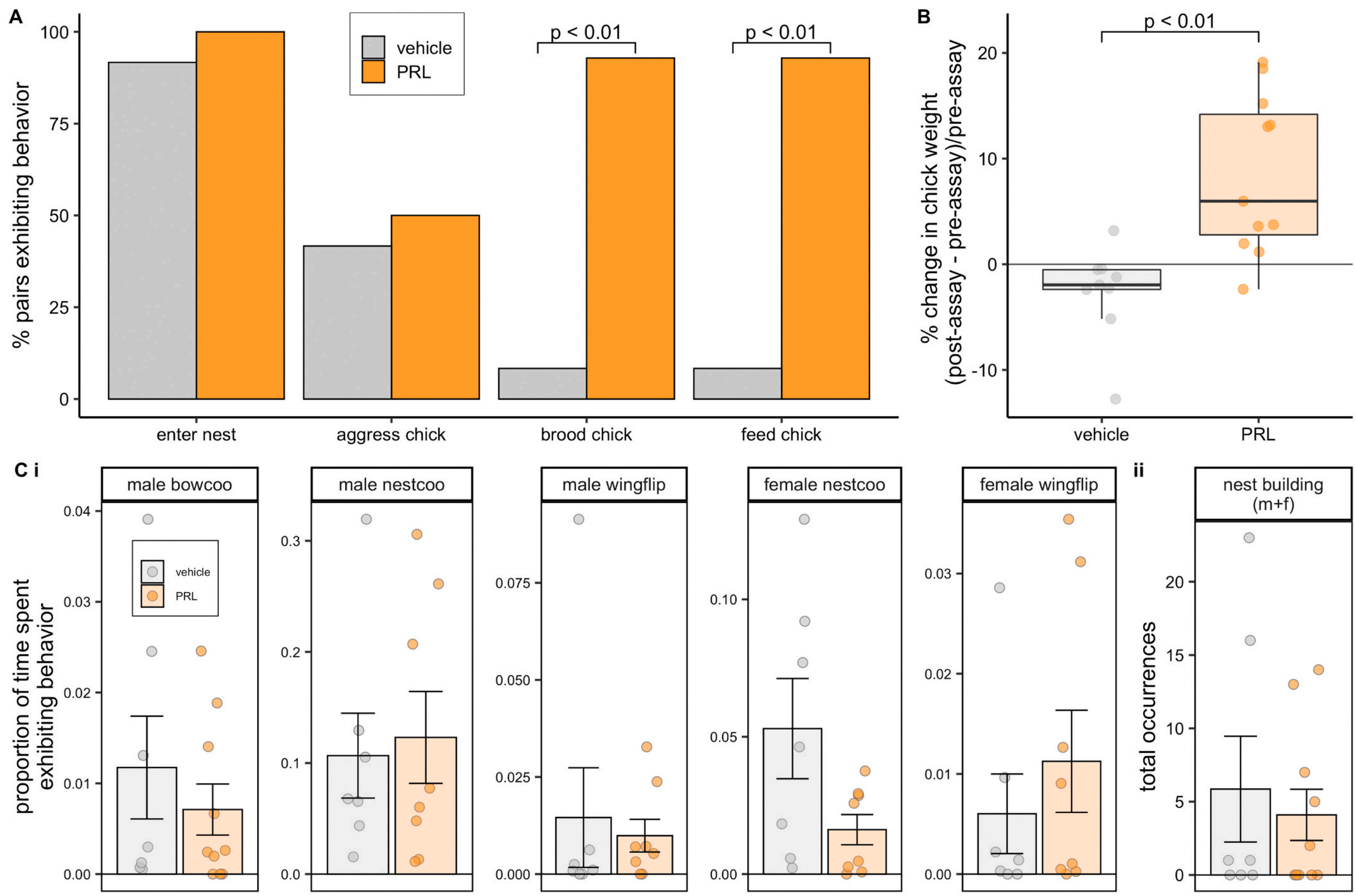

Fig. 2. Behavioral responses to a novel chick and observed parental behavior after several days of experimental treatment (vehicle or PRL). A novel chick was placed in a pair's home nest box. (A) The percent of pairs that entered the nest or showed behavioral responses to the chick as a percent of pairs assayed is shown, as well as (B) the percent change in chick weight at the time which chick was measured post-assay minus pre-assay, divided by pre-assay weight. (C) After 6 days of treatment, the pairs were observed, with the proportion of the observation time exhibiting the behavior (out of 1.0 max, or 100% of the observation window) (i) or the number of occurrences of behavior (ii) shown. Means ± SEM are shown, points represent individual birds (Ci) or pairs (B, Cii).

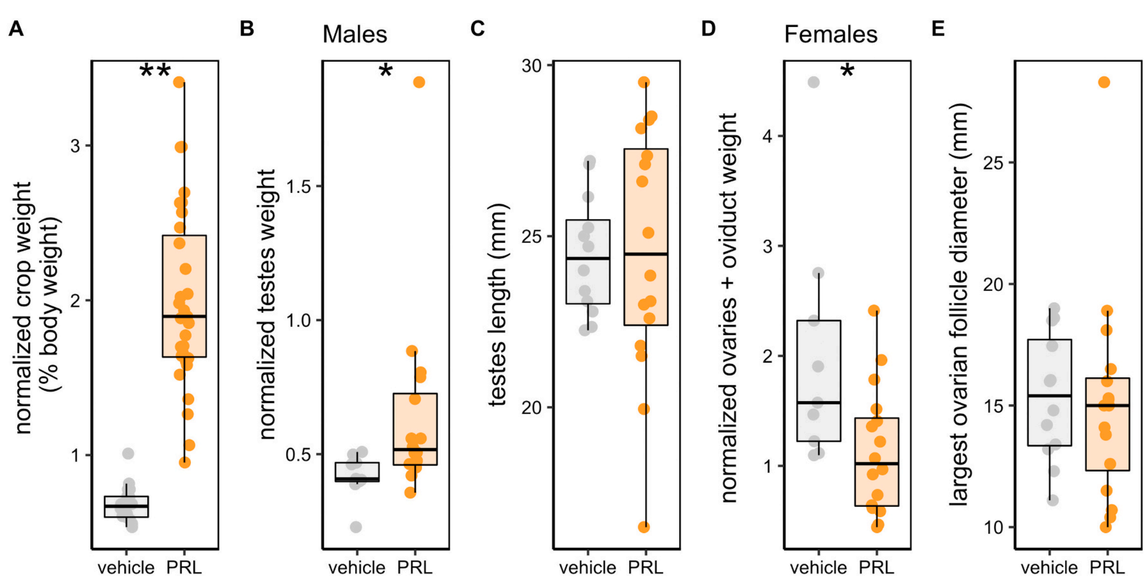

Fig. 3. Measurements of crop and gonadal traits of birds treated with PRL or vehicle. (A) Crop weight normalized to body weight (% of overall body weight), (B) male testes weight (normalized to body weight), (C) testes length (in millimeters), (D) female ovary and oviduct weight together normalized to body weight, and (E) the diameter (mm) of the largest ovarian follicle were measured at dissection. ** $p_{adj} < 0.01$ and * $p_{adj} < 0.05$ in Mann-Whitney U tests comparing vehicle-treated and PRL-treated birds. Boxplots show median and first and third quartiles, dots represent individual samples.

Table 2
Reproductive behaviors assessed across days of experimental treatment. Courtship and pair-bond-maintenance behaviors were scored for one randomly chosen male and female from each nest; nest-building and copulation behaviors were scored from 1 h of video recording per day. Percentage (number) of birds that exhibited each behavior is shown for all behaviors. For state behaviors (with duration), average proportion of time spent (± SEM) is shown; for point behaviors, occurrence rate (± SEM) is shown. Raw p values and p values adjusted using the Benjamini-Hochberg method for correcting for multiple comparisons are shown.

State behavior	Vehicle		Prolactin		t	p
	Percent (number) of birds exhibiting behavior	Proportion of time spent	Percent (number) of birds exhibiting behavior	Proportion of time spent		
Courtship behaviors						
Male bow-coo	100% (7)	0.012 ± 0.006	87.5% (7)	0.007 ± 0.003	−0.46	0.652
Male nest coo	100% (7)	0.107 ± 0.038	100% (8)	0.123 ± 0.041	0.30	0.769
Male wing flip	71.4% (5)	0.016 ± 0.013	75% (6)	0.01 ± 0.004	−0.34	0.745
Female nest coo	100% (7)	0.053 ± 0.018	87.5% (7)	0.016 ± 0.005	−1.93	0.095
Female wing flip	71.4% (5)	0.006 ± 0.004	87.5% (7)	0.010 ± 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 allofeeding (bill)	42.9% (3)	0 ± 0	62.5% (5)	0.002 ± 0.001	1.40	0.195

Point behavior	Vehicle		Prolactin		t	p
	Percent exhibiting behavior	Occurrences	Percent exhibiting behavior	Occurrences		
Female nest building	42.9% (3)	1.71 ± 1.392	50% (4)	1.33 ± 0.678	0.25	0.811
Male nest building	28.6% (2)	4.14 ± 2.694	37.5% (3)	2.08 ± 1.282	0.69	0.508
Copulation behaviors						
Mount (male)	100% (7)	2.93 ± 0.7	100% (8)	2.57 ± 0.953	0.30	0.766
Solicit copulation (female)	28.6% (2)	0.71 ± 0.474	50% (4)	1 ± 0.423	−0.45	0.660

0.516, p_{adj} = 0.645; Cohen's d = −0.08). Females had ... follicles ... Similarly, there was no significant difference in testes mass between treatment groups (t = 0.11, p = 0.909, p_{adj} = 0.909; Cohen's d = 0.04). No birds laid eggs during the experimental period, and no females were laying at collection.

3.4. Hypothalamic gene expression

In the POA, there was no significant effect of treatment on GnRH or GnIH expression (Table 3; Fig. 4). Similarly, there was no significant effect of treatment on GnIH expression in the PVN (Table 3; Fig. 4C). As for sex steroid receptors in the POA and the PVN, there was no effect of treatment on nuclear AR expression. ESR1 tended to be expressed at lower levels in PRL-treated birds compared to vehicle (p = 0.044), but after Benjamini-Hochberg correction, this effect was no longer significant (p_{adj} = 0.136; Supplementary Table 4). There was no effect of treatment on nuclear ESR1 expression; ESR2 did not significantly differ with treatment in the POA, but appeared to have marginally lower expression in the PVN (p = 0.045); however, this effect did not persist after Benjamini-Hochberg correction (p_{adj} = 0.251; Supp. Table 4). We did not find any effect of treatment on nuclear PRLR expression (Supp. Table 4).

3.5. Pituitary gene expression

In the pituitary, there was a significant effect of treatment on FSHB (p_{adj} < 0.001; Cohen's d = 1.99), where PRL significantly increased expression compared to vehicle controls. There was no significant effect on CGA (p_{adj} > 0.05; Table 3; Fig. 5). PRL treatment significantly affected GnRHR and GnIHR expression (Table 3; Fig. 5), with PRL-treated birds expressing higher levels of these receptor mRNAs (GnRHR, Cohen's d = 0.77; GnIHR, Cohen's d = 0.85).

3.6. Gonadal gene expression

In the testes, we found a significant effect of treatment on FSHR and LHCGR expression, where PRL-treated males had higher expression levels of both genes (Table 4; Fig. 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 pre-ovulatory follicles in females (Table 4; Fig. 6B). There was also no significant effect of treatment on AROM or GnIH expression in either the testes or ovary (Table 4; Fig. 6).

In the ovary, we found a significant effect of treatment on both estrogen receptors, ESR1 and ESR2, in that PRL treatment reduced expression of both genes compared to vehicle birds (Table 4; Fig. 6C; Cohen's d = −2.02 and −0.84 for ESR1 and ESR2, respectively).

4. Discussion

We found that providing prolactin (PRL) to experienced mated parents did not substantially alter courtship or copulation behaviors compared to vehicle controls. On the gene expression level, we found no overall negative effect of PRL treatment, but we found increased FSHB in the hypothalamic gene expression in the pituitary, as well as specific responses in the testes and ovary in gonadotropin receptor expression and estrogen receptor expression. To our knowledge, this study is the first to measure the effect of PRL on courtship and copulation in the context of HPG-wide gene expression.

Overall, using experimental PRL manipulation, we found mixed support for the hypothesis that PRL acts as an "anti-gonadal" hormone in HPG axis regulation. Our results contribute to a literature of laboratory studies with mixed results; some found PRL treatment to inhibit gonadal function (Bates et al., 1937; Buntin et al., 1991, 1998, p. 199; Buntin and Tesch, 1985; Janik and Buntin, 1985; Meier, 1969), whereas others did not (Foreman et al., 1990; Hamner, 1968; Jones, 1969; Li et al., 2011; Meier and Dusseau, 1968; Opel and Proudman, 1984). Below we discuss methodological considerations for these differences, including the use of multiple-brooding/clutch

Table 3
Linear model test results for differences in gene expression after treatment, sex, and their interaction in the hypothalamus and pituitary. Estimates (β) and standard errors shown in log(fold change) calculated using the Livak and Schmittgen (2001) method. Females and vehicle were the reference levels for each [illegible] of the gene linear model. p-values adjusted using the Benjamini-Hochberg false discovery rate correction (p_{adj}); significant values ($p < 0.05$) in bold.

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

explain why we did not observe the predicted "anti-gonadal" effect.

4.1. Effects on parental and reproductive behaviors

We found that PRL promoted a parental phenotype, increasing the likelihood that doves would show parental responses towards a novel chick (i.e., feeding, [illegible]). Supporting this finding, chicks [illegible] PRL-treated [illegible] (presumably to regurgitate crop milk [illegible]), whereas chicks [illegible] vehicle-treated [illegible]. Further, PRL treatment did not increase crop weights, which is [illegible] crop milk production, a classic indicator of PRL bioactivity (Lebovitz and Nicoll, 1992; Riddle and Braucher, 1931). Together, these results suggest that systemic PRL administration [illegible] PRL receptor [illegible] crop growth and milk production, effects [illegible] behaviors. Our results are consistent with previous studies in ring doves, where systemic PRL administration [illegible] regurgitative feeding and chick-[illegible] in reproductively-experienced birds (Buntin et al., 1991; Wang and Buntin, 1999).

Unlike parental responses, however, our results show that population-level reproductive behavior remained unaffected by PRL treatment. The typical courtship cycle of doves consists of bow-cooing to attract a mate's attention (males), nest-cooing to point to nest sites (males), nest-cooing if she accepts the nest site (females), then both male and female participating in nest building before oviposition (Cheng, 1992; Goodwin, 1983). All birds, regardless of treatment, appeared to express courtship behaviors at similar [illegible]. Similarly, we observed no differences in population rates [illegible] established pairs [illegible] (Cheng et al., 1981).

The similar courtship and copulation rates between groups suggests PRL manipulation did not affect these reproductive behaviors in established pairs. There are several possible explanations. First, our PRL doses may not have been high enough to inhibit non-parental reproductive behaviors. Our exogenous PRL doses, calculated as described in [illegible] et al. (2020), approximated levels seen during mid-chick-rearing (Austin et al., 2021). The mid-to-late chick-rearing period can include clutch overlap in rock doves, especially [illegible] experienced (Burley, 1980). Thus, PRL levels in this study may have been similar to a period when mating and courtship co-occur with parental care. Second, stimulatory cues such as the presence of a mate, nest, and nesting material may have overridden any inhibitory effect of PRL, especially [illegible] photostimulated [illegible] behaviors, [illegible] (Cheng and Balthazart, 1982; Lehrman [illegible]; Shields et al., 1989). Previous studies showing anti-gonadal PRL effects in doves were conducted [illegible] (Buntin et al., 1991, 1988; [illegible] et al., 1990). Third, the lack of treatment effect [illegible] behavior expression (i.e., [illegible]) [illegible] measured courtship behaviors [illegible] (Cheng and Balthazart, 1982). While established pairs may [illegible] (unpublished data), [illegible] experiment [illegible]. Alternatively, [illegible] measured courtship behaviors [illegible] where these behaviors occur relatively frequently. Comparing this [illegible] unmanipulated [illegible] typical nest-establishment [illegible] manipulated [illegible] courtship and copulation [illegible] likely.

Lastly, [illegible] behavioral [illegible] population [illegible] (Hunt and Wingfield, 2004), and [illegible] (Fusani and Hutchison, 2003). Future studies [illegible] PRL [illegible] in conjunction with other hormones [illegible] possibilities.

4.2. Effects on hypothalamic gene expression

In the hypothalamus, exogenous PRL did not significantly alter GnRH, GnIH, or GnRH-R expression. Although this is [illegible] previous studies in rock doves [illegible] hypothalamic [illegible] significant changes [illegible] during parental care (Harris, 2020; Harris et al., 2020). The absence of differential [illegible] reproductive behavior [illegible] explanation [illegible] hypothalamic gene expression [illegible] courtship [illegible] POA GnRH activity in doves (Mantei et al., 2008). Our

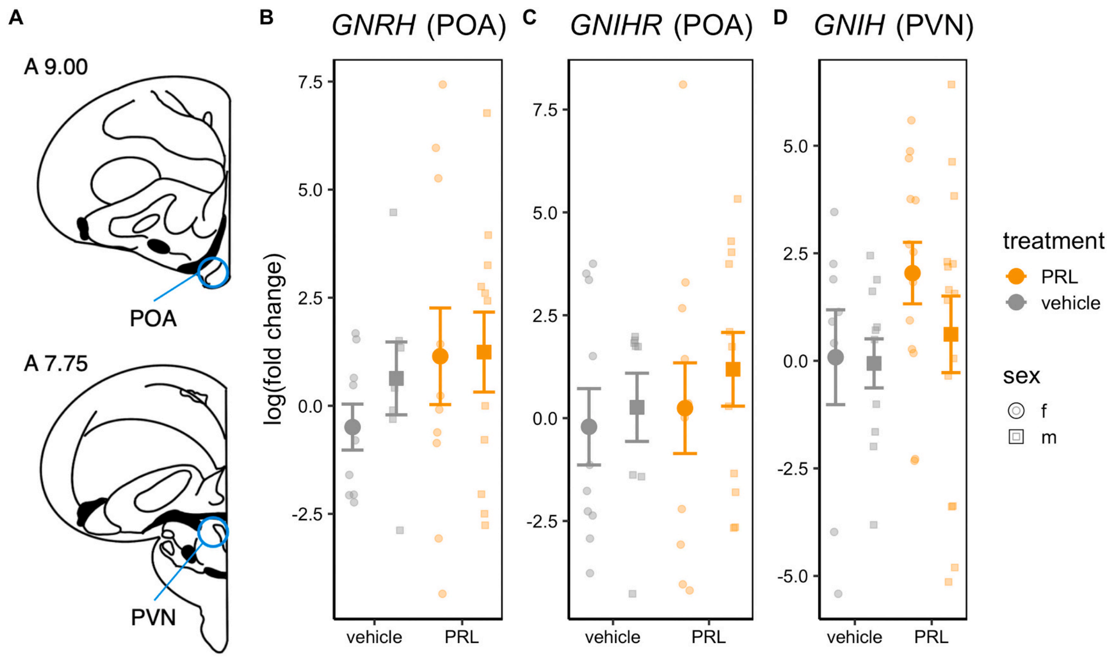

Fig. 4. HPG gene expression in hypothalamic (A). Genes of interest were measured in microdissected punches of the preoptic area (POA) and paraventricular nucleus (PVN) using (Karten and Hodos, 1967) atlas (representative illustrations with plate numbers shown). (B) Gonadotropin releasing hormone (GNRH) expression and (C) gonadotropin inhibitory hormone receptor (GNIHR) expression were measured in the POA, and (D) GnIH was measured in the PVN, where GnIH-neurons are found. Circles represent females and squares represent males. Means and SEM are shown for each sex and treatment.

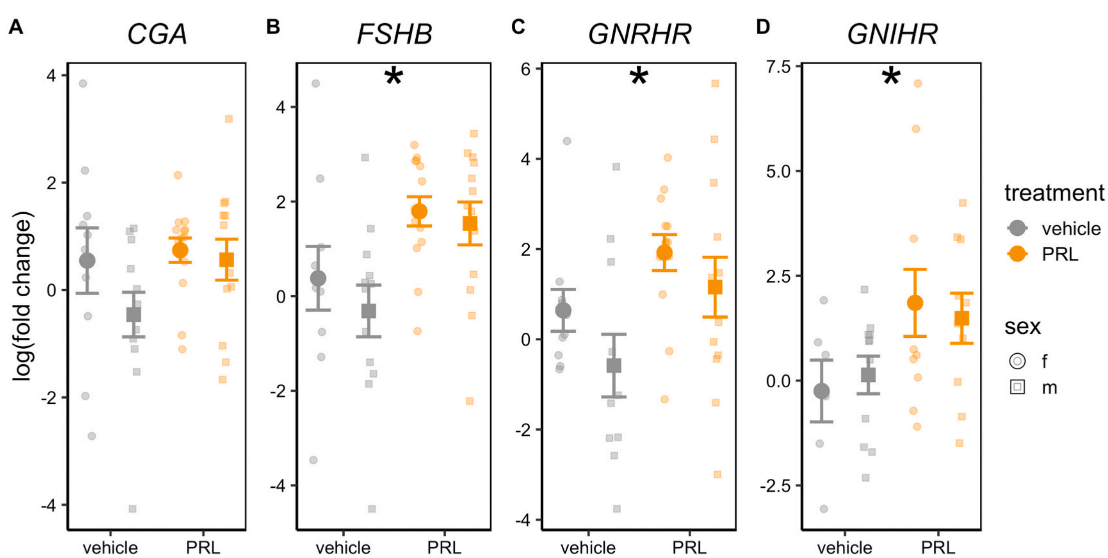

Fig. 5. Pituitary gene expression. (A) Luteinizing hormone (CGA), (B) follicle stimulating hormone (FSHB), (C) GnRH receptor (GNRHR), (D) GnIH receptor (GNIHR) expression in the pituitary of males and females that were given either vehicle or PRL. Means and SEM are shown for females and males in each treatment group, circles and squares respectively. Significantly increased expression is indicated by asterisks (*) for main effect of treatment ($p_{adj} < 0.05$).

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studies showing anti-gonadal ... ... altered expression ... manipulations
cerebroventricular ... mechanism ... PRL ... altered ... changing HPG gene

Table 4
Linear model testing differences in gene expression between treatment groups in gonadal tissues. Gene expression was measured in testes, and small white follicles and oviducts of females. Estimates (β) and standard errors shown in log(fold change), calculated using the Livak and Schmittgen (2001) method. Vehicle treatment was the reference group. Degrees of freedom come from the linear model. p-values adjusted using the Benjamini-Hochberg correction; significant values ($p_{adj} \leq 0.05$) 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

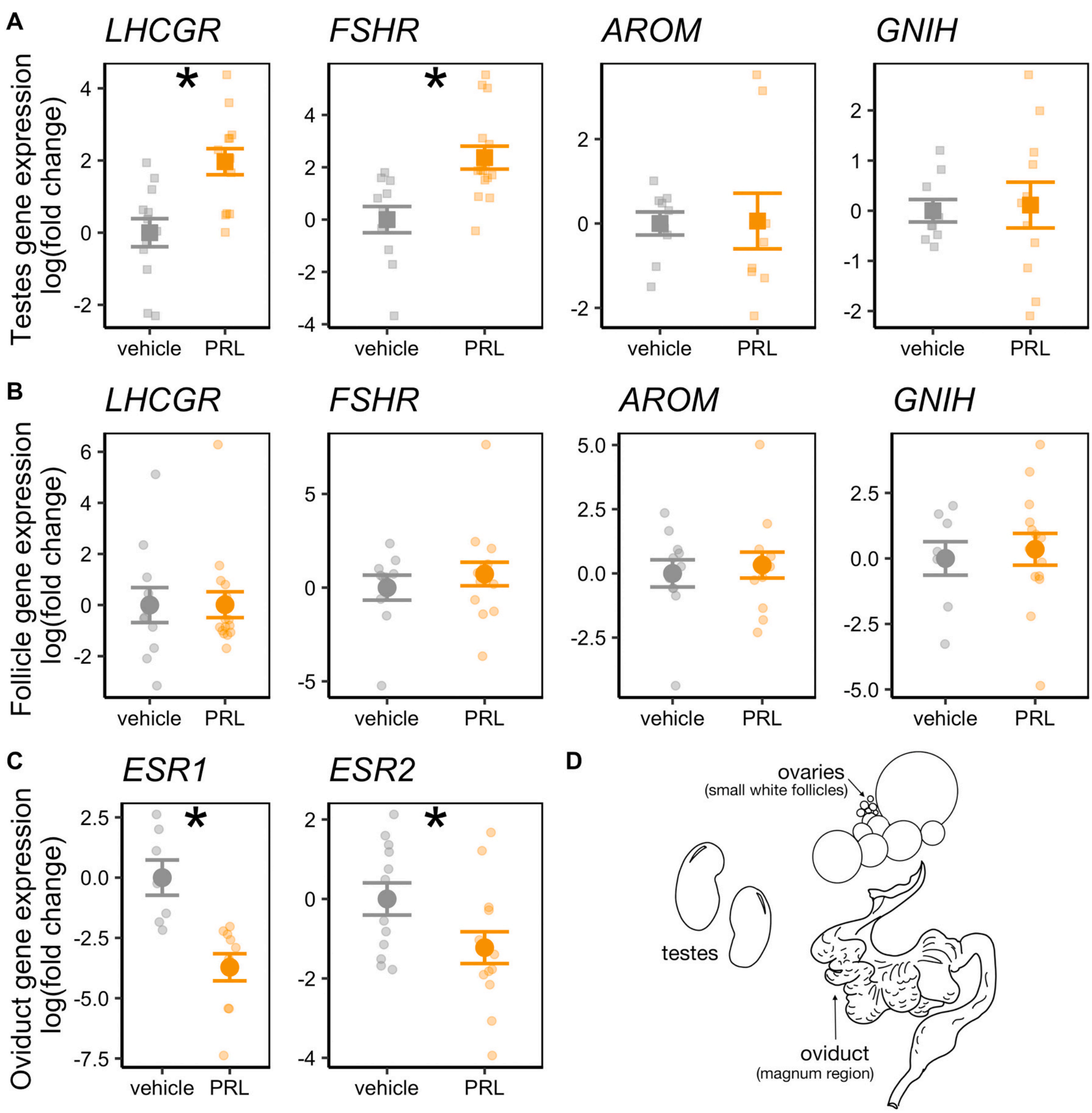

Fig. 6. Gonadal gene expression. Relative expression of luteinizing hormone receptor (LHCGR), follicle stimulating hormone receptor (FSHR), aromatase (AROM) and gonadotropin inhibitory hormone (GNIH) mRNA levels in (A) male testes, (B) small white follicles, as well as (C) estrogen receptor 1 (ESR1) and 2 (ESR2) in female oviducts treated with PRL (orange) or vehicle control (grey). Means ± SEM are shown. Small points represent individual birds. Asterisks (*) represent significant effect of treatment ($p_{adj} < 0.05$). Males are shown with squares, females with circles.

expressions observed were also mediated through the hypothalamus.

4.3. Effects on pituitary gene expression

In the pituitary, we found significant decreases in hypothalamic hormone receptor (GnRHR and GnIHR) and FSHB with oPRL treatment. The fact that FSHB expression decreased, and was inhibited by oPRL, was surprising and does not support our original hypothesis. Gonadotropins are stimulated by external stimuli, such as presence of mates or reproductively receptive females (Cheng and Balthazart, 1982; Cheng, 1979; Cheng and Follett, 1976; Shields et al., 1989; Silver et al., 1980). In birds, as in mammals, FSH serves to stimulate steroidogenesis and enhance follicle development in ovaries (Johnson, 2015) and testes (Deviche et al., 2011). Increased FSHB expression combined with increased GnRH receptors may act as a compensatory mechanism under potentially inhibitive effects of PRL and maintain courtship behavior and gonad size as observed in our study. Lastly, lack of changes in LHB (via CGA expression) is consistent with previous studies where immunization against 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, this hypothesis requires further testing, ideally directly measuring gonadotropin release.

Importantly, while oPRL led to changes in HPG gene expression compared to vehicle controls, none of our PRL "maintaining" gene expression patterns were the same as unmanipulated comparison groups that were removed and allowed to hatch. It is possible that PRL treatment regime may have led to an alternative route to maintain HPG gene expression as it is difficult to compare, but it is possible that we did not include a group of birds at day 4 post-hatching as a comparison. This is important in terms of gene expression at tissues related to behavior and direct comparisons of vehicle birds in this study.

4.4. Effects on GnIH 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. While most studies show that GnIH administration affects pituitary PRL gene expression (Son et al., 2009), no studies have examined the causal relationship between PRL and GnIH expression to our knowledge. GnIH inhibits the HPG axis during stress and seasonal transitions (Calisi et al., 2008, 2011; Kirby et al., 2009); we hypothesized that GnIH may play a role in any inhibitory effects of PRL on avian reproductive function. We found no change in hypothalamic or gonadal GnIH expression after oPRL treatment, but pituitary GnIHR increased in both sexes given PRL. Classically, hypothalamic GnIH inhibits gonadotropin release and inhibits GnRH synthesis and release from POM neurons (Tsutsui and Bentley, 2009; Ubuka et al., 2013), but GnIH also directly inhibits gonadotropin release at the pituitary (Ciccone et al., 2004; Clarke et al., 2008) or by local expression in gonads (Bentley et al., 2017). Our results suggest that PRL does not affect the HPG axis via hypothalamic or gonadal GnIH expression when PRL levels are equivalent to those at mid-chick rearing. GnIHR in the pituitary of the HPG axis is regulated (Maddineni et al., 2008), but we did not observe any concordant changes in gonadotropin expression. It is possible that PRL manipulation only regulates HPG axis gene expression in a way that would be detrimental to the mate (Bosson and Cox, 2020). However, in European starlings, GnIH and PRL have been possibly co-elevated during PRL treatment, and PRL levels also rose and are required for GnIH expression during incubation, suggesting that avian GnIH systems are definitively dependent on PRL.

4.5. Effects on gonadal gene expression

In the gonads, we found sex-specific differences in morphology as well as gene expression. We observed significantly decreased testes size, and no effect on ovarian follicle size, which is contradicting the reported "anti-gonadal" role of PRL and contradicts previous studies (Janik and Buntin, 1985). PRL has been associated with the end of laying and ovulation (Ryan et al., 2015; Sockman et al., 2000), but other studies show that PRL manipulation inhibited or delayed laying or ovulation (Li et al., 2011; Opel and Proudman, 1984).

Differences in our study may be explained by why we found "anti-gonadal" effects in males; our study was done with a colonial avian species with high breeding density, similar to previously described access of females to mates, growth and maintenance in cowbirds (Dufty and Wingfield, 1986), and spermatogenesis testes to release (Pinxten et al., 2003; Schwabl and Lott, 1996). In females, exposure to mate courtship stimulates gonadotropin release and ovarian development (Barfield, 1971; Cheng, 1979; Lehrman et al., 1961). Male presence alone can stimulate ovarian development, even males that do not typically show courtship behaviors, including vocalization, improve ovarian development (Cheng, 1974). Females also stimulate themselves via vocalization (Cheng, 1992). Social cues and the maintenance of courtship behavior may have led to increased testis size in males to compensate for effects of PRL on their own reproductive axis and may also explain a lack of differences. These results highlight the importance of environmental and social cues in hormone regulation in a semi-natural aviary (Calisi and Bentley, 2009), in addition to highly-controlled studies.

Gene expression in the gonads was consistent with morphology. In testes, which increased in size, we found PRLR increased, and LHCGR expression increasing, suggesting increased responsiveness to gonadotropins. LH acts upon Leydig cells to promote androgen synthesis, and FSH stimulates spermatogenesis (Deviche et al., 2011). While both contribute to growth, at minimum FSH has been shown to stimulate testes growth and development in the absence of LH (Brown et al., 1975; Deviche et al., 2011). In ovaries, FSH stimulates follicular development and granulosa cells, whereas LH stimulates ovulation and steroidogenesis (Johnson, 2015; Mishra et al., 2020). However, we found no change in gonadotropin receptor expression in females. Taken together with increased FSHB expression in both sexes, increased LHCGR and FSHR in PRL-treated testes is consistent with stimulation and a lack of differences in reception in females is consistent with similar ovarian size. Similarly, we found no treatment effects on aromatase, suggesting that sex steroid production may be maintained, which would be consistent with the similar reproductive behaviors observed. We did not directly measure sex steroid levels, however, so these expected sex steroid differences are likely affected by the immediate gene expression and reproductive behaviors at the time. Overall, these gene expression results suggest that males may be compensating for "anti-gonadal" effects of PRL treatment to maintain reproductive behavior, though more work, including direct hormone measurements, is required to test this hypothesis.

In contrast to the gonads (ovaries and testes), PRL induced female-specific decreases in oviduct weight and individual variation in estrogen receptor gene expression. Typically, oviduct growth and development are regulated by ovarian estrogen (Barfield, 1971; Hutchison, 1974). The developed oviduct is then released, stimulating growth of the oviduct in different regions and in preparation for laying (Johnson, 2015). Thus, reduced estrogen responsiveness (via reduced ESR1 and ESR2 expression in PRL-treated females) corresponds with the observed reduction in oviduct weight. Other studies have reported that PRL reduces oviduct weight (Jeong et al., 2012; Yi et al., 2020); however, we did not find increased expression of PRLR in the main

unexplored, we cannot rule out the possibility that hormone manipulations could elicit developmental or transient dysregulation of the HPG axis in a way that would reduce fecundity (Bonier and Cox, 2020).

4.6. Multiple-brooding and productivity despite elevated prolactin

In addition to methodological considerations, we hypothesized the reason we did not observe an anti-gonadal effect of PRL may be due to the multiple-brooding strategy employed by rock doves. Many birds engage in multiple breeding attempts over the course of a breeding season (Burley, 1988; Westmoreland et al., 1986), including seasonal breeders (Grüebler and Naef-Daenzer, 2010; Morton, 2002; Szpiniewski and Halupka, 2021; Walsh and Bock, 1997). This reproductive strategy results in the 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 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 relatively low levels, not high enough to impair gonadal function in the HPG axis. Activity may also naturally increase as PRL declines. In fact, the PRL doses we used approximate levels observed at mid-to-late post-hatching in rock doves (Austin et al., 2021), a period where clutch overlap occurs in our population (pers. obs.). The PRL levels we used were lower than those seen around late-incubation/hatch, however, when around-the-clock parental care is maintained. Thus, PRL's effects on the HPG axis appear dependent on the breeding context, with this species' multiple-brooding strategy represented by circulating PRL. PRL has often been studied in animals in the context of seasonal photorefractoriness (e.g., Sharp et al., 1998), such that PRL responsiveness may change when hormone mediates reproductive transitions in species where multiple-brooding occurs.

Taken together, our results support that in mutually-parental species (Stiver and Alonzo, 2009), and that proximate mechanisms involved in parental care, like prolactin, may not always inhibit reproduction. When we consider the context in which parental care occurs during multiple-brooding, the subsequent HPG axis regulation seems to be breeding-stage dependent. Our study highlights the importance of studying hormone effects in the context of pro- and reproductive responses of the HPG axis to unveil complex reproductive regulation during breeding transitions.

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CRediT authorship contribution statement

V.S. Farrar: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - Original draft. L. Flores: Methodology, Validation, Investigation. R.C. Viernes: Methodology, Validation, Investigation. L. Ornelas Pina: Investigation. S. Mautshon: Investigation. R.M. Calisi: Resources, Writing - Review & Editing, Supervision, Funding acquisition.

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