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The Behavioral, Neurobiological, and Physiological Effects of Single Parenthood on Single Parents in the *Columba livia* Model System

By

APRIL MEIYEE BOOTH-GRIFFITHS
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

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DAVIS

Approved:

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Committee in Charge

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Abstract

Various species across the animal kingdom utilize a biparental strategy to rear offspring to adulthood and to maximize fitness. Various studies have been conducted to date that demonstrate the effects of single parenthood on offspring. However, much less is known about the physiological, neural, and longer-term effects of single parenthood on the parents. For this dissertation, I set out to increase our understanding on this subject using the biparental avian species *Columba livia* (pigeon, rock dove). Both females and males of this species are capable of rearing offspring to adulthood after the loss of a mate, and both sexes are capable of pseudo-lactation to feed the offspring during the early stages post-hatching. For these reasons, pigeons present us with a powerful opportunity to examine sex-specific behavioral, neural, and physiological changes of single parenting in a biparental species. This present dissertation sets out to elucidate: 1) The general behavioral and physiological short term effects of single parenthood, 2) The differences in crop milk quality and provisioning between single and paired parents, as well as the similarities and differences between single mother and single father crop milk quality and provisioning, and 3) The neural and physiological differences between single and paired parents and the similarities and differences between single mothers and fathers over the course of rearing offspring to independence.

In chapter 1, I report my findings from a short-term study I conducted on the behavioral, neural, and physiological effects of single parenting on single parents at day 5 post their chicks hatching. My team and I found that single parents maintained similar provisioning levels to paired parents but spent less time brooding their offspring. The chicks of single parents were smaller than paired-parented chicks at 3 days post-hatching. Mothers exhibited higher glucocorticoid receptor (*GR*) gene expression than fathers in general. Single parents exhibited

lower prolactin (*PRL*) gene expression in the pituitary gland compared to paired parents. These findings collectively provided a robust foundation for understanding the dynamics at play within avian parenting after a major parental disturbance, offering a steppingstone for further exploration into the molecular and physiological underpinnings that shape the parenting landscape in avian species.

In chapter 2, we assessed variations in crop milk quality and offspring development among single-mothered, single-fathered, and paired-parented nests. Single fathered chicks exhibited reduced size compared to those from paired-parented and single-mothered nests, with second-hatched chicks of single parents being particularly affected. Single-fathered chicks also received less crop milk with a lower dry weight percentage compared to paired-parented and single-mothered chicks. The crop tissue of fathers was heavier than mothers and fathers retain crop milk with a higher dry weight percentage than mothers. Fathers also expressed more crop tissue mesotocin receptors (*OxtR*) than mothers. Paired fathers demonstrated higher prolactin receptor (*PRLR*) gene expression when compared to paired mothers, single mothers, and single fathers, and single parents once again expressed less pituitary *PRL* than paired parents. Single mothers expressed higher paraventricular nucleus (PVN) *GR* expression compared to paired mothers. Two-chick brood single parents exhibited higher baseline corticosterone (CORT) concentrations than their paired counterparts. Our comprehensive data demonstrate that single parents, regardless of sex, undergo physiological and neurobiological changes to sustain crop milk production and offspring care. However, these changes do not fully compensate for the absence of a partner. Our findings will open avenues for further investigation of potential trade-offs and sex-specific disparities in avian pseudo-lactation.

In chapter 3, we manipulated presence of parental partners and measured offspring growth and gene expression associated with glucocorticoids, prolactin, mesotocin, and gonadotropins at different stages of parenting from the early (day 5 post-hatching), middle (day 15 post-hatching), and late stages of parenting (day 3 post-fledging) in the pituitary and PVN. Additionally, we measured baseline circulating plasma concentrations of CORT. Single-fathered chicks were the smallest chicks overall at day 5 post-hatching, while single-mothered chicks were the smallest overall at day 15 post-hatching. Single mothers had more PVN *GR* gene expression than paired mothers, and single parents had higher baseline circulating CORT compared to paired parents in general at day 5 post-hatching. Single parents at day 15 post-hatching had lower baseline circulating CORT than paired parents. Mothers expressed more PVN mineralocorticoid receptors (*MR*) than fathers at day 15 post-hatching, and paired mothers expressed more PVN *MR* than paired fathers, single mothers, and single fathers at day 3 post-fledging. Paired fathers expressed more pituitary *PRLR* than paired mothers, single mothers, and single fathers. Single parents experienced lower *PRL* gene expression in the pituitary as compared to paired parents at day 5 post-hatching. Fathers at day 15 post-hatching and day 3 post-fledging had higher PVN *PRLR* than mothers. Mothers expressed less PVN *OxtR* than fathers at day 5 post-hatching, Single parents at day 15 post-hatching expressed less pituitary *GnRHR* than paired parents. Single mothers at day 15 post-hatching also expressed less PVN *GnIH* than paired fathers. This investigation, conducted from the initial stages of parental care through to the point of offspring achieving independence, reveals the myriad of neurological and physiological changes that male and female parents undergo in the face of a major disturbance in their parenting strategy.

As a whole, the comprehensive data I present for my dissertation work demonstrates that single parents, regardless of sex, undergo profound physiological and neurobiological changes in their journey to rear offspring. However, these changes, while remarkable, do not completely compensate for the absence of a partner. My findings open up exciting new avenues for future research of potential trade-offs and sex-specific disparities in parenting behaviors following a major disturbance in parent-offspring dynamics. This work holds the promise of furthering our understanding of the complex world of avian parenting and promises to shed light on broader aspects of reproductive biology.

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Before I joined the UC Davis Molecular Cellular and Integrative Physiology Graduate Program, I completed a master's in biology at California State University Fresno, mentored by Dr. David Lent. David allowed me to be creative and design my own research project, and I am so thankful for his advice and guidance. I am forever grateful for David, Dr. Saeed Attar, and Dr. Honora Chapman for encouraging me and helping me see I could pursue a career in science and thrive when I was at a point of wanting to quit and give up.

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Chapter 1 Sex-Specific Behavioral And Physiological Changes During Single Parenting In A Biparental Species, *Columba Livia*

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April Booth performed experiments presented in chapter 1: Figures 1-1 – 1-6. She also analyzed data for those figures, co-designed all experiments, drafted the manuscript, and revised, edited and approved the manuscript.

Abstract

Many species exhibit biparental care to maximize fitness. When a partner is lost, the surviving partner may alter their behavior to compensate offspring. Whether both sexes use the same physiological mechanisms to manifest their change in behavior remains elusive. We investigated behaviors and mechanisms associated with the alteration of parental care post partner removal in a biparental avian species, the rock dove (*Columba livia*). We hypothesized that rock dove single parents experience sex-biased changes in neural genomic transcription and reproductive behaviors, and these changes are related to chick development. We manipulated parental partner presence and measured parental attendance, offspring growth, gene expression of glucocorticoid receptors (*GR*) and mineralocorticoid receptors (*MR*) in the pituitary, and *GR*, *MR*, and estrogen receptor beta (*ER-β*) in the hypothalamus. We also measured circulating plasma concentrations of the stress-associated hormone corticosterone and the parental care-associated hormone prolactin. We also quantified prolactin gene (*PRL*) expression changes in the pituitary, as well as prolactin receptor (*PRLR*) expression in the hypothalamus and pituitary. We found that single mothers and fathers maintained similar provisioning levels as paired parents, but spent less cumulative time brooding chicks. Chicks of single parents were smaller than paired-parented chicks after three days post-hatch. Mothers in both treatment groups experienced higher expression of hypothalamic *GR* as compared to fathers. Single parents experienced lower *PRL* gene expression in the pituitary as compared to paired parents. No significant differences were found for the circulating hormones or other genes listed.

Keywords: parental care, hypothalamus, pituitary, hypothalamus-pituitary-adrenal axis, prolactin

Introduction

Biparental care is a common reproductive strategy in birds, and it likely reflects the high energetic demands of rearing rapidly-growing offspring (Burley and Johnson, 2002). In species exhibiting biparental care, loss of one parent can be detrimental to both the remaining parent and offspring (Banerjee et al., 2012; Chary et al., 2015; Helmeke et al., 2009; Rogers and Bales, 2019). While this topic has become a popular area of investigation, there is a dearth of knowledge regarding how single mothers and single fathers alter their behavior following the loss of their opposite-sex partner. Similarly, very little is known about the physiological response of these single parents following the loss of their mate. A general phenomenon reported across multiple species, including burying beetles, fish, mammals, and birds, is that single parents can behaviorally compensate for the loss of a partner, (e.g. Parker et al., 2015; Pilakouta et al., 2018; Silverin and Wingfield, 1998; Wang and Novak, 1992; Zhao et al., 2019). An illuminating study conducted with insects revealed transcriptome-wide, sex-specific gene expression differences in the brains of male and female single parent burying beetles (*Nicrophorus vespilloides*) as compared to their paired counterparts (Parker et al., 2015). However, little is understood regarding how and why these changes manifest in biparental vertebrate species.

We experimentally examined whether sex-biased behavioral and physiological effects occur in both single fathers and single mothers in a biparental avian species in which both sexes pseudo-lactate to feed their young. Mammalian studies have historically dominated the neural landscape of vertebrate parental behavior investigations (e.g. Dulac et al., 2014; Saltzman et al., 2017; Zhao et al., 2019). However, the physiological events involved in gestation and lactation are highly sex-biased in that only females can gestate and, later, provision offspring via lactation. In order to control for the physiological complexities that surround gestation and lactation, we

conducted our investigations using the biparental species of the rock dove (*Columba livia*)(pigeon). All avian species lay eggs, with embryo development occurring external to the body. Rock doves exhibit a biparental strategy in that both mother and father incubate their eggs until hatch. Rock doves also exhibit a lactation-based parental care strategy in that they, like mammals, 'lactate' to feed their young (Chadwick, 1983; Gillespie et al., 2011; Gillespie et al., 2012; Horseman and Buntin, 1995). However, unlike mammals, *both* sexes lactate. This pseudo-lactation, more precisely termed crop milk production, consists of the production of cells that proliferate and detach from the inner lining of the crop sac of both sexes, creating a fat- and protein-rich milk-like substance on which they rear their chicks. Many functional similarities between rock dove and mammalian lactation exist, including the mediation of this event by the hormone prolactin (Austin et al., 2021b; Buntin, 1996; Dumont, 1965; Farrar et al., 2022a; Leash et al., 1971), and the delivering of essential immunoglobulins and nutritional benefits to young (Gillespie et al., 2012). Upon the death of a partner, the surviving male or female parent will continue to care for chicks alone, and without re-pairing until chicks have fledged (Booth et al., 2018; Burley, 1980). For all of these reasons, the rock dove presents us with a rare and powerful opportunity to examine sex-specific behavioral and physiological changes of single parenting in a biparental species.

We hypothesized that rock dove single parents experience sex-biased changes in neural genomic transcription and reproductive behaviors, and these changes are related to chick development. To investigate the physiological and behavioral similarities and differences of conspecific male and female parents upon their transition to a single parenthood state, we experimentally removed either the male or female parent permanently from a nest. To determine the behavioral responses to mate loss and single parenthood, we measured key parental care

behaviors that included brooding, feeding, and parental recesses from the nest and compared them between single and paired birds. In altricial birds like rock doves, brooding behavior is required during early development because young chicks cannot thermoregulate (Hetmanski and Wolk, 2005). However, brooding parents cannot forage while they are warming nestlings, which requires parents, particularly single parents, to make trade-offs. The number of feeding bouts and nest recesses (time away from the nest) also help to provide information about how much parents invest in their offspring. Additionally, we measured offspring condition and size.

On day 5 post-hatch, we collected tissues from the single-parent experimental group and paired-parent control group. We measured the hypothalamic and pituitary gene expression of single parents and compared it to that of single parents of the opposite sex, as well as that of their same-sex paired counterparts. The genes we pinpointed for investigation are well known mediators of the stress response, reproduction, and their associated behaviors, are expressed in tissues vital for regulating these behaviors, and appear to be highly conserved in vertebrates (Buntin, 1996; Calisi et al., 2018; Calisi et al., 2008): mineralocorticoid receptor (*MR*), glucocorticoid receptor (*GR*), estrogen receptor beta (*ER-β)*, prolactin (*PRL*) and prolactin receptor (*PRLR*). Circulating corticosterone (CORT) in the plasma binds to its nuclear receptors, including *MR* and *GR*, in the hypothalamus as part of an endocrine cascade that influences subsequent behavior and physiology (De Kloet et al., 1998). CORT naturally increases during breeding, and in response to a stressor, it collaborates with other stress-associated components of the 'HPA' axis, involving the hypothalamus, pituitary, and adrenal glands. This collaboration triggers the emergency life history stage, prioritizing individual survival over reproduction (Wingfield and Sapolsky, 2003). Additionally, an increase in *ER-β* expression in the paraventricular nucleus (PVN) within the hypothalamus can result in the depression of the stress

response (Handa et al., 2012; Lund et al., 2006). Prolactin, an important hormone in mediating parental care behaviors (Austin et al., 2021b; Chastel et al., 2005; Farrar et al., 2022b; Smiley, 2019), is secreted by the pituitary. PRL receptors (*PRLR*) in the pituitary and hypothalamus play a pivotal role in facilitating parental behaviors and maintaining crop milk production in Columbid species (Farrar et al., 2022a; Smiley and Adkins-Regan, 2016). Our findings reveal sex-biased physiological and behavioral phenotype plasticity as a result of single parenthood within a biparental avian species.

Material and Methods

Animal Care

Birds were housed at the University of California, Davis in large and covered outdoor aviaries (5'x4'x7'), protected from inclement weather (e.g. major storms, harsh winds, sleet, hail, extreme heat, etc). All birds were captive-bred. As rock doves are a naturally social species, an average of 8 reproductively experienced pairs were housed in each aviary. The pairs were allowed to form naturally on their own after introduction into a given aviary. All birds were sexually mature, determined by the occurrence of at least one prior nesting event, and 1 to 2 years old. Along with natural light exposure, the aviaries also had artificial lights set to 12L:12D to help control for any daylight fluctuations. 16 nest boxes (13.5''x15''x13.25'') were offered in each aviary to birds in which to choose their nest. Birds were maintained on an *ad libitum* diet of whole corn (Farmers), commercial poultry food (Farmers Best Turkey/Game Bird Starter Crumbles), grit (Winner's Cup Pigeon Grit), and water. All husbandry procedures and experimental protocols were approved by the UC Davis Institutional Animal Care and Use Committee (IACUC) (Protocol #18895) and have been successfully used in our lab to measure

rock dove reproduction (Calisi et al., 2018; MacManes et al., 2017; Austin et al., 2021a, Austin et al., 2021b).

Experimental Design

On the day of hatching in the morning (0800-1100) (considered day 1 post-hatching), one parenting partner was removed from our treatment group to create a single-mothered or single-fathered nest (Figure 1-1A). Paired control nests were left with the parenting pair intact. The parents in each treatment group were then left to care for their offspring for 4 days before tissue collection on day 5 post-hatching. Rock doves normally have broods of 1 to 2 chicks.

Chick Morphometrics

The mass, gram, (using an electronic scale) and tarsus length, mm, (using analog calipers) of the chicks were measured over the course of the study to determine differences in nestling growth as a proxy of parental provisioning. These measurements were collected in the morning (0800-1100) in an adjoining room. The measurements from the chicks were collected in less than 5 minutes to decrease handling stress to the chicks and stress to the parents (e.g. Wingfield, Vleck, and Moore, 1992; Wingfield, O'Reilly, and Astheimer, 1995). Chicks were measured on days 1, 3 and 4-post hatching. Sample sizes of chicks per treatment group were as follows: single-mothered nests (n= 10 chicks, from 7 nests), single-fathered nests (n=17 chicks, from 10 nests), and paired-parented control (n=13 chicks, from 9 nests). There were 4 single-mother nests, 3 single-father nests, 5 paired-parent nests with one chick nests and 3 single-mother nests, 7 single-father nests, 4 paired-parent nests with two chicks nests.

Behavioral Scoring

Six-hour videos of single and paired parents were recorded on day 4 post-hatching (the day before tissue sample collection). Handheld video cameras (Sony HDR-CX440 and Canon

VIXIA HF R800) on tripods were used to record the behavior of the parents on the nest from the late morning to late afternoon. The video cameras were set up between 1030 and 1100. Time spent brooding, feeding, and away from the nest were scored using the program BORIS (Friard and Gamba, 2016). Scorers were blind to whether or not a single-parented nest was a single-mothered nest or a single-fathered nest. To identify the single parent or the parents in our paired-parent treatment group in each nest, the parents were identified via unique colored leg-band combinations and/or unique colored sharpie markings on their head and/or wings. Brooding was defined as the time that parents were actively sitting on the offspring. Feeding was defined as the time when the parent would regurgitate crop milk to their offspring. Off, or "away from the nest", was defined as the time the parent was off the nest and away from the chicks, including perching at the edge of the nest-box. In instances involving nests with paired parents, simultaneous execution of parenting behaviors, such as concurrent chick brooding, did not occur. The total duration of each behavior was calculated in minutes across a six-hour period. Additionally, the cumulative time spent by paired mothers and fathers in both brooding and feeding activities was combined for comparison against single-mothered and single-fathered nests. Moreover, the cumulative time spent on all the scored behaviors was separately compiled for paired mothers and fathers, aiming to identify potential shifts in behavior among single mothers and/or single fathers. For comprehensive information, refer to the statistical methods section.

Tissue Collection

Our experiment occurred over 24 consecutive months, from 2016-2018, which was the length of time needed to acquire the following sample sizes: 8 single mothers, 9 single fathers, 9 paired mothers, and 8 paired fathers. One paired father was removed from the study because it

was not collected at the same time as its paired mother counterpart; thus, exposure to a stressor (human disturbance within the cage and capture of its mate) could have influenced results. Tissue collections always occurred between 0800-1200, and birds were humanely euthanized within three minutes of entering the aviary using an overdose of isoflurane anesthesia prior to decapitation (MacManes et al., 2017). Trunk blood was immediately collected and placed on ice for 2 hours or less until being transferred to our nearby lab and centrifuged at 4°C for 10 minutes to extract plasma for hormone assay. Plasma was then stored at -80°C until assayed. Brains and pituitaries were also collected and immediately flash frozen on dry ice, then transported to the lab within 2 hours and stored at -80°C until further processing was performed. Following methods from Calisi et al (2021a; 2018; 2017), hypothalami were punch-biopsied coronally in a -20°C cryostat (Leica CM 1860) at 100μM and stored in RNAlater at -80°C. In brief, a stereotaxic atlas of the pigeon brain was used to confirm the identity and location of the hypothalamus, using the bifurcation of the septopallio-mesencephalic tract and the cerebellum or supramamillary nucleus as landmarks (Karten and Hodos, 1967; Kuenzel and van Tienhoven, 1982) (Figure 1-1B). Lateral septum tissue attached to the hypothalamus was also collected.

Quantitative PCR

Before beginning RNA extraction, an average of 35 mg (± 8.7) of hypothalamus tissue or 8 mg (± 2.6) pituitary in RNALater was washed with 1X phosphate buffered saline (PBS) three times. After the washing procedure, the RNA was then extracted from the tissue using a Direct-zol RNA MiniPrep kit (Zymo research, California) using a modified protocol for lipid-rich tissues (Farrar and Calisi, 2022b), similar to previous work in our research group (Farrar et al., 2022a; Farrar, Morales Gallardo and Calisi, 2022c). We measured RNA purity and concentration using a NanoDrop 2000c (Thermo Scientific, Massachusetts). We did not use samples with a

260/280 reading of 1.67 or below and a 260/230 reading of 0.79 or below. Extracted RNA was then treated with DNase (Perfecta DNase, Quanta Biotech, Massachusetts) to remove any genomic DNA contamination. DNase-treated RNA was converted into cDNA using the qScript cDNA synthesis script (Quanta Biotech) and samples were diluted 1:5 for qPCR.

cDNA samples were run in triplicate using species-specific primers (see Table 1-1). Reactions (10 μL total) included 1 μL cDNA template (diluted 1:5), 5 μL 2X SSOAdvanced SYBR Green PCR mix (BioRad, California), and 10 μM of each primer. Reactions were run on a BioRad CFX384 qPCR machine under the following cycling conditions: 50℃ for 2 min, 95℃ for 10 min, and then 40 cycles of 95℃ for 15 sec and 60℃ for 30 sec. Reference genes, *rpl4* and *ACTB* were used for hypothalamic tissue and *HPRT1* and *rpL4* were used for pituitary tissue (Zinzow-Kramer et al., 2014). Because we did not find significant differences in mean reference gene expression between the treatment groups for the hypothalamus or pituitary (see *Results* for details), we were able to quantify the relative expression of each gene of interest relative to the geometric mean of the reference genes using the ddCt method (Livak and Schmittgen, 2001; Mayer et al., 2019). Normalized expression (dCt) was calculated as the average Ct 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 dCt of a randomly chosen paired female control. Fold change (Rq) was then determined (2^{-ddCt}). We then obtained a normalized Rq value by dividing Rq by the average Rq value of the paired female treatment group.

Hormone Assays

Corticosterone Assay

Circulating concentration of CORT was quantified (ng/mL) using radioimmunoassay as described by Wingfield et al. (1992). The method specifically for measuring corticosterone in rock doves is described in detail by Austin et al (2021a, 2021b). Final hormone values were corrected using the individual recovery and volume correction for each sample. Mean recoveries were 80.69% (sd = 4.36) and 84.32% (sd = 4.43). Intra-assay variations ranged from 3.31-3.77 and inter-assay variations were 2.94 (sd = 2.94). The mean for the detection limits of the assays was 8.90 and 8.52 pg per tube.

Prolactin Assay

The circulating concentration of prolactin was determined using a competitive enzyme-linked immunosorbent assay (ELISA) designed by Dr. Zhiyong Wang (ADS Biosystems) (Wang, Farrar, and Calisi, 2022; Farrar, 2022c). In brief, goat anti-rabbit antibody from Jackson ImmunoResearch (Cat#111-005-003 stock at 2.3mg/ml) was diluted 1:2000 in 1xPBS (prepared with 10xPBS solution, Affymetrix 75889). 0.1 mL was added into each well of an ELISA plate (Nunc MaxiSorp) and incubated at 4°C for 24 hours. The plate was then washed with 1xPBS with 0.05% Tween20 (Sigma-Aldrich) four times, and then blocked with 0.4% Casein and 0.4xPBS (diluted from Casein and 1xPBS buffer from Biorad, #161-0783) at room temperature for 2 hours. The plate was washed again with 1xPBS with 0.05% Tween20 for 4 times each. Bird plasma was diluted 1:5 in assay buffer (0.4%Casein+0.4xPBS+0.05% Tween20) and then added to the designated wells along with the standards. Biotinylated PRL tracer (20ng/ml, diluted with assay buffer) was then added, and then rabbit anti-chicken PRL antibody (1:20000, diluted with assay buffer, stock was 0.05 mg/mL) provided by A.F. Parlow and the National Hormone and

Peptide Program (NHPP, Torrance, CA). The plate was then incubated at 4°C overnight. The next day the plate was washed with 1xPBS with 0.05% Tween 20 four times, and then streptavidin horseradish peroxidase was added (1:5000 diluted with assay buffer, stock was 1mg/mL) (Jackson ImmunoResearch, #016-030-084) and incubated at room temperature for two hours. The plate was then washed with 1xPBS with 0.05% Tween20 four times. TMB (BioLegend, #421501) substrate was added to each well and incubated in the dark for 15 minutes. H_2SO_4 at 0.18M was then added to stop the reaction, and the plate was read with a BioRad iMark microplate reader at 450 nm, with 580 nm as the background. All samples were tested on the same plate. The intra-assay CV was 4.6% (sd = 3.4).

Statistical Analysis

The R statistical language (version 3.6.0) was used for all statistical testing (Wickham and Grolemund, 2017). We used the packages 'tidyverse', 'car', 'cowplot', 'effsize', 'effectsize', 'lme4', 'emmeans' and 'extrafont' for data analysis and plotting. All data in terms of the covariables described below were checked for homogeneity of variance (Levene's test for homogeneity of variance). If the variance was significantly different in terms of a given covariable, then the response variable data was rank-transformed. We included brood size (one-chick nest, two-chick nest) in our models as a covariable to account for any variation that could be attributed to variation in broods. Unless otherwise noted in the results section, there were no significant differences related to brood size for the response variables measured. Our alpha level was 0.05 and all tests were one-tailed. All findings (Figures 2-6) are presented in the form of standard boxplots.

Chick Analysis:

To assess the relationship between chick size and covariables, two distinct analyses of variance (ANOVA) tests were employed, utilizing the effect size measure Eta Squared. These analyses sought to examine the impact of treatment groups (comprising paired-parented nests, single-mothered nests, and single-fathered nests), nest types (encompassing paired-parent nests and single-parented nests), and brood size on chick size. One ANOVA test included the covariables treatment and brood size, and the other included nest-type and brood size. This had to be done because nest-type was an aliased coefficient with treatment groups. Tukey multiple comparisons of means was used for post-hoc analysis (effect size test: Cohen's *d* test). Since pigeon chicks hatch asynchronously, a two-sample t-test (effect size test: Cohen's *d* test) was used to compare size of the first hatched chicks to the second hatched chick of two chick nests. An ANOVA test was also used to determine if there was an interaction between chick-hatching order and nest-type. A two-sample t-test was also used to compare the size of paired-parented and single-parented one-chick nests. These last three analyses were only performed in terms of nest-type due to sample size constraints.

Adult Analysis:

In the domain of adult examination, our analytical models were designed to juxtapose response variables against several factors: treatment groups, specifically paired mothers, paired fathers, single mothers, and single fathers; nest types, including paired-parent and single-parent configurations; individual sex; and brood size. A linear mixed-effects model was used to compare response variables to treatment group and brood size, using Type II Wald F tests with Kenward-Roger degrees of freedom (effect size test: Eta Squared). Given the inherent

interdependence of paired mothers and fathers, we introduced PairID as a random effect within the linear mixed-effects model. Computed estimated marginal means (least-squares means) with a Tukey adjustment was used as a post-hoc analysis (effect size test : Cohen's *d* test). An ANOVA test was used to compare response variables with nest-type, sex, and brood size. Similar to the situation with the chick analysis, these two types of analyses had to be conducted because nest-type and sex were aliased coefficients with treatment groups. In addition, nest-type and sex were analyzed as separate covariables because there was a significant interaction between nest-type and sex in terms of the behaviors scored in the parents (brooding ((rank-transformed) $F_{1,19} = 12.6$, $p < 0.05$, $\eta^2 = 0.40$), feeding ($F_{1,19} = 5.7$, $p < 0.05$, $\eta^2 = 0.23$), and time spent off the nest ((rank-transformed) $F_{1,19} = 20.0$, $p < 0.05$, $\eta^2 = 0.51$).

The analysis described in the prior paragraph were the methods used in general, but in the case of the parental brooding and feeding behavior, three models were used. One mixed-effects model described in the last paragraph was used to ascertain if there were any differences between how much paired mothers, paired fathers, single mothers and single fathers exhibited brooding and feeding behaviors (this analysis kept cumulative time for paired mothers and fathers separate). Two ANOVA models were used to assess how much parental attendance the chicks were receiving for each treatment group (paired-parented nest (cumulative time of paired mothers and fathers added together), single-mothered nest, single-fathered nest), nest-type (paired-parented nest, single-parented nest.) and brood size. These analyses were similar to the ones described in the "Analysis regarding chicks" section. In the case of time spent off the nest, only one mixed-effects model described in the last paragraph was used since this behavior was scored to specifically assess differences in time spent away and not attending the chicks for paired mothers, paired fathers, single mothers and single fathers.

Since the sample sizes were too small to measure by treatment group for circulating prolactin (6 paired mothers, 3 paired fathers, 4 single mothers, 8 single fathers) only one ANOVA testing nest-type, sex, and brood size was conducted. In the case of measuring hypothalamic and pituitary gene expression using qPCR, outliers were removed from all analyses if they were two or more-fold above or below the distribution of a given treatment group.

Results

Chicks raised by single parents were smaller

To assess differences by treatment group and nest-type, the average chick size (weight/tarsus ratio) of the two chicks on day 3 post hatching for two-chick nests were used in the analysis, while the chick size of the single chick of one-chick nests were used. The analysis revealed that chicks reared in both single-fathered and single-mothered nests exhibited significantly smaller sizes compared to chicks nurtured in paired-parent control nests ($F_{2,17} = 8.8$, $p < 0.05$, $\eta^2 = 0.47$). This distinction was evident through the Tukey HSD test, which demonstrated statistical significance between paired-parented and single-fathered chicks ($d = 2.2$), as well as between paired-parented and single-mothered chicks ($d = 1.5$) (Figure 1-2A). Notably, no significant difference in chick size was observed between single-mothered and single-fathered chicks (Tukey HSD test, $p = 0.91$, $d = 0.98$). Moreover, a general trend emerged where chicks nurtured within single-parented nests displayed smaller sizes when compared to those raised in paired-parented nests ($F_{1,18} = 17.5$, $p < 0.05$, $\eta^2 = 0.45$).

Further examination unveiled a significant disparity in size between first and second-hatched chicks ($t_{20} = 2.2$, $p < 0.05$, $d = 0.75$), with the former exhibiting larger proportions (Figure 1-2B). The interaction effect between chick hatching order and nest-type was also found

to be statistically significant ($F_{1,18} = 6.6$, $p < 0.05$, $\eta^2 = 0.27$). It is also important to note that in one-chick nests, the size of single-parented chicks was notably smaller than their paired-parented counterparts ($t_8 = 3.4$, $p < 0.05$, $d = 0.69$) (Figure 1-2C).

Parental behavior patterns and care allocation

The parental care dynamics exhibited distinct patterns in terms of brooding and feeding care. Single-parented chicks received notably reduced brooding care in comparison to their paired-parented counterparts. However, the allocation of feeding time remained relatively consistent between the two groups. Single mothers, in particular, demonstrated heightened investment in tending to their offspring compared to paired mothers.

Brooding care allocation and differences

Comparing brooding behaviors, a comprehensive analysis revealed intriguing distinctions. Paired mothers exhibited less time dedicated to brooding their chicks relative to paired fathers, single mothers, and single fathers ((rank transformed) $F_{3,10.2} = 16.4$, $p < 0.05$, $\eta^2 = 0.89$, estimated marginal means, $p < 0.05$ for paired mothers versus paired fathers ($d = 219.9$), single mothers ($d = 110.3$), and single fathers ($d = 109.9$)) (Figure 1-3A). When the cumulative time of paired mothers and fathers were added together and compared to the cumulative time of single mothers and fathers, single-mothered and single-fathered chicks experienced significantly less brooding time compared to their paired-parented counterparts ($F_{2,14} = 7.9$, $p < 0.05$, $\eta^2 = 0.53$, Tukey HSD test, $p < 0.05$ for paired parents vs single fathered ($d = 64.7$) and vs single mothered ($d = 81.3$)) (Figure 1-3B). Moreover, single-parented chicks as a whole received less brooding care in comparison to paired-parented chicks, indicating a consistent trend ($F_{1,15} = 13.7$, $p < 0.05$, $\eta^2 = 0.47$).

Feeding time, treatment group and brood size effects

The dynamics of feeding time exhibited intricate dependencies. Notably, brood size exerted a significant influence on the time parents spent feeding their chicks, with two chick broods being fed more than one chick broods in general ($F_{1,11.7} = 8.6$, $p < 0.05$, $\eta^2 = 0.35$). To accommodate this, feeding time in two-chick nests was adjusted by halving the time. This data, along with that from one-chick nests, was used for subsequent analyses. Following this adjustment, brood size ceased to yield significant differences ($F_{1,12.8} = 0.0045$, $p = 0.95$, $\eta^2 = 0.00046$). Distinct trends emerged among the treatment groups concerning feeding behavior, with single mothers dedicating more cumulative time to feeding their chicks compared to paired mothers ($F_{3,10.7} = 4.6$, $p < 0.05$, $\eta^2 = 0.51$, estimated marginal means, $p < 0.05$ for paired mothers versus single mothers ($d = 38.5$)) (Figure 1-3C).

Total time devoted to feeding chicks and brood size effects

The interplay between brood size and feeding time was further illuminated. An evident distinction arose with respect to cumulative time spent feeding the chicks, where parents from across the treatment nests (paired parents, single mothers, and single fathers) invested more time in two-chick nests compared to one-chick nests ((rank-transformed) $F_{1,14} = 8.2$, $p < 0.05$, $\eta^2 = 0.38$). Similar adjustments, as mentioned earlier, were made for this dataset as well. Following these adjustments, no significant variation emerged concerning brood size and the total time allocated to feeding chicks ($F_{1, 14} = 0.035$, $p = 0.85$, $\eta^2 = 0.0025$). Notably, no significant distinctions emerged when comparing single-mothered, single-fathered, and paired-parented nests ($F_{2, 14} = 1.3$, $p = 0.31$, $\eta^2 = 0.15$) (Figure 1-3D). Additionally, combining nests by nest-type yielded no significant differences ($F_{1, 15} = 0.30$, $p = 0.59$, $\eta^2 = 0.020$).

Time away from the nest

Distinct patterns emerged in terms of time away from the nest. Paired mothers exhibited more off-nest time compared to paired fathers, single mothers, and single fathers within the recorded time frame ($F_{3, 11.4} = 17.1$, $p < 0.05$, $\eta^2 = 0.75$, estimated marginal means, $p < 0.05$ for comparisons, paired mothers vs paired fathers ($d = 236.2$), paired mothers vs single mothers ($d = 176.4$), paired mothers vs single fathers ($d = 139.2$)) (Figure 1-3E).

Differential hypothalamic GR expression between mothers and fathers, with no evident pituitary disparities

There was no significant difference in mean reference gene expression between the treatment groups for the hypothalamus ($F_{3,21.2} = 1.9$, $p = 0.16$, $\eta^2 = 0.17$), nor by nest type ($F_{1,30} = 0.50$, $p = 0.48$, $\eta^2 = 0.01$) or sex ($F_{1,30} = 0.99$, $p = 0.33$, $\eta^2 = 0.03$), indicating stable reference genes for the hypothalamus. There was also no significant difference in mean reference gene expression between the treatment groups for the pituitary ((rank-transformed) $F_{3,20.2} = 1.7$, $p = 0.20$, $\eta^2 = 0.16$) nor by nest type ($F_{1,28} = 3.1$, $p = 0.091$, $\eta^2 = 0.09$) or sex ($F_{1,28} = 1.4$, $p = 0.24$, $\eta^2 = 0.04$).

There was no significant difference in hypothalamic *MR* expression between treatment groups, nest-type, or sex ((rank-transformed, treatment groups), $F_{3,20.7} = 0.30$, $p = 0.83$, $\eta^2 = 0.03$, after removal of one single-mother outlier) ((rank-transformed, nest-type), $F_{1,29} = 0.76$, $p = 0.39$, $\eta^2 = 0.02$) ((rank-transformed, sex), $F_{1,29} = 0.17$, $p = 0.68$, $\eta^2 = 0.01$) (Figure 1-4A). There was a significant difference by sex in hypothalamic *GR* expression ($F_{1,28} = 5.9$, $p < 0.05$, $\eta^2 = 0.19$, after removal of one paired-mother outlier), with mothers expressing *GR* more than fathers (Figure 1-4B). There was also a significant difference by treatment groups ($F_{3,18.1} = 3.3$, $p < 0.05$, $\eta^2 = 0.33$). However, there were no significant differences in the post-hoc analysis between

the treatment groups (the closest to the 0.05 threshold: estimated marginal means, single mothers versus single fathers $p = 0.083$ ($d = 0.85$)). There was no significant difference in hypothalamic *GR* expression by nest-type ($F_{1,28} = 0.54$, $p = 0.47$, $\eta^2 = 0.0092$). There was also no statistical difference in hypothalamic *ER-β* expression between any of the treatment groups, nest-type, or sex ((treatment), $F_{3,18.3} = 0.74$, $p = 0.54$, $\eta^2 = 0.09$) ((rank-transformed, nest-type), $F_{1,30} = 0.24$, $p = 0.63$, $\eta^2 = 0.0086$) ((rank-transformed, sex), $F_{1,30} = 0.020$, $p = 0.89$, $\eta^2 = 0.00064$) (Figure 1-4C).

There was no significant difference in pituitary *MR* gene expression ((treatment groups), $F_{3,19.4} = 1.3$, $p = 0.29$, $\eta^2 = 0.14$) ((rank-transformed, nest-type), $F_{1,29} = 1.2$, $p = 0.28$, $\eta^2 = 0.04$) ((rank-transformed, sex), $F_{1,29} = 0.046$, $p = 0.83$, $\eta^2 = 0.0027$)(Figure 1-5A). Pituitary *GR* expression did not differ between treatment groups, nest-type, or sex ((rank-transformed, treatment groups), $F_{3,20.2} = 0.49$, $p = 0.69$, $\eta^2 = 0.05$) ((rank-transformed, nest-type), $F_{1,29} = 1.2$, $p = 0.28$, $\eta^2 = 0.04$) ((rank-transformed, sex), $F_{1,29} = 0.24$, $p = 0.62$, $\eta^2 = 0.0096$) (Figure 1-5B).

Reduced pituitary PRL expression observed in single-parent individuals

There were no statistically significant differences in hypothalamic *PRLR* expression by treatment groups, nest-type, or sex ((rank-transformed, treatment groups), $F_{3,\ 20.7} = 0.65$, $p = 0.59$, $\eta^2 = 0.07$, after removal of one single-father outlier)((rank-transformed, nest-type), $F_{1,\ 29} = 0.057$, $p = 0.81$, $\eta^2 = 0.000047$) ((rank-transformed, sex), $F_{1,\ 29} = 1.6$, $p = 0.21$, $\eta^2 = 0.03$) (Figure 1-4D). When pooled by nest-type, single parents had lower pituitary *PRL* expression compared to paired parents ($F_{1,\ 28} = 4.5$, $p < 0.05$, $\eta^2 = 0.14$) (Figure 1-5C). There were no statistically significant differences in pituitary *PRL* expression between the treatment groups ($F_{3,\ 19.0} = 1.9$, $p = 0.16$, $\eta^2 = 0.19$), nor by sex ($F_{1,\ 28} = 0.024$, $p = 0.88$, $\eta^2 = 0.0015$). There were no significant differences in pituitary *PRLR* expression ((treatment groups), $F_{3,19.4} = 1.0$, $p = 0.40$, $\eta^2 = 0.11$)

((rank-transformed, nest-type), $F_{1, 29} = 0.16$, $p = 0.69$, $\eta^2 = 0.0054$) ((rank-transformed, sex), $F_{1, 29} = 1.5$, $p = 0.23$, $\eta^2 = 0.04$) (Figure 1-5D).

Circulating corticosterone and prolactin did not differ between groups or sexes

Circulating corticosterone concentrations were not significantly different between treatment groups, nest-type, or sex ((treatment groups), $F_{3, 19.0} = 0.54$, $p = 0.66$, $\eta^2 = 0.06$) ((nest-type), $F_{1, 27} = 0.26$, $p = 0.62$, $\eta^2 = 0.02$) ((sex), $F_{1, 27} = 1.1$, $p = 0.30$, $\eta^2 = 0.040$) (Figure 1-6A). Circulating prolactin did not differ between nest-type or sex ((nest-type), $F_{1, 17} = 0.020$, $p = 0.66$, $\eta^2 = 0.02$) (ANOVA(sex), $F_{1, 17} = 0.067$, $p = 0.80$, $\eta^2 = 0.0022$) (Figure 1-6B).

Discussion

The outcomes of this study highlight a significant difference in chick sizes between those reared by single parents and those nurtured by paired parents. While single parents exhibited comparable feeding levels to their paired counterparts, a notable disparity emerged in terms of cumulative brooding and thermoregulation times. Paired parents were observed to collectively invest more time in these behaviors than their single-parent counterparts. Moreover, a distinct pattern was discerned in pituitary *PRL* gene expression, where single parents exhibited lower expression levels compared to paired parents. In addition, a general trend emerged wherein mothers demonstrated elevated hypothalamic *GR* expression relative to fathers. These findings collectively shed light on the intricate interplay between parental strategies and hormonal dynamics, contributing to a more comprehensive understanding of avian parenting behaviors.

Implications of mate loss on prolactin pathways

Circulating prolactin concentrations exhibited no discernible distinctions between single and paired parents, a pattern mirrored in the pituitary *PRLR* gene expression. Intriguingly, a marked divergence was observed in pituitary *PRL* gene expression, indicating lower levels in

single parents compared to their paired counterparts. This finding echoes prior observations within this species from our research cohort, thereby underscoring the intriguing phenomenon that *PRL* gene expression does not necessarily mirror circulating prolactin levels (Farrar et al., 2022a).

One plausible conjecture for the reduced *PRL* gene expression in single parents may involve the autocrine function of prolactin within the pituitary (Ferraris et al., 2013). Existing knowledge establishes that the prolactin receptor (*PRLR*) partakes in a negative feedback loop within the anterior pituitary in rodent model systems (Ferraris, et al., 2013; Ferraris et al., 2011). It is conceivable that the reduction in *PRL* gene expression is orchestrated to mitigate autocrine interactions between prolactin and *PRLR*, potentially aiming to sustain or augment pseudo-lactation and associated parental behaviors among single parents. This hypothesis seeks to elucidate how the modulation of gene expression might contribute to the maintenance of crucial parenting mechanisms under altered circumstances.

Notably, the absence of discernible shifts in hypothalamic *PRLR* gene expression across treatment groups could potentially be attributed to the inherent high levels of circulating prolactin in parents, a physiological requirement for the production of crop milk (Austin et al., 2021b; Buntin, 1996; Farrar et al., 2022a). However, the current observations accentuate the need for further investigation, warranting future studies that directly manipulate prolactin and PRLR expression within the pituitary. Such endeavors are essential to definitively determine the functional role of these gene expression alterations in sustaining parental behaviors, particularly in the context of single parents.

It is important to acknowledge a discrepancy between our results and those of Austin et al. (2021b) regarding the measurement of circulating prolactin. While Austin et al. (2021b)

employed a heterologous radioimmunoassay, our current study utilized an enzyme-linked immunosorbent assay (ELISA). This methodological divergence accounts for the observed dissimilarities in the recorded levels of circulating prolactin, even though the measurements were taken at similar time points.

Comparative feeding levels of single and paired parents: reduced brooding and impaired offspring growth in single-parent nests

Although single parents maintained feeding levels akin to those of paired parents, this equilibrium could not counterbalance the absence of the co-parents, as evidenced by the reduced size of their offspring. It is plausible that the decline in brooding time contributed to the lower body temperatures of chicks within single-parented nests, thereby potentially influencing their smaller size. This reduced brooding time might have concurrently spurred an elevation in chicks' metabolism and energy expenditure, ultimately leading to compromised growth rates. This could be attributed to the inability of single parents to augment their feeding rates as a compensatory measure.

This finding resonates with observations made by Lendvai and Chastel (2008) in house sparrows (*Passer domesticus*), where maternal investment increased after the temporary removal of male partners for 48 hours. Despite the heightened provisioning efforts, the brood value, as indicated by chick mass, remained reduced. It is noteworthy that our experimental birds enjoyed ad libitum access to food and water, which likely mitigated the impacts of single parenting. However, such mitigating factors might not be as effective in the wild or under resource-constrained conditions, potentially magnifying the observed effects. For instance, Stock and Haag-Wackernagel (2016) discovered diminished fledging success within a free-living rock dove colony after a sudden decline in food availability. Although our data collection time frame does

not particularly align with increased foraging activity, as parents primarily relied on crop milk provisioning (Mondloch and Timberlake, 1991), differences in chick size could still signify a physiological limit on the quality and quantity of crop milk that parents can produce for chick provisioning. Pigeon chicks, known for their rapid growth facilitated by crop milk production (Blockstein, 1989; Vandeputte-Poma, 1980), might encounter limitations in sustaining this accelerated growth due to the combined effects of reduced brooding time and potential constraints on crop milk production.

In the context of two-chick broods, a clear distinction emerged: second-hatched chicks exhibited smaller sizes relative to their first-hatched counterparts. This discrepancy was influenced by an interaction between hatching order and nest type, with single-parented chicks prominently contributing to this trend. Nevertheless, it is important to acknowledge the limitations imposed by the dataset, particularly the collection of day-3 post-hatching chick size data from only two paired-parented nests. The averaging of chick sizes within two-chick nests might have introduced an element contributing to the observed disparity between single- and paired-parented nests. Comparable results were documented by Silverin (1982), who experimentally altered brood sizes in pied flycatchers (*Ficedula hypoleuca*), resulting in similar outcomes. Similarly, single-parented chicks within one-chick nests exhibited smaller sizes compared to their paired-parented counterparts. This pattern is likely explained by the diminished brooding behavior exhibited by single parents, as previously discussed. As we look forward, conducting more expansive studies could offer deeper insights into the intricate effects of brood size on chick quality and size concerning single- and paired-parented nests.

When considering the overall allocation of time, it was evident that paired mothers spent more time away from the nest and their offspring compared to single mothers, single fathers, and

paired fathers during the recorded time frame (Day 4 Post-hatching, 1000-1100 to 1600-1700). Within our pigeon colony, a shift change typically occurs around 11:30 am to 12:00 pm, during which females leave the nest to be replaced by their male partners—a temporal pattern consistent with observations made by Johnston and Janiga (1995) for feral pigeons and Levi (1986) for domesticated pigeons. Consequently, paternal care extends for 5-6 hours before the female's return. This transition accounts for the father's caretaking 'shift' primarily captured in our video footage, potentially resulting in underestimation of the paired mothers' investment. However, these data underscore the comparable commitment of single mothers and fathers to attending their chicks on the nest. Furthermore, it reveals how single mothers compensated for their partner's absence by elevating their engagement levels when they would typically be off the nest. Specifically, single mothers increased both brooding and feeding durations, while also reducing time spent away from the nest in contrast to paired mothers. These findings collectively contribute to our understanding of avian parenting strategies under altered circumstances.

Potential role of enhanced hypothalamic GR expression in facilitating maternal care

At five days post-hatching, we discerned a heightened expression of hypothalamic glucocorticoid receptors (*GR*s) in female parents compared to males. This finding aligns with the general inclination of paired mothers to invest more time in chick provisioning and care than their male counterparts, corroborated by previous studies (Johnston and Janiga, 1995; Levi, 1986). Comparative research conducted by De Vries (2004) in prairie voles (*Microtus ochrogaster*) indicated distinct pathways for male and female parental care behaviors, possibly reflecting differential neurobiological mechanisms.

The observed disparity in hypothalamic *GR* expression among parents at Day 5 post-hatch hints at a sex-specific responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis.

Glucocorticoid receptors play a pivotal role in the HPA axis' negative feedback loop within the hypothalamus (De Kloet et al., 1998). This suggests that mothers might necessitate a more finely tuned HPA negative feedback pathway, possibly explaining the divergence in *GR* expression. This pathway might also be instrumental for single mothers in maintaining consistent parental care behaviors. Corroborating this notion, studies on barn swallows revealed that individuals with lower stress-induced corticosterone (CORT) levels during early reproductive stages exhibited heightened offspring provisioning and increased fledgling success (Vitousek, Jenkins, and Safran, 2014).

Considering the role of glucocorticoids in metabolic processes, it is conceivable that heightened hypothalamic GR expression contributed to mothers' altered foraging behavior, potentially reducing time spent away from the nest (Kitaysky et al., 2001). To further comprehend the persistence of this sex-specific *GR* expression discrepancy as chick rearing progresses and the male's contribution to parental care intensifies (Johnston and Janiga, 1995), future investigations should focus on examining *GR* expression in single parents of both sexes at later stages. Similarly to the proposed manipulations for *PRL* and *PRLR* expression, exploring hypothalamic *GR* expression through targeted interventions could provide crucial insights into its functional significance.

Minimal role of MR and ER-β gene expression

Contrary to our observations of heightened hypothalamic *GR* expression in female parents, we found no significant differences in gene expression related to mineralocorticoid receptors (*MR*) or estrogen receptor-beta (*ER-β*) between treatment groups. Krause et al. (2015) previously suggested that *MR* might exert its influence in the hippocampus rather than the hypothalamus in the regulation of parental care behaviors. This perspective may partly account

for our inability to discern differences in *MR* expression within our tissue of interest. Moreover, hypothalamic *ER-β*'s potential contribution to our observed outcomes appears limited. In rodent models, *ER-β* activity has been linked to dampened paraventricular nucleus (PVN) activity in response to stressors (Lund et al., 2006). Our failure to detect significant differences in *ER-β* expression could be attributed to our whole hypothalamus assay, which may not capture specific changes within nuclei like the PVN. Future investigations could consider employing nuclei-specific assays to provide a more nuanced perspective.

CORT dynamics and parental care

Intriguingly, following partner removal, we did not observe significant changes in baseline circulating corticosterone (CORT) levels at Day 5 post-separation. This contrasts with findings by Remage-Healey et al. (2003) and Madison et al. (2018) in other avian species (*Taeniopygia guttata*), who detected alterations in CORT levels 24-48 hours post-partner separation. Our measurement time frame differed, capturing circulating CORT levels five days post separation. This discrepancy could suggest a temporal lag in CORT response or emphasize the significance of acute stressors. Lendvai and Chastel (2008) observed CORT increases in single parent house sparrows (*Passer domesticus*) following acute stressors, indicating potential variations in CORT dynamics between different contexts.

Conclusion

The unique attributes of pigeons, characterized by external embryo development and the capacity for both male and female parents to produce crop milk, offered an exceptional avenue for exploring potential sex-biased neurogenetics and physiological responses within the context of single parenthood. Leveraging the behavioral and physiological parallels between the sexes in our study system, we gained valuable insights into the intricacies of avian parenting strategies.

In light of our investigation, we substantiate our initial hypothesis that rock dove parents encounter sex-biased shifts in glucocorticoid receptor gene expression, a phenomenon that potentially underlies differential behavioral trajectories for single parents. Our empirical evidence highlights the interconnectedness between parental care behaviors and crop milk production, indirectly modulated through pituitary prolactin gene expression. These findings collectively provide a robust foundation for understanding the intricate dynamics at play within avian parenting, offering a stepping stone for further exploration into the molecular and physiological underpinnings that shape the parenting landscape in avian species.

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Figures

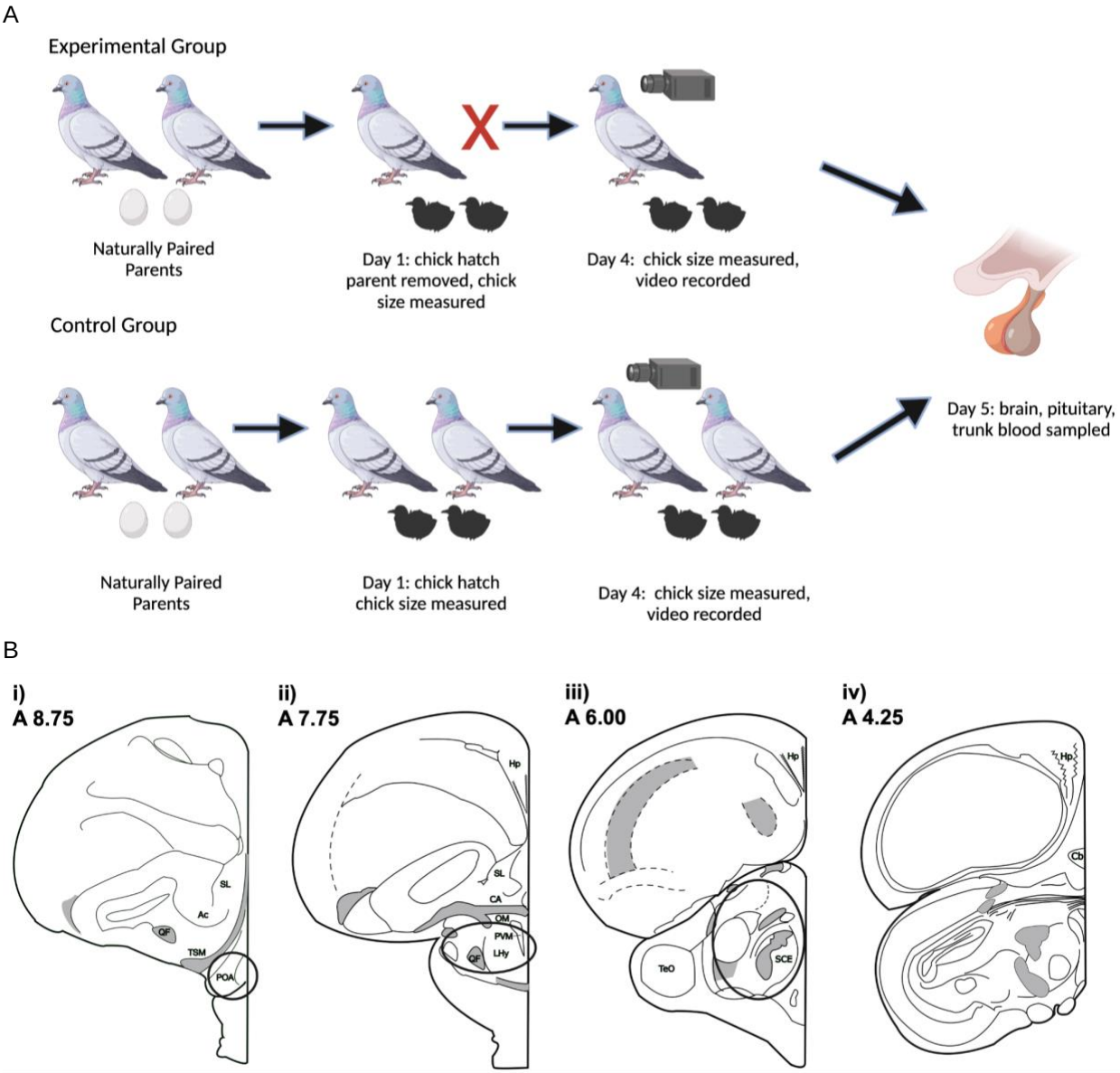

Figure 1-1. Experimental Setup and Tissue Collection

A) Experimental Design: We implemented a controlled manipulation by randomly removing one parental partner, either male or female, from the nest on Day 1 post-chick hatch. The paired parent control group remained undisturbed post-hatching. Chick size measurements were obtained on Day 4 post-hatching, while behavioral recordings were conducted on the same day.

Brain, pituitary, and trunk blood samples were collected on Day 5 post-hatching (Figure generated using BioRender.com).

B) Representative coronal slices through the hypothalamus. The hypothalamus was microdissected using multiple punches of a 2.00 (preoptic Area (POA)) or 3.00 (Starting at the paraventricular nucleus (PVN), onwards) mm punch (as shown in black circles on each atlas slice). Hypothalami punching began when the tractus septomesencephalicus (TSM) extended to the bottom of the brain (i), continued through when the cloudy tractus quintofrontalis (QF) (ii) and optic tecta (TeO) appeared (iii) and ended when the cerebellum (Cb) was visible (iv). Hypothalamic punches included nuclei such as the POA (i), PVN (ii), lateral hypothalamus (LHy) (ii), and other nuclei not pictured. Coronal slice images are recreated from Karten and Hodos (1968) (Figure further modified using BioRender.com).

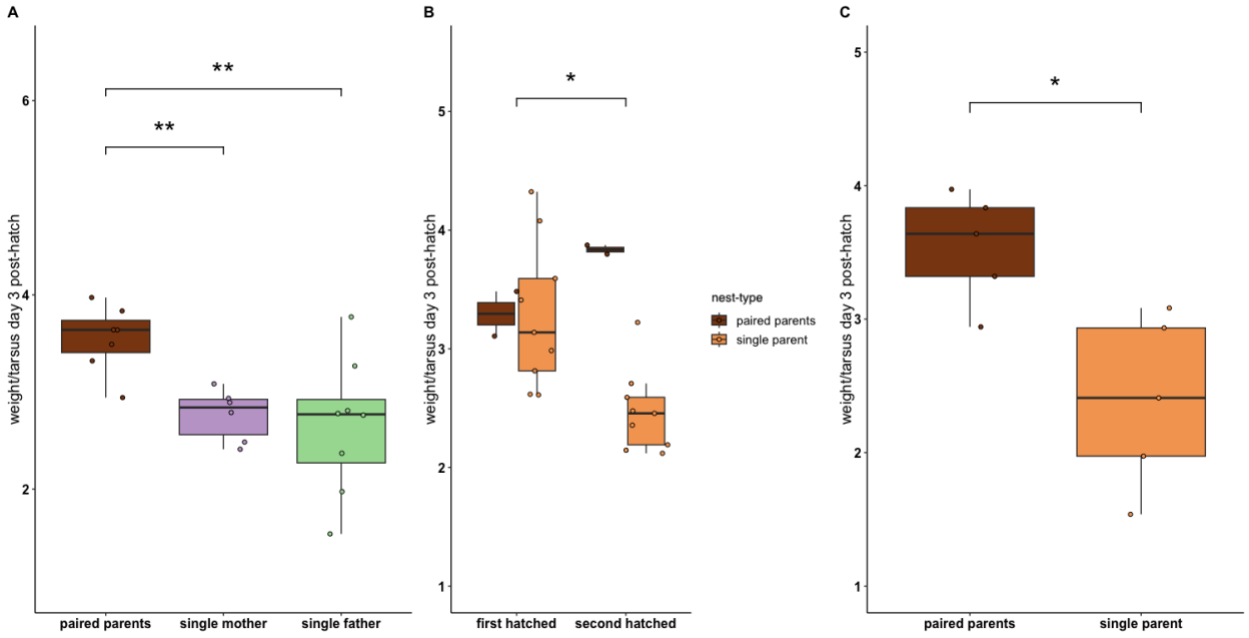

Figure 1-2. Offspring size (weight/tarsus ratio) at 3 days post-hatch. Box plots show A.) Difference in chick size between treatment-group nests. B.) Difference in chick size between first-hatched chicks and second-hatched chicks in regards to two-chick nests. The chicks are also categorized by nest-type. C.) Difference in chicks size between paired parented and single parented one-chick nests. Statistical significance denoted as follows: *: $p < 0.05$, **: $p < 0.01$.

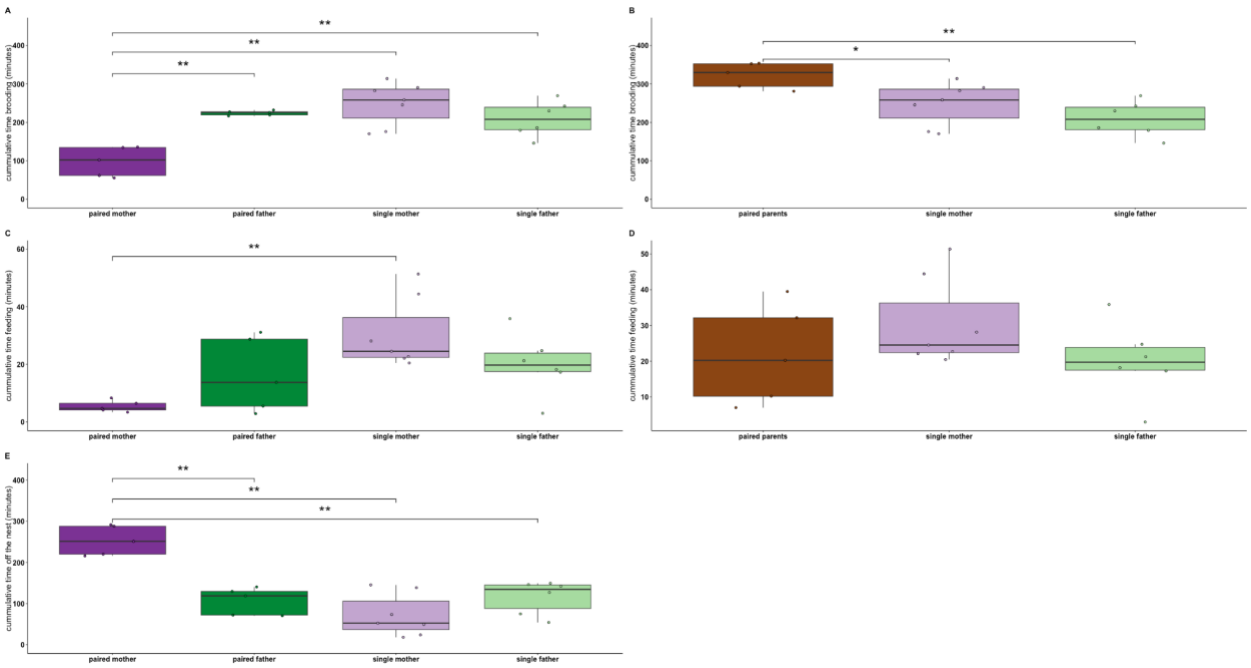

Figure 1-3. Parent Behavior Duration (total minutes across 6 hours). In Figures 3B and 3D, the cumulative time spent by paired mothers and fathers was combined to present the overall time dedicated to parental behaviors (brooding and feeding) with regard to the chicks. The box plots illustrate: A.) cumulative time allocated to brooding chicks within each treatment group, B.) overall time expended on brooding chicks, C) cumulative time devoted to feeding chicks within each treatment group, D.) total time invested in feeding chicks, and E.) cumulative time spent off the nest for each treatment group. Statistical significance denoted as follows: *: $p < 0.05$, **: $p < 0.01$.

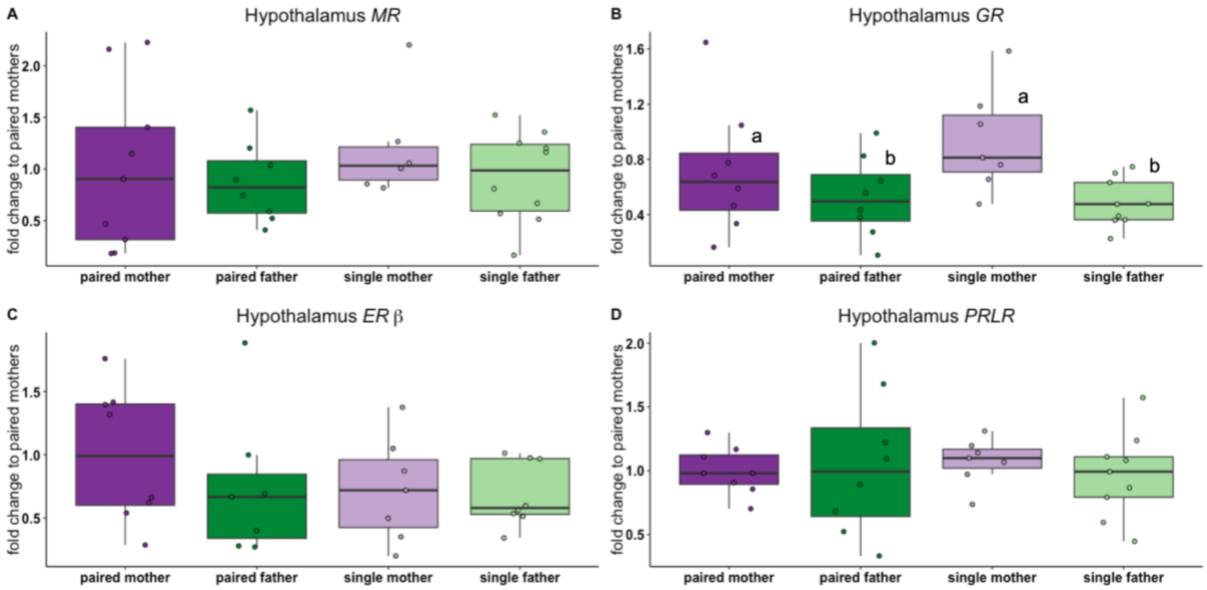

Figure 1-4. Hypothalamic Gene Expression. Box plots represent the gene expression levels of: A. Mineralocorticoid Receptor (*MR*), B.) Glucocorticoid Receptor (*GR*), C.) Estrogen Receptor Beta (*ER-β*), D.) Prolactin Receptor (*PRLR*). Gene expression analysis was conducted in the hypothalamus. While no significant differences were observed by treatment groups, a notable distinction emerged in *GR* expression based on sex. Specifically, females exhibited significantly higher *GR* expression compared to males, as indicated by differing letters in plot B.

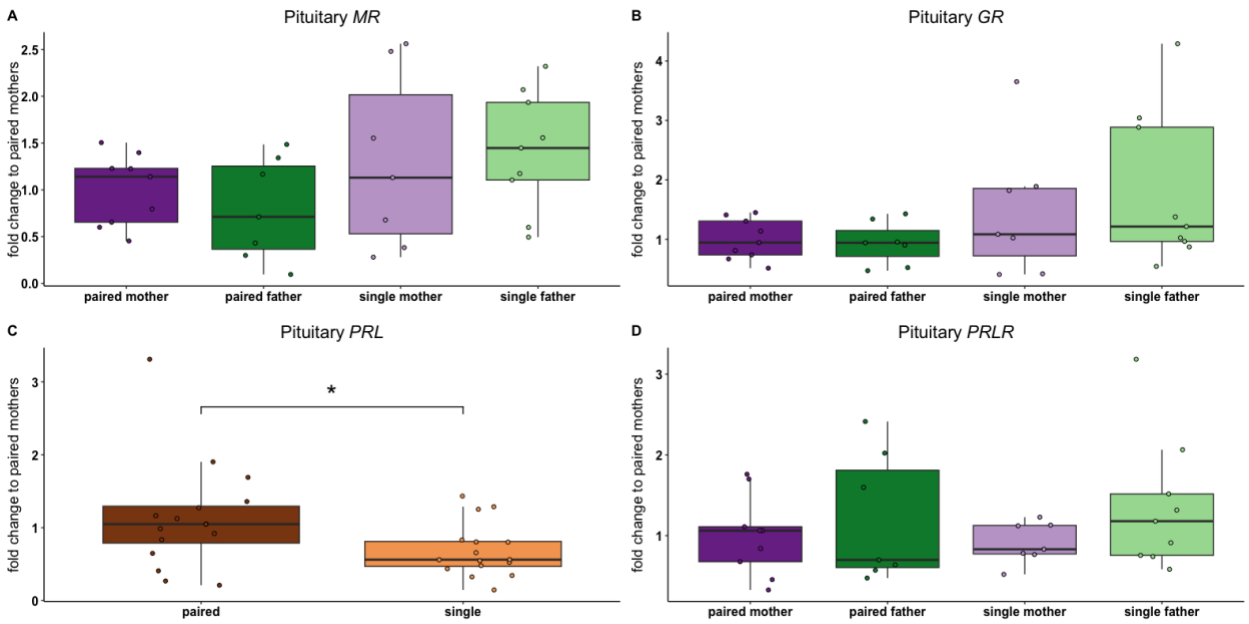

Figure 1-5. Pituitary Gene Expression Box plots illustrate gene expression levels of: A.) Mineralocorticoid Receptor (*MR*), B.) Glucocorticoid Receptor (*GR*), C.) Prolactin (*PRL*), D.) Prolactin Receptor (*PRLR*). The examination of gene expression took place in the pituitary. Despite the absence of significant differences among treatment groups, a significant disparity surfaced in PRL expression. Notably, single parents displayed markedly lower PRL gene expression in contrast to paired parents Statistical significance denoted: *: $p < 0.05$.

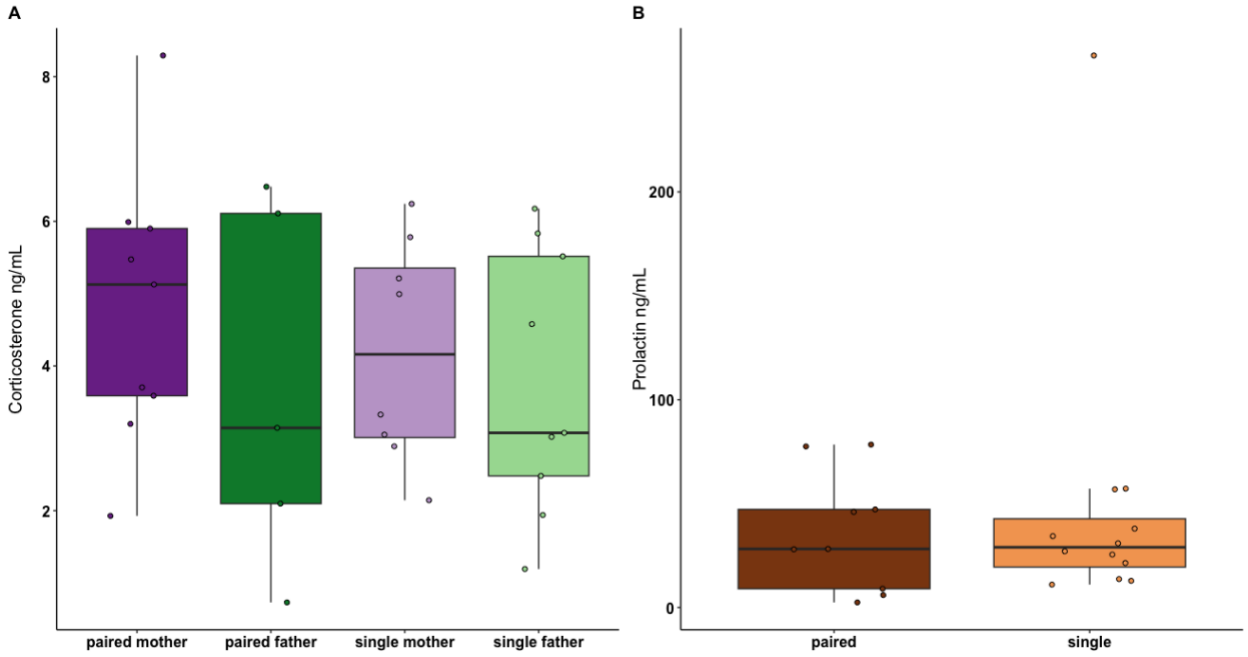

Figure 1-6. Circulating Hormones of Single and Paired Parents. Box plots portray circulating hormone concentrations for: A.) Corticosterone, B.) Prolactin**.** No discernible differences of significance were detected in circulating hormone concentrations between single and paired parents.

Tables

Table 1-1.

Gene	GenBank Accession No.	Primer Sequence	Efficiency (%)
Glucocorticoid Receptor, *GR*	XM_021301096.1	F:TGCTTAACTCGTCGGATCAA R:AAAGTCCATCACGATCCCTC	90.5
Mineralocorticoid receptor, *MR*	XM_021296726.1	F:AGAACATGGCTTCCTCGGTG R:CTAGAAAGCGGAGACCCGAC	103.9
Estrogen Receptor beta, *ER-β*	NM_001282841.1	F:GGGAATGATGAAATGTGGCTC R:GATCTCTTTTACGCGGGTTG	100.6
Prolactin, *PRL*	XM_005506024.2	F:GGCGGGTTCATACTGGTGAG R:TGGATTAGGCGGCACTTCAG	92.6
Prolactin Receptor, *PRLR*	NM_001282822.1	F:TCTTCCTTGCACACATGAAACC R:TCCAGGGTATGATTGACCAGT	95.2
Beta actin, *ACTB*	XM_005504502.2	F:ATGTGGATCAGCAAGCAGGAG R:CATTTCATCACAAGGGTGTGGG	95.8
Ribosomal protein L4, *rpL4*	XM_005511196.1	F:GCCGGAAAGGGCAAAATGAG R:GCCGTTGTCCTCGTTGTAGA	105.1
hypoxanthine phosphoribosyltransferase 1, *HPRT1*	XM_005500563.2	F:GCCCCATCGTCATACGCTTT R:GGGGCAGCAATAGTCGGTAG	94.7

Chapter 2 Sex-Specific Differences in Crop Milk Quality and Neurobiological Profiles of Single-Parent Pigeons (*Columba livia*)

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April Booth performed experiments presented in chapter 2: Figures 2-1 - 2-11. She also analyzed data for those figures, co-designed all experiments, drafted the manuscript, and revised, edited, and approved the manuscript.

Abstract

Crop milk provisioning is an essential aspect of the bi-parental care strategy of both male and female columbids, including pigeons (*Columba livia*). Despite its considerable energy cost, both sexes can produce crop milk during the early parenting stages. However, the nuances of crop milk quality and associated shifts in the neurobiological and physiological profiles of single mothers and fathers remain relatively unknown. In this investigation, we assessed variations in crop milk quality and offspring development among single-mothered, single-fathered, and paired-parented nests. Simultaneously, we examined alterations in gene expression in crop tissue, pituitary, and the paraventricular nucleus (PVN) of the hypothalamus, as well as circulating corticosterone (CORT) concentrations in single mothers and fathers compared to their paired counterparts at Day 5 post-hatching. Our findings reveal that single-fathered chicks exhibit reduced size compared to those from paired-parented and single-mothered nests, with second-hatched chicks of single parents being particularly affected. Single-fathered chicks also receive less crop milk with a lower dry weight percentage compared to paired-parented and single-mothered chicks. Interestingly, fathers display heavier crop tissue and retain crop milk with a higher dry weight percentage than mothers. Furthermore, fathers express higher levels of crop mesotocin receptors (*OxtR*). In the pituitary, paired fathers demonstrate elevated prolactin receptor (*PRLR*) expression in comparison to paired mothers, single mothers, and single fathers, and single parents expressed less pituitary prolactin (*PRL*) than paired parents. Additionally, single mothers exhibit greater PVN glucocorticoid receptor (*GR*) expression than paired mothers. Finally, two-chick brood single parents exhibit higher baseline CORT concentrations than their paired counterparts. Our comprehensive data demonstrate that single parents, regardless of sex, undergo physiological and neurobiological changes to sustain crop milk production and offspring

care. However, these changes do not fully compensate for the absence of a partner. Our findings open avenues for further investigation of potential trade-offs and sex-specific disparities in avian pseudo-lactation.

Introduction

The crop in birds, situated between the esophagus and proventriculus (Johnston and Janiga, 1995; Levi, 1986), serves as a food storage organ responsible for moistening food before further digestion. Both male and female columbids like pigeons (*Columba livia*) have the ability to produce a substance called "crop milk" during the early stages post-hatch of offspring, a phenomenon termed "pseudo-lactation". Both mother and father pigeon milk is notably rich in nutrients (Gillespie et al., 2013), comprising approximately 60% protein, 32-36% lipids, and a small amount of carbohydrates (1-3%) (Davies, 1939). Unique attributes of dove crop milk including the presence of IgA antibodies (Goudswaard et al., 1979; Kocianová et al., 1993), make it essential for chick growth and development. Artificial attempts to replicate this diet in pigeon squabs result in poor growth and increased mortality (Gillespie et al., 2011).

Although prior investigations have identified key genes and transcriptomic aspects governing crop milk production (Gillespie et al., 2013; Xie et al., 2019), there is a paucity of research concerning longer-term changes in crop milk quality and the consequent alterations in gene expression in associated tissues, such as the crop and pituitary gland. While earlier studies touched upon the role of the hormone prolactin (PRL) in crop tissue ((Folley, 1939) 1939) and explored sex differences in regurgitation behavior during pseudo-lactation (*Streptopelia risoria*) (Buntin et al., 1977), comprehensive investigations into these aspects are lacking. Notably, it has been established that single-parent pigeons, regardless of sex, can effectively provide crop milk and rear offspring to independence following the loss of a partner (Booth, 2022; Burley, 1980).

However, the potential changes in crop milk quality resulting from physiological and neurobiological adaptations remain largely uncharted territory.

In this study, we hypothesized that single parent birds would experience sex-biased differences in crop milk quality concurrent with alterations in the neurobiological and physiological profiles of single mothers and fathers. To test this hypothesis, we experimentally manipulated parent presence and scrutinized differences in crop milk and offspring quality within single-mothered, single-fathered and paired-parented nests. Concurrently, we delved into variations in crop tissue, pituitary, and paraventricular nucleus (PVN) gene expression in single mothers and fathers in comparison to their paired counterparts, all at day 5 post-hatching. Our focus encompassed genes pivotal to pseudo-lactation (Austin et al., 2021b; Farrar et al., 2022b, 2022c), encompassing prolactin (*PRL*) and its receptor (*PRLR*) in crop, pituitary, and PVN tissues, as well as vasoactive intestinal peptide receptor (*VIPR*) in pituitary tissue. While less examined in the context of pseudo-lactation, we also assessed mesotocin receptors (*Oxt-R*) in crop and pituitary tissue due to their mammalian analog (oxytocin) playing a vital role in facilitating lactation (Uvnäs-Moberg et al., 2001).

Additionally, we measured glucocorticoid receptor (*GR*) and mineralocorticoid receptor (*MR*) in crop, pituitary, and PVN tissue, as well as estrogen receptor beta (*ER-β*), specifically in PVN tissue, as these genes are known to modulate stress and metabolism (Handa et al., 2012; Lund et al., 2006; Wingfield and Sapolsky, 2003). Furthermore, we investigated differences in circulating corticosterone (CORT) concentrations among parents within each treatment group. To our knowledge, this study represents one of the few endeavors examining sex-biased content in avian crop milk in response to a social manipulation, all while considering chick and parent condition.

Methods

Animal Care

Birds were housed at University of California, Davis, in large, covered outdoor aviaries (dimensions: 5'x4'x7'), which were protected from inclement weather. All birds were captive bred rock dives. As rock doves are a naturally social species, an average of 8 mature pairs were housed in each aviary. All birds were 1 to 2 years old and sexually mature, as evidenced by at least one prior nesting event. Pairs naturally formed upon introduction to the aviaries. The aviaries were equipped with both natural and artificial lighting, maintained on a 12-hour light:12-hour dark cycle to help control for any daylight fluctuations. Sixteen nest boxes (dimensions:13.5''x15''x13.25'') were offered in each aviary for the birds to select their nests. Birds were maintained on an *ad libitum* diet consisting of whole corn (Farmers), protein (Farmers Best Turkey/Game Bird Starter Crumbles), grit (Winner's Cup Pigeon Grit), and water. All animal husbandry procedures and experimental protocols were approved by the UC Davis Institutional Animal Care and Use Committee (IACUC, Protocol #20618). This protocol has been previously employed successfully in our lab to study rock dove reproduction (MacManes et al., 2017; Austin et al., 2021a; Calisi et al., 2018). Typically, rock doves have one to two chick broods.

Experimental Design

On the morning of hatching, between 800-1100 hours; considered "Day 1 post-hatching"), one parenting partner was removed from the treatment group, resulting in either a single mother or single father parented nest (see Figure 2-1). Paired control nests remained intact

with both parents. The single parents in each treatment group were then responsible for caring for their offspring for 4 days before collection on Day 5 post-hatching.

Chick Morphometrics

To assess chick growth as an indicator of parental provisioning, chick weight was measured using a scale and tarsus lengths using a caliper throughout the study. These measurements took place in the morning (0800-1100 hours) in an adjacent room and were completed in less than five minutes to minimize handling stress (e.g. (Wingfield et al., 1995, 1992). Chick measurements were recorded on Days 1, 3 and 4 post-hatching. Sample sizes of chicks per treatment group were as follows: single-mothered nests (n = 5 one chick nests, n = 9 two chick nests), single-fathered nests (n = 4 one chick nests, n = 7 two chick nests), and paired-parented nests (control) (n = 3 one chick nests, n = 13 two chick nests).

Parent Morphometrics

We determined the weight of the parents by weighing them inside a cloth bag. Similar to chick morphometrics, these measurements occurred in the morning (0800-1100) in an adjacent room and were completed in less than 5 minutes. Parent weights were recorded on Day 1 post-hatching and Day 4 post-hatching.

Tissue Collection

Our experiment spanned from September 2018 to September 2021, allowing sufficient time to achieve the following sample sizes: 14 single mothers, 11 single fathers, 16 paired mothers, and 16 paired fathers. Data collection was temporarily halted between February and July of 2020 due to Covid-19 restrictions and California wildfires.

To obtain fresh crop milk, tissue collection began at 0730 hours. Birds were humanely euthanized within three minutes of entering the aviary using inhaled isoflurane anesthesia,

followed by decapitation (MacManes et al., 2017). Some collections occurred between 0800 and 1100 hours, comprising 3 paired mothers, 3 paired fathers, and 4 single mothers. While their brain, pituitary, and circulating CORT data were included in subsequent analyses, unfortunately, we were unable to collect crop tissue or crop milk quality from these individuals.

Trunk blood was immediately collected and placed on ice for a maximum of 2 hours before being transported to our nearby laboratory. It was then centrifuged at 4°C for 10 minutes to extract plasma for hormone assays. Plasma samples were stored at -80°C until assayed. Brains and pituitaries were also collected and immediately flash frozen on dry ice. These frozen tissues were transported to the laboratory within 2 hours and stored at -80°C until further processing. Following established methods by Calisi et al. (Calisi et al., 2018; Farrar et al., 2022a; MacManes et al., 2017), paraventricular nuclei were coronally punch-biopsied at 100μm in a -20°C cryostat (Leica CM 1860) and stored in homogenizer tubes for RNA extractions at -80°C. The identification and location of the PVN were confirmed using a stereotaxic atlas of the pigeon brain, with reference to landmarks such as the anterior commissure and occipitomesencephalic tract (Karten and Hodos, 1967; Kuenzel and van Tienhoven, 1982). Tissue collection ceased once the optic tectum became apparent. The number of parents from which PVN tissue was obtained per group were as follows: 14 single mothers, 11 single fathers, 16 paired mothers, and 16 paired fathers.

The entire crop, along with a sample of the crop milk inside the parent, was collected and assessed for quality in terms of color and consistency. The crop milk quality metric was based on past work scoring for stool quality (Jackson and Jewell, 2019) (Table 2-1). After rinsing the whole crop with water and patting it dry with paper towels, it was weighed. A sample of crop

tissue and crop milk was temporarily stored on dry ice before being transported and stored at -80℃.

Crop tissue weights and/or crop milk data were collected from the following numbers of subjects per group: 10 single mothers (two females had completely empty crops upon collection), 11 single fathers, 11 paired mothers (one female had a completely empty crop upon collection), and 11 paired fathers.

Chick crop milk was obtained following the procedure described by Ma et al (2018). Briefly, after euthanizing the chicks as previously described for the parents, their crops were surgically incised with a 2 cm-long opening. Crop milk was weighed using a scale, assessed for quality as described for the parents (Table 2-1), and then aliquoted into 5mL Eppendorf tubes., These samples were temporarily stored on dry ice before being transported and stored at -80℃. The number of chicks from which crop milk data was collected included: single-mothered nests (n = 3 for one chick nests, n = 7 for two chick nests), single-fathered nests (n = 4 for one chick nests, n = 7 for two chick nests), and paired-parented nests (n = 2 for one chick nests, n = 9 for two chick nests).

Crop milk dry weight

Crop milk dry weight was determined by weighing approximately 1 mg of wet crop milk for each chick sample in duplicate using tin weighboats. Subsequently, the samples underwent a 48-hour drying process at 98℃, followed by an overnight cooling period in a vacuum desiccator. The weighboats containing the dehydrated samples were weighed the following day (CV replicate average = 6.1%, sd = 13.6).

For single parents and paired mothers, obtaining only ~200 μL of crop milk was often feasible as they were collected shortly after their first daily offspring feeding. To address this

limitation, methods outlined by Hinde (2007) for determining the dry matter of small milk quantities were adapted for parental crop milk samples. Tin capsules (Elemental Microanalysis D1008) were used to determine the wet weight of the crop milk samples in duplicate, utilizing a Mettler Toledo XS64 scale. The samples were stored in a 96-well cell-culture plate (Nunclon Delta Surface, Thermo Scientific 167008) and subjected to uncovered drying at 98℃ for 3 hours. Following the drying phase, the samples were left to cool overnight in a vacuum desiccator containing desiccant. The capsule, along with the dehydrated sample, was weighed the subsequent day (CV replicate average = 9.2%, sd = 15.6).

Quantitative PCR

For RNA extraction, pituitary tissue (mean weight 6.2 mg ±2.3), which was preserved in RNALater, was initially rinsed three times with 1X phosphate buffered saline (PBS). Each sample of PVN tissue (mean weight 5.7 mg ± 1.1) and crop tissue (mean weight of 9.7 mg ± 1.8) did not require washing. All samples were randomly selected across treatment groups for each RNA extraction experiment. Tissue RNA extraction employed a Direct-zol RNA MiniPrep kit (Zymo research, California) with a modified protocol tailored for lipid-rich tissues (Farrar and Calisi, 2022). RNA purity and concentration were assessed using a NanoDrop One (Thermo Scientific, Massachusetts). Samples with a 260/280 reading below 1.82 and a 260/230 reading below 1.2 for the crop, 1.62 and 0.74 for the pituitary, and 1.43 and 0.2 for the PVN were excluded. 3 crop tissue samples were re-extracted when found to be below the 260/280 and/or 260/230 readings described. 8 pituitary samples were discarded due to being below the 260/280 and/or 260/230 reading indicated, and/or due to contamination warnings indicated by the NanoDrop One. 8 PVN samples were discarded for similar reasons. DNase treatment (Perfecta DNase 95150-01K, Quanta Biotech, Massachusetts for pituitary and crop tissue, VWR Quanta

kit 18068-015 for PVN tissue) removed genomic DNA contamination from the RNA. The cDNA was synthesized from DNase-treated RNA using the qScript cDNA SuperMix (Quanta Biotech) and diluted 1:5 for qPCR.

Species-specific primers (see Table 2-2) were used for triplicate cDNA sample runs. Each 10 μL reaction included 1 μL of cDNA template (diluted 1:5), 5 μL 2X SSOAdvanced SYBR Green PCR mix (BioRad, California), and 1 μL of 10 μM of each primer. Reactions underwent cycling conditions on a BioRad CFX384 qPCR machine: 50℃ for 2 min, 95℃ for 10 min, and then 40 cycles of 95℃ for 15 sec and 60℃ for 30 sec. Reference genes *HPRT1* and *rpL4* (Zinzow-Kramer et al., 2014) were used for the crop tissue and pituitary, and *HPRT1*, *rpL4*, and *ACTB* (Zinzow-Kramer et al., 2014) for the PVN, as they showed no significant differences in mean reference gene expression between treatment groups (see Results). Relative gene expression was quantified using the ddCt method (Livak and Schmittgen, 2001; Mayer et al., 2019). Normalized expression (dCt) was calculated as the average Ct value between technical replicates of each gene minus the geometric mean of the reference genes for each sample. Relative expression (ddCt) was calculated as dCt minus the dCt of a randomly chosen paired female control. Fold change (Rq) was then determined (2^{-ddCt}), and a normalized Rq value was obtained by dividing Rq by the average Rq value of the paired female treatment group. Outliers, defined as values 2 or more fold above or below the distribution of a given treatment group, were removed. As the PVN and pituitary data were a part of a larger study, candidate genes were measured across two qPCR plates, yielding an inter-assay control CV average of 1.82% (sd=1.25) for the PVN and 1.7% (sd = 1.1) for the pituitary.

Corticosterone Assay

Corticosterone concentrations (ng/mL) were determined using radioimmunoassay (RIA) as previously described (Calisi et al., 2018). A 1:20 dilution was applied to commercially available Corticosterone RIA kits (MP Biomedicals, Orangeburg, NY). Intra-assay variation averaged 4.5, and the inter-assay variation averaged 5.2.

Statistical Analysis

The R statistical language (version 3.6.0) was used for all statistical analyses (Wickham and Grolemund, 2017). Packages utilized included 'tidyverse', 'car', 'cowplot', 'effectsize', 'lme4', 'emmeans' and 'extrafont' for data analysis and plotting. Data were checked for homogeneity of variance regarding the specified covariables (Levene's test for homogeneity of variance). In cases where variance significantly differed for a given covariable, the response variable data were rank-transformed. Brood size (one chick nest, two chick nest) was included as a covariable in our models to account for potential variation attributable to brood size differences. Unless otherwise indicated in the Results section, brood size did not significantly affect the response variables. The alpha level was set at 0.05, and all tests were conducted using one-tailed analyses.

<u>Chick Analysis:</u>

Two distinct analyses of variance (ANOVA) tests were employed, utilizing the effect size measure Eta Squared, to assess the relationship between response variables and covariables. These analyses sought to examine the impact of treatment groups (comprising paired-parented nests, single-mothered nests, and single-fathered nests), nest-types (encompassing paired-parent nests and single-parented nests), and brood size on chick size. One ANOVA test included the covariables treatment and brood size, while the other incorporated nest-type and brood size,

necessitated by nest-type aliasing with treatment groups. Post-hoc analysis utilized Tukey multiple comparisons of means (effect size test: Cohen's d test). Response variables were averaged between the chicks of two-chick nests for these analyses.

Given asynchronous pigeon chick hatching, an ANOVA test determined if an interaction existed between chick-hatching order and nest-type for two chick broods concerning chick size. Additionally, an ANOVA compared the response variables of first-hatched chicks of two-chick nests between treatment groups. To assess the general size difference between first-hatched single-parented and paired-parented chicks, a two sample t-test (or Welch's t-test for unequal variances) was conducted. Similar ANOVAs and t-tests were employed for second-hatched chicks of two-chick nests and for one-chick nests regarding chick size. For all other chick-related response variables, only the chicks from two-chick nests were analyzed, due to sample size constraints.

Adult Analysis:

For adults, analytical models were designed to juxtapose response variables against several factors: treatment groups (paired mothers, paired fathers, single mothers, and single fathers), nest-type (paired-parent and single-parent configurations), individual sex, and brood size. A linear mixed-effects model, utilizing Type II Wald F tests with Kenward-Roger degrees of freedom (effect size test: Eta Squared), was employed to compare response variables to treatment group and brood size. PairID was introduced as a random effect within the linear mixed-effects model to account for the inherent interdependence of paired mothers and fathers. Post-hoc analysis utilized computed estimated marginal means (least-squares means) with a Tukey adjustment (effect size test : Cohen's d test). An ANOVA test compared response variables with nest-type, sex, and brood size. These two types of analyses were conducted

because nest-type and sex were aliased coefficients with treatment groups. Additionally, nest-type and sex were analyzed as separate covariables due to a significant interaction between nest-type and sex in terms of parental behaviors, as indicated by previous research (Booth et al., 2023)

Circulating CORT was the only response variable significantly different by brood size (see Results). Since circulating CORT also significantly differed by nest-type, a follow up ANOVA compared CORT concentrations to nest-type and sex for the parents of two-chick nests. A similar analysis was conducted for parents of one-chick nests.

To score for crop milk quality in terms of color and consistency, a Fisher's exact test for count data was employed (effect size test: Cramer's *V*). This analysis was only performed with data that included two-chick brood color and consistency scores averaged (along with one chick brood data).

Results

Parent Weight

Overall, a decrease in body weight was observed from Day 1 to Day 4 for the pseudo-lactating parents (Figure 2-2). There were no significant differences in parent weight observed from Day 1 to Day 4 among treatment groups, nest types, or sexes (Table 2-3).

Chick Size on Day 3 Post-Hatching - Single fathered chicks were smaller than both paired parented and single mothered chicks, while second-hatched chicks of single parents exhibited smaller sizes compared to their second-hatched counterparts from paired parents.

The average chick size (weight to tarsus ratio) on Day 3 post-hatching was determined for two-chick nests, and the weights of the one chick from one-chick nests were used in this analysis (Table 2-4). Single-fathered chicks on Day 3 post-hatching were smaller than paired-

parented and single-mothered chicks (treatment groups, Tukey HSD $p < 0.05$: single-fathered versus paired-parented chicks $d = 1.6$, single-fathered versus single-mothered chicks $d = 0.91$) (Figure 2-3A). Chicks reared in single-parented nests were smaller than those in paired-parented nests in general.

A significant interaction between chick-hatching order and nest-type was found concerning chick size ($F_{1,63} = 5.0$, $p < 0.05$, $\eta^2 = 0.07$). First-hatched chicks from two-chick broods exhibited no significant differences between treatment groups or nest-types (Table 2-4) (Figure 2-3B). However, second-hatched single-parented chicks were smaller than their second-hatched paired-parented counterparts (treatment groups, Tukey HSD $p < 0.05$: paired-parented versus single-mothered chicks $d = 1.6$, paired-parented versus single-fathered chicks $d = 2.1$) (Figure 2-3C). No significant differences were observed between treatment groups or nest-types for one-chick nests (Figure 2-3D).

Chick Crop Milk Quality - Single-fathered chicks received less crop milk than paired-parented chicks and single-mothered chicks. Single-parented chicks received lower dry-weight crop milk compared to paired-parented chicks.

When analyzing crop milk quality data, it was averaged for two chick nests, similar to the data analysis for chick size. A significant difference in the amount of crop milk received was found, with single-fathered chicks receiving less crop milk than paired-parented chicks and single-mothered chicks (after removal of a single father outlier) (Table 2-4) (treatment groups, Tukey HSD test $p < 0.05$ for comparisons described, single fathered versus paired parented $d = 1.4$, single fathered versus single mothered $d = 1.3$) (Figure 2-4A). There was no significant difference in crop milk provisioned by nest type. Crop milk color showed no significant difference (Fisher's Exact Test for Count Data $p = 0.15$, Cramer's $V = 0.28$), while crop milk

consistency revealed a significant difference between treatment groups, with single fathers having crop milk with a consistency less like “cottage cheese” (Fisher’s Exact Test for Count Data $p < 0.05$, Cramer’s $V = 0.29$) (Figure 2-5).

The ingested crop milk weight of first-hatched single-fathered chicks was less than that of first-hatched paired-parented chicks (Table 4) (treatment groups, Tukey HSD test $p < 0.05$ for single fathered versus paired parented $d = 1.8$; Figure 2-4B). No significant difference by nest-type was observed. Second-hatched single-fathered chick crop milk weight was also less than their second-hatched paired counterparts (treatment groups, Tukey HSD test $p < 0.05$ for single fathered versus paired parented $d = 1.7$; Figure 2-4C), and second-hatched single parented chicks received less crop milk in general.

Significant differences in crop milk dry weights were observed between chicks fed by paired parents and those fed by single fathers, with single fathered chicks receiving crop milk with lower dry weights (Table 4) (treatment groups, Tukey HSD $p < 0.05$: single fathered versus paired parented chicks $d = 1.10$)(Figure 2-6A). When data from both single mothered and fathered chicks were combined, a significant difference emerged between single-parented chicks as a group and paired-parented chicks, with single-parented chicks receiving crop milk with lower dry weights than their paired counterparts (Figure 2-6B).

For first-hatched chicks, there was no significant difference in crop-milk dry weight between treatment groups or by nest-type (Table 2-4). Similarly, for second-hatched chicks in two-chick broods, there was no significant difference in crop-milk dry weight between treatment groups or by nest-type.

Parent Crop Tissue Weight and Crop Milk Quality - Paired fathers exhibited heavier crop tissue compared to both single mothers and single fathers. Additionally, fathers, in general, retained crop milk with a higher dry-weight percentage than mothers.

A significant disparity in the whole crop tissue weight relative to the entire body weight was evident when comparing paired parents to single parents (Table 3; Figure 2-7A). Specifically, paired fathers had notably heavier whole crops than single mothers and single fathers (estimated marginal means, $p < 0.05$ for paired fathers versus single mothers $d = 2.1$, paired fathers versus single fathers $d = 1.7$). Additionally, a significant difference was observed concerning nest type and sex, with paired parents having heavier crops, and males generally exhibiting heavier crops than females (Table 2-3). No significant differences were found between treatment groups in terms of crop milk color or consistency (Fisher's Exact Test for Count Data, $p = 0.74$ for color (Cramer's V = 0.0), $p = 0.075$ for consistency (Cramer's V = 0.23)).

Concerning crop milk dry weight, a notable sex difference was observed within the parent group, with fathers demonstrating a higher percentage of dry weight compared to mothers (Table 2-3) (treatment groups, estimated marginal means, $p < 0.05$ for paired fathers versus paired mothers $d = 2.2$, paired fathers versus single mothers $d = 1.6$, single mothers versus single fathers $d = 1.2$, single fathers versus paired mothers $d = 1.5$) (Figure 2-7B). There was no significant difference in crop milk dry weight based on nest-type.

Parent Crop Tissue Gene Expression - Fathers expressed a higher level of OxtR compared to mothers

For the reference genes used in the qPCR analysis of crop tissue, no significant differences were found between treatment groups (treatment groups, $F_{3,32.1} = 1.9$, $p = 0.15$, $\eta^2 = 0.12$) (nest-type, $F_{1,44} = 1.6$, $p = 0.22$, $\eta^2 = 0.03$)(sex, $F_{1,44} = 3.1$, $p = 0.09$, $\eta^2 = 0.07$). There was no significant variation in *PRLR* expression between treatment groups, nest-type, or sex (Table 2-3) (Figure 2-8A). Similarly, no significant difference was detected in *PRL* expression (after removal of outliers: 2 paired mothers, 1 paired father, and 2 single mothers) (Figure 2-8B).

Concerning mesotocin receptor (*OxtR*) gene expression , there were no significant differences between treatment groups (after removal of outliers: 1 paired father and 1 single mother) or nest-type (Table 2-3). However, a significant difference was observed by sex, with fathers displaying higher *Oxt-R* gene expression compared to mothers in general (Figure 2-8C). No statistically significant differences were observed in terms of *GR* gene expression (Table 2-3) (Figure 2-8D). Similarly, there were no significant differences in *MR* gene expression (Figure 2-8E).

Parent Pituitary Gene Expression - Paired fathers exhibited more PRLR expression compared to paired mothers, single mothers, and single fathers. Single parents showed lower PRL expression than paired parents

No significant differences were detected among treatment groups for the reference genes used for qPCR analysis of pituitary tissue (rank-transformed, treatment groups, $F_{3,35.2} = 0.18$, $p = 0.91$, $\eta^2 = 0.01$) (rank-transformed, nest-type, $F_{1,47} = 0.020$, $p = 0.89$, $\eta^2 = 0.0015$) (rank-transformed, sex, $F_{1,47} = 0.50$, $p = 0.48$, $\eta^2 = 0.01$).

Significant differences were detected in *PRLR* expression among treatment groups. Paired fathers exhibited significantly higher *PRLR* expression than paired mothers and single mothers (after excluding 1 paired mother and 2 single father outliers) (Table 2-3) (treatment groups, estimated marginal means, $p < 0.05$ for comparisons described, paired fathers versus paired mothers $d = 1.3$, paired fathers versus single mothers $d = 1.5$) (Figure 2-9A). Although there was no significant difference between paired fathers and single fathers when brood size was included as a covariate (estimated marginal means, $p = 0.059$, $d = 1.4$), the removal of brood size as a covariate from the model (brood size not significant, $F_{1,33.5} = 0.51$, $p = 0.48$, $\eta^2 = 0.02$) revealed a significant difference similar to paired fathers versus paired mothers and single mothers (estimated marginal means, $p < 0.05$, $d = 1.4$). Additionally, a significant difference was observed by nest-type and sex, with paired parents and males expressing more *PRLR* (Table 2-3).

Single mothers expressed less PRL than paired parents (after removal of a single mother outlier) (Table 2-3) (treatment groups, estimated marginal means, $p < 0.05$, paired mothers versus single mothers $d = 1.3$) (Figure 2-9B). Single parents in general expressed less *PRL* than paired parents (Figure 2-9C), with no significant difference detected by sex.

No significant differences were found in *VIPR* gene expression according to treatment groups, nest-type, or sex (after removal of 2 paired mothers, 1 paired father, and 2 single mother outliers)(Table 2-3). Similarly, no significant differences were observed in *OxtR* gene expression.

No significant differences were detected in *GR* gene expression (after removal of 1 paired mother and 1 paired father outlier)(Table 2-3). Additionally, no significant differences were observed in *MR* gene expression (after removal of 2 paired fathers and 1 single father outliers).

Parent PVN Gene Expression - Single mothers exhibited higher expression of GR compared to paired mothers.

No significant differences were found between treatment groups, nest-types, or sexes for the reference genes used for the PVN (treatment groups, $F_{3,38.8} = 2.1$, $p = 0.11$, $\eta^2 = 0.11$) (nest-type, $F_{1,53} = 0.051$, $p = 0.82$, $\eta^2 = 0.00010$)(sex, $F_{1,53} = 3.9$, $p = 0.054$, $\eta^2 = 0.07$). A significant difference was observed between paired mothers and single mothers in *GR* expression, with single mothers showing higher *GR* expression than paired mothers (after excluding 1 single mother and 1 single father outlier)(Table 2-3)(treatment groups, estimated marginal means, $p < 0.05$, $d = 1.1$) (Figure 2-10A). No significant differences were found by nest-type or sex. There were no significant differences in *MR* expression (Figure 10B) or *ERβ* expression (Figure 2-10C).

Circulating CORT in Parents - Single parents rearing two-chick broods exhibited higher baseline CORT concentrations compared to their paired parent counterparts

Although a significant difference in baseline circulating CORT was detected among treatment groups (Table 2-3), a post-hoc analysis did not reveal any significant differences between specific treatment groups. In general, single parents had higher baseline CORT concentrations compared to paired parents (Figure 2-11A). There was no significant difference by sex.

Additionally, a significant difference was observed based on brood size ($F_{1,30.1} = 5.7$, $p < 0.05$, $\eta^2 = 0.16$), with parents rearing two-chicks showing higher baseline CORT concentrations than those rearing a single chick (Figure 2-11B). Specifically, two-chick single parents had higher baseline CORT than two-chick paired parents (nest-type, $F_{1,31} = 7.8$, $p < 0.05$, $\eta^2 = 0.20$),

while there were no significant differences between one-chick single parents and one-chick paired parents (rank-transformed, nest-type, $F_{1,10} = 1.8$, $p < 0.05$, $\eta^2 = 0.13$).

Discussion

Impact of Single Parenting on Chick Growth and Crop Milk Quality

The results of this study underscore the profound influence of parenting dynamics on chick growth and crop milk quality in pigeons. Single-parented chicks, particularly those raised by single fathers, received lower quality crop milk and consequently exhibited smaller sizes compared to their counterparts reared in paired-parented nests. When considering two-chick broods, the average size of single-fathered chicks was notably smaller than that of paired-parented and even single-mothered chicks, suggesting that single fathers struggled to provision their offspring as effectively as paired parents or single mothers. Furthermore, we discovered that second-hatched chicks of single parents were consistently smaller than their paired-parented counterparts. This finding suggests that at least in the context of two-chick nests, single mothers also faced challenges in adequately providing for their second-hatched chicks.

These disparities in chick size between single parents and paired parents may be largely attributed to the differences in the quality of crop milk provided by the chicks. Single-fathered chicks received significantly less crop milk compared to both paired-parented and single-mothered chicks, which likely contributed to their reduced growth. Additionally, the crop milk received by single-parented chicks had a lower dry-weight content, including lipids and proteins, when compared to paired parents. This nutritional deficit helps explain why second-hatched chicks of single parents, including single mothers, were smaller, as they depended on crop milk for their growth and development.

Crop Tissue Changes in Pseudo-Lactation

The investigation into crop tissue changes during pseudo-lactation unveils intriguing sex-biased patterns. Paired fathers had heavier crop tissue compared to single mothers and single fathers. It is noteworthy that the data collection occurred in the morning, before the typical shift-change between parents. Pigeon fathers typically assume parental duties around 1100 to 1200 hours, tending to and feeding the offspring (as similarly described by Levi (1986) and Johnston and Janiga (1995)). This finding underscores the effort of single fathers to engage in feeding behaviors when they would not normally do so. Remarkably, there were no significant differences in crop tissue between single fathers, single mothers, and paired mothers, indicating that single fathers were attempting to compensate for the loss of their partner in provisioning offspring. Given that second-hatched chicks tended to be smaller for single parents in general, this emphasizes the importance of both parents, particularly fathers, in providing crop milk for growth and development.

The dry-weight percentage of crop milk remaining inside the fathers' crop after collection was higher than that of mothers. This may further explain why single-fathered chicks were smaller and why single fathers could not entirely provision their offspring to the extent of a paired mother, or even a single mother. Although single fathers did attempt to provision their offspring when mothers normally do, they seemed unable to provide all their crop milk for the first meal of the day.

Role of Oxytocin Receptor (OxtR) in Pseudo-Lactation

Fathers expressed a higher level of *OxtR* in their crop tissue compared to mothers in general. This finding suggests that pigeon fathers might employ mesotocin to facilitate pseudo-lactation, a phenomenon akin to lactating mammals and marsupials (Sebastian et al., 1998;

Uvnäs-Moberg et al., 2001) (though mesotocin's role in enhancing production of crop milk similar to milk "letdown" in mammals is yet to be established). The lack of significant difference in *OxtR* expression between paired fathers and single fathers could explain why single fathers could not entirely compensate for the loss of their partner, leading to a reduced crop milk provisioning and smaller chick sizes. However, further experiments manipulating mesotocin and mesotocin receptor expression in the crop tissue are warranted to confirm these hypotheses.

Prolactin (PRL) and Prolactin Receptor (PRLR) in Pituitary as Facilitators of Pseudo-Lactation

In the pituitary gland, paired fathers exhibited significantly higher *PRLR* expression compared to paired mothers, single mothers, and single fathers. This suggests that *PRLR* may play a role in suppressing the negative feedback mechanism to enhance pseudo-lactation and parental behaviors, especially after the loss of a partner. Previous research in rodents has suggested that *PRLR* in the pituitary gland likely functions in negative feedback (Ferraris et al., 2013, 2012). We postulate that in single fathers, *PRLR* expression may be lower than in paired fathers to reduce the inhibitory effect of this negative feedback mechanism. This reduction could potentially enhance pseudo-lactation and stimulate parental behaviors, compensating for the absence of their partner. This difference in *PRLR* expression might also have contributed to the lighter crop tissue observed in single fathers compared to paired fathers. While a previous study from our research group did not find significant differences in *PRLR* gene expression (Booth et al., 2023), the larger sample size in this study could explain the detection of what may be subtle differences.

Moreover, single parents, both mothers and fathers, generally expressed less pituitary *PRL* compared to paired parents. This result is repeated in past work published by our research group (Booth et al., 2023) supporting the notion that reduced pituitary *PRL* gene expression in

single parents could limit autocrine binding of this hormone, thereby diminishing the signaling of the negative feedback mechanism. Additional research involving interventions to manipulate circulating PRL, such as with bromocriptine (Ruiz-Raya et al., 2021) and the manipulation of *PRLR* expression in the pituitary gland is necessary to gain a better understanding of how PRLR and PRL contribute to pseudo-lactation and the behaviors of single parents.

CORT and Its Influence on Single Parenting

Single mothers exhibited higher expression of *GR* in the PVN compared to paired mothers. These receptors play a crucial role in regulating the negative feedback loop of the hypothalamic-pituitary-adrenal (HPA) axis (De Kloet et al., 1998). This difference suggests that single mothers may require a more finely tuned negative feedback pathway to facilitate increased provisioning and parental care. For instance, although barn swallows (*Hirundo rustica erythrogaster*) do not pseudo-lactate like pigeons, a study by Vitousek, Jenkins, and Safran (2014) revealed that parents with lower circulating stress-induced CORT exhibited increased provisioning of offspring. This suggests that the higher presence of *GR* in the PVN of single mothers in our study might have contributed to increased crop milk provisioning overall. Notably, our findings contrast with past research conducted by our group, which measured *GR* expression in whole hypothalamus samples. In that previous study (Booth et al., 2023), we postulated that this difference might enable mothers to have a more responsive negative feedback pathway along the HPA axis. Our focused examination of the PVN, a specific hypothalamic nucleus integral to the HPA axis, has revealed a specific disparity between single mothers and paired mothers. To further understand how single mothers provision their offspring, future research should involve manipulations of circulating CORT and *GR* expression.

Furthermore, our study revealed that two-chick brood single parents had higher baseline CORT concentrations than their paired-parented counterparts, which aligns with findings by Bonier et. al (2011) in tree swallows (*Tachycineta bicolor*). In the enlarged broods of tree swallows, mothers displayed higher baseline CORT concentrations compared to those with reduced broods. These tree swallow mothers with elevated baseline CORT also provisioned their offspring more frequently. In our study, single parents of two-chick nests may have adjusted baseline CORT concentrations to augment crop milk provisioning to their chicks, compensating for the absence of their partner. Although our research did not directly investigate this, prior work from our laboratory (Booth et al., 2023) at a similar stage of development suggests that single parents can at least maintain provisioning levels similar to paired parents.

In addition to this difference between the single parents and paired parents in two-chick broods, we observed a significant difference in brood size overall. Parents of one-chick broods exhibited lower baseline CORT concentrations compared to their counterparts with two-chick broods. In particular, this difference did not exist between single parents and paired parents with one-chick broods. Although we did not analyze differences in crop milk quality between the treatment groups with one-chick broods, we did not observe differences in chick size based on treatment groups or nest types. It's plausible that tending to a single offspring instead of two provided a buffer for single parents of one-chick nests. As mentioned previously, future studies manipulating CORT should be conducted to confirm its role in facilitating crop milk provisioning to offspring at this developmental stage. It should be acknowledged that previous research from our group did not find significant differences between single parents at the same time point (Booth et al., 2023). Sample sizes likely hindered our ability to detect these subtle distinctions between single and paired parents and brood size.

Conclusions

Our research uncovered compelling differences in *PRL*, *PRLR*, *GR* gene expression, and circulating CORT between single and paired parents. However, despite these variations, single parents fell short in fully compensating for the absence of their partner, as revealed by smaller chick size and reduced crop milk quality. These findings shed light on the existence of an apparent physiological limit to crop milk production. Even though single fathers made remarkable efforts to provision their chicks, surpassing their typical contributions at this developmental stage, their offspring were still smaller compared to those raised by single mothers, especially when considering two-chick broods. Furthermore, single fathers provisioned less crop milk than single mothers. These results underscore a sex-related disparity in the capacity to supply vital crop milk to offspring, a factor that warrants consideration in the design of future studies involving pseudo-lactation of species like Colombids.

This study represents the pioneering examination of sex-biased crop milk composition in response to social dynamics, considering parent and chick condition alongside neurobiological and physiological changes. We anticipate that these findings will serve as a catalyst for further investigations into avian pseudo-lactation, unlocking new dimensions in our understanding of parental care in birds.

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Figures

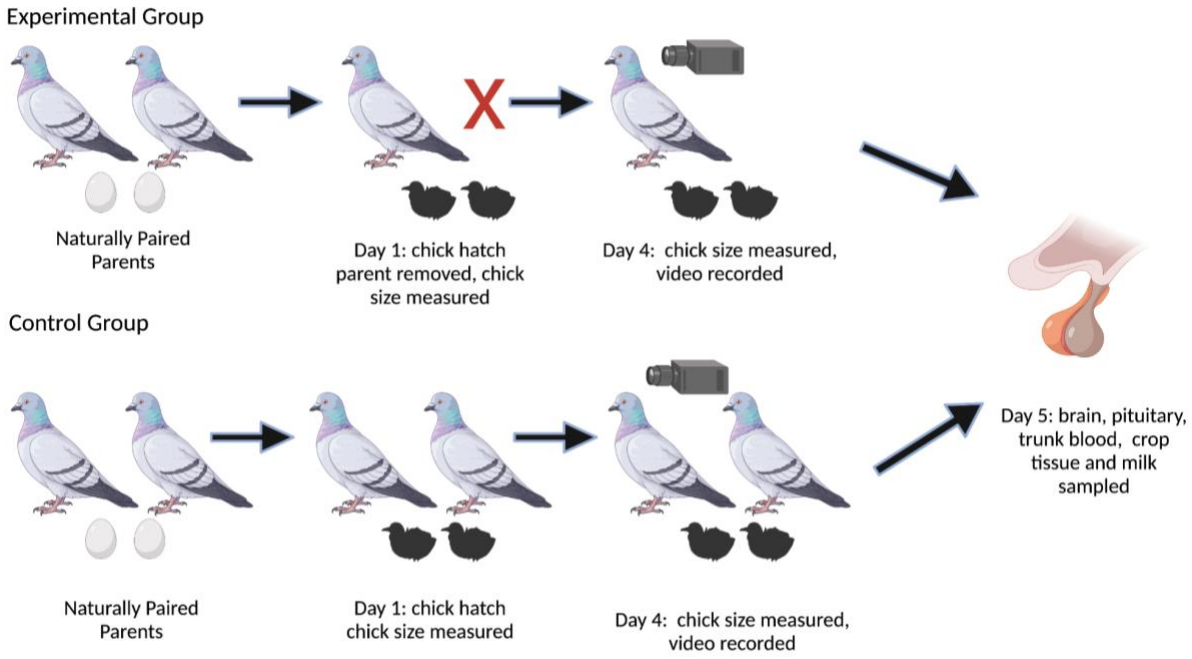

Figure 2-1. Experimental Design. The presence of a parental partner was manipulated by randomly removing one parent, male or female, from a nest Day 1 post chick hatch. Post-hatching, the paired parent control group was left unmanipulated. Chick size was determined on Day 1 and Day 4 post-hatching and behavioral videos were recorded on Day 4 post-hatching. Brains, pituitaries, trunk blood, crop milk and crop tissue were collected on Day 5 post-hatching. Figure created with BioRender.com.

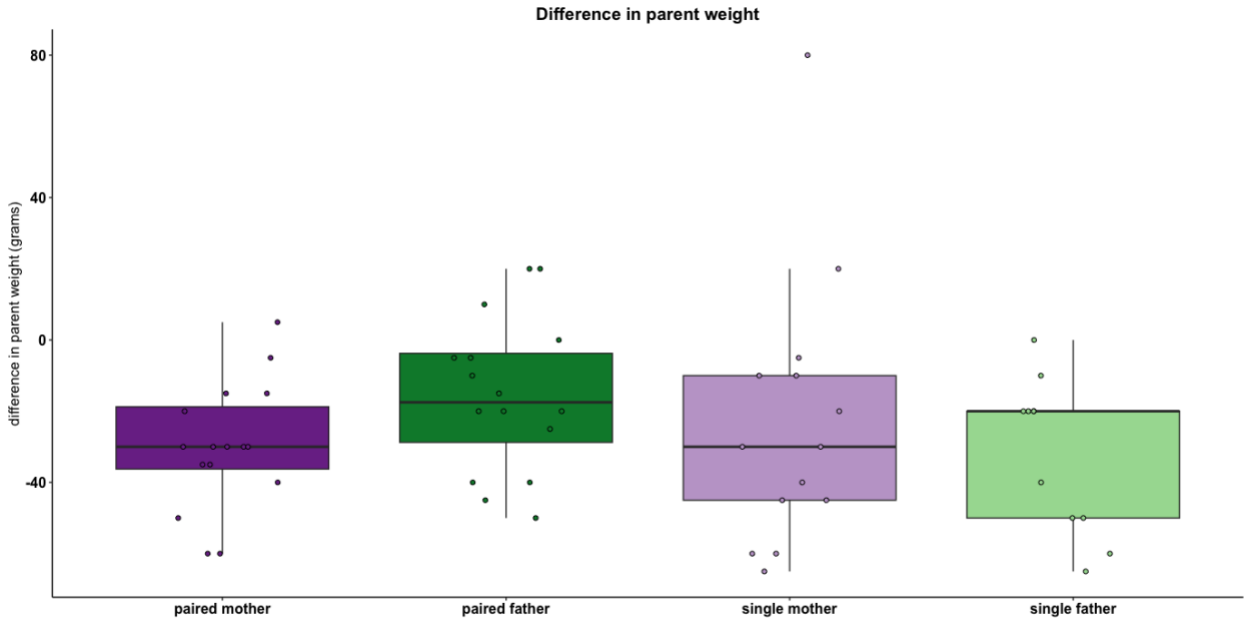

Figure 2-2. Change in parent weight from day 1 post-hatching to day 4 post-hatching in terms of treatment groups. No significant differences were detected. Points represent individual birds, and boxplots represent where the first quartile, median, and third quartile for each treatment group. The whiskers indicate the minimum to the first quartile and the maximum of the third quartile.

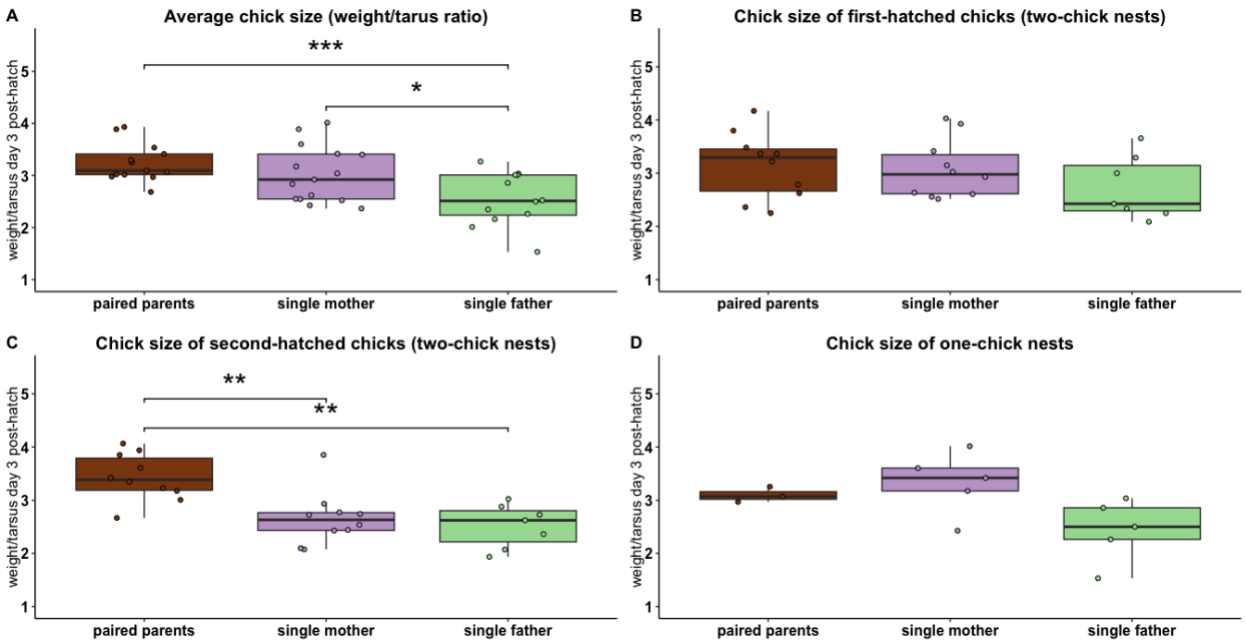

Figure 2-3. Chick size (weight/tarsus ratio) on Day 3 post-hatching. A) Chi Difference in chick size between treatment groups (chicks from two-chick broods averaged). Single-parented chicks were smaller than paired-parented chicks. Single-mothered chicks were larger than single-fathered chicks. B) Difference in chick size of first-hatched chicks from two chick nests. C) Difference in chick size of second-hatched chicks of two chick nests. Single-parented chicks were smaller than paired-parented chicks. D) Difference in chick size of one-chick nests. Points represent individual chicks (chicks from two-chick broods averaged for A), and boxplots represent where the first quartile, median, and third quartile for each treatment group. The whiskers indicate the minimum to the first quartile and the maximum of the third quartile. Statistical significance denoted as follows: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

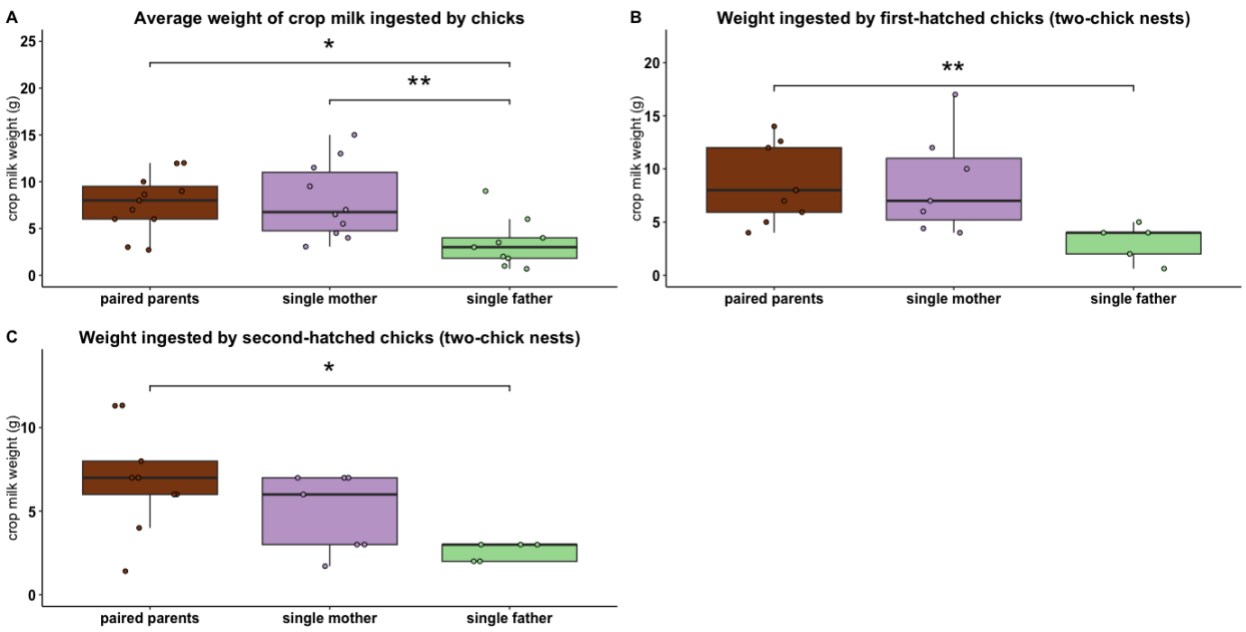

Figure 2-4. Weight of crop milk ingested by chicks in terms of treatment groups. A) Chick crop milk weight (crop-milk weight of two-chick nests averaged). B) First-hatched chick of two-chick nests crop milk weight . C) Second-hatched chick of two-chick nests crop milk weight. Single-fathered chicks received less crop milk compared to paired-parented and single-mothered chicks. First-hatched and second-hatched single-fathered chicks received less crop milk compared to their paired-parented chicks. Second-hatched chicks also received less crop milk general. Points represent individual chicks (chicks from two-chick nests averaged for plot A), and boxplots represent where the first quartile, median, and third quartile for each treatment group. The whiskers indicate the minimum to the first quartile and the maximum of the third quartile. Statistical significance denoted as follows: *: $p < 0.05$, **: $p < 0.01$

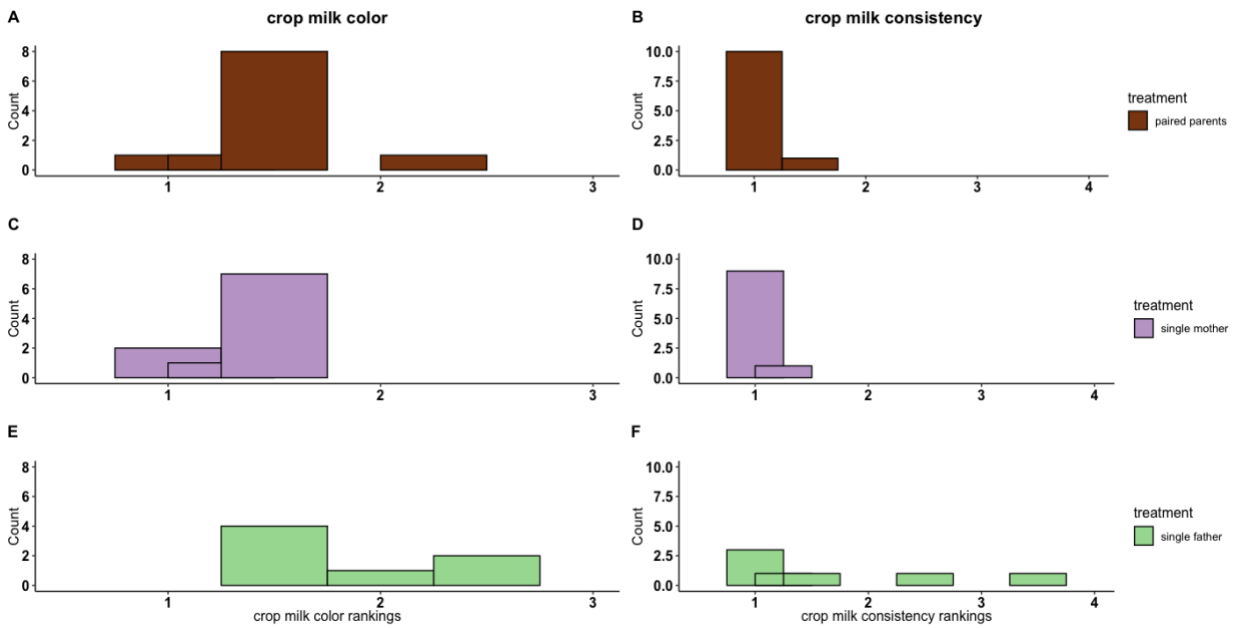

Figure 2-5. Crop milk ingested by chicks quality rankings (two-chick broods are averaged). A,C,E) Crop milk color. The x-axis of each histogram are the rankings (1 being white/freshest to 3 being yellowish/least fresh). The y-axes indicate the count of samples in each ranking. B,D,F) Crop milk consistency. The x-axis of each histogram are the rankings (1 being like cottage cheese/freshest to 4 being like liquid/least fresh). The y-axes indicate the count of samples in each ranking.

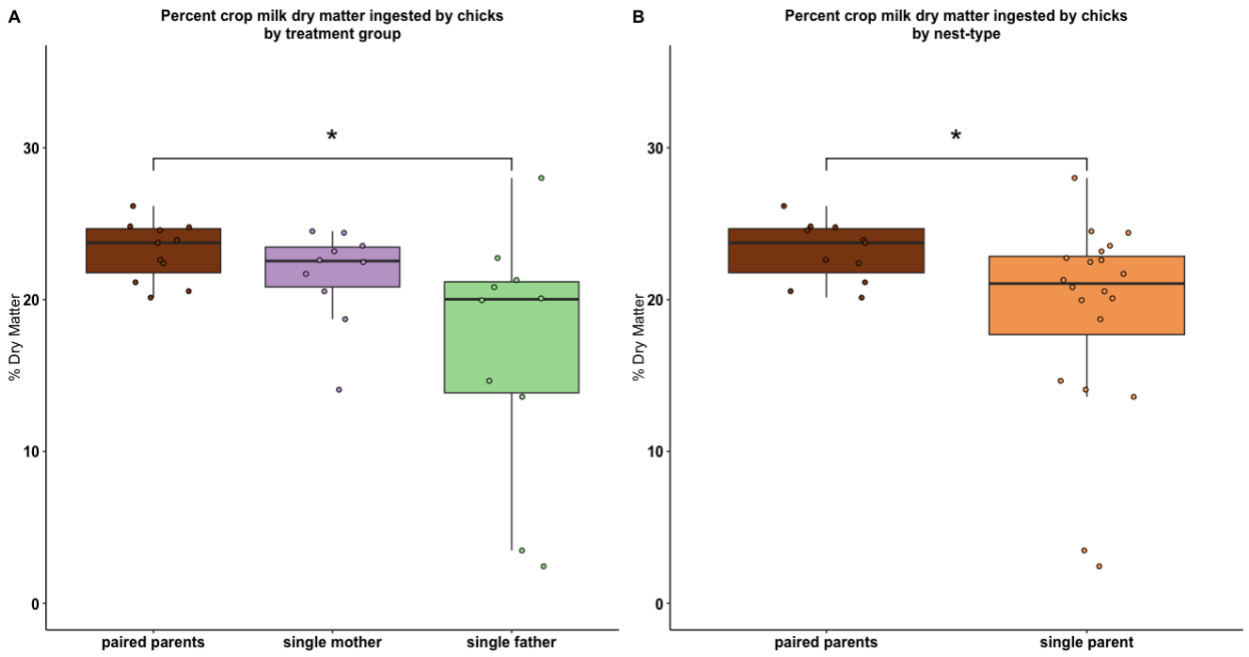

Figure 2-6. Crop milk ingested by chicks dry weight (two-chick nests averaged). A) Chick-ingested crop milk dry matter percentage on chicks originating from paired parents (left), single mothers (middle), or single fathers (right). B) Chick-ingested crop milk dry matter percentage pooled by nest-type. Single-fathered chicks received crop milk with a lower dry matter percentage compared to paired parents, and single parented chicks in general received crop milk with less dry matter compared to paired parents. Points represent individual chicks (chicks from two-chick nests averaged for both plots), and boxplots represent where the first quartile, median, and third quartile for each treatment group/nest-type. The whiskers indicate the minimum to the first quartile and the maximum of the third quartile. Statistical significance denoted as follows: *: $p < 0.05$

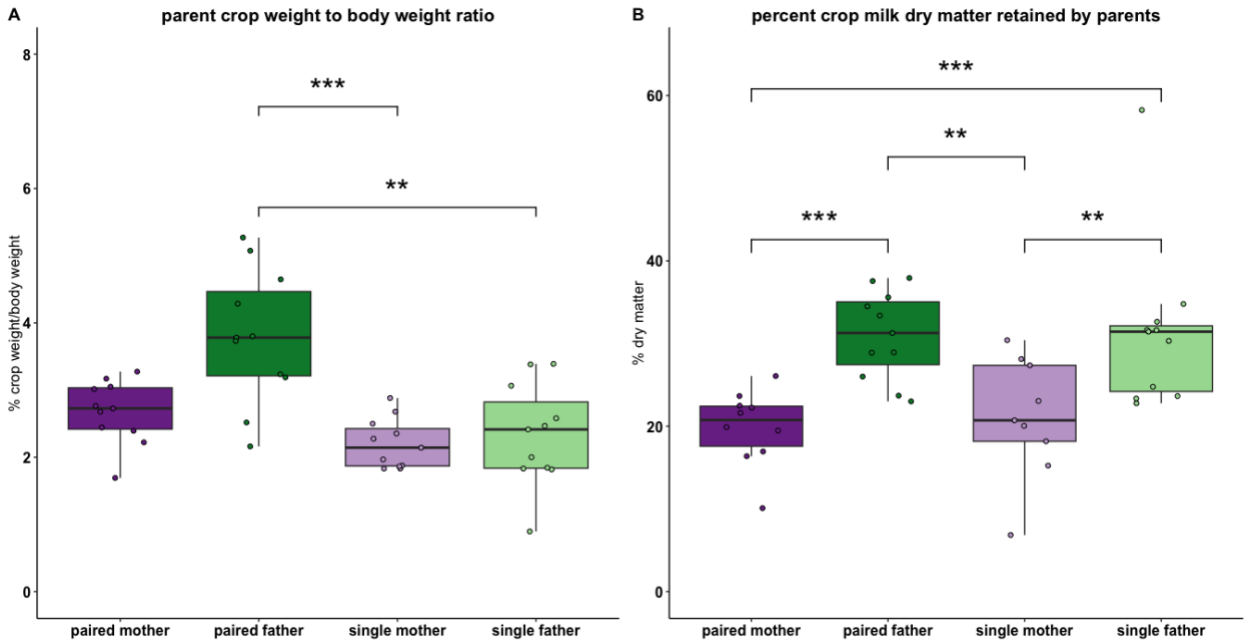

Figure 2-7. Parent crop tissue and crop milk quality by treatment group. A) Parent percent crop weight to body weight. Paired fathers had heavier crops than single parents. B) Parent crop milk dry matter percentage retained by parents. Fathers retained crop milk within their crops with a higher dry matter percentage than mothers in general. Points represent individual birds, and boxplots represent where the first quartile, median, and third quartile for each treatment group. The whiskers indicate the minimum to the first quartile and the maximum of the third quartile. Statistical significance denoted as follows: **: $p < 0.01$, ***: $p < 0.001$

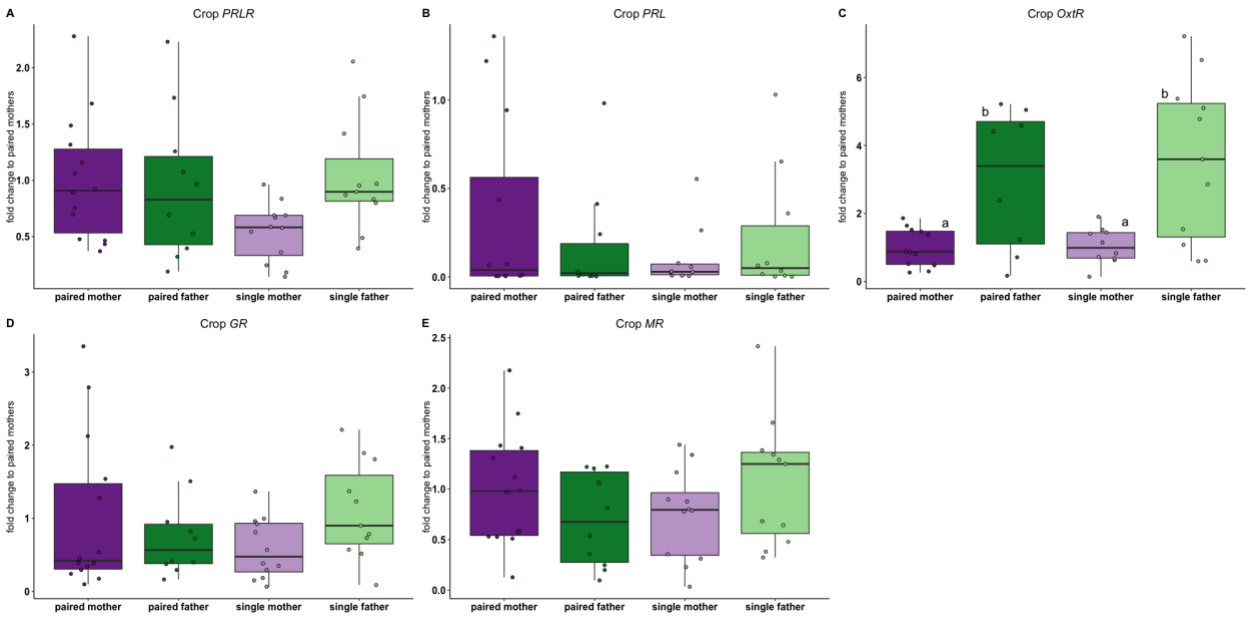

Figure 2-8. Parent crop tissue gene expression A) *PRLR* B) *PRL* C) *OxtR*, different lowercase letters indicate a significant difference by sex. D) *GR* E) *MR*. Points represent individual birds, and boxplots represent where the first quartile, median, and third quartile for each treatment group. The whiskers indicate the minimum to the first quartile and the maximum of the third quartile.

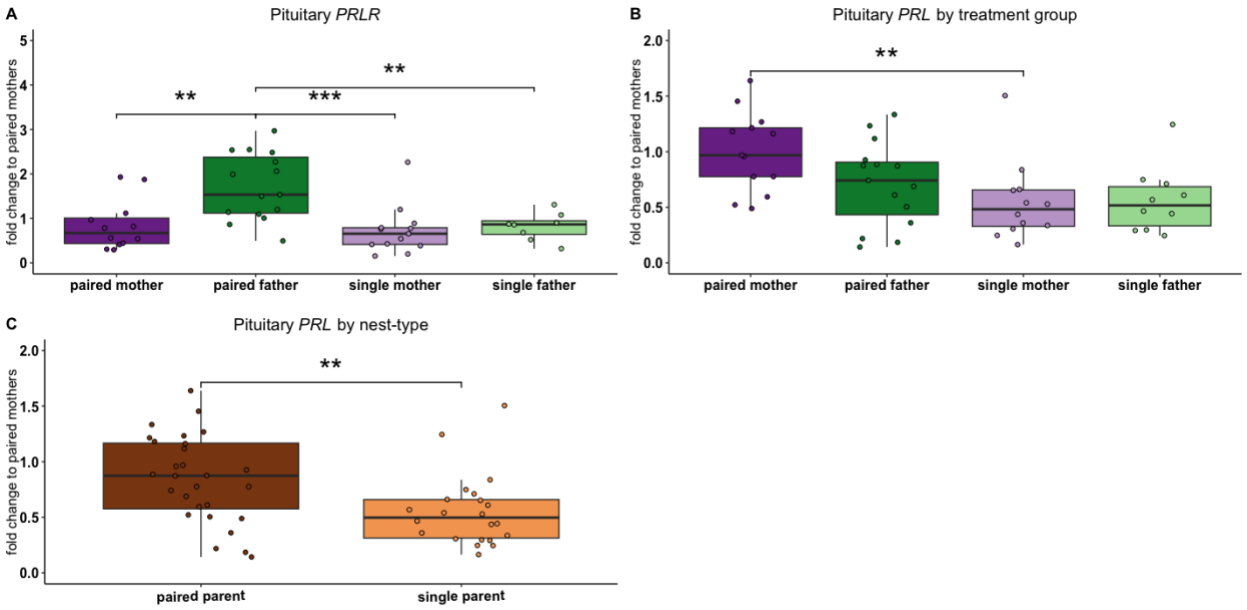

Figure 2-9. Parent pituitary gene expression. A) *PRLR* expression. Paired fathers had higher expression than paired mothers, single mothers and single fathers (after brood size removed as co-variable). B) *PRL* expression. Paired mothers expressed more *PRL* than single mothers C.) PRL expression as it relates to nest-type. Paired parents expressed more PRL in general than single parents. Points represent individual birds, and boxplots represent where the first quartile, median, and third quartile for each treatment group. The whiskers indicate the minimum to the first quartile and the maximum of the third quartile. Statistical significance denoted as follows: **: $p < 0.01$, ***: $p < 0.001$

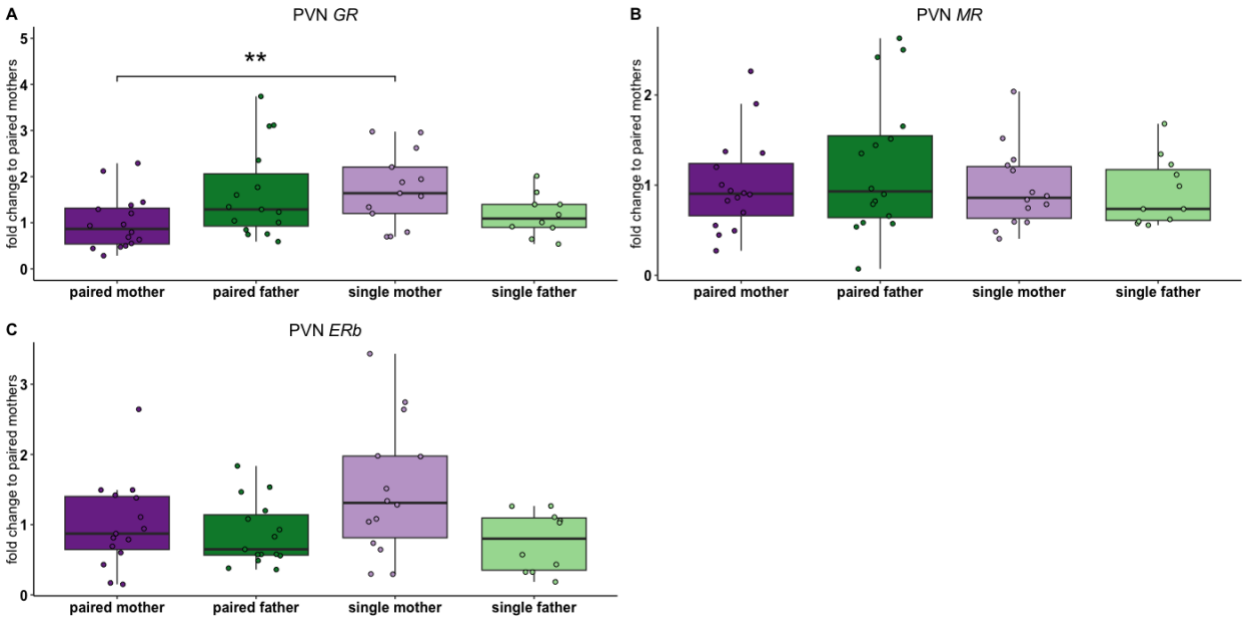

Figure 2-10. Parent PVN gene expression. A) *GR* expression. Single mothers expressed more *GR* than paired mothers. B) *MR* expression. C) ER-β expression. Points represent individual birds, and boxplots represent where the first quartile, median, and third quartile for each treatment group. The whiskers indicate the minimum to the first quartile and the maximum of the third quartile. Statistical significance denoted as follows: **: $p < 0.01$

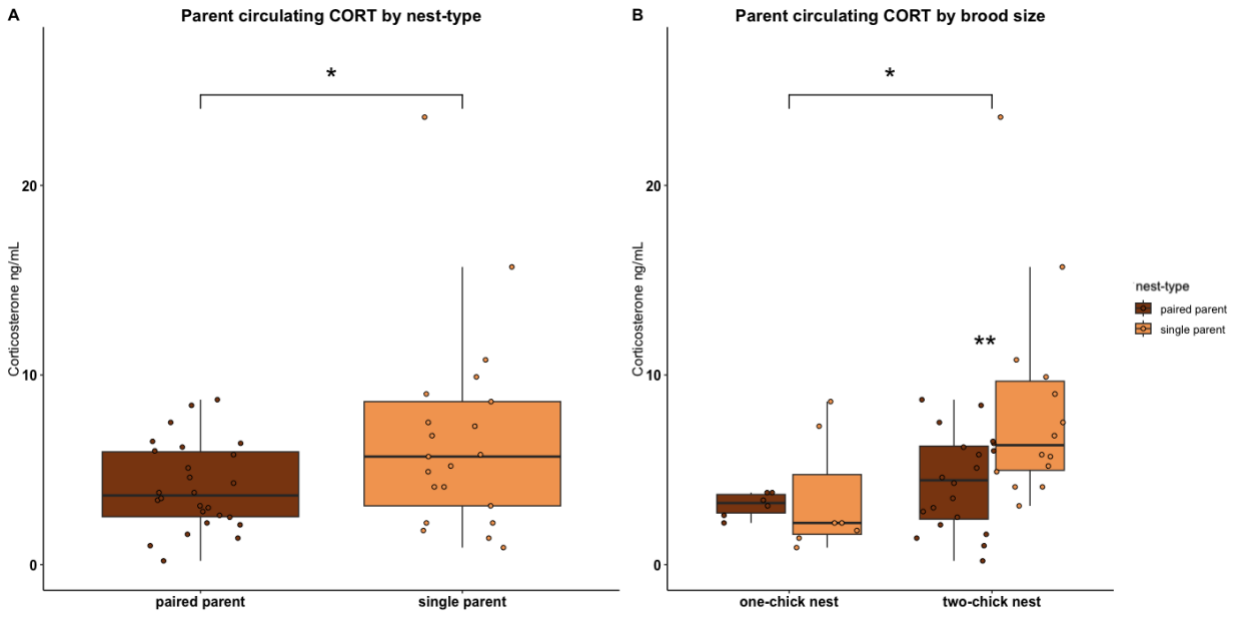

Figure 2-11. Parent baseline circulating CORT. A) Circulating CORT in terms of nest type. Single parents had higher circulating CORT than paired parents. B) Circulating CORT in terms of brood size. Parents of two-chick broods had higher circulating CORT than the parents of one-chick broods. Single parents with two-chick broods had higher circulating CORT than paired parents with two-chick broods. Points represent individual birds, and boxplots represent where the first quartile, median, and third quartile for each treatment group. The whiskers indicate the minimum to the first quartile and the maximum of the third quartile. Statistical significance denoted as follows: *: $p < 0.05$, **: $p < 0.01$

Tables

Table 2-1. Example crop milk quality scoring data sheet. Below are examples of data collected to score for crop milk quality for the chicks and adults.

Chicks

Chick ID:

Crop milk mass (g):

Crop milk consistency and color:

color:	1- white	1.5 between white and off-white	2- off-white	2.5 between off-white and yellowish	3- yellowish		
Consistency:	1- cottage cheese (freshest)	1.5- between cottage cheese and grainy	2- grainy	2.5- between grainy and smooth	3- smooth	3.5- between smooth and like liquid	4- like liquid (in the crop the longest)

Adults:

Adult ID:

Crop sac mass (g):

Crop milk consistency and color:

color:	1- white	1.5 between white and off-white	2- off-white	2.5 between off-white and yellowish	3- yellowish		
Consistency:	1- cottage cheese (freshest)	1.5- between cottage cheese and grainy	2- grainy	2.5- between grainy and smooth	3- smooth	3.5- between smooth and like liquid	4- like liquid (in the crop the longest)

Table 2-2. Primers used for quantitative PCR.

Gene	GenBank Accession No.	Primer Sequence	Efficiency (%)
Prolactin, *PRL*	XM_005506024.2	F:GGCGGGTTCATACTGGTGAG R:TGGATTAGGCGGCACTTCAG	92.6
Prolactin receptor, *PRLR*	NM_001282822.1	F:TCTTCCTTGCACACATGAAACC R:TCCAGGGTATGATTGACCAGT	95.2
Vasoactive intestinal peptide receptor, *VIPR*	XM_013369762.2	F:AGGGATTTGTGGTGGCTGTT R:TGCCTAAGGAAGGGTGGTGA	100.9
Mesotocin receptor, *Oxt-R*	XM_021296600.1	F:GGTCTGTGTGGGACACGAAT R:GGCCGGTGTAGAGCATGTAG	92.7
Glucocorticoid receptor, *GR*	XM_021301096.1	F:TGCTTAACTCGTCGGATCAA R:AAAGTCCATCACGATCCCTC	90.5
Mineralocorticoid receptor, *MR*	XM_021296726.1	F:AGAACATGGCTTCCTCGGTG R:CTAGAAAGCGGAGACCCGAC	103.9
Estrogen Receptor beta, ER-β	NM_001282841.1	F:GGGAATGATGAAATGTGGCTC R:GATCTCTTTTACGCGGGTTG	100.6
Ribosomal protein L4, *rpL4*	XM_005511196.1	F:GCCGGAAAGGGCAAAATGAG R:GCCGTTGTCCTCGTTGTAGA	105.1
hypoxanthine phosphoribosyltransferase 1, *HPRT1*	XM_005500563.2	F:GCCCCATCGTCATACGCTTT R:GGGGCAGCAATAGTCGGTAG	94.7
Beta actin, *ACTB*	XM_005504502.2	F:ATGTGGATCAGCAAGCAGGAG R:CATTTCATCACAAGGGTGTGGG	95.8

Table 2-3. Linear mixed models and ANOVAs regarding the parents

	treatment					**nest-type**					**sex**				
Response Variable	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
parent body weight	1.5	0.24	3	37.6	0.10	0.54	0.47	1	53	0.0071	0.35	0.55	1	53	0.0067
crop tissue weight	8.5	< **0.05**	3	27.7	0.4	17.2	< **0.05**	1	40	0.32	6.6	< **0.05**	1	40	0.14
crop milk dry weight	7.2	< **0.05**	3	26.1	0.38	0.1	0.75	1	37	0.0062	22.8	< **0.05**	1	37	0.38
circulating CORT	3.1	< **0.05**	3	28	0.33	5.1	< **0.05**	1	43	0.08	2.1	0.15	1	43	0.04
crop tissue gene expression	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
PRLR	2.3	0.10	3	31.4	0.14	1.4	0.24	1	43	0.04	2.0	0.16	1	43	0.04
PRL	0.36	0.78	3	27.4	0.03	0.84	0.36	1	39	0.01	0.016	0.9	1	39	0.000022
OxtR	2.4	0.09	3	29.7	0.16	0	1.0	1	42	0.00012	7.0	< **0.05**	1	42	0.14
GR	1.1	0.35	3	32.1	0.07	0.047	0.83	1	43	0.0021	0.36	0.55	1	43	0.0082
MR	1.4	0.26	3	31.4	0.09	0.15	0.7	1	43	0.0010	0.0038	0.95	1	43	0.000061
pituitary gene expression	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
PRLR	7.3	< **0.05**	3	33.1	0.37	4.1	< **0.05**	1	44	0.17	12	< **0.05**	1	44	0.22
PRL	3.6	< **0.05**	3	32.2	0.22	6.8	< **0.05**	1	46	0.15	2.6	0.12	1	46	0.05
VIPR	0.88	0.46	3	32.1	0.07	0.18	0.67	1	42	0.01	2.4	0.13	1	42	0.06
OxtR	0.91	0.45	3	33.0	0.06	0.0022	0.96	1	47	0.0019	1.8	0.18	1	47	0.04
GR	0.47	0.71	3	33.3	0.03	0.007	0.98	1	45	0.00069	0.54	0.47	1	45	0.01
MR	1.1	0.35	3	33.5	0.08	1.6	0.22	1	44	0.06	0.87	0.36	1	44	0.02
PVN gene expression	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
GR	3.4	< **0.05**	3	37.1	0.17	1.6	0.21	1	50	0.01	0.29	0.59	1	50	0.0068
MR	0.26	0.85	3	38.0	0.02	0.35	0.55	1	53	0.0082	0.17	0.68	1	53	0.0032
ERβ	1.5	0.23	3	38.1	0.08	0.87	0.35	1	52	0.01	3.0	0.087	1	52	0.06

Table 2-4. ANOVAs and t-tests regarding the chicks

Response Variable	**treatment**					**nest-type**				
analysis where two chick nests were averaged	***F***	***p***	**df**	**df residual**	***η2***	***F***	***p***	**df**	**df residual**	***η2***
chick size	6.7	< **0.05**	2	36	0.27	6	< **0.05**	1	37	0.14
crop milk quantity	6.5	< **0.05**	2	26	0.32	2.3	0.14	1	27	0.07
crop milk dry weight	4.2	< **0.05**	2	27	0.24	5.3	< **0.05**	1	28	0.16
first hatched chick analysis (from two chick broods)	***F***	***p***	**df**	**df residual**	***η2***	***t***	***p***	**df residual**	***d***	
chick size	1.2	0.33	2	24	0.09	0.88	0.39	25	0.35	
crop milk quantity	4.1	< **0.05**	2	18	0.31	1.2	0.26	19	0.52	
crop milk dry weight	1.9	0.19	2	19	0.16	2.1	0.054	14.2	0.77	
second hatched chick analysis (from two chick broods)	***F***	***p***	**df**	**df residual**	***η2***	***t***	***p***	**df residual**	***d***	
chick size	10.5	< **0.05**	2	24	0.47	4.6	< **0.05**	25	1.8	
crop milk quantity	4.8	< **0.05**	2	18	0.35	2.5	< **0.05**	19	1.1	
crop milk dry weight	2.0	0.16	2	19	0.18	1.8	0.082	20	0.79	
one chick nest analysis	***F***	***p***	**df**	**df residual**	***η2***	***t***	***p***	**df residual**	***d***	
chick size	3.7	0.063	2	10	0.42	0.49	0.63	11	0.41	

Chapter 3 - Neurobiological and physiological changes during single parenthood in pigeons (*Columba livia*): A study of glucocorticoids, prolactin, mesotocin, and gonadotropins across early to late stages of parental care

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April Booth performed experiments presented in chapter 3: Figures 3-1 – 3-10. She also analyzed data for those figures, co-designed all experiments, drafted the manuscript, and revised, edited, and approved the manuscript.

Abstract

Biparental care is a strategy employed across the animal kingdom in which both parents work together to rear their offspring. While the adverse effects of single parenthood on offspring have been extensively studied to date, less is understood regarding the physiological, neural, and longer term effects of single parenting on single parents. We investigated whether single rock dove (*Columba livia*, "pigeon") parents experience sex-specific physiological and neural genomic transcription over the course of rearing their offspring to independence, and whether these changes are related to chick development. We manipulated parental partner presence and measured offspring growth and gene expression for key hormones and receptors related to parental care and the stress response: glucocorticoids, prolactin, mesotocin, and gonadotropins. We conducted these assessments during the early (Day 5 post-hatching), middle (Day 15 post-hatching), and late stages of parenting (Day 3 post-fledging). Our focus areas included the pituitary and paraventricular nucleus (PVN), and we also measured circulating plasma concentrations of the stress-associated metabolic hormone corticosterone (CORT). We report that at Day 5 post-hatching, single-fathered chicks were the smallest among all groups, while Day 15 post-hatching, single-mothered chicks exhibited the smallest size. Notably, single mothers exhibited elevated gene expression of glucocorticoid receptors (*GR*) in the PVN compared to paired mothers, and single parents, in general, had higher baseline circulating CORT than paired parents at Day 5 post-hatching. At Day 15 post-hatching, single parents displayed lower baseline circulating CORT than paired parents. Furthermore, mothers had higher expression of mineralocorticoid receptors (*MR*) in the PVN at Day 15 post-hatching, with paired mothers displaying the highest *MR* expression compared to paired fathers, single mothers, and single fathers at Day 3 post-fledging. Paired fathers had higher pituitary prolactin receptor

(*PRLR*) expression than paired mothers, single mothers, and single fathers at Day 5 post-hatching. Interestingly, single parents demonstrated reduced pituitary prolactin (*PRL*) gene expression compared to paired parents at Day 5 post-hatching. Fathers exhibited higher PVN *PRLR* at Day 15 post-hatching and Day 3 post-fledging than mothers. At Day 5 post-hatching, mothers expressed fewer PVN mesotocin receptors (*OxtR*) than fathers, and single parents at Day 15 post-hatching exhibited lower pituitary gonadotropin-releasing hormone receptor (*GnRHR*) expression than paired parents. Single mothers at Day 15 post-hatching also expressed less PVN gonadotropin-inhibitory hormone (*GnIH*) expression than paired fathers. These findings reveal the neurological and physiological responses that male and female parents undergo in the face of a major disturbance in their parenting strategy over the course of parenting.

Introduction

For many species in the animal kingdom, parental care of offspring is essential to maximize fitness. This is a strategy that is prevalent in birds, with 81-90% of species exhibiting a biparental care strategy (Burley and Johnson, 2002; Cockburn, 2006; Lack, 1968). Unpredictable conditions in the wild, such as sickness, predation, and inclement weather, can result in the loss of a partner and subsequently single parenthood (Lima, 2009). Across various species, single parents and/or parents with enlarged broods can and will continue to rear offspring (Banerjee et al., 2012; Bonier et al., 2011; Rogers and Bales, 2019). However, much remains to be understood regarding the physiological, neural and longer term consequences of single parenting on single parents, particularly in how single parenthood may similarly or differentially affect mothers versus fathers.

In this study, we examined if sex-biased physiological and neurobiological effects occur in both single mother and single father pigeons (*Columba livia*), as compared to paired mothers

and paired fathers, over the course of parenting. Specifically, we examined these effects during the early (Day 5 post-hatching), middle (Day 15 post-hatching), and late stages (Day 3 post-fledging) of the parenting process.

The monogamous, biparental system of pigeons presents a powerful model for this examination. This is due to our capacity to partially control gestation, which occurs externally as both male and female pigeons incubate their eggs, and pseudo-lactation, a process in which both male and female pigeons feed their offspring. In addition, upon the loss of a partner, the remaining pigeon parent, either male or female, will continue to rear their young to independence (Booth, 2022; Burley, 1980).

We hypothesized that single mothers and fathers would have distinct neurobiological and physiological profiles, which would align with the parent primarily responsible for offspring care at specific stages. During the course of parenting, mothers initially allocate more time tending to the offspring than fathers (Johnston and Janiga, 1995; Levi, 1986). However, as the chicks mature and the mother begins preparing for a future clutch, the father's role in chick care intensifies. This shift is most prominent after the chicks have fledged when the father becomes the primary caregiver, responsible for feeding the fledglings until they reach approximately 5 weeks old.

Based on their parental roles, we predicted that single fathers at Day 5 post-hatching would exhibit physiological and neurobiological phenotypes akin to those of paired mothers at Day 5 post-hatching. Likewise, we anticipated that single mothers at Day 15 post-hatching and Day 3 post-fledging would respectively demonstrate physiological and neurobiological phenotypes resembling those of paired fathers at Day 15 post-hatching and Day 3 post-fledging. Our findings revealed differences between single and paired parents in general and sex biased

differences in regards to glucocorticoids, prolactin, mesotocin, and gonadotropins that likely aid in single parent care.

Methods

General

Initially, we assessed offspring condition and size as indicators of parental provisioning and investment, comparing single parents to paired parents on three specific time points: Day 5 post-hatch, Day 15 post-hatch, and Day 3 post-fledging. Tissue samples were collected from both the single mother and single father experimental groups, as well as from the paired mothers and fathers constituting the paired-parent control group. We conducted gene expression analysis in two key areas: the paraventricular nucleus (PVN) and the pituitary, aiming to discern any distinctions between single mothers and fathers and their paired-parent counterparts. The specific genes we focused on are recognized for their roles in stress response and metabolism, including mineralocorticoid receptor (*MR*), glucocorticoid receptor (*GR*), and estrogen receptor beta (*ER-β*) (Lund et al., 2006). We also quantified the levels of circulating corticosterone (CORT). Additionally, we investigated genes associated with reproduction and parental care, including prolactin (*PRL*), prolactin receptor (*PRLR*), vasoactive intestinal peptide receptor (*VIPR*), gonadotropin releasing hormone I (*GnRH-I*), gonadotropin releasing hormone receptor (*GnRHR*), gonadotropin inhibitory hormone (*GnIH*), gonadotropin inhibitory hormone receptor (*GnIHR*), and mesotocin receptor (*OxtR*). It is worth noting that *PRL*, its associated receptor, and *VIPR* play a pivotal role in facilitating lactation and pseudo-lactation and parental care behaviors (Farrar et al., 2022a, 2022b, 2022c). Similarly, *GnRH-I* and *GnIH*, along with their respective receptors, play pivotal roles in regulating reproduction and associated behaviors (Calisi et al., 2011, 2008; Counis et al., 2005). Notably, increased mesotocin immunoreactive neurons have

been observed in regions such as the PVN during parenting in avian models such as Thai hens (*Gallus domesticus*) (Chokchaloemwong et al., 2013).

Animal Care

Birds were housed at University of California, Davis in spacious outdoor aviaries (measuring 5'x4'x7'), equipped with protective coverings to shield them from inclement weather. All the birds used in this study were captive-bred. Given the naturally social nature of rock doves, each aviary accommodated an average of 8 sexually reproductive pairs. These birds were between 1 and 2 years old, meeting the criteria for sexual maturity, as evidenced , by their prior nesting experiences. Pair formation occurred spontaneously after the birds were introduced to their respective aviaries. To maintain consistent lighting conditions, in addition to natural daylight exposure, artificial lights were set to a 12-hour light, 12-hour dark cycle (12L:12D).

Within each aviary, 16 nest boxes (sized 13.5''x15''x13.25'') were provided in each aviary, affording the birds ample choices for selecting their preferred nest sites. Birds were maintained on an *ad libitum* diet of whole corn (Farmers), protein (Farmers Best Turkey/Game Bird Starter Crumbles), grit (Winner's Cup Pigeon Grit), and water.

All aspects of animal care and experimental protocols were conducted in compliance with the guidelines and regulations set forth by the UC Davis Institutional Animal Care and Use Committee (IACUC) under Protocol #20618. These procedures have been previously employed successfully in our laboratory for assessing rock dove reproduction (MacManes et al., 2017; Austin et al., 2021; Calisi et al., 2018). Typically, rock doves have one to two chick broods as part of their natural reproductive cycle.

Experimental Design

On the morning of hatching (between 0800 and 1100, designated as Day 1 post-hatching), one of the parenting partners was removed from the treatment group, resulting in either a single female or single male parent caring for the nest (see Figure 3-1). In contrast, the paired control nests retained both parenting partners throughout the study. Subsequently, the single parents in each treatment group assumed the sole responsibility for nurturing their offspring until specific time points: either Day 5 post hatching, Day 15 post hatching, or Day 3 post-fledging.

Chick Morphometrics

Throughout the study, chick weight was determined using a scale, and tarsus lengths were measured using a caliper to assess growth as an indicator of parental provisioning. These measurements were conducted in an adjacent room during the morning hours (between 0800 and 1100). To minimize stress related to handling (Wingfield et al., 1995, 1992), each chick's measurements were completed in under 5 minutes.

For the Day 5 post-hatching time point, chick measurements were recorded on days 1, 3 and 4 post-hatching. For the Day 15 post-hatching time point, measurements were recorded on days 1, 4, 8, 12 and 13 post-hatching. For the Day 3 post-fledging time point (note that some chicks fledged before day 30), measurements were obtained on days 1, 4, 8, 12, 13, 20, and/or Day 30 post hatching and Day 2 post-fledging. Latency to fledging was also calculated for the Day 3 post-fledging time point, with the first chick to fledge from 2-chick nests being considered for analysis.

Sample sizes of chicks within each treatment group were as follows:

Day 5 post-hatching: single-mothered nests (n = 5 one-chick nests, n = 9 two-chick nests), single-fathered nests (n = 4 one-chick nests, n = 7 two-chick nests), and paired parented-control (n = 3 one-chick nests, n = 13 two-chick nests);

Day 15 post hatching: single-mothered nests (n = 6 one-chick nests, n = 8 two-chick nests), single-fathered nests (n = 5 one-chick nests, n = 5 two-chick nests), and paired parented-control (n = 5 one-chick nests, n = 11 two-chick nests);

Day 3 post fledging: single-mothered nests (n = 3 one-chick nests, n = 2 two-chick nests), single-fathered nests (n = 2 one-chick nests, n = 4 two-chick nests), and paired parented-control (n = 2 one-chick nests, n = 4 two-chick nests).

Tissue Collection

The duration of our experiment spanned from September 2018 to September 2021. Collection efforts were temporarily suspended between February and July of 2020 due to Covid-19 restrictions and California wildfires. This timeframe was necessary to accumulate the requisite sample sizes: Specifically, for the Day 5 post-hatching time point, we collected samples from 14 single mothers, 11 single fathers, 16 paired mothers, and 16 paired fathers. For the Day 15 post-hatching time point, we obtained samples from 14 single mothers, 10 single fathers, 15 paired mothers, and 15 paired fathers. For the Day 3 post-fledging time point, our sample sizes comprised 5 single mothers, 6 single fathers, 6 paired mothers, and 6 paired fathers.

Tissue collection consistently commenced between 0730-1000 hr. Birds were euthanized humanely within three minutes of entering the aviary using inhaled isoflurane anesthesia immediately prior to decapitation (MacManes et al., 2017). Trunk blood was promptly collected and placed on ice for a duration of 2 hours or less before being transferred to our nearby

laboratory. Subsequently, the blood samples were centrifuged at 4°C for 10 minutes to obtain plasma for hormone assays. Plasma samples were then stored at -80°C until the time of assay.

Brains and pituitaries were also collected and immediately flash frozen on dry ice. These samples were then transported to the laboratory within a 2-hour timeframe and stored at -80°C until further processing. Following the methods outlined by Calisi et al. (2018; 2017), coronal punch biopsies of the paraventricular nuclei (PVN) were performed at -20°C in a cryostat (Leica CM 1860) with a thickness of 100μm. These biopsy samples were stored in homogenizer tubes for RNA extractions at -80°C. Conformation of the identity and location of the PVN was conducted using a stereotaxic atlas of the pigeon brain, with the anterior commissure and occipitomesencephalic tract serving as landmarks (Karten and Hodos, 1967; Kuenzel and van Tienhoven, 1982). Collection of PVN tissue ceased once the optic tectum became apparent.

Quantitative PCR

Before beginning RNA extraction, an average of 6.5 mg (± 2.8) of pituitary tissue preserved in RNALater underwent three washes with 1X phosphate buffered saline (PBS). For PVN tissue, an average of 5.9 mg (±1.2) was extracted, requiring no additional washing. RNA was then extracted from the tissue using a Direct-zol RNA MiniPrep kit (Zymo research, California), employing a modified protocol designed for lipid-rich tissues (Farrar and Calisi, 2022). RNA purity and concentration were assessed using a NanoDrop One (Thermo Scientific, Massachusetts). Samples with a 260/280 reading below 1.62 and a 260/230 reading below 0.74 for the pituitary were excluded. For PVN samples, those with a 260/280 reading below 1.43 and a 260/230 reading below 0.2 were omitted. 8 pituitary samples were discarded due to being below the 260/280 and/or 260/230 reading indicated, and/or due to contamination warnings indicated by the NanoDrop One. 8 PVN samples were discarded for similar reasons.

Extracted RNA underwent DNase treatment (Perfecta DNase 95150-01K, Quanta Biotech, Massachusetts- for the pituitary, VWR Quanta kit 18068-015 - for the PVN tissue) to remove genomic DNA contamination. DNase-treated RNA was converted into cDNA using the qScript cDNA SuperMix (Quanta Biotech), with subsequent samples diluted 1:5 for qPCR.

Categorized as triplicates, cDNA samples were subjected to qPCR with species-specific primers (see Table 3-1). Each reaction (10 µL total) contained 1 µL cDNA template (diluted 1:5), 5 µL 2X SSOAdvanced SYBR Green PCR mix (BioRad, California), and 1 µL of 10 µM of each primer. Reactions were executed on a BioRad CFX384 qPCR machine under the following cycling conditions: 50℃ for 2 min, 95℃ for 10 min, and then 40 cycles of 95℃ for 15 sec and 60℃ for 30 sec.

Reference genes *hprt1* and *rpl4* (Zinzow-Kramer et al., 2014) were employed for the pituitary, While reference genes *hprt1, rpl4, and ACTB* (Zinzow-Kramer et al., 2014) were used for the PVN (Table 3-1). Because no significant differences in mean reference expression were detected among the treatment groups for the PVN and pituitary (see Results for details), relative expression of each gene of interest was quantified relative to the geometric mean of the reference genes using the ddCt method (Livak and Schmittgen, 2001; Mayer et al., 2019). Normalized expression (dCt) was calculated as the average Ct value between technical replicates of each gene minus the geometric mean of the reference genes for each sample. Relative expression (ddCt) was computed as the normalized value (dCt) minus the dCt of a randomly chosen paired female control for each time point. Fold change (Rq) was then determined (2^{-ddCt}). A normalized Rq value was obtained by dividing Rq by the average Rq value of the paired female treatment group for each time point.

Outliers were excluded if their fold difference exceeded 2 or more fold compared to the distribution of a given treatment group. The candidate genes measured for the PVN and pituitary were assessed across two qPCR plates each. The average inter-assay control coefficient of variation (CV) average was 1.8% (sd = 1.2) for the PVN, and 1.4% (sd = 1.2) for the pituitary.

Corticosterone Assay

Circulating corticosterone concentrations were determined using radioimmunoassay (RIA), following the procedures outlined in a prior publication (Calisi et al., 2018). A 1:20 dilution was applied to assess CORT concentrations (ng/mL) used in a commercially available Corticosterone RIA kit (MP Biomedicals, Orangeburg, NY). The intra-assay variation averaged 4.5, while the inter-assay variation averaged 5.2.

Statistical analysis

For all statistical analyses, the R statistical language (version 3.6.0) was employed (Wickham and Grolemund, 2017). Data analysis and plotting were conducted using a range of packages, including 'tidyverse', 'car', 'cowplot', 'effectsize', 'lme4', 'emmeans' and 'extrafont'.

To ensure data quality, an initial assessment was made for homogeneity of variance in all data with respect to the covariables described below, using Levene's test for homogeneity of variance. In cases where variance significantly differed for a given covariable, the data for the corresponding response variable was rank-transformed. Brood size (either one chick nest, two chick nest) was included as a covariable in our model to account for potential variations attributable to differences in broods. Unless otherwise specified in the results section, no significant differences related to brood size were observed for the measured response variables.

An alpha level of 0.05 was set as the significant threshold, and all tests were one-tailed. The results, as illustrated in Figures 2-9, are presented in the form of standard boxplots. The analyses described below were conducted separately for each time point.

Chick Analysis:

To assess the relationship between response variables and covariables, two distinct analyses of variance (ANOVA) tests were employed, utilizing the effect size measure Eta Squared. These analyses aimed to assess the impact of treatment groups (comprising paired-parented nests, single-mothered nests, and single-fathered nests), nest types (encompassing paired-parent nests and single-parented nests), and brood size on chick size. One ANOVA test included the covariables treatment and brood size, and the other included nest-type and brood size. This distinction was necessary because nest-type was an aliased coefficient with treatment groups. Post-hoc analysis was performed using Tukey multiple comparisons of means (effect size test: Cohen's d test). For these analyses, the response variable was averaged between the chicks of two-chick nests. In the case of Day-3 post-fledging chicks, these were the sole models examined due to sample size constraints (4 paired-parented two-chick nests, 2 paired-parented one-chick nests).

Given that pigeon chicks hatch asynchronously, an ANOVA test was employed to ascertain whether there existed an interaction between chick-hatching order and nest-type for two chick broods in terms of chick size for Days 5 and 15 post-hatching. An ANOVA was also used to compare the response variables of the first-hatched chicks in two chick nests across the treatment groups. To compare the size of first-hatched single-parented and paired-parented chicks overall, a two-sample t-test (or a Welch's t-test in the case of unequal variances) was

conducted. Similar ANOVA and t-test analyses were conducted for second-hatched chicks in two-chick nests, as well as for one-chick nests in terms of chick size.

Adult Analysis:

In the domain of adult examination, our analytical models were designed to juxtapose response variables against several factors: treatment groups (paired mothers, paired fathers, single mothers, and single fathers); nest types (paired-parent and single-parent configurations); individual sex; and brood size. To compare response variables with treatment group and brood size, we employed a linear mixed-effects model, utilizing Type II Wald F tests with Kenward-Roger degrees of freedom (effect size test: Eta Squared). To account for the inherent interdependence of paired mothers and fathers, PairID was introduced as a random effect within the linear mixed-effects model. Post-hoc analysis was conducted using computed estimated marginal means (least-squares means) with a Tukey adjustment (effect size test: Cohen's d test).

To compare response variables with nest-type, sex, and brood size, an ANOVA test was utilized. Similar to the chick analysis, these two types of analyses had to be conducted because nest-type and sex were aliased coefficients with treatment groups. In addition, nest-type and sex were analyzed as separate covariables due to a significant interaction between nest-type and sex regarding parental behaviors, as determined through our prior research (Booth et al., 2023)

Regarding the analysis of *OxtR* gene expression in the PVN of Day 3 post-fledging parents, only one ANOVA was conducted, incorporating \nest-type, sex, and brood size as covariables. This limitation was due to sample size constraints, as fold change relative to paired mothers could only be determined for 3 paired mothers and 3 single mothers. Circulating CORT was the sole response variable that exhibited significant differences by brood size at Day 5 post-hatching (see Results). As circulating CORT also displayed significant differences by nest-type,

a follow up ANOVA was subsequently conducted to compare CORT concentrations with nest-type and sex for the parents of two-chick nests. A similar analysis was conducted for the parents of one-chick nests.

Results

Chick Size

Day 5 post-hatching - Single fathered chicks were smaller than both paired parented and single mothered chicks, whereas second-hatched chicks of single parents exhibited smaller sizes compared to their second-hatched counterparts from paired parents.

For two-chick nests on Day 5 post-hatching, the average chick size (weight to tarsus ratio) on Day 3 post-hatching was determined and used for further analysis, along with the chick size of one-chick nests. On Day 3 post-hatching, smaller sizes were observed in single-fathered chicks compared to both paired-parented and single-mothered chicks (Table 3-2) (treatment groups, Tukey HSD $p < 0.05$: single-fathered versus paired-parented chicks $d = 1.6$, single-fathered versus single-mothered chicks $d = 0.91$) (Figure 3-2A). In general, smaller sizes were exhibited by chicks reared in single-parented nests compared to those in paired-parented nests.

A significant interaction effect was observed between chick-hatching order and nest-type in terms of chick size ($F_{1,63} = 5.0$, $p < 0.05$, $\eta^2 = 0.07$). When first-hatched chicks from two-chick broods were examined across treatment groups, no significant differences were observed between the treatment groups, or nest-types (Table 3-2). Conversely, second hatched single-parented chicks were smaller than second-hatched paired-parented counterparts (treatment groups, Tukey HSD $p < 0.05$: paired-parented versus single-mothered chicks $d = 1.6$, paired-parented versus single-fathered chicks $d = 2.1$) (Figure 3-2B). No significant differences were found between treatment groups or nest-types for one-chick nests.

Day 15 post-hatching - Single-mothered chicks tended to exhibit the smallest sizes, and single-parented chicks were generally smaller than their paired-parented counterparts

A significant difference was observed between single-mothered chicks versus both paired-parented chicks and single-fathered chicks around the Day 15 timepoint, with single-mothered chicks being smaller on Day 13 post-hatching (averaging two-chick data averaged) (Table 3-2) (treatment groups, Tukey HSD, $p < 0.05$, single-mothered versus paired-parented chicks $d = 1.8$, single-mothered versus single-fathered chicks $d = 1.2$) (Figure 3-3A). When data from single-parented chicks were pooled together, they exhibited smaller sizes overall than paired-parented chicks (Figure 3-3B).

While there was no significant interaction observed between chick hatching order and nest-type ($F_{1,60} = 1.7$, $p = 0.19$, $\eta^2 = 0.03$), a significant difference was noted by brood size ($F_{1,38} = 6.9$, $p < 0.05$, $\eta^2 = 0.15$). No significant differences were found between treatment groups or nest-types for first-hatched chicks of two-chick broods (Table 3-2). Second-hatched single-mothered chicks were significantly smaller than second-hatched paired-parented chicks (Tukey HSD, $p < 0.05$, $d = 1.7$) (Figure 3-3C). Similarly, second-hatched single-parented chicks were significantly smaller than their paired-parented counterparts (Figure 3-3D). Single-mothered chicks from one-chick nests exhibited smaller sizes than both paired-parented chicks and single-fathered chicks (Tukey HSD, $p < 0.05$, single-mothered versus paired-parented chicks $d = 2.3$, single-mothered versus single-fathered chicks $d = 2.3$) (Figure 3-3E). When pooled by nest-type, single-parented chicks from one-chick nests were smaller than paired-parented chicks (Figure 3-3F).

Day 3 post-fledging - No differences in chick size were detected; however, single-parented fledglings took longer to fledge compared to their paired-parented counterparts.

When the sizes of two-chick nests were averaged, there was no significant difference between treatment groups or nest-types at Day 30 post-hatching for chicks reared to Day 3 post-fledging (Table 3-2). A significant difference was noted in latency to fledging between treatment groups, with single mothered chicks taking on average 35.2 (s.d. = 2.5) days compared to 30.5 (s.d. = 2.3) days (Tukey HSD, $p < 0.05$, $d = 2.0$) (Figure 3-4A). When data from single mothers and fathers were pooled together by nest-type, single-parented fledglings also took longer, on average, to fledge compared to paired-parented fledglings (34.6 (s.d. = 2.6) days for single-parented fledglings versus 30.5 (s.d. = 2.3) days for paired-parented fledglings) (Figure 3-4B).

Parent pituitary gene expression

Day 5 post-hatching - Paired fathers exhibited higher *PRLR* expression compared to paired mothers, single mothers, and single fathers, whereas single parents displayed had less *PRL* expression than paired parents

No significant differences were detected among treatment groups for the reference genes used for qPCR analysis of pituitary tissue (rank-transformed, treatment groups, $F_{3,35.2} = 0.18$, $p = 0.91$, $\eta^2 = 0.01$) (rank-transformed, nest-type, $F_{1,47} = 0.020$, $p = 0.89$, $\eta^2 = 0.0015$) (rank-transformed, sex, $F_{1,47} = 0.50$, $p = 0.48$, $\eta^2 = 0.01$).

No significant differences were detected in *GR* gene expression (after removal of 1 paired mother and 1 paired father outlier, as described in Results section “Quantitative PCR”) (Table 3-3). Additionally, no significant differences were observed in *MR* gene expression (after removal of 2 paired fathers and 1 single father outliers).

Significant differences were detected in *PRLR* expression among treatment groups. Paired fathers exhibited significantly higher *PRLR* expression than paired mothers and single mothers (after excluding 1 paired mother and 2 single father outliers) (Table 3-3) (treatment groups, estimated marginal means, $p < 0.05$, paired fathers versus paired mothers $d = 1.3$, paired fathers versus single mothers $d = 1.5$) (Figure 3-5A). Although no significant difference was found between paired fathers and single fathers when brood size was included as a covariate (estimated marginal means, $p = 0.059$, $d = 1.4$), the removal of brood size as a covariate from the model (brood size not significant, $F_{1,33.5} = 0.51$, $p = 0.48$, $\eta^2 = 0.02$) revealed a significant difference similar to paired fathers versus paired mothers and single mothers (estimated marginal means, $p < 0.05$, $d = 1.4$). Additionally, a significant difference was observed by nest-type and sex, with paired parents and males expressing more *PRLR* (Table 3-3).

Single mothers expressed less *PRL* than paired mothers (after removal of a single mother outlier) (Table 3-3) (treatment groups, estimated marginal means, $p < 0.05$, paired mothers versus single mothers $d = 1.3$) (Figure 3-5B). Single parents in general expressed less *PRL* than paired parents (Figure 3-5C), with no significant difference detected by sex.

No significant differences were found in *VIPR* gene expression concerning treatment groups, nest-type, or sex (after removal of 2 paired mothers, 1 paired father, and 2 single mother outliers) (Table 3-3). Similarly, no significant differences were observed in *OxtR, GnRHR* (after removal of 2 paired mother and 3 paired father outliers) and *GnIHR* (after removal of one paired mother and 4 paired father outliers) gene expression.

Day 15 post-hatching - *GnRHR* expression was lower in single fathers compared to paired mothers and fathers, and single parents expressed less *GnRHR* than paired parents.

No significant differences were detected among treatment groups for the reference genes used for qPCR analysis of the Day 15 post-hatching pituitary tissue (treatment groups, $F_{3,33.8}$ = 0.23, p = 0.87, η^2 = 0.02) (nest-type, $F_{1,44}$ = 0.012, p = 0.91, η^2 = 0.000061) (sex, $F_{1,44}$ = 0.12, p = 0.73, η^2 = 0.0029). No significant differences in the pituitary were detected for the genes listed in Table 3-4, except *GnRHR* (*GR*: after removal of 2 paired mother, 2 single mother, and 1 single father outliers; *MR*: after removal of 2 paired mother and 1 single mother outliers; *PRLR*: after removal of 1 single mother outlier; *PRL*: after removal of one paired father outlier; *VIPR*: after removal of one paired mother outlier; *OxtR*: after removal of 1 paired mother, 1 paired father, and 1 single mother outlier; *GnRHR*: after removal of 1 paired mother and 1 single mother outlier; *GnIHR*: after removal of 2 paired mother and 1 single mother outlier). *GnRHR* expression was significantly less for single fathers when compared to paired mothers and paired fathers (estimated marginal means, $p < 0.05$, single fathers versus paired mothers d = 1.1, single fathers versus paired fathers d = 1.1) (Figure 3-6A). Single parents also expressed less *GnRHR* than paired parents in general (Figure 3-6B).

Day 3 post-fledging - No significant differences were observed.

No significant differences were detected among treatment groups for the reference genes used for qPCR analysis of the day 3 post-fledging pituitary tissue (treatment groups, $F_{3,12.2}$ = 0.16, p = 0.92, η^2 = 0.03) (nest-type, $F_{1,18}$ = 0.35, p = 0.56, η^2 = 0.0098) (sex, $F_{1,18}$ = 0.19, p = 0.66, η^2 = 0.0048). No significant differences were detected for the genes listed on Table 5 (*MR*: after removal of 1 paired mother outlier; *PRL*: after removal of 1 paired mother outlier; *VIPR*:

after removal of 1 paired mother outlier; *GnIHR*: after removal of 1 paired mother and 1 paired father outlier).

Parent PVN gene expression

Day 5 post-hatching - *GR* expression was found to be higher in single mothers compared to paired mothers, and mothers expressed less *OxtR* than fathers.

No significant differences were found between treatment groups, nest-types, or sex for the reference genes used for the PVN (treatment groups, $F_{3,38.8} = 2.1$, $p = 0.11$, $\eta^2 = 0.11$) (nest-type, $F_{1,53} = 0.051$, $p = 0.82$, $\eta^2 = 0.00010$)(sex, $F_{1,53} = 3.9$, $p = 0.054$, $\eta^2 = 0.07$).

A significant difference was observed between paired mothers and single mothers in *GR* expression, with single mothers showing higher *GR* expression than paired mothers (after excluding 1 single mother and 1 single father outlier) (Table 3-3) (treatment groups, estimated marginal means, $p < 0.05$, $d = 1.1$) (Figure 3-7A). No significant differences were found by nest-type or sex.

There was a significant difference in *OxtR* gene expression between paired mothers and paired fathers (estimated marginal means, $p < 0.05$, $d = 1.5$)(after removal of 1 single mother and 1 single father outlier) (Table 3-3) (Figure 3-7B). There was also a significant difference in *OxtR* expression by sex. All other genes listed on Table 3 were not significantly different (*Erβ*: after removal of 1 paired father outlier; *PRL*: after removal of 3 single mother and 3 single father outliers; *GnRH-I*: after removal of 1 paired mother, 1 paired father, and 1 single father outlier; *GnIH*: after removal of 2 paired mother and 1 single father outlier; *GnIHR*: after removal of 1 paired father outlier)

Day 15 post-hatching - Mothers expressed more *MR* than fathers, and fathers expressed more *PRLR* than mothers. Single mothers expressed less *GnIH* than paired fathers.

No significant differences were found between treatment groups, nest-types, or sex for the reference genes used for the PVN (treatment groups, $F_{3,36.4} = 0.091$, $p = 0.96$, $\eta^2 = 0.0056$) (nest-type, $F_{1,50} = 0.019$, $p = 0.89$, $\eta^2 = 0.0024$)(sex, $F_{1,50} = 0.24$, $p = 0.63$, $\eta^2 = 0.0040$).

Although a significant difference was detected for *MR* expression in terms of treatment groups (after removal of 1 paired father and 1 single father outlier)(Table 3-4), no significant differences were detected after a post-hoc analysis. The closest comparison to the $p < 0.05$ threshold was between paired mothers and paired fathers (estimated marginal means, $p = 0.075$, $d = 0.82$). There was a significant difference by sex, with mothers expressing more *MR* than fathers in general (Figure 3-8A).

There was significant difference by sex overall in terms of *PRLR* expression, with fathers expressing more *PRLR* than mothers (Table 3-4) (treatment groups, estimated marginal means, $p < 0.05$, paired fathers versus paired mothers $d = 0.73$, paired fathers versus single mothers $d = 0.79$, single fathers versus paired mothers $d = 0.92$, single fathers versus single mothers $d = 0.97$) (Figure 3-8B). There was also a significant difference between paired fathers and single mothers in terms of GnIH expression, with single mothers expressing less *GnIH* than paired fathers (after removal of 1 paired mother, 1 paired father, 2 single mother, and 1 single father outlier)(treatment groups, estimated marginal means, $p < 0.05$, $d = 1.3$) (Figure 3-8C). No significant differences were detected for the other genes listed on Table 3-4 (*GR*: after removal of 1 paired father and 1 single mother outlier; *Erβ*: after removal of 1 paired father and 1 single father outlier; *PRL*: after removal of 3 paired father outliers; *OxtR*: after removal of 1 paired

father outlier; *GnRH-I*: after removal of 1 paired father and 1 single mother outlier; *GnIHR*: after removal of 2 paired father outliers).

Day 3 post-fledging - *MR* expression was higher in paired mothers as compared to paired fathers. Single mothers and single fathers expressed higher *PRLR* than single mothers, and fathers, in general, demonstrated higher *PRLR* expression compared to mothers.

No significant differences were found between treatment groups, nest-types, or sex for the reference genes used for the PVN (treatment groups, $F_{3,11.4} = 0.7$, $p = 0.56$, $\eta^2 = 0.20$) (nest-type, $F_{1,19} = 0.58$, $p = 0.45$, $\eta^2 = 0.02$)(sex, $F_{1,19} = 0.58$, $p = 0.46$, $\eta^2 = 0.04$). PVN *MR* gene expression was higher in paired mothers compared to paired fathers, single mothers, and single fathers (Table 3-5) (treatment groups, estimated marginal means, $p < 0.05$, paired mothers versus paired fathers $d = 1.9$, paired mothers versus single mothers $d = 1.6$, paired mothers versus single fathers $d = 1.5$) (Figure 3-9A).

PVN *PRLR* expression was higher in single fathers compared to single mothers (treatment groups, estimated marginal means, $p < 0.05$, $d = 3.0$) (Table 3-5), and there was also a sex difference in general, with fathers expressing more *PRLR* than mothers (Figure 3-9B). There were no significant differences for the other genes listed in Table 5 (*GnRH-I*: after removal of 1 paired father outlier; *GnIH*: after removal of 1 paired mother outlier; *GnIHR*: after removal of 1 paired father and 1 single father outlier)

Circulating CORT

Day 5 post-hatching - Single parents rearing two-chick broods exhibited higher baseline circulating plasma CORT concentrations when compared to their paired parent counterparts

Although a significant disparity in baseline circulating CORT was identified among treatment groups (Table 3-3), a subsequent post-hoc analysis failed to reveal any significant

differences between specific treatment groups. In general, single parents exhibited higher baseline CORT concentrations in comparison to paired parents (Figure 3-10A), with no significant variations based on sex.

Furthermore, a notable distinction was observed concerning brood size ($F_{1,30.1} = 5.7$, $p < 0.05$, $\eta^2 = 0.16$), with parents rearing two chicks demonstrating higher baseline CORT concentrations than those rearing a single chick (Figure 3-10B). Specifically, two-chick single parents had higher baseline circulating CORT concentrations than two-chick paired parents (nest-type, $F_{1,31} = 7.8$, $p < 0.05$, $\eta^2 = 0.20$), while no significant differences were identified between one-chick single parents and one-chick paired parents (rank-transformed, nest-type, $F_{1,10} = 1.8$, $p < 0.05$, $\eta^2 = 0.13$).

Day 15 post-hatching - Single parents exhibited lower baseline circulating plasma CORT concentrations than paired parents.

Paired mothers displayed higher baseline circulating CORT concentrations compared to single fathers at Day 15 post-hatching (Table 3-4) (treatment groups, estimated marginal means, $p < 0.05$, paired mothers versus single fathers $d = 1.2$) (Figure 3-10C). In general, single parents exhibited lower baseline CORT concentrations than their paired counterparts (Figure 3-10D).

Day 3 post-fledging -No significant differences were detected.

No significant differences in baseline circulating CORT were detected concerning treatment groups, nest-type, or sex (Table 3-5).

Discussion

Trade-offs in single parenting revealed by chick size.

We examined the dynamics of single parenting in pigeons and the trade-offs associated with it, focusing on chick size during the early and middle stages of development. We found that

single-parented chicks, both on Day 5 and Day 15 post-hatching, were smaller than paired-parented counterparts. This observation underscores the challenges single parents face in compensating for the absence of their partners, even when food resources are abundant.

Furthermore, we noticed that the nature of these trade-offs depended on the age of the chicks being cared for by single parents. On Day 5 post-hatching, single-fathered chicks were consistently smaller than paired-parented and single-mothered chicks. We explored this phenomenon further in previous work (Booth et al. *in prep*), highlighting that pigeon fathers typically invest less time in offspring care during this stage, an event also documented in previous studies (Johnston and Janiga, 1995; Levi, 1986). We also discussed how differences in the quality of crop milk, the primary chick food at Day 5 post-hatching, might contribute to the observed size disparity in single-fathered chicks at Day 5 post-hatch.

In contrast, by Day 15 post-hatching, single-mothered chicks tended to be smaller than their counterparts raised by paired parents as well as by single fathers. During this later stage, pigeons typically have overlapping nests (Hetmański and Wołk, 2005; Johnston and Janiga, 1995), and fathers become more involved in chick care while mothers prepare for the next clutch of eggs. This shift in parental roles may explain why single mothered-chicks at Day 15 post-hatching were the smallest among all treatment groups. Despite single mothers continuing to care for their offspring, they might not have been capable of maintaining the provisioning level of a father during this phase.

Additionally, although we found no significant differences in chick size at Day 3 post-fledging, it's noteworthy that single-parented chicks took approximately 4.1 days longer on average to fledge compared to paired-parented chicks. This result aligns with prior studies involving experimentally enlarged broods. For example, De Kogel (1997) observed delayed

development in zebra finch (*Taeniopygia guttata*) nestlings when brood size was increased experimentally. These collective findings suggest that while single parent pigeons can successfully rear their offspring to independence, they might not fully compensate for the loss of their partner in the long-term.

The role of glucocorticoids

Our results uncovered the intriguing role of glucocorticoids in modulating parenting behaviors in single parents, with their influence varying depending on the parenting stage. Our previous findings (Booth et al., *in prep*) indicated that single mothers expressed more *GR* in the PVN compared to paired mothers. We speculated that this could suggest a more finely tuned negative feedback pathway for the hypothalamic-pituitary-adrenal (HPA) axis (as proposed by De Kloet et al., 1998) in single mothers. This adaptation might enable them to enhance their provisioning of offspring, as suggested by Vitousek et al. (2014).

In contrast, by Day 15 post-hatching, mothers in general, exhibited higher PVN *MR* expression than fathers. Additionally, at Day 3 post-fledging, paired mothers expressed more PVN *MR* compared to paired fathers, single mothers, and single fathers and no significant differences in *GR* expression. Considering that *MR* is speculated to play a more substantial role in regulating the HPA axis in the hippocampus, as demonstrated in studies by Dickens et al. (2009) and Krause et al. (2015), the exact implications of these differences in *MR* and *GR* expression at this stage of parental care remain unclear. Future investigations could involve manipulating *MR* and *GR* expression in the PVN and hippocampus of single parents to gain a deeper understanding of their potential roles in sustaining single-parent care behaviors.

Our study also revealed intriguing patterns of baseline circulating plasma CORT concentrations in single parents at different stages of parenting. On Day 5 post-hatching, single

parents rearing two-chick broods exhibited higher baseline circulating CORT compared to paired parents. This elevation in CORT might be associated with an increased provisioning effort, akin to findings in prior studies involving enlarged brood sizes, as suggested by Bonier et al. (2011) as well as in our previous work (Booth et al. *in prep*).

Conversely, on Day 15 post-hatching, we observed that single parents had lower baseline circulating CORT compared to paired parents, in contrast to earlier findings at Day 5 post-hatching. This shift in CORT concentrations may serve to prevent nest abandonment. Previous research by Ouyang et al. (2012) in female great tits (*Parus major*) found that those with higher baseline circulating CORT were more prone to nest abandonment, particularly during harsh weather conditions.

However, by Day 3 post-fledging, no significant differences were detected in terms of circulating baseline CORT among the parenting groups. To gain further insights into the role of CORT in regulating parental care behaviors following a significant parental disturbance like single parenting, future studies could explore the effects of acute stressors (e.g. as demonstrated by Austin et al., 2021; Calisi et al., 2018) as well as prolonged elevation of CORT (e.g. Spée et al., 2011) across various parenting stages. This could shed more light on the dynamic and shifting role of CORT in the context of parental care behaviors.

The role of prolactin and mesotocin

At Day 5 post-hatching, we noted some intriguing patterns in pituitary gene expression related to *PRL* and its receptor, *PRLR.* Paired fathers exhibited higher pituitary *PRLR* expression compared to paired mothers, single mothers, and single fathers. Moreover, single parents, in general, displayed lower *PRL* gene expression in comparison to paired parents. This finding may suggest an effort to reduce the inhibitory effect of *PRLR* in a negative feedback mechanism, a

concept supported by previous research in rodents (Ferraris et al., 2013, 2012) as well as by our prior research (Booth et al., *in prep*). However, it's worth highlighting that these differences were only evident at Day 5 post-hatching, coinciding with the period when pigeon parents pseudo-lactate, and not at the later stages we investigated.

Considering this, it is possible that the shifts in *PRLR* and *PRL* expression in the pituitary are crucial primarily during the early stages of single parenting when pseudo-lactation is most relevant. To gain a deeper understanding of how *PRL* and *PRLR* in the pituitary contribute to provisioning and parenting behaviors in single parents, future experiments involving manipulations of prolactin should be considered.

Turning to the expression of *PRLR* in the PVN, we found no significant differences at Day 5 post-hatching. However, at Day 15 post-hatching, fathers expressed more *PRLR* than mothers, and at Day 3 post-fledging, single fathers expressed more *PRLR* than single mothers, with fathers, in general, displaying higher *PRLR* compared to mothers. The PVN is a nucleus where *PRLR* increases in breeding birds, as observed in studies involving *Taeniopygia guttata* (Smiley et al., 2021), yet *PRLR*'s exact role in parental care behaviors in the PVN has not been definitively established to date (Smiley, 2019).

We speculate that the higher PVN *PRLR* expression observed in fathers at Day 15 post-hatching and Day 3 post-fledging might be related to the maintenance of parental behaviors. However, because there are no significant differences between paired and single parents in this regard, this might explain why single-parented chicks tended to be smaller than paired-parented chicks at day 15 post-hatching. To confirm whether *PRLR* indeed plays a role in sustaining single parenting behaviors, future studies involving manipulations of PVN *PRLR* should be conducted.

Finally, at Day 5 post-hatching, fathers expressed more PVN *OxtR* than mothers. Previous research in male Thai hens (*Gallus domesticus*) has demonstrated that mesotocin-immunoreactive neurons are found in high concentrations in the PVN (Kamkrathok et al., 2017), a phenomenon also observed in breeding females (Chokchaloemwong et al., 2013). This higher expression of *OxtR* in fathers might be associated with the maintenance of parental care behaviors in general. Future investigations involving manipulations of mesotocin and its receptor should be conducted to ascertain if that is the case and whether it plays a role in sustaining single parenting behavior specifically.

The role of gonadotropins

At Day 15 post-hatching, we observed a notable difference wherein single parents displayed lower pituitary *GnRHR* expression compared to paired parents. *GnRH*, released from the hypothalamus, binds to *GnRHR* in the pituitary gland, prompting the secretion of gonadotropic hormones as part of the HPG axis (Counis et al., 2005). We speculate that single parents, who cannot initiate another clutch as typically done by paired parents at this stage of parenting (Hetmański and Wołk, 2005), reduce the expression of pituitary *GnRHR* to dampen the stimulation of the HPG axis. These findings offer a practical illustration of gonadotropin regulation in response to a real-life parental disturbance, namely single parenthood.

Interestingly, at Day 15 post-hatching, we observed a surprising contrast: single mothers expressed less PVN *GnIH* compared to paired fathers. Our initial expectation was that *GnIH* expression would increase in single parents, particularly single mothers, given their inability to commence a new clutch, which is customary for paired parent nests. It is worth noting that during our study, the birds were housed socially, with a selection of nest boxes and other environmental factors at their disposal. Therefore, these results may be influenced by factors

beyond single parenting, such as the availability of nesting materials or the presence of reproductively receptive males in the socially housed aviary (Cheng and Balthazart, 1982; Cheng and Follett, 1976). Further investigations are warranted to explore these potential influences on PVN *GnIH* expression.

Conclusions

In the course of this study, we characterized the intricacies of parental care behaviors, neural transcription, and physiological changes in both single and paired mother and father pigeons. Our investigation, conducted from the initial stages of parental care through to the point of offspring achieving independence, has illuminated several critical aspects of avian parental care dynamics. It is our hope that these insights will serve as a catalyst for an expanded examination of sex-specific reproductive behaviors. Additionally, we anticipate that our findings will inspire further inquiries into the intricate biological parallels and distinctions underlying parental care roles, recognizing that the realm of reproductive biology may encompass a spectrum beyond the traditional binary framework of male and female. Such investigations hold the promise of deepening our understanding of the complex world of avian parenting and may shed light on broader aspects of reproductive biology, transcending conventional boundaries and encompassing diverse biological manifestations in avian species and beyond.

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Figures

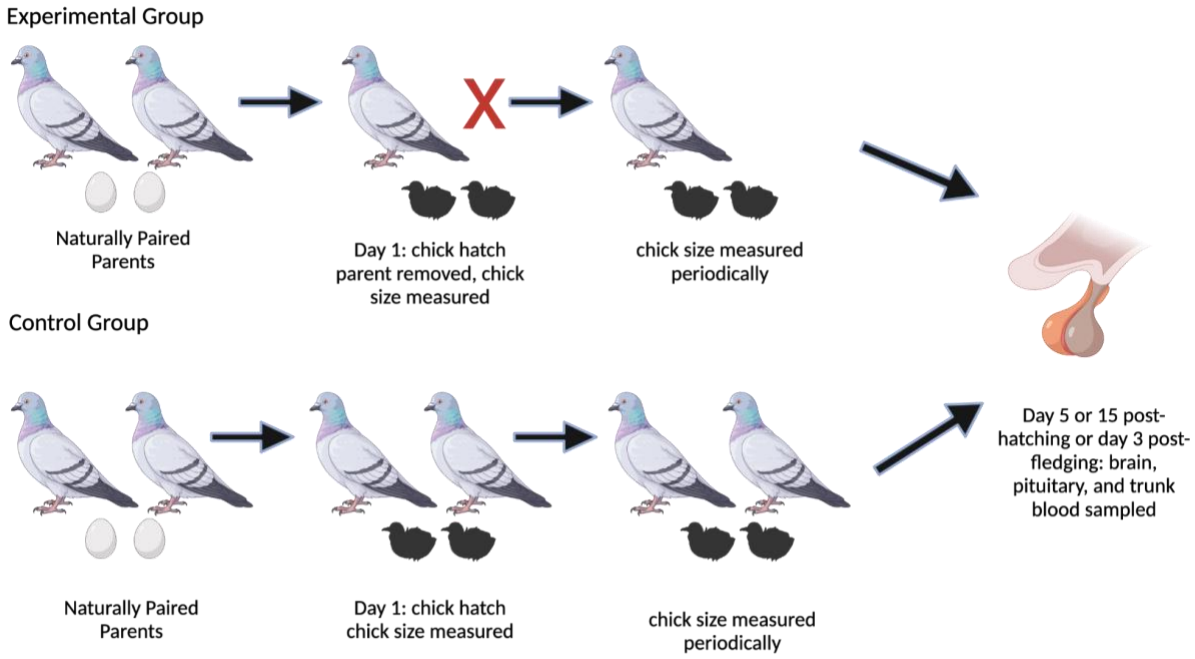

Figure 3-1. Experimental design. The presence of a parental partner was manipulated by randomly removing one parent, male or female, from a nest Day 1 post-chick hatch. Post-hatching, the paired parent control group was left unmanipulated. Chick size was periodically measured until the birds were collected. Brains, pituitaries, and trunk blood were collected on Day 5 post-hatching, Day 15 post-hatching, or Day 3 post-fledging. Created with BioRender.com.

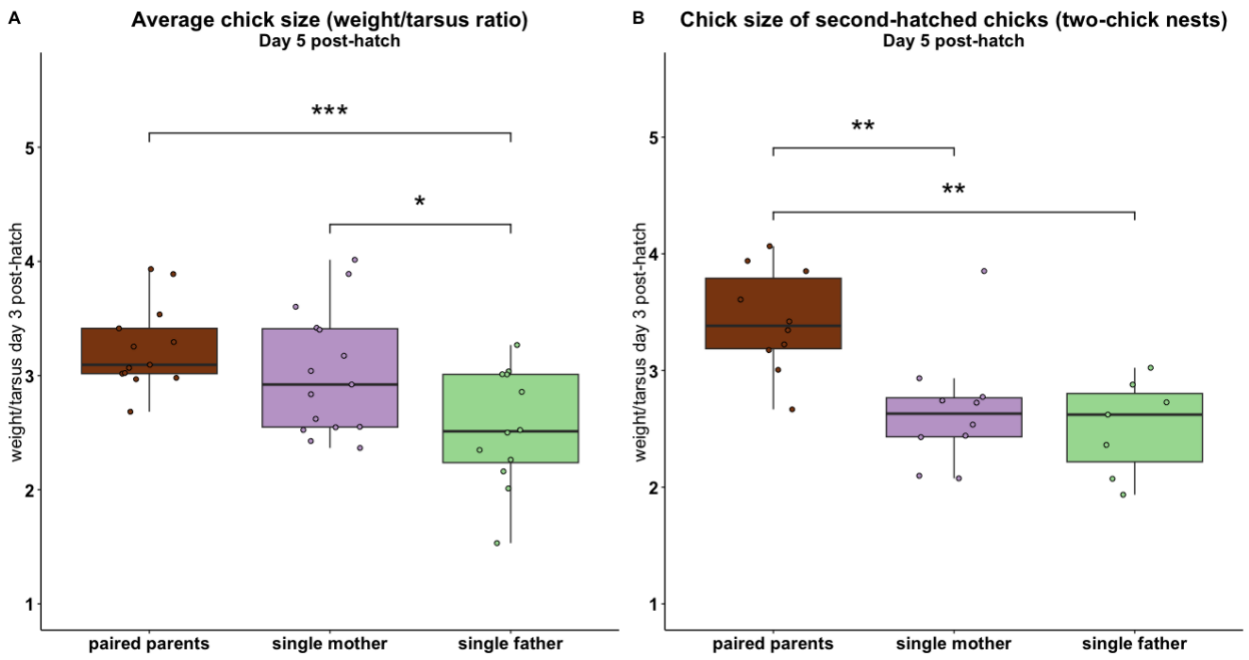

Figure 3-2. Chick size of Day 5 post-hatching chicks. A.) Chick size at Day 3 post-hatching. The chicks of two-chick nests were averaged for this data set. B.) Chick size at Day 3 post-hatching for second-hatched chicks of two-chick broods. In both cases, single-parented chicks were smaller than paired-parented chicks. Statistical significance denoted as follows: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

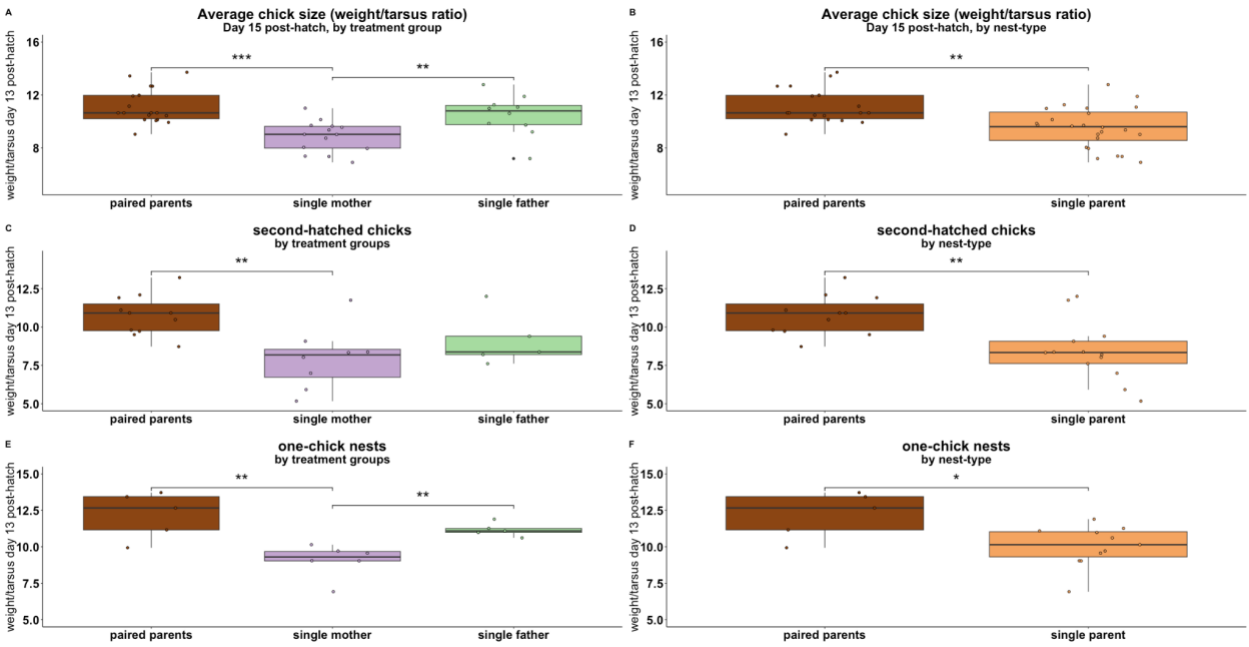

Figure 3-3. Chick size day of day 15 post-hatching chicks. A.) Chick size at Day 13 post-hatching in terms of treatment groups. The chicks of two-chick nests were averaged for this data set. B.) Chick size at Day 13 post-hatching in terms of nest-type. C.) Chick size at Day 13 post-hatching in terms of treatment groups for second-hatched chicks of two-chick nests. D.) Chick size at Day 13 post-hatching in terms of nest-type for second-hatched chicks of two-chick nests. E.) Chick size at Day 13 post-hatching in terms of treatment groups for the chicks of one-chick nests. F.) Chick size at Day 13 post-hatching in terms of nest type for the chicks of one-chick nests. Single-mothered chicks were smaller than paired-parented and single-fathered chicks in general, and when pooled by nest-type, single-parented chicks were smaller than paired-parented chicks in general. Statistical significance denoted as follows: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

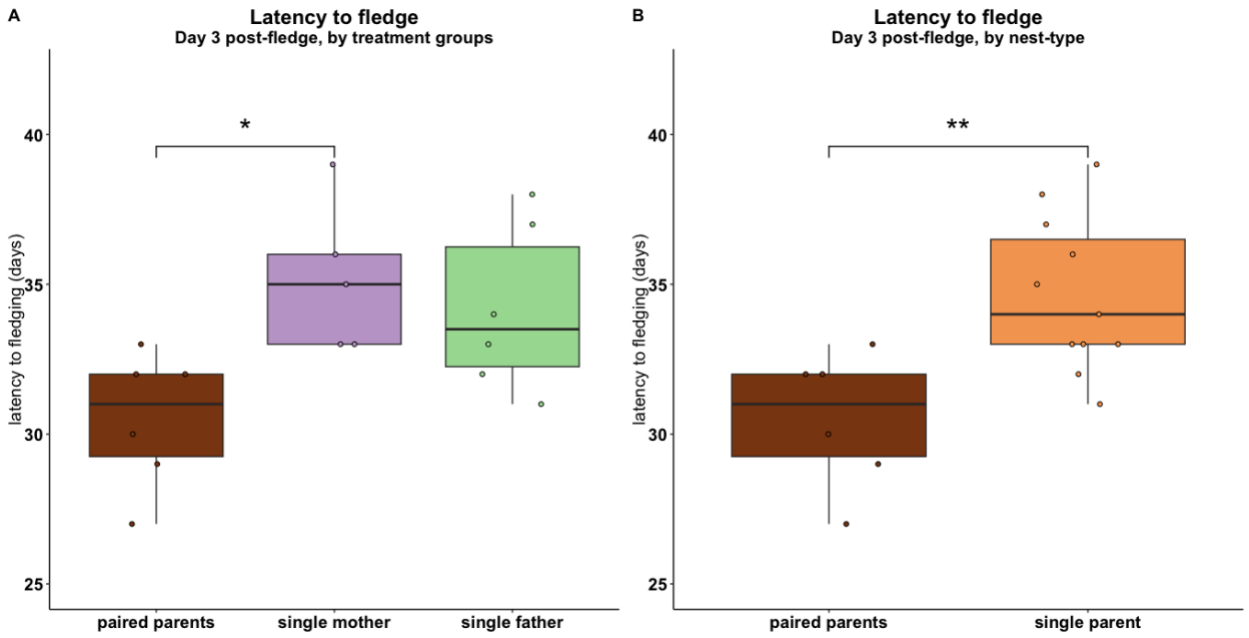

Figure 3-4. Latency to fledge for Day 3 post-fledging chicks (for two-chick nests, the time of the first-fledged chick was used). A.) Latency to fledge in terms of treatment groups. Single-mothered chicks took longer to fledge than paired-parented chicks. B.) Latency to fledge in terms of nest-type. Single-parented chicks took longer to fledge than paired parented chicks in general. Statistical significance denoted as follows: *: $p < 0.05$, **: $p < 0.01$

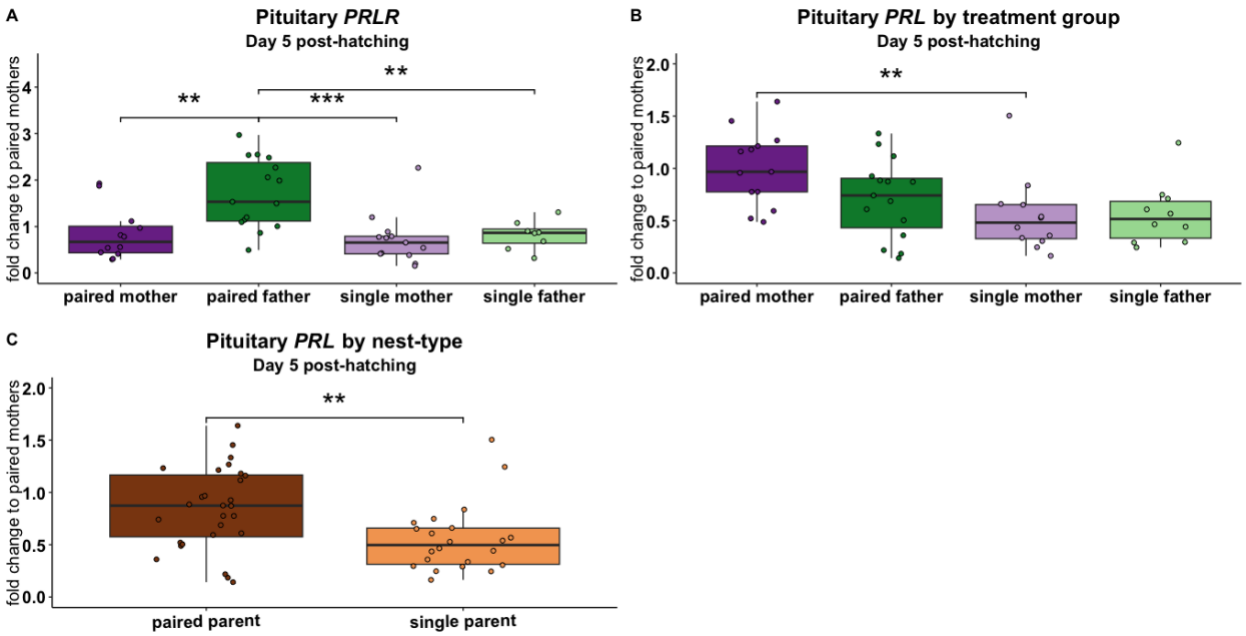

Figure 3-5. Parent pituitary gene expression Day 5 post-hatching. A.) *PRLR* expression. Paired fathers had higher expression than paired mothers, single mothers and single fathers (after brood size removed as co-variable). B.) *PRL* expression. Paired mothers expressed more *PRL* than single mothers C.) *PRL* expression in terms of nest-type. Paired parents expressed more *PRL* in general than single parents. Statistical significance denoted as follows: **: $p < 0.01$, ***: $p < 0.001$

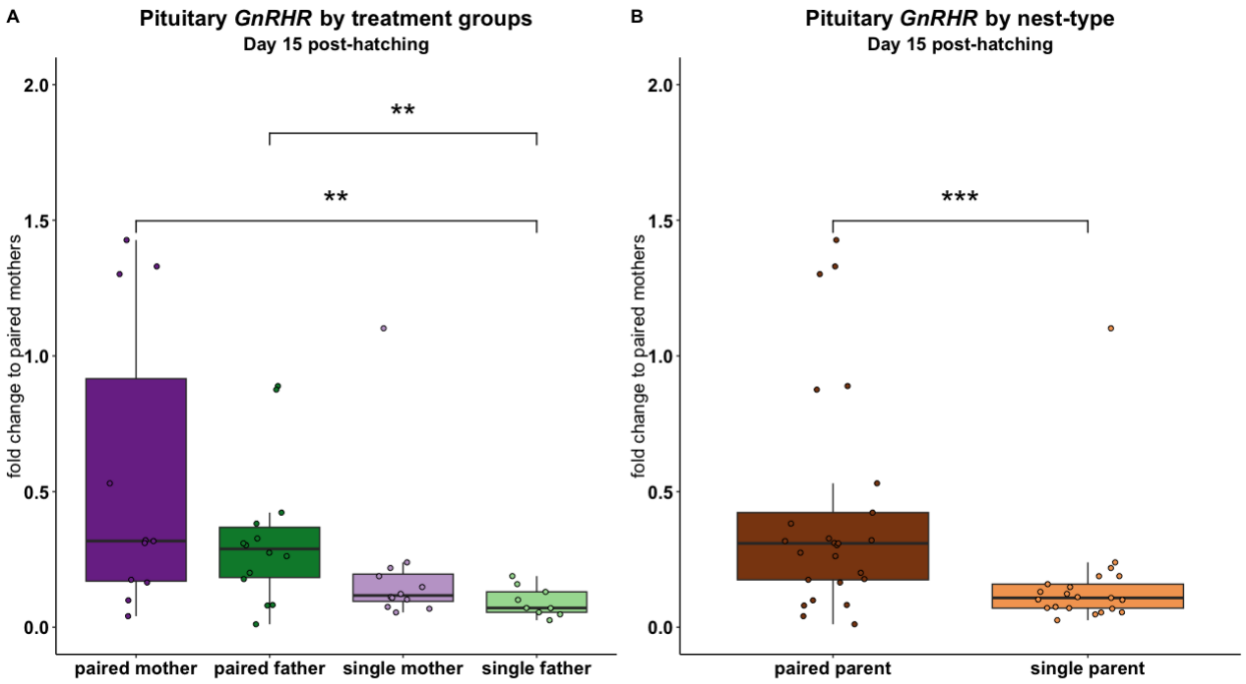

Figure 3-6. Parent pituitary gene expression Day 15 post-hatching. A.) *GnRHR* gene expression in terms of treatment groups. Single fathers expressed less *GnRHR* than paired mothers and paired fathers. B.) *GnRHR* gene expression in terms of nest-type. Single parents expressed less *GnRHR* than paired parents. Statistical significance denoted as follows: **: $p < 0.01$, ***: $p < 0.001$

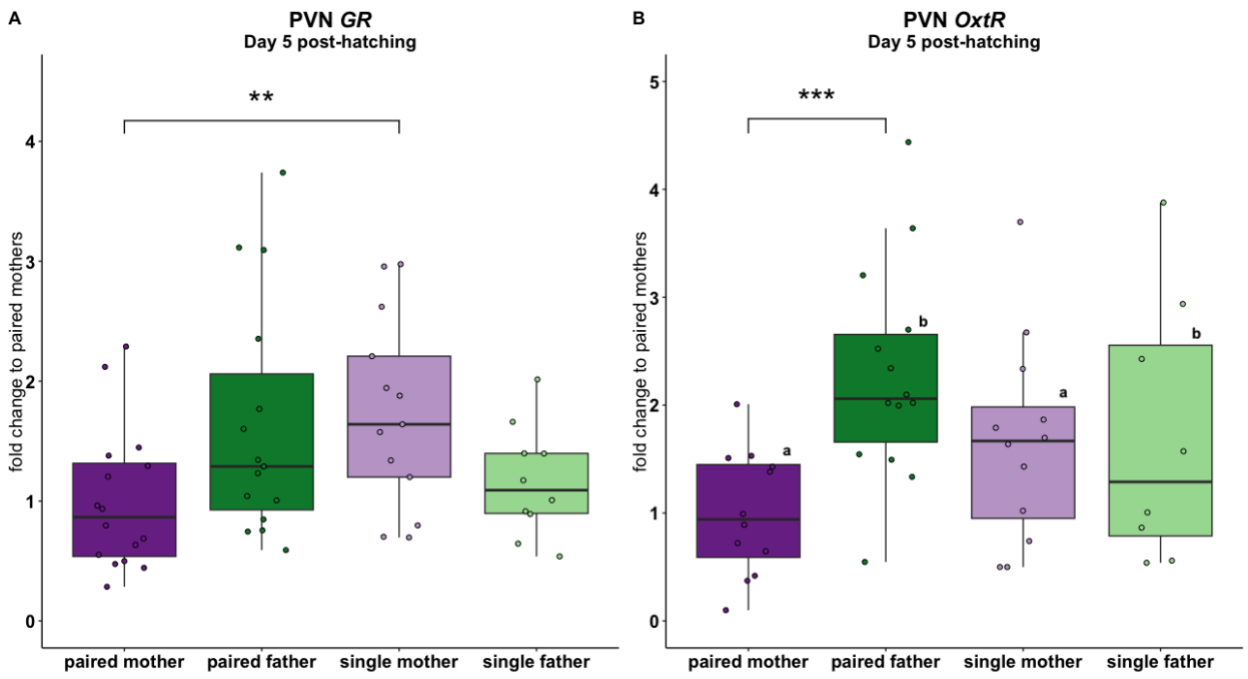

Figure 3-7. Parent PVN gene expression Day 5 post-hatching. A.) *GR* gene expression in terms of treatment groups. Single mothers expressed more *GR* than paired mothers. B.) *OxtR* gene expression in terms of treatment groups. Paired mothers expressed less *OxtR* than paired fathers, and mothers in general expressed less *OxtR* than fathers. Statistical significance denoted as follows: **: $p < 0.01$, ***: $p < 0.001$, sex differences indicated by different letters.

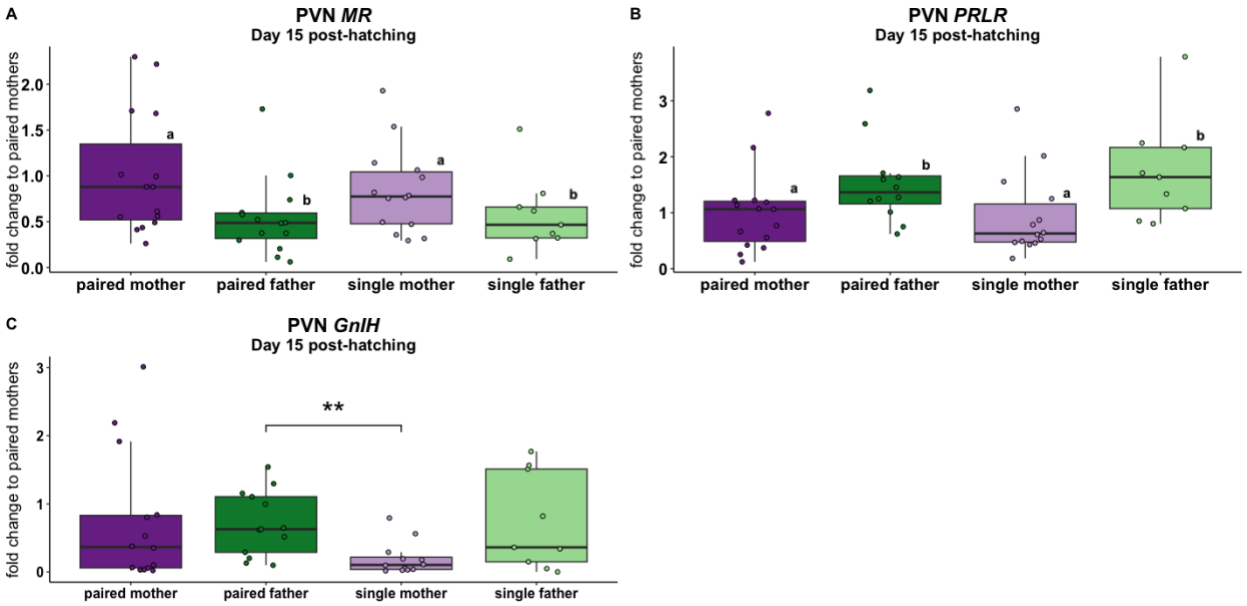

Figure 3-8. Parent PVN gene expression Day 15 post-hatching. A.) *MR* gene expression in terms of treatment groups. Mothers expressed more *MR* than fathers in general. B.) *PRLR* gene expression in terms of treatment groups. Fathers expressed more *PRLR* than mothers in general. C.) *GnIH* gene expression in terms of treatment groups. Single mothers expressed less *GnIH* than paired fathers. Statistical significance denoted as follows: **: $p < 0.01$, sex differences indicated by different letters.

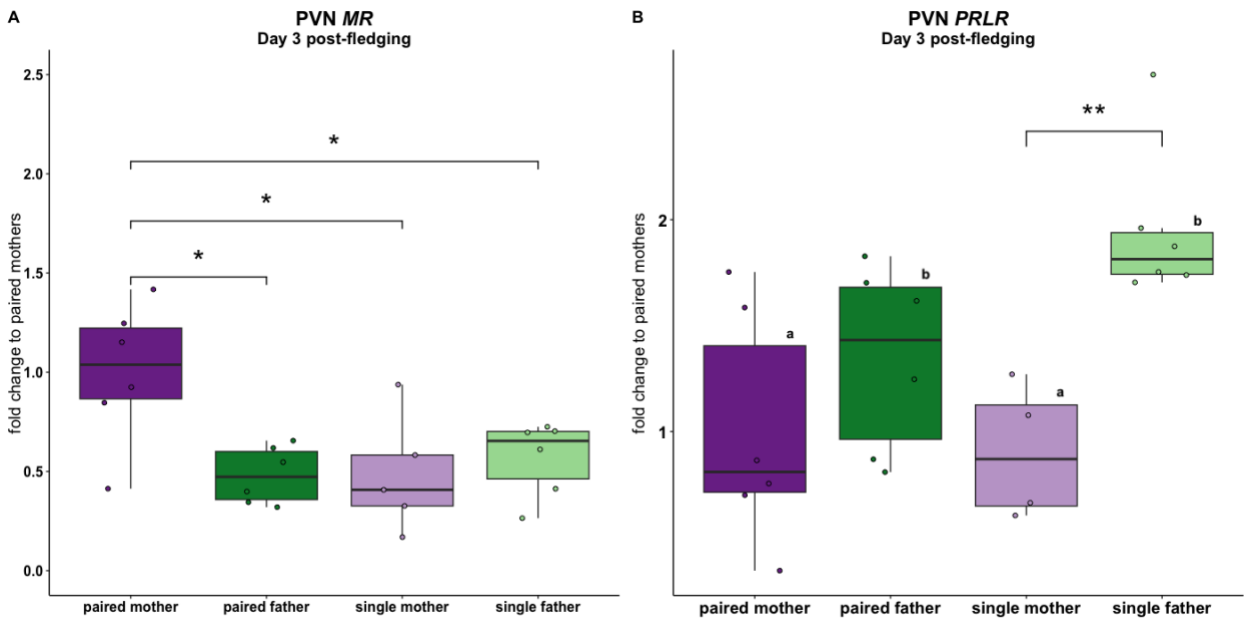

Figure 3-9. Parent PVN gene expression Day 3 post-fledging. A.) *MR* gene expression in terms of treatment groups. Paired mothers expressed more MR than paired fathers, single mothers and single fathers. B.) *PRLR* gene expression in terms of treatment groups. Single fathers expressed more *PRLR* than single mothers, and fathers expressed more *PRLR* than mothers in general. Statistical significance denoted as follows: **: $p < 0.01$, *: $p < 0.05$, sex differences indicated by different letters.

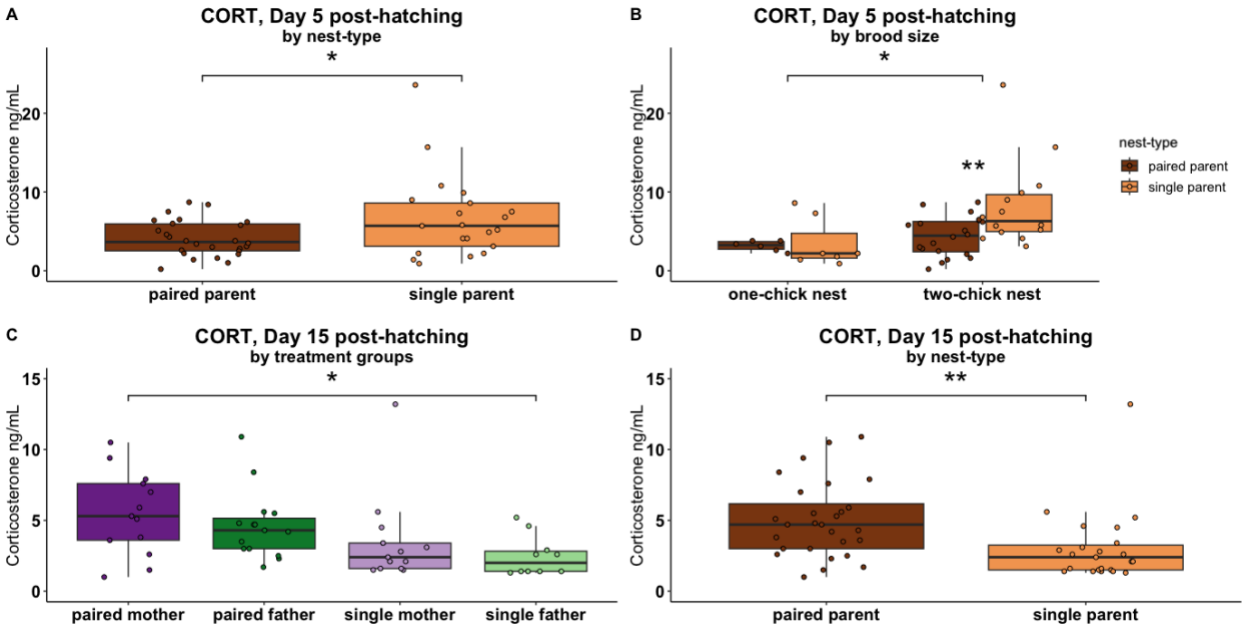

Figure 3-10. Circulating Baseline CORT. A.) Circulating CORT in terms of nest-type at Day 5 post-hatching. Single parents had higher CORT than paired parents. B.) Circulating CORT in terms of brood size and nest type. Single parents rearing two-chick nests had higher CORT than their paired-parent counterparts, and no differences were detected between single and paired parents rearing one-chick nests. C.) Circulating CORT in terms of treatment groups at Day 15 post-hatching. Paired mothers had higher CORT than single fathers D.) Circulating CORT in terms of nest-type at Day 15 post-hatching. Single parents had less CORT than paired parents in general. Statistical significance denoted as follows: **: $p < 0.01$, *: $p < 0.05$

Tables

Table 3-1. Primers used for quantitative PCR.

Gene	GenBank Accession No.	Primer Sequence	Efficiency (%)
Glucocorticoid receptor, *GR*	XM_021301096.1	F:TGCTTAACTCGTCGGATCAA R:AAAGTCCATCACGATCCCTC	90.5
Mineralocorticoid receptor, *MR*	XM_021296726.1	F: AGAACATGGCTTCCTCGGTG R: CTAGAAAGCGGAGACCCGAC	103.9
Estrogen receptor beta, ER-β	NM_001282841.1	F:GGGAATGATGAAATGTGGCTC R:GATCTCTTTTACGCGGGTTG	100.6
Prolactin, *PRL*	XM_005506024.2	F: GGCGGGTTCATACTGGTGAG R: TGGATTAGGCGGCACTTCAG	92.55
Prolactin receptor, *PRLR*	NM_001282822.1	F:TCTTCCTTGCACACATGAAACC R:TCCAGGGTATGATTGACCAGT	95.24
Vasoactive intestinal peptide receptor, *VIPR*	XM_013369762.2	F: AGGGATTTGTGGTGGCTGTT R: TGCCTAAGGAAGGGTGGTGA	100.9
Gonadotropin releasing hormone I, *GnRH-I*	XM_005513520.3	F: GAAGTGCAGAAGAGCGAATG R: AATCTTCCGTCTGGCTTCTC	94.01
Gonadotropin releasing hormone receptor, *GnRHR*	XM_013369955.2	F: GGCACGAGACCCTCTACAAC R: TGTGAGGAGAAGAGGCTGGA	96.12
Gonadotropin inhibitory hormone, *GnIH*	XM_005513478.2	F:AAGGTATCACACACAGGCTTGG R:TAGTCTTCATTTCCCTGGTTCA	96.29
Gonadotropin inhibitory hormone receptor, *GnIHR*	XM_021283542.1	F:CTGGACACTGACGCTGCTGA R:GGTTGGCACTGCTGTTGAAG	99.4
Mesotocin receptor, *Oxt-R*	XM_021296600.1	F: GGTCTGTGTGGGACACGAAT R: GGCCGGTGTAGAGCATGTAG	92.7
Ribosomal protein L4, *rpL4*	XM_005511196.1	F: GCCGGAAAGGGCAAAATGAG R: GCCGTTGTCCTCGTTGTAGA	105.1
hypoxanthine phosphoribosyltransferase 1, *HPRT1*	XM_005500563.2	F:GCCCCATCGTCATACGCTTT R: GGGGCAGCAATAGTCGGTAG	94.72
Beta actin, *ACTB*	XM_005504502.2	F:ATGTGGATCAGCAAGCAGGAG R:CATTTCATCACAAGGGTGTGGG	95.8

Table 3-2. ANOVAs and t-tests regarding the chicks

Day 5 post-hatching	**treatment**					**nest-type**				
Chick size	***F***	***p***	**df**	**df residual**	***η2***	***F***	***p***	**df**	**df residual**	***η2***
analysis where two chick nests were averaged	6.7	**< 0.05**	2	36	0.27	6	**< 0.05**	1	37	0.14
	F	***p***	**df**	**df residual**	***η2***	***t***	***p***	**df residual**	***d***	
first hatched chick analysis (from two chick broods)	1.2	0.33	2	24	0.09	0.88	0.39	25	0.35	
second hatched chick analysis (from two chick broods)	10.5	**< 0.05**	2	24	0.47	4.6	**< 0.05**	25	1.8	
one chick nest analysis	3.7	0.063	2	10	0.42	0.49	0.63	11	0.41	
Day 15 post-hatching	**treatment**					**nest-type**				
Chick size	***F***	***p***	**df**	**df residual**	***η2***	***F***	***p***	**df**	**df residual**	***η2***
analysis where two chick nests were averaged	15.1	**< 0.05**	2	38	0.42	17.7	**< 0.05**	1	39	0.27
	F	***p***	**df**	**df residual**	***η2***	***t***	***p***	**df residual**	***d***	
first hatched chick analysis (from two chick broods)	1.9	0.18	2	21	0.15	1.5	0.14	22	0.63	
second hatched chick analysis (from two chick broods)	6.8	**< 0.05**	2	21	0.39	3.4	**< 0.05**	22	1.4	
one chick nest analysis	10.4	**< 0.05**	2	13	0.61	2.8	**< 0.05**	14	1.4	
Day 3 post-fledging	**treatment**					**nest-type**				
Response Variable	***F***	***p***	**df**	**df residual**	***η2***	***F***	***p***	**df**	**df residual**	***η2***
Chick size - analysis where two chick nests were averaged	1.6	0.25	2	12	0.19	3.3	0.092	1	13	0.19
latency to fledging (days)	4.8	**< 0.05**	2	13	0.44	9.8	**< 0.05**	1	14	0.43

Table 3-3. Linear mixed models and ANOVAs regarding the parents at Day 5 post-hatching

	treatment					**nest-type**					**sex**				
Response Variable	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
circulating CORT	3.1	< **0.05**	3	28	0.33	5.1	< **0.05**	1	43	0.08	2.1	0.15	1	43	0.04
pituitary gene expression	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
GR	0.47	0.71	3	33.3	0.03	0.007	0.98	1	45	0.00069	0.54	0.47	1	45	0.01
MR	1.1	0.35	3	33.5	0.08	1.6	0.22	1	44	0.06	0.87	0.36	1	44	0.02
PRLR	7.3	< **0.05**	3	33.1	0.37	4.1	< **0.05**	1	44	0.17	12	< **0.05**	1	44	0.22
PRL	3.6	< **0.05**	3	32.2	0.22	6.8	< **0.05**	1	46	0.15	2.6	0.12	1	46	0.05
VIPR	0.88	0.46	3	32.1	0.07	0.18	0.67	1	42	0.01	2.4	0.13	1	42	0.06
OxtR	0.91	0.45	3	33.0	0.06	0.0022	0.96	1	47	0.0019	1.8	0.18	1	47	0.04
GnRHR	0.55	0.65	3	32.5	0.04	0.79	0.38	1	42	0.0092	1.12	0.30	1	42	0.03
GnIHR	0.56	0.64	3	31.7	0.04	0.37	0.54	1	42	0.0048	1.4	0.24	1	42	0.03
PVN gene expression	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
GR	3.4	< **0.05**	3	37.1	0.17	1.6	0.21	1	50	0.01	0.29	0.59	1	50	0.0068
MR	0.26	0.85	3	38.0	0.02	0.35	0.55	1	53	0.0082	0.17	0.68	1	53	0.0032
ERβ	1.5	0.23	3	38.1	0.08	0.87	0.35	1	52	0.01	3.0	0.087	1	52	0.06
PRLR	0.49	0.69	3	36.0	0.03	0.0043	0.95	1	49	0.00029	0.76	0.39	1	49	0.01
PRL	0.39	0.76	3	34.7	0.03	1.0	0.32	1	46	0.04	0.023	0.88	1	46	0.00056
OxtR	8.6	< **0.05**	3	27.5	0.55	0.23	0.64	1	42	0.0000067	6.6	< **0.05**	1	42	0.15
GnRH-I	2.8	0.056	3	34.4	0.13	0.19	0.66	1	50	0.0094	0.0094	0.92	1	50	0.00010
GnIH	0.64	0.59	3	36.7	0.07	0.25	0.62	1	50	0.00086	0.71	0.40	1	50	0.01
GnIHR	0.69	0.56	3	35.5	0.05	0.16	0.69	1	52	0.0026	1.3	0.26	1	52	0.02

Table 3-4. Linear mixed models and ANOVAs regarding the parents at Day 15 post-hatching

	treatment					**nest-type**					**sex**				
Response Variable	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
circulating CORT	2.9	< **0.05**	3	36.1	0.16	8.1	< **0.05**	1	47	0.13	1.6	0.21	1	47	0.03
pituitary gene expression	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
GR	0.75	0.53	3	26.0	0.08	0.46	0.50	1	39	0.01	0.075	0.79	1	39	0.0016
MR	1.1	0.35	3	26.3	0.13	1.1	0.31	1	41	0.02	0.76	0.39	1	41	0.02
PRLR	0.92	0.44	3	29.8	0.08	3.4	0.073	1	43	0.07	0.20	0.66	1	43	0.005
PRL	1.5	0.24	3	33.8	0.10	1.9	0.18	1	43	0.020	3.0	0.091	1	43	0.07
VIPR	0.81	0.49	3	32.7	0.06	0.0056	0.94	1	43	0.00025	0.002	0.96	1	43	0.000051
OxtR	1.4	0.26	3	28.9	0.11	0.086	0.77	1	40	0.0041	0.22	0.64	1	40	0.0055
GnRHR	5.3	< **0.05**	3	30.3	0.29	16.1	< **0.05**	1	42	0.26	1.9	0.18	1	42	0.04
GnIHR	0.75	0.53	3	28.2	0.06	0.67	0.42	1	40	0.02	0.066	0.80	1	40	0.0016
PVN gene expression	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
GR	0.84	0.48	3	33.3	0.06	1.1	0.30	1	45	0.03	0.49	0.49	1	45	0.01
MR	3.0	< **0.05**	3	34.0	0.17	0.092	0.76	1	48	0.000018	7.9	< **0.05**	1	48	0.14
ERβ	1.6	0.20	3	28.418	0.19	0.21	0.65	1	45	0.0034	2.5	0.12	1	45	0.05
PRLR	7.0	< **0.05**	3	36.4	0.3	0.11	0.75	1	50	0.0057	21.2	< **0.05**	1	50	0.30
PRL	0.96	0.42	3	27.4	0.08	3.0	0.094	1	35	0.08	0.11	0.75	1	35	0.0030
OxtR	0.81	0.50	3	24.4	0.07	0.67	0.42	1	33	0.02	0.90	0.35	1	33	0.02
GnRH-I	1.3	0.28	3	34.0	0.11	0.70	0.41	1	48	0.0049	3.0	0.087	1	48	0.06
GnIH	3.2	< **0.05**	3	32.7	0.19	2.0	0.17	1	44	0.05	1.2	0.28	1	44	0.03
GnIHR	0.86	0.47	3	35.1	0.05	2.5	0.12	1	48	0.04	0.017	0.90	1	48	0.00082

Table 3-5. Linear mixed models and ANOVAs regarding the parents at Day 3 post-fledging

	treatment					**nest-type**					**sex**				
Response Variable	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
circulating CORT	0.89	0.47	3	13.2	0.14	0.35	0.56	1	19	0.04	0.34	0.57	1	19	0.0035
pituitary gene expression	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
GR	0.11	0.95	3	11.8	0.02	0.13	0.73	1	18	0.01	0.16	0.70	1	18	0.02
MR	0.28	0.84	3	11.1	0.06	0.012	0.91	1	17	0.00018	0.27	0.61	1	17	0.01
PRLR	0.29	0.83	3	11.6	0.05	0.78	0.39	1	18	0.040	0.20	0.66	1	18	0.0099
PRL	0.59	0.63	3	9.8	0.18	0.17	0.68	1	17	0.00026	0.47	0.50	1	17	0.05
VIPR	0.23	0.87	3	11.3	0.04	0.0070	0.93	1	17	0.00098	0.36	0.56	1	17	0.02
OxtR	1.7	0.23	3	11.1	0.32	1.7	0.21	1	18	0.07	0.84	0.37	1	18	0.04
GnRHR	0.43	0.74	3	12.2	0.07	0.28	0.6	1	18	0.04	1.1	0.31	1	18	0.04
GnIHR	0.29	0.83	3	8.7	0.12	0.24	0.63	1	16	0.01	0.0056	0.94	1	16	0.00045
PVN gene expression	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**	***F***	***p***	**df**	**df residual**	**η2**
GR	0.039	0.99	3	11.6	0.010	0.012	0.92	1	19	0.0062	0.024	0.88	1	19	0.00035
MR	6.4	**< 0.05**	3	11.9	0.55	0.93	0.35	1	19	0.08	2.8	0.11	1	19	0.10
ERβ	0.51	0.68	3	11.7	0.10	0.62	0.44	1	19	0.03	0.071	0.79	1	19	0.00523
PRLR	5.7	**< 0.05**	3	12.1	0.51	2.2	0.16	1	18	0.15	10.0	**< 0.05**	1	18	0.37
PRL	0.22	0.88	3	12.6	0.04	0.48	0.49	1	19	0.02	0.049	0.83	1	19	0.0083
OxtR	NA	NA	NA	NA	NA	0.70	0.42	1	12	0.06	1.2	0.30	1	12	0.1
GnRH-I	0.068	0.98	3	10.4	0.34	0.021	0.89	1	18	0.00047	0.12	0.73	1	18	0.01
GnIH	0.89	0.47	3	11.8	0.14	0.093	0.76	1	18	0.0064	0.24	0.63	1	18	0.02
GnIHR	0.24	0.87	3	11.3	0.05	0.67	0.42	1	17	0.05	0.012	0.91	1	17	0.0013