THE ROLE OF PROLACTIN AND REPRODUCTIVE EXPERIENCE IN THE ONSET OF PARENTAL CARE IN THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*)

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THE ROLE OF PROLACTIN AND REPRODUCTIVE EXPERIENCE IN THE ONSET OF PARENTAL CARE IN THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*)

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Parental care is a widespread phenomenon observed in many diverse taxa and is an important component of fitness. Birds are the largest parental vertebrate clade as 98% of avian species provide some variation of parental care. Despite this, we still know surprisingly little about the neuroendocrine regulation of avian parental care. Neuroendocrine systems have long been thought to play an important role in the onset of parental care as they are known to regulate various aspects of both physiology and behavior. In virtually all birds that raise altricial young, circulating prolactin (PRL) levels are generally low during non-breeding times, but significantly increase during late incubation and early post-hatch care. Because of this pattern, PRL has been suggested to be involved in the initiation of parental care in birds, but rarely has this hypothesis been causally tested. This dissertation provides the first descriptive studies of PRL's relationship with breeding cycle stages and reproductive experience (chapter 1) and the relationship between PRL and variation in post-hatch parental behavior (chapter 2) in the socially monogamous and biparental zebra finch. In chapters 3 and 4, we provide the first causal evidence that PRL plays a role in the onset of zebra finch parental behavior and show that reproductive experience positively affects parental

behavior. Lastly, in chapter 5, we provide the first description of the central PRL receptor (PRLR) in the brains of male and female zebra finches and show how the PRLR distribution is significantly affected by breeding status by comparing the PRLR distribution between breeding and non-breeding brains. This information is essential for continuing to test for a causal role in central PRL in parental care, for generalizing the role of PRL to other avian species, and for comparative analyses to help elucidate the evolution of parental care and other PRL-mediated behaviors.

BIOGRAPHICAL SKETCH

I was born in Royal Oak, MI and grew up in Troy, MI. After high school, I enrolled in Oakland Community College and worked part time as a Subway restaurant manager. After earning my Associates degree in liberal arts, I transferred to Wayne State University in the heart of Detroit, MI where I began to pursue a psychology major. There I met Dr. Michelle Tomaszycki and discovered my passion for hormones and behavior while working in her lab. After graduating from Wayne State with a B.S. in honors psychology, I moved to Ithaca, NY and began my Ph.D. program at Cornell University with Dr. Elizabeth Adkins-Regan in the psychology department.

This dissertation is dedicated to my grandparents, who were all teachers.

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ABBREVIATIONS

AC	nucleus accumbens
AOB	accessory olfactory bulb
AVP	vasopressin
AVT	vasotocin
BC	body condition
BLA	basolateral nucleus of the amygdala
BML	basomedial nucleus of the amygdala
BNST	bed nucleus of the stria terminalis
BR	bromocriptine
CB	cerebellum
CNS	central nervous system
CohortID	a unique number to denote which cohort birds were used in
CON	control
CV	coefficient of variation
DA	dopamine
ELISA	enzyme-linked immunosorbent assay
EXP	experienced
GEE	generalized estimation equation model
GLMM	general linear mixed model
ICV	intracerebroventricular
IHC	immunohistochemistry
IM	intramuscular
IN	tractus infundibularis
INEX	inexperienced
ir	immunoreactivity
IV	intravenous
LHA	lateral hypothalamic area (mammals)
LHy	nucleus lateralis hypothalami
LMM	linear mixed model
LS	lateral septum
LSD	least significant difference
LSi	intermediate subdivision of the lateral septum
LSM	least square mean
LSv	ventral lateral septum
MeA	medial amygdala
MPOA	medial preoptic area
mRNA	messenger ribonucleuc acid
MT	mesotocin

NAcc	nucleus accumbens (mammals)
NestID	a unique number to identify each nest was occupied in the study
nST	nucleus striaterminalis
OT	oxytocin
PairID	a unique number to identify each pair used in the study
POA	preoptic area
POM	nucleus preopticus medialis
PRL	prolactin
PRLR	prolactin receptor
PVM	nucleus paraventricularis magnocelluaris
ReproExp	reproductive experience
SD/sd	standard deviation
SEM/SE	standard error of the mean
SL	nucleus septalis lateralis
StudyRoomID	a unique number to denote which room birds were tested in
SubjectID	a unique number to identify individual subjects
TIDA	tuberoinfundibular dopamine
TnA	nucleus taeniae of the amygdala
Tu	nucleus tuberis
TV	nucleus tegmenti ventralis
VIP	vasoactive intestinal peptide
VMH	ventromedial hypothalamus
VNO	vomeronasal organ
VTA	ventral tegmental area

GENERAL INTRODUCTION

Parental care is undoubtedly important for offspring survival, offspring quality, and future offspring reproductive success (Klug and Bonsall, 2014), and hence, is an important component of fitness for parents. Birds are the largest parental vertebrate clade, and 98% of avian species provide some variation of parental care, with over 80% providing biparental care (Cockburn, 2006). Despite this, we still know surprisingly little about the neuroendocrine regulation of avian parental care. Offspring directed behaviors are often novel for first time parents and require a series of neural, endocrine, and other physiological changes to promote their onset. Neurondocrine systems have long been thought to play an important role in the onset of parental care as they are known to regulate various aspects of both physiology and behavior. Prolactin (PRL), a peptide hormone produced in the pituitary, may be a mechanism that has conserved functions in stimulating parental care across vertebrate species. PRL was initially discovered as a hormone involved in milk production in lactating female mammals (reviewed in Bole-Feysot et al., 1998) and plays a causal role in the onset of mammalian maternal behaviors (reviewed in Bridges, 2015). Increased PRL is also positively related to mammalian paternal care, though evidence for a causal relationship between PRL and paternal care is less conclusive (Bales and Saltzman, 2016; Saltzman and Ziegler, 2014; Wynne-Edwards and Timonin, 2007). PRL's role in parental care likely dates back even further in evolutionary time, however. In fish, pituitary and plasma levels of PRL increase during the late stages of brooding and early egg hatching and this increase in PRL promotes oral egg carrying (mouth brooding), nest building, and egg fanning behavior in a variety of fish species

(reviewed in Whittington and Wilson, 2013). PRL is also important for proliferation of skin mucous cells and mucus production, or discus milk, which is used to provide nutrients to offspring in discus fish (reviewed in Whittington and Wilson, 2013).

Similarly, in virtually all birds that raise altricial young, circulating PRL levels are generally low during non-breeding times, but significantly increase during late incubation and early post-hatch care (reviewed in Angelier et al., 2016; Smiley and Adkins-Regan, 2016a; reviewed in Wingfield and Farner, 1993). High PRL concentrations in breeding birds have been positively correlated with increased parental care behaviors (Chastel et al., 2005; Khan et al., 2001; Miller et al., 2009; Schoech et al., 1996; Smiley and Adkins-Regan, 2016b; Vleck et al., 1991) and increased reproductive success (Ouyang et al., 2011, 2013; Riechert et al., 2014; Smiley and Adkins-Regan, 2016b), while low PRL concentrations have been related to poor body condition, nest abandonment, low rates of parental care, and offspring mortality (reviewed in Angelier et al., 2016; reviewed in Angelier and Chastel, 2009; Smiley and Adkins-Regan, 2016b). Because of these relationships, there has been a growing interest in PRL's role in regulating life history stages and trade-offs, environmental change produced stress during breeding, and control of breeding decisions (reviewed in Angelier et al., 2016; reviewed in Angelier and Chastel, 2009). However, most studies on PRL in birds to date have been primarily correlational, describing patterns of PRL secretion during the breeding cycle and its relationship with parental behavior. There remains a striking lack of causal experiments that demonstrate PRL's role in regulating various aspects of avian post-hatch parental care.

Moreover, the causal studies that have manipulated PRL during post-hatch

parental care have produced mixed results. The most prominent work on the role of PRL during post-hatch care has been conducted in ring doves (*Streptopelia risoria*), in which PRL has been shown to be causally related to squab feeding and brooding (Buntin et al., 1991; Wang and Buntin, 1999). However, both male and female ring doves feed squabs crop milk which is produced by the epithelial mucosal cells along the wall of the crop sac in response to PRL, an evolved trait unique to pigeons and doves (Buntin, 1996; Patel, 1936), making these findings difficult to generalize to other kinds of birds. One study in house finches (*Carpodacus mexicanus*) showed that pharmacologically increasing or decreasing PRL respectively increased and decreased nestling feeding rates (Badyaev and Duckworth, 2005). Additionally, reducing PRL in black-legged kittiwakes (*Rissa tridactyla*) caring for young reduced nest attendance and motivation to return to the nest following a disturbance (Angelier et al., 2009). Conversely, injections of mammalian PRL in willow ptarmigans (*Lagopus l. lagopus*) during incubation resulted in hatching twice as many chicks as controls and increased post-hatch parental behavior (Pedersen, 1989), and induced squab feeding in ring doves (Buntin et al., 1991). On the other hand, while reducing PRL in male Adélie penguins (*Pygoscelis adeliae*) during the chick-rearing period reduced their diving efficiency, it did not affect chick growth or survival or food foraging durations (Cottin et al., 2014), suggesting that parental behavior, particularly feeding, was not disrupted by inhibiting PRL. Therefore, although PRL is clearly *related* to parental care, it is currently unclear whether PRL has a direct role in parental care behavior of birds that do not produce crop milk or, alternatively, is involved in some other physiological process that coincides with the time of parental care and breeding. Testing this

hypothesis in a wider range of birds will help generalize the role of PRL and will help clarify what the PRL-parental care relationship is.

Importantly, in all species mentioned above that provide care to young (mammals, birds, and fish), an increase in circulating PRL is generally observed in parents *before* the arrival of offspring, which might ensure that the mechanisms are in place to show parental behavior as soon as offspring appear. In many species, high PRL is maintained by the presence of sensory stimuli associated with eggs or young. For example, Silverin and Goldsmith (1990) showed that the PRL peak observed at hatching in pied fly catchers (*Ficedula hypoleuca*) could be prolonged for up to 12 days by repeatedly swapping out chicks for younger ones. On the contrary, PRL was reduced when newly hatched chicks were swapped out for older ones (Silverin and Goldsmith, 1990), demonstrating that the presence of young chicks can stimulate and maintain high PRL levels. In contrast, PRL levels remain high during long foraging trips that can last up to 8-11 days in Adélie penguins which are devoid of all egg or chick contact, a pattern observed in many penguins and other sea birds (Vleck et al., 2000). Therefore, it has been suggested that the role of PRL is to maintain the motivation to consume enough food and return to the chicks, as opposed to directly affecting parental care behavior itself. In support of this, while reducing PRL in male Adélie penguins did not affect foraging trip duration, it did affect diving and foraging effort (Cottin et al., 2014), suggesting males were less motivated to provide food to chicks. In addition, although egg or nest removal is known to drastically reduce PRL in many avian species, treating ring doves with PRL for 10 days following nest removal maintained the interest in resuming incubation, if provided the opportunity to

do so (Buntin, 1996; Sharp, 2009). These studies illustrate the need for more work to decipher what role PRL plays prior to, and after the time of hatching.

In addition, birds with different levels of reproductive experience may have different sensitivities to PRL. Circulating PRL levels have been found to be greater in reproductively experienced birds, compared to inexperienced birds, during egg laying in pigeons (*Columba livia*; Dong et al., 2013), early incubation in wandering albatrosses (*Diomedea exulans*; Angelier et al., 2006), the middle of incubation in common terns (*Sterna hirundo*; Riechert et al., 2012), and during early post-hatch care in black-browed albatrosses (*Thallasarche melanophris*; Angelier et al., 2007). Additionally, seasonal elevation in PRL, which corresponds to breeding stage, is greater in experienced male dark-eyed juncos (*Junco hyemalis*), compared to first time breeding males (Deviche et al., 2000), which together, suggests that experienced birds may have a higher sensitivity to PRL, relative to inexperienced birds. Notably, treating reproductively experienced ring doves with PRL resulted in a greater frequency of regurgitation feedings, greater squab weight gain, and more time spent sitting in the nest than treating inexperienced ring doves with PRL (Wang and Buntin, 1999), suggesting ring doves have an increased sensitivity to PRL as a result of reproductive experience. Aside from ring doves, however, it is currently unknown whether the increase in PRL or PRL sensitivity that comes with experience also regulates any behavior changes that accompany reproductive experience, such as increased chick feeding. Alternatively, PRL may not be as important for experienced birds, if they can rely on learning and memory from previous reproductive cycles, and their behavior may be less hormonally dependent.

One way to increase hormonal sensitivity is through the upregulation of receptors during a particular behavioral or breeding state. For example, in female rats, central prolactin receptor (PRLR) mRNA is either undetectable or is at very low concentrations during diestrus, when circulating PRL is low, but significantly increases 2-3-fold during pregnancy, when circulating PRL is high (reviewed in Grattan, 2001). PRLR upregulation is mediated by pup contact and pup suckling (Sugiyama et al., 1996) and PRLRs are maintained at high concentrations during lactation in the POA, VMH, PVM, arcuate nucleus of the hypothalamus, and the olfactory bulb during times of maternal care (de Moura et al., 2015; reviewed in Grattan, 2001). In addition, peripheral injections of PRL increased central PRL receptor mRNA expression in the POA and arcuate nucleus, areas known to be involved in maternal care and PRL secretion, respectively (Anderson et al., 2006).

Similar to lactating rats, it is plausible that an upregulation in central PRL receptors may occur in zebra finches that are near the end of the incubation cycle and beginning of post-hatch care, when PRL is highest, which may increase the sensitivity to PRL. PRL crosses the blood brain barrier through a specialized protein transport and uptake system at the choroid plexus (reviewed in Buntin, 1996). Part of the mechanism of how PRL affects behavior may involve changes in central PRLR abundance. Although this hypothesis has not yet been directly tested, a central specific binding study using radio-active labeled PRL in Wilson's Phalarope (*Phalaropus tricolor*) suggests that incubating males have increased sensitivity to central PRL binding in the POA and SL (Buntin et al., 1998). In addition, brown headed cowbirds (*Molothrus ater*), which are parasitic breeders and lay their eggs into other birds'

nests, have a much lower density of PRLRs in the POA, relative to ring doves and other birds which do provide care, despite showing an increase in circulating PRL after egg laying, which may explain why they do not provide any parental care (reviewed in Buntin, 1996)[1]. However, in domestic turkeys (*Meleagris gallopavo*) and bantam chickens (*Gallus domesticus*), which do not provide extensive post-hatch parental care, increasing blood levels of PRL during incubation were associated with *decreasing* levels of PRLR mRNA in the hypothalamus, but increasing levels in the pituitary gland (Ohkubo et al., 1998; Zhou et al., 1996). Thus, it is unclear whether there would be an up or down regulation of PRLR, or no pattern of change in PRLR, to increase sensitivity to PRL in avian species that provide extensive parental hatch altricial young.

In addition to upregulating PRLR during certain breeding cycle stages, it is also possible that there are permanent changes in the PRLR distribution occur as a result of reproductive experience. Permanent neural and/or other physiological changes that occur during an animal's first reproductive bout may increase the sensitivity to, or enhance the effectiveness of, the mechanisms that promote parental care. For instance, while experienced, non-breeding female rats have lower circulating PRL levels than age-matched virgin females, they show increased central responsiveness to PRL (Anderson et al., 2006), which may explain why experienced females are quicker to initiate care and may be more responsive to stimuli such as pup

[1] To my knowledge this work was never published beyond a conference abstract, so this should be interpreted cautiously.

odors. On the contrary, circulating PRL levels in breeding zebra finches *increase* after gaining reproductive experience, consistent with a variety of other avian species (reviewed in Smiley and Adkins-Regan, 2016a). In support of this, Christensen and Vleck (2015) found that reproductively experienced zebra finches had nearly 50% more PRL producing cells in the anterior pituitary gland than did age-matched, inexperienced zebra finches. Changes in central nervous system sensitivity to PRL and PRLR abundance may also help explain why plasma levels of PRL increase with experience.

Testing the hypothesis that PRLRs upregulate during post-hatch parental care is hindered by the considerable lack of information on the distribution of PRLR in the avian CNS. Central specific binding sites for PRL have been detected in pigeons, Wilson's phalarope, redwing blackbird (*Agelaius phoeniceus*), European starlings (*Sturnus vulgaris*), and dark-eyed juncos (reviewed in Buntin, 1996), but a detailed mapping and characterization of PRLRs is only complete in ring doves (Buntin et al., 1993; Buntin and Ruzycki, 1987), chickens (Ohkubo et al., 1998b), and turkeys (Zhou et al., 1996). In ring doves, the highest concentrations of PRLRs for both males and females were found in the POA, LHy, TU, VMH, and PVM, as well as several extrahypothalamic brain regions such as the SL, and AC (Buntin et al., 1993; Buntin and Ruzycki, 1987) . Buntin and Buntin (2014) showed that PRLR activation, as measured by pSTAT5-ir (one of the transcription factors that is expressed when the PRLR is activated), is greatest in the POA, LHy, PVM, TU, VMH, BNST, and the SL in ring doves that were in the late incubation and early post-hatch care stage of breeding, relative to non-breeding or incubating birds. A detailed description and

mapping of the PRLR in birds that do not produce crop milk, but care for altricial young, is necessary to make informed hypotheses and predictions in future studies that will systematically manipulate PRL to test for the effects on parental care in other avian species.

In addition, there are very few studies which manipulate PRL in the brain of birds that care for altricial young to know which areas are most important for PRL to affect parental care. To date, ring doves are the only avian species with altricial young in which the neural mechanisms of parental care have been extensively studied (reviewed in Buntin, 1996). Both systemic and central injections of PRL induce parental behavior in male and female ring doves (reviewed in Buntin, 1996). Specifically, ICV injections of PRL into the POA, VMH, or TU stimulate regurgitation and hyperphagia (increased eating) in ring doves (reviewed in Buntin, 1996). Studies which manipulate central PRL in birds other than ring doves, which do not produce crop milk, are necessary to understand PRL's role in avian parental care more generally.

Overall, the lack of causal studies of PRL in birds with altricial young that do not produce crop milk prevents the generalization of a role for PRL in avian parental care and precludes comparative analyses between species to understand to the evolution of parental care. **The hypothesis tested in this dissertation is that PRL plays a causal role in stimulating the onset of parental care, in interaction with reproductive experience, in both sexes of the biparental zebra finch.** The specific **predictions** were that:

1) Circulating PRL concentrations would be low in non-breeding birds, but gradually increase over incubation, peaking highest during late incubation and early post-hatch care, similar to other avian species which hatch altricial young. In addition, circulating PRL would be higher in reproductively experienced birds, compared to inexperienced birds.
2) The variation in parental care behavior and reproductive success would be positively related to variation in plasma PRL levels.
3) Manipulations that increase PRL in non-breeding birds (when PRL is normally low) will stimulate parental care and PRL-treated subjects will provide more care to foster chicks, relative to controls. In addition, reproductively experienced birds will show more care than inexperienced birds.
4) Manipulations that decrease PRL in normally breeding birds (when PRL is high) will decrease the amount of parental care and reproductively experienced birds will be less affected by the decrease in PRL, relative to inexperienced birds.
5) The PRLR distribution will be similar to that of ring doves, having the highest concentrations of PRLRs for both males and females in the POA, LHy, TU, VMH, and PVM, as well as several extrahypothalamic brain regions such as the SL, and AC.
6) Lastly, we predicted that there would be an upregulation in PRLR in breeders, relative to non-breeders, and that is upregulation would be permanent in reproductively experienced birds.

This dissertation provides the first descriptive studies of PRL's relationship with breeding cycle stages and reproductive experience (chapter 1) and the relationship between PRL and variation in early post-hatch parental behavior (chapter 2) in the socially monogamous and biparental zebra finch. In chapters 3 and 4, we provide the first causal evidence that PRL plays a role in the onset of zebra finch parental behavior and show that reproductive experience positively affects parental behavior. Lastly, in chapter 5, we provide the first description of the PRLR distribution in the brains of male and female zebra finches and show that PRLRs are significantly affected by breeding status by comparing the PRLR distribution between breeding and non-breeding brains.

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CHAPTER 1

RELATIONSHIP BETWEEN PROLACTIN, REPRODUCTIVE EXPERIENCE, AND PARENTAL CARE IN A BIPARENTAL SONGBIRD, THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*)

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1.1. ABSTRACT

Parental care encompasses a wide variety of offspring directed behaviors that require major physiological and neurobiological modifications to undertake. Elevations in the peptide hormone prolactin (PRL) have been repeatedly correlated with the onset and maintenance of parental care across vertebrate species. Although a causal role for PRL in parental care has been established in some mammals, ringdoves, chickens, and turkeys, it is unknown whether PRL plays a causal role in the parental care of other bird species, particularly songbirds. The zebra finch, a socially monogamous, biparental songbird, is an exceptionally useful animal model to study parental care and other close social relationships. Both sexes share parental care equally, exhibit the same parental behaviors, and show a marked improvement in breeding success with experience. Nothing is currently known, however, about PRL's involvement in the expression of zebra finch parental behavior or the mechanisms

underlying the improvement in breeding success. We hypothesize that PRL is critically involved in the expression of zebra finch parental care and predict that circulating PRL levels will increase with breeding experience. To begin testing this, we measured plasma PRL concentrations in 14 male-female zebra finch pairs (N=28), using a repeated measures design across two breeding cycles: the first with no previous reproductive experience and the second as experienced parents who had successfully fledged at least one chick. We found that plasma PRL is significantly elevated from non-breeding baseline concentrations during late incubation and early post-hatch care and that this elevation is greater in reproductively experienced birds compared to inexperienced birds. Findings of this study will be used to inform hypotheses and predictions for future experimental manipulations of PRL during parental care.

1.2. INTRODUCTION

The pituitary hormone prolactin (PRL) is involved in many physiological functions and behavioral processes in vertebrates, including osmoregulation, immune response, growth, development, metabolism, the stress response, and reproduction (Ben-Jonathan et al., 2002; Bole-Feysot et al., 1998; Freeman et al., 2000). PRL also plays a role in feeding, the sleep-wake cycle, sexual behavior, and other reproductive behaviors (Ben-Jonathan et al., 2002; Bole-Feysot et al., 1998; Freeman et al., 2000; Whittington and Wilson, 2013). In particular, PRL has a strong relationship with parental behavior and other offspring-directed nurturance behaviors in a wide range of vertebrate taxa.

In female mammals, including humans, circulating PRL is significantly elevated during pregnancy and remains high during lactation (Ben-Jonathan et al., 2008). This rise in PRL has been shown to stimulate the onset of maternal care, such as pup retrieval and pup licking and grooming, in rodents, and has been shown to play a role in increased responsiveness to offspring in some primates (Bridges, 2015; Saito, 2015). The elevation in PRL *before* birth is critical because PRL, in part with a suite of other neural and hormonal mechanisms, primes the mother to be able to show maternal behavior before the offspring arrive, ensuring immediate care upon birth.

Similar to mammals, in virtually all birds studied to date, PRL is low during non-breeding times but is significantly elevated near the end of egg incubation and also during early post-hatch care in both males and females that hatch altricial young (Buntin, 1996; Schradin and Anzenberger, 1999). Because elevated PRL is so tightly linked with the time of intense chick care, researchers have become increasingly interested in using PRL as hormonal predictor of individual variation in reproductive success and parental investment in free-living birds, particularly in passerines. For example, higher PRL has been correlated with earlier egg laying dates (Ouyang et al., 2013a, 2011), higher parental feeding rates and greater numbers of fledglings per year (Ouyang et al., 2011) in free-living great tits (*Parus major*) and house sparrows (*Passer domesticus)* and greater nestling weights in mourning doves (*Zenaida macroura*; Miller et al., 2009). While there are an impressive number of bird species that show a close association between PRL and parental care, most of these species are photosensitive seasonal breeders restricted to the northern temperate climate zone. Furthermore, to our knowledge, there are currently no published experiments

manipulating PRL in any passerine bird during parental care to determine what, if any, causal role PRL is playing in promoting parental behavior. Songbirds, part of the passerine clade, are the most speciose group of birds, the majority of which display intensive parental care. Therefore it would be beneficial to have more information about PRL release during the breeding cycle of an experimentally tractable songbird species in order to design experiments in which PRL is manipulated to determine if it is playing a causal role in behavior.

Zebra finches, a socially monogamous, biparental songbird species, are an excellent model for studying PRL secretion and its potential relationship to parental behavior. Native to the semi-arid regions of Australia, which has unpredictable weather patterns, zebra finches are opportunistic breeders, as they are capable of reproducing anytime of the year following heavy rainfall, when high temperatures and humidity levels occur (Zann, 1996). In the wild, zebra finches are always paired, even when they are not actively breeding, and both members of the pair participate equally in nest building, egg incubation, and post-hatch chick care (Zann, 1996). The average brood size is four chicks, which rely heavily on parental brooding for thermoregulation for at least the first seven days of life and parental feedings for 16-18 days post-hatch, until they fledge from the nest (Zann, 1996). Offspring continue to rely on parental feeding for some time after fledging until they fully transition to self-feeding by 30-40 days post-hatch (Zann, 1996).

Zebra finches are one of the few passerines that breed extremely well in captivity. They perform all of their natural social behaviors, including parental behaviors, in captivity, which makes them ideal for performing experiments that are

both controlled and likely translatable to wild populations. In addition, zebra finches have a sequenced and well-annotated genome, enabling a wide range of advanced genetic and molecular techniques to be used to probe the mechanisms underlying complex social behaviors.

Christensen and Vleck (2008) have previously shown that circulating PRL concentrations are greater in breeding zebra finches compared to paired but non-breeding zebra finches, regardless of sex. Additionally, those researchers found that non-breeding zebra finches with reproductive experience had higher non-breeding baseline PRL concentrations than inexperienced birds. Our study sought to determine the pattern of PRL release across the different stages of an entire breeding cycle, beginning at courtship and pairing and extending until chicks fledged from the nest. Additionally, we tested if this pattern differed with experience within birds across two different cycles: the first cycle when they had no prior reproductive experience and the second as reproductively experienced breeders. We predicted that PRL would remain low throughout pairing and would show a steady rise over the course of incubation, peaking at chick hatching, as previously established in other avian species (Buntin, 1996). However, because songbirds may potentially have a different physiology than non-songbirds and physiological and reproductive mechanisms of opportunistic breeding are often different from seasonal breeders (Perfito et al., 2007), it is critical to determine the pattern of PRL secretion in zebra finches to inform the design of causal experiments. Additionally, we predicted that experienced zebra finches would show higher levels of PRL throughout the breeding cycle compared to inexperienced breeders and that there would be no sex differences in PRL concentrations.

1.3. METHODS

Study population

Subjects included 16 male and 16 female zebra finches (*Taeniopygia guttata*) that were bred in the lab. Subjects were raised in social breeding aviaries (0.94 m × 0.76 m × 0.94 m) consisting of eight adult male-female breeding pairs until they reached parental independence (day 40 post-hatch), at which time they were transferred to sex-specific aviaries. Seed, grit, cuttlebone, and water were available *ad libitum* in all aviaries and boiled egg supplement was provided once a week to the breeding aviaries. All birds were kept on 14L:10D schedule, in a temperature and humidity controlled room. Birds were identified by a unique sequence of colored leg bands and one silver metal leg band engrained with a unique number. Subjects were on average 146 days old (s.d. ± 21.7 days) at the beginning of the experiment. All subjects had no pairing or reproductive experience prior to the experiment.

Study Design

All methods and procedures were approved by the Cornell University IACUC. Prior to the start of the experiment, subjects were randomly selected and assigned to be housed in one of four aviaries such that each aviary housed four males and four non-related, unfamiliar, age-matched females. Each aviary (0.94 m × 0.76 m × 0.94 m) was equipped with four empty nest boxes and coconut fiber nest material, seed, grit, cuttlebone and water *ad libitum*. Boiled egg supplement was provided once a week. Each aviary was located in a separate room with another aviary of 10 adult males hidden behind a white curtain that extended the width of the room. Zebra

finches are highly gregarious and prefer to be in large groups (Goodson and Kingsbury, 2011; Zann, 1996); therefore, the additional male aviaries in the rooms served to provide background conspecific sounds but were hidden to prevent visual contact and interaction with subjects. Subjects' aviaries were set up several days apart to stagger breeding cycles between subjects.

On the first day an aviary was set up, four males and four females were moved from their sex-specific aviary, placed into the new aviary and given five minutes to acclimate. Following the five-minute acclimation period, courtship and pairing behavior was observed and recorded for one hour by two observers who sat behind a curtain with window cut outs to reduce any potential disturbance to the birds. Behavioral observations were performed thereafter for 1hr/day for 10 consecutive days to determine breeding pairs and nest box occupancy. Pair bonds form quickly (typically 1-3 days; Zann, 1996) and allowing zebra finches to choose a partner and a nest box tends to yield greater reproductive success (e.g., Smiley et al., 2012). Each aviary had an assigned observation period, which took place between 0830 and 1100, such that multiple rooms could be observed on the same day. For a detailed description of the behavior observation method, behaviors recorded, and how pair bonds were determined please see Smiley et al., 2012; Vahaba et al., 2013. If birds did not show pairing behavior with one partner exclusively at the end of the 10-day observation period they were removed from the aviary, in addition to removing the unoccupied nest boxes. A total of four birds, two males and two females, and two nest boxes were removed resulting in a final N=28 (14 male-female pairs). After the 10-day observation period, daily nest checks were performed to monitor the breeding

status for each pair for the rest of the experiment.

We left subjects in the aviaries until they completed two consecutive breeding cycles. For each breeding cycle, fledged offspring were kept in the aviary until they reach full independence (day 40 post-hatch). After day 40, offspring were removed and placed into sex-specific aviaries. The male offspring were kept in a small cage (0.6 m × 0.4 m × 0.35 m) next to the parents' aviary so that they could be exposed to their father song-tutors, to avoid disrupting song learning (Eales, 1985), but in a way that would not interfere with the parents' subsequent breeding or behavior. The female offspring were housed in a separate room. When subject pairs completed their second cycle and all offspring had fledged, nest boxes were removed and the experiment concluded.

Blood Sampling

Subjects were repeatedly sampled both within and between their two breeding cycles. Due to zebra finches' small body size (average 13.86 g in this study), each pair was randomly assigned to one of four bleeding schedules such that each bird had a minimum of two weeks to recover between blood samples to avoid physiological stress. Bleeding schedules were staggered such that all time points of interest during the breeding cycle could be collected using the minimum number of subjects. All four bleeding schedules were represented in each aviary, except for one aviary that only housed two pairs, in which case only two randomly assigned bleeding schedules were represented. Thus, each bird was sampled two or three times *per* breeding cycle, according to its scheduled bleeding days. Each bird was sampled at the same time

points in both its first and second breeding cycles. Because we wanted to sample subjects across two consecutive breeding cycles, we did not alter the aviaries between breeding cycles and instead let subjects continue onto a second cycle undisturbed. Therefore, baseline, courtship, and nest building were not represented in the second cycle since partners remained paired and reused the same nest.

To measure baseline PRL concentrations, all subjects had a blood sample taken at least two weeks prior to the start of the experiment, while still in sex-specific aviaries, to allow enough time to recover before being sampled again during the study. At baseline sampling all subjects were sexually mature and had not interacted with opposite-sex conspecifics since separation from the parents. To measure PRL during courtship, two subjects, one male and one female, which had been randomly selected from each aviary prior to the start of the experiment, were bled immediately after the one-hour observation period on the first day of the study. In the lab, zebra finches show the most robust courtship behaviors during the first hour of interacting with opposite-sex conspecifics. Nest-building samples were collected immediately after one of the observation periods during the 10-day observation period in which the subject was observed bringing nest material into a nest box (on average five days after birds were put together in this study). All other blood samples were taken from subjects on the day of the breeding cycle assigned to them from their bleeding schedule. In addition to baseline, courtship, and nest building, blood samples were collected during early incubation (day 4 post-laying), middle of incubation (day 9 post-laying), end of incubation (day 13 of incubation), the day chicks hatched, the middle of post-hatch care (day 7 post-hatch) and the end of post-hatch care, when chicks fledge from the

nest (day 19 post-hatch). All 14 male-female pairs successfully completed their first breeding cycle, defined as raising at least one chick to fledging, and 12 pairs successfully completed their second cycle.

Blood sample collection and processing

We captured birds for bleeding by turning the lights off in the room and locating subjects with flashlights. Blood samples were taken by pricking the alar vein with a 26G needle (BD PrecisionGlide™, Becton, Dickinson and Company) and collecting approximately 100µl of blood in heparinized microhematocrit capillary tubes (Fisherbrand™). PRL concentrations decline with handling stress (Christensen and Vleck, 2008), hence, blood was collected as quickly as possible, which took no longer than three minutes from turning lights off in the room. To control for stress and time-of-day effects we only sampled birds from each room once per day and all birds were bled between 0930 and 1130. Collected blood samples were immediately put on ice and then centrifuged for 4-5 min at 5125*g*. Plasma was extracted using a Hamilton syringe and was stored at -80ºC until assayed for PRL.

Hormone Analysis and Validation

ELISA validation

There is a history of using a RIA to measure PRL in the zebra finch (Vleck and Patrick, 1999). Here we validated a heterologous competitive enzyme linked immunosorbent assay (ELISA) method used by Rochester et al. (2008) as an alternative way to measure plasma PRL. Validation included assessments of intra- and

inter-assay coefficients of variation (CVs), serial dilution of a pooled sample to demonstrate parallelism, and a spike-recovery of a known amount of chicken PRL standard into a pooled zebra finch sample. We collected a pool of plasma from late incubators ("Hi-pool") and non-mated zebra finches ("Lo-pool") based on results from Christensen and Vleck (2008) for intra-assay CV comparison and biological validation. For the intra-assay CV, five samples from each pool were run in duplicate. For the inter-assay CV, the Hi-pool was run in duplicate in each of the five assays used in this study. We used the same purified chicken PRL standard (Dr. A.F. Parlow, National Hormone and Peptide Program, USA) as the PRL RIA validated in Vleck and Patrick (1999).

ELISA protocol

Plasma PRL samples were analyzed following similar methods as Rochester et al. (2008) with some minor modifications as described below. Briefly, 96 well ELISA plates (Nunc MaxiSorp) were coated with 0.1 ml of goat anti-rabbit IgG (Jackson Immunoresearch) diluted 1:2000 in 0.05M phosphate buffer (pH = 7.4) and incubated overnight at 4ºC. Twenty-four hours later, wells were blocked by adding 0.1 ml of blocking solution containing 0.15 M PBS (pH = 7.2), 0.4% Casein and 0.25M EDTA and incubated for two hours at room temperature. After blocking, plates were washed three times (ELX 405 AutoPlate Washer, Biotek Instruments, Inc.) using wash buffer containing 10X PBS diluted 1:50 and 0.05% Tween-20. Fifty microliter samples, either 10 µl of plasma diluted in 40 µl of assay buffer containing 0.15M PBS (pH = 7.2), 0.1% casein, and 0.25M EDTA, or serially diluted chicken PRL standard (Dr.

A.F. Parlow, National Hormone and Peptide Program) in assay buffer were added to wells. We then dispensed 25 µl of biotinylated PRL tracer (generously provided by Dr. I. Rozenboim and Dr. R. Heiblum, The Hebrew University of Jerusalem) diluted 1:50,000 in assay buffer across the plate, followed by 25 µl of rabbit anti-chicken PRL (Dr. A.F. Parlow, National Hormone and Peptide Program) diluted 1:20,000 in assay buffer across wells. Plates were incubated overnight at 4ºC. Following incubation, plates were washed three times with the wash buffer and 0.1 ml of streptavidin horseradish peroxidase diluted 1:5000 in assay buffer was added to each well and incubated for two hours at room temperature. Plates were washed three times and 0.1ml of ABTS reagent was dispensed across all wells. The color reaction was read 30 minutes later (450 µm; Synergy HT plate reader, Biotek). All samples were run in duplicate across five plates.

Statistical Analysis

All statistical analyses were performed using IBM SPSS software, version 21.0 (Armonk, NY: IBM Corp.). We used a generalized linear mixed model (GLMM) to analyze data, which allows for both fixed and random variables to be fitted into the model. The random variables address the non-independence of the data and the repeated measures of subjects. Fixed variables included sample day, experience, and sex. Random variables included cage, pair ID nested within a cage (which also accounts for which bleeding schedule they were assigned too), and subject ID nested within a pair ID nested within an aviary. We initially thought that baseline PRL concentration might contribute to variance in later PRL concentration measures but

when included as a fixed variable it was non-significant (p=0.12). Including baseline PRL as a random variable does not explain any additional variance and as such, was not included in the final model. All main effects and possible interactions, including all two- and three-way interactions, were initially modeled. We then removed all non-significant main effects and interactions via stepwise backwards elimination until only significant terms remained.

1.4. RESULTS

ELISA Validation

The serial dilution was parallel to the standard curve for chicken PRL (Fig. 1.1.). The limit of detection (i.e., sensitivity) was 0.8 ng/ml based on 2 standard deviations from the mean for the B0, run 8 times, which is approximately equal to the lowest chicken standard. The spike-recovery resulted in 74% recovery. The intra-assay coefficient of variation (CV) for the Hi-pool, with a mean of 17.3 ng/ml, was 9.1% and for the Lo-pool, with a mean of 4.9 ng/ml, was 20.3%. These means are similar to the results achieved by Christensen and Vleck (2008) using the RIA. The inter-CV for the study plates was 30.19%.

Relationship between PRL, reproductive experience, and breeding cycle stage

We found a significant main of effect of sample day on PRL concentrations ($F_{5, 24} = 15.34$, $p < 0.001$). Pairwise comparison analysis with a Bonferroni correction applied revealed significant differences between specific days as displayed in figure 1.2.

Figure 1.1. Standard curve for chicken prolactin standard and zebra finch prolactin dilution

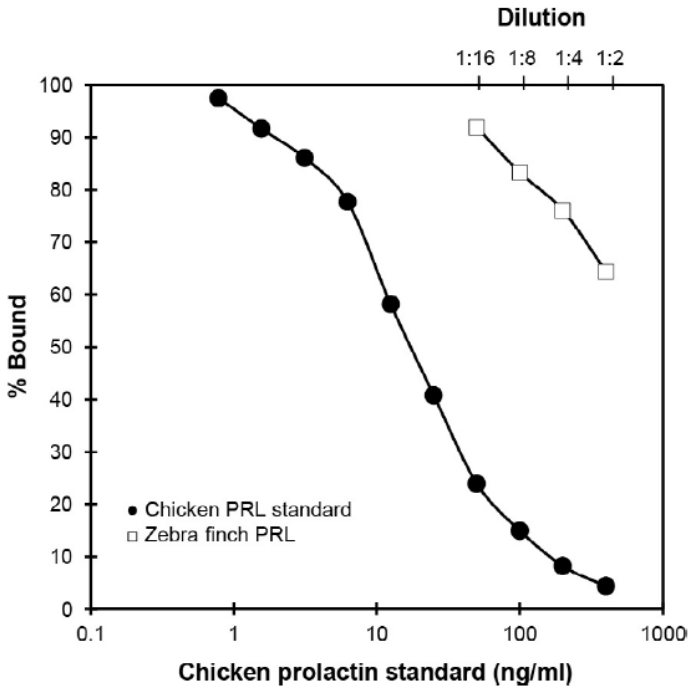

Figure 1.1. The zebra finch prolactin dilution curve is relatively parallel to the chicken prolactin standard reference hormone, showing that this heterologous ELISA can be used to assay relative levels of plasma prolactin in zebra finches.

We also found a significant main effect of experience ($F_{1, 24} = 9.955$, $p = 0.004$) such that experienced subjects had greater overall mean PRL than when they were inexperienced, but following the same general pattern as when inexperienced (Fig. 1.3.). There was no significant effect of sex on PRL, nor any significant interactions between any variables. Additionally, neither pair ID nor cage ID (random effects) explained any variance in PRL values. Reproductive outcomes based on reproductive experience are listed in table 1.1.

Even though samples were assayed as they were collected, i.e., they were not randomized across ELISA plates; we do not believe the relatively high inter-assay CV

Figure 1.2. PRL concentrations across different breeding cycle stages in the zebra finch

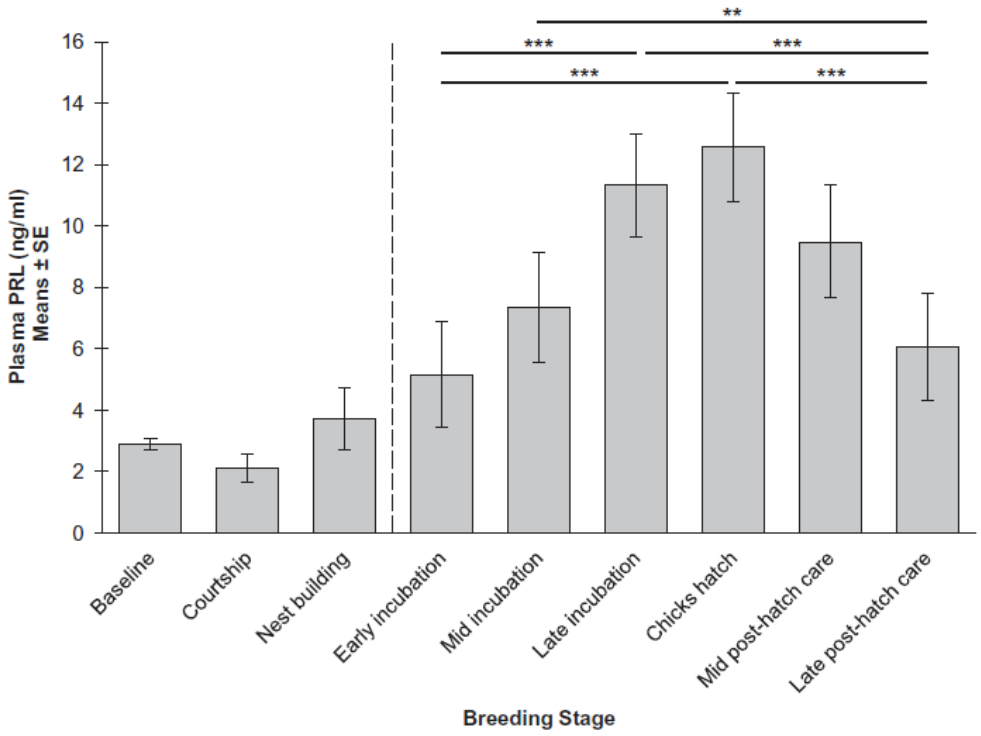

Figure 1.2. Shaded bars represent average PRL concentrations (y-axis) measured at various times in breeding cycle (indicated on the x-axis). Error bars represent standard error (SE). Solid bars with asterisks denote significant differences in PRL concentrations between breeding cycle stages (***$p < 0.001$; **$p < 0.01$). Breeding stages to the left of the dashed line were only measured during the first of two breeding cycles in this study. Breeding stages to the right of the dashed line were measured in both the first and second breeding cycle. Values shown for repeatedly measured time points are estimated marginal means from the GLMM to account for repeated measures. Sample sizes are listed in Table 1.2.

Figure 1.3. PRL concentrations by reproductive experience across the zebra finch breeding cycle

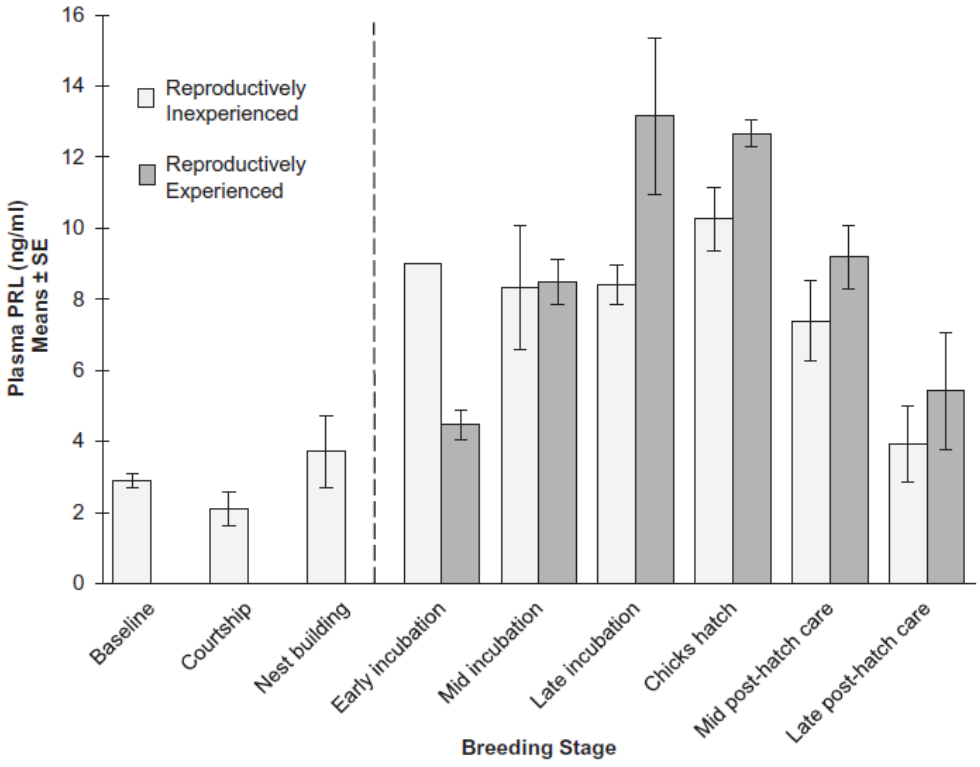

Figure 1.3. Lightly shaded bars represent average PRL concentrations (y-axis) measured in reproductively inexperienced zebra finches at various times in breeding cycle (indicated on the x-axis). Darkly shaded bars represent average PRL concentrations in reproductively experienced zebra finches. Error bars represent standard error (SE). Breeding stages to the left of the dashed line were only measured in reproductively inexperienced birds. Breeding stages to the right of the dashed line were repeatedly measured in subjects during their first (reproductively inexperienced) and second (reproductively experienced) breeding cycles. Values shown for repeatedly measured time points are estimated marginal means from the GLMM to account for repeated measures. Sample sizes are listed in Table 1.2.

influenced our results for several reasons. First, our mean PRL values for breeders during late incubation and baseline non-breeders in this study and in our assay validation are very similar to the values obtained by Christensen and Vleck (2008), who sampled birds at identical time points ("breeders" were sampled during late incubation or just after chicks hatched and "non-breeders" were paired, but not actively breeding). To rule out the possibility that the high inter-assay CV accounted for our results, we corrected for plate variation in the PRL data by calculating a correction value for each plate's data by dividing the mean value of all five Hi-pool samples used to calculate the inter-assay CV by the Hi-pool value for each plate. We then standardized our PRL data by multiplying each PRL value by the correction value to adjust each plate's value accordingly. We then re-ran the same statistical model as described in section 2.6. Using the standardized data, both main effects of sample day ($F_{5, 27} = 14.72$, $p < 0.001$) and experience ($F_{1, 28} = 5.90$, $p = 0.022$) remain significant. All pairwise comparisons with a Bonferroni correction applied remain significant with the addition of a significant difference between beginning incubation and middle incubation ($p = 0.045$). Because our data remain significant when standardized, we are confident we are capturing real effects and thus report results from our original (non-standardized) data in the figures.

Table 1.1. Reproductive outcomes based on reproductive experience.

Panel A describes data collected in the 12 pairs that successfully completed two breeding cycles. Panel B shows the comparison of clutch size, chicks hatched, and chick survival between cycles using a Wilcoxin signed-ranks tests. ***Indicates significance at $p<0.01$.

			Latency to pair-bond (days)	Latency to build nest (days)	Clutch size	Number of chicks hatched	Number of chicks that survived to fledging
A.	Cycle 1: Reproductively Inexperienced	Mean	2.07	5.00	5.67	3.50	3.25
		Standard Deviation	1.27	1.71	1.30	1.57	1.91
	Cycle 2: Reproductively Experienced	Mean	--	--	7.45	3.75	3.50
		Standard Deviation	--	--	1.67	1.54	1.83
B.	Difference between cycle 1 and cycle 2	Z-score	--	--	-2.976	-0.284	-0.06
		N =			12	12	12
		p-value	--	--	0.003 **	0.776	0.952

Table 1.2. Sample sizes for effect of breeding cycle stage and reproductive experience on plasma PRL.

Samples sizes, broken down by sex, correspond with Fig. 1.2. (total N) and Fig. 1.3. (cycle 1 and cycle 2).

		Sample Day						
		Early incubation	Mid incubation	Late incubation	Chicks hatch	Mid post-hatch care	Late post-hatch care
Cycle 1: Reproductively Inexperienced	Female	1	1	2	2	2	3
	Male	0	1	3	3	3	3
	N =	1	2	5	5	5	6
Cycle 2: Reproductively Experienced	Female	2	3	2	1	2	2
	Male	3	3	1	2	2	2
	N =	5	6	3	3	4	4

1.5. DISCUSSION

Pattern of PRL across the breeding cycle

The pattern of circulating plasma PRL during the breeding cycle of the zebra finch is similar to previous findings in non-opportunistically breeding songbirds and other non-passerine avian species that hatch altricial young (Buntin, 1996; Crossin et al., 2012; Riou et al., 2010; Setiawan et al., 2006; Sockman et al., 2006). Our findings show that PRL remains at low levels until egg laying, after which it begins to gradually increase, peaking at the time of hatching. PRL remains high for at least the first week of post-hatch care and then slowly declines back to non-breeding baseline levels by the time the chicks fledge. These patterns have been observed in both males and females in species that provide post-hatch care (Buntin, 1996). Interestingly, in cooperatively breeding avian species, both breeders and non-breeding helpers also show similar patterns of PRL elevation across the breeding cycle as described above. In non-breeding helpers, elevated PRL concentrations during the post-hatch period have been associated with levels of feeding nestlings (Schoech et al., 1996; Vleck et al., 1991) and nest provisioning (Khan et al., 2001). In fact, during the post-hatch period in Harris' hawks (*Parabuteo unicinct*) male helpers, who provide more food to nestlings then breeders do, have higher circulating PRL concentrations than breeding males and females (Vleck et al., 1991). In contrast, in birds hatching precocial young, which require little post-hatch care, PRL tends to return to baseline levels almost immediately after hatching, a striking difference compared to breeders hatching altricial young. Taken together, these patterns suggest that PRL may play a crucial role in promoting offspring-directed behaviors.

In many seasonally breeding birds, PRL is thought to become elevated in response to increasing day length (Buntin, 1996), which signals an appropriate time to begin breeding. Zebra finches are opportunistic breeders and breeding is not strictly limited to any particular season or time of year (Bentley et al., 2000; Perfito, 2010; Zann, 1996). Instead, breeding is primarily triggered by high humidity and rainfall. PRL may be stimulated by these environmental cues, in response to egg and/or nest stimuli, or some other internal physiological response that corresponds with incubation. Mechanistically, PRL seems to be under similar stimulatory control by the vasoactive intestinal polypeptide system as in other passerines including white-crowned sparrows (*Zonotrichia leucophrys gambelii*), dark-eyed juncos (*Junco hyemalis*), Florida scrub-jays (*Aphelocoma coerulescens*), western scrub-jays (*Aphelocoma californica*), Mexican jays (*Aphelocoma ultramarine*), and blue jays (*Cyanocitta cristata*) (Christensen and Vleck, 2008; Maney et al., 1999; Vleck and Patrick, 1999). However, understanding which environmental, social, or other external or internal cues trigger this cascade of events which leads to increased PRL secretion during the breeding cycle may differ across species and will be important to determine.

We found no sex differences in PRL during the breeding cycle. This was expected as zebra finches are biparental and both males and female contribute roughly equally to nest building, incubation, and chick care. While we did not collect behavioral data in the parents during incubation or post-hatch care, other studies in zebra finches have found that nest visits are generally synchronized between breeding pair partners (Mariette and Griffith, 2012; van Rooij and Griffith, 2013) and that pair

synchrony increases with greater parental investment (i.e., larger brood sizes; Mariette and Griffith, 2015). If our hypothesis that PRL promotes parental behavior (including nest visits) is correct, then one could predict that PRL concentrations would be more strongly correlated in reproductively successful male-females pairs, compared with less successful breeding pairs. However, it is not always the case that PRL concentrations are similar between sexes even with biparental care (Buntin, 1996). For example, in wandering albatrosses (*Diomedea exulans*), females have higher PRL than males, even though both participate in parental care at similar rates (Angelier et al., 2006). This may indicate that PRL is playing a role in some other physiological sex difference that coincides with parental care, and not necessarily in the behavior itself.

Given that PRL only remains elevated after hatching in species with altricial young, it is tempting to say that PRL must be causally involved in post-hatch parental care. However, the only strong evidence for PRL playing a significant role in post-hatch parental care in any bird with altricial young comes from experiments with ring doves (*Streptopelia risoria*). Both systemic and central injections of PRL increased offspring feeding invitations, regurgitation feeding, and crop-sac milk production, a unique adaptation of the crop-sac organ in ring doves which provides a food source for the offspring, analogous to lactation in mammals (Buntin, 1996). However, crop-milk production is PRL-dependent and unique to ring doves, so it is not known whether the role of PRL in avian post-hatch parental care can be generalized to other avian species with altricial young such as passerines. Thus, evidence for a causal role of PRL is needed in more avian species before we can conclude that PRL plays a role in avian parental care generally. Given that PRL peaks just before hatching (late incubation)

and right at the time of hatching in zebra finches, one could hypothesize that PRL may play a causal role in promoting the onset of parental behavior in zebra finches as well.

Experience effects

While the pattern of PRL secretion during the breeding cycle has been established in many species of birds, fewer researchers have systemically compared circulating PRL levels in both reproductively inexperienced and experienced birds at similar time points during the breeding cycle. Our study found a significant main effect of experience on PRL concentrations, such that reproductively experienced birds have an overall greater increase in PRL than inexperienced birds between egg laying and chick fledging. Circulating PRL levels have also been found to be greater in reproductively experienced birds, compared to inexperienced birds, during egg laying in pigeons (*Columba livia*; Dong et al., 2013), early incubation in wandering albatrosses (*Diomedea exulans*; Angelier et al., 2006), the middle of incubation in common terns (*Sterna hirundo*; Riechert et al., 2012), and during early post-hatch care in black-browed albatrosses (*Thallasarche melanophris*; Angelier et al., 2007). Additionally, seasonal elevation in PRL, which correspond to breeding stage, is greater in experienced male dark-eyed juncos (*Junco hyemalis*), compared to first time breeding males (Deviche et al., 2000), suggesting this phenomenon is not limited to zebra finches or songbirds necessarily. We used a repeated measures design in order to see the effects of experience within subjects. However, we did not interrupt the continuation onto the second breeding cycle after the first clutch of chicks fledged, so our repeated measures begin at egg-laying since zebra finches remain paired and reuse

the same nest. However, Christensen and Vleck (2008) found that reproductively experienced zebra finches had higher non-breeding baseline PRL concentrations than did inexperienced breeders, so conceivably circulating PRL levels could also have been higher during courtship and nest building in experienced birds as well, had we measured that time point twice.

A more recent study by Christensen and Vleck (2015) reported that reproductively experienced zebra finches had nearly 50% more PRL producing cells in the anterior pituitary gland than did age-matched, inexperienced zebra finches. This effect was observed in both experienced breeders and non-breeders, suggesting that reproductive experience permanently alters the morphological structure of the anterior pituitary, which may be a potential mechanism for the elevated secretion of PRL observed during subsequent breeding attempts in this study.

There were no sex differences in the experience effect on plasma PRL in the zebra finches. Both males and females showed a similar pattern of PRL secretion across both breeding cycles, as well as similar PRL concentrations at each time point measured. Since both males and females participate in the incubation of eggs and rearing of the young roughly equally and are gaining similar amounts of experience, this result was expected. Interestingly, in both wandering albatrosses and common terns, where females have higher circulating PRL then males, PRL continues to increase in males with experience whereas females show no further increase with gained experience (Angelier et al., 2006; Riechert et al., 2012). We only measured two breeding cycles (in only one of which birds were experienced) so it is unknown whether PRL would continue to increase with further experience. However, both

seabirds mentioned above are long-lived avian species (40-50 years) that breed once a year or every other year, so the seabird effect may not generalize to a shorter-lived songbird that breeds more frequently and opportunistically.

In many studies reproductive experience is confounded with age, which may be playing a large factor in parental behavior and PRL secretion. All the studies cited above, with the exception of the pigeons (Dong et al., 2013), have been conducted on free-living species, so it is impossible to disentangle the effects of age independent of reproductive experience on hormones and behavior. Even in our study, subjects were of similar age but inevitably subjects were older when they underwent their second breeding cycle (on average subjects were 49 days ± 13 days older when they started their second clutch in this study). Age affects parental investment decisions (Royle et al., 2012), which may affect parental behavior and possibly influence PRL secretion patterns (Préault et al., 2005). Additional laboratory experiments which measure hormones in age-matched individuals varying in reproductive experience would likely separate the effects of age and reproductive experience.

The next question is whether this increase in circulating PRL in reproductively experienced birds has any functional significance. In many birds, including zebra finches, reproductive success generally improves with experience as determined by fitness measures such as earlier lay dates (Adkins-Regan and Tomaszycki, 2007; Baran and Adkins-Regan, 2014; Ouyang et al., 2013a), increased hatching success (Riechert et al., 2014), and increased nestling weights (Miller et al., 2009). However, the physiological mechanisms underlying these changes have received much less attention. High PRL titers have been correlated with increased reproductive success

generally throughout the breeding cycle, and thus, may be a candidate mechanism for the improvement in reproductive success. For example, higher pre-breeding baseline PRL correlates with earlier laying dates in free-living great tits (*Parus major*; Ouyang et al., 2013b) and earlier egg laying dates and total numbers of fledgings for the breeding season in free-living house sparrows (*Passer domesticus*; Ouyang et al., 2011). Additionally, higher PRL during the middle of incubation in male and female common terns predicted increased hatching success (Riechert et al., 2014) and PRL measurements taken 2-4 days post-hatch in male and female mourning doves (*Zenaida macroura*) correlate with early post-hatch nestling weight (Miller et al., 2009). Conversely, low circulating PRL levels are associated with nest abandonment, poor body condition, poor environmental conditions (Angelier and Chastel, 2009), and egg predation (Angelier et al., 2013).

Are any of these changes in reproductive success mediated by behavioral changes induced by PRL? Despite the significant correlations, a causal role for PRL in increased reproductive success with experience has yet to be determined in any bird other than the ring dove. Reproductively experienced ring doves treated with PRL show a greater frequency of regurgitation feedings, greater squab weight gain, and spend more time sitting in the nest than PRL-treated inexperienced ring doves (Wang and Buntin, 1999), suggesting there is increased sensitivity to PRL as a result of reproductive experience. If PRL is indeed facilitating reproductive success behaviorally then PRL could affect parental care behaviors in two ways: either quantitatively, whereby the overall amount of parental behavior increases, or by affecting care qualitatively, whereby the changes would be more subtle, such as

increased intensity or quality of care via increased chick attentiveness, pair synchrony, and more energy efficient behaviors. Interestingly, in the present study, the experience effects are most prominent during late incubation and throughout the post-hatch period. Previous studies from our lab have shown that reproductive success improves with experience (Adkins-Regan and Tomaszycki, 2007; Baran and Adkins-Regan, 2014), which taken together, suggest the hypothesis that behaviors related to increased reproductive success may be influenced PRL secretion, or vice-versa. Experimental manipulations of PRL in age-matched birds that vary in reproductive experience would be illuminating to as to which factor(s) influence avian parental care.

Conclusions

In conclusion, elevated PRL is highly correlated with the parental phase of the breeding cycle in zebra finches, a pattern found in many birds that hatch altricial chicks requiring intensive parental care. Considering the relationship between PRL and parental care across species, there may be conserved functions of PRL that are important for promoting parental care. However, the role of PRL parental care is still largely unknown in passerines and most other avian groups, despite birds being the most biparental vertebrates and the extensive correlational evidence that suggest this relationship. Clearly more experimental work with PRL should be done to establish the role of PRL in avian parental care. In addition, comparative analyses across taxa can elucidate the degree of commonality between hormonal mechanisms and between mammals and birds, which is important for understanding the evolution of this hormone-behavior relationship.

In addition, there is a positive association between reproductive experience and PRL. However, it is unclear what the functional role, if any, this has in the increased reproductive success that tends to come with experience and/or age. Considering the similar associations between PRL and parental care across species, one could hypothesize that PRL has some conserved roles in promoting various aspects of offspring responsiveness and care, but until the right manipulation experiments are conducted in variety of species differing in social life histories, ecological backgrounds, and reproductive systems we cannot perform the comparative analyses needed to investigate this possibility. Understanding the role of PRL in more avian species will provide fruitful insights into the evolution of prolactin-mediated behavior and parental care generally.

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CHAPTER 2

PROLACTIN IS RELATED TO INDIVIDUAL DIFFERENCES IN PARENTAL BEHAVIOR AND REPRODUCTIVE SUCCESS IN A BIPARENTAL PASSERINE, THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*)

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2.1. ABSTRACT

Variation in parental care can lead to important fitness consequences. The endocrine system is known to regulate physiological and behavioral reproductive traits that are important contributors to lifetime reproductive success. However, the hormonal basis of variation in avian parental care is still not well understood. Plasma prolactin (PRL) concentrations are generally high during post-hatch parental care in birds, and may be a candidate mechanism that regulates variation in parental care and other reproductive success outcomes. Here we analyze the relationship between PRL, parental behavior (chick brooding and feeding), and reproductive success outcomes (clutch size, number of chicks hatched, and chick survival) for the first time in the zebra finch (*Taeniopygia guttata*). Birds were given cabergoline, a dopamine agonist traditionally used to lower prolactin in mammals, or vehicle in their food. Cabergoline had no effect on prolactin concentrations, but across both groups we found that PRL is

positively correlated with parental behavior, number of chicks hatched, and chick survival, but not clutch size. Results from this study will inform hypotheses and predictions for future manipulation studies which test for a causal role for PRL in parental traits.

2.2. INTRODUCTION

Variation in parental care can play a critical role in determining an offspring's phenotype and survival (Royle et al., 2012), and so has important fitness consequences. Therefore, it is important to understand the underlying sources and mechanisms behind this variation in order to gain a fuller understanding of the evolution of this reproductive behavior. The neuroendocrine systems are known to regulate various reproductive traits that make up aspects of an individual's reproductive phenotype, as well as to coordinate physiological and behavioral processes in response to internal and external cues to maximize fitness. As such, hormonal measurements have become an increasingly popular tool to predict reproductive effort and success in free-living birds. However, our understanding of the physiological and hormonal basis of phenotypic variation in reproductive traits and behaviors that contribute to lifetime fitness is still rudimentary (Williams, 2012). A more in-depth understanding of the dynamics of hormone-behavior relationships will inform hypotheses and predictions for future experimental manipulations which test for a causal role of hormonal contributions to individual variation in parental care.

One potential source of individual variation in parental care and other reproductive traits may come from prolactin (PRL) during breeding. Plasma PRL is

significantly elevated above the low, non-breeding baseline levels during late incubation and post-hatch care in many birds that hatch altricial young (Angelier et al., 2016; Buntin, 1996; Smiley and Adkins-Regan, 2016; Sockman et al., 2006) and is thought to play a significant role in promoting the onset of parental behavior (Angelier et al., 2016). Because of this pattern, researchers have become increasingly interested in using PRL as hormonal predictor of individual variation in reproductive success and parental investment in free-living birds, particularly in passerines. For example, higher pre-breeding baseline PRL concentrations correlate positively with earlier laying dates in free-living great tits (*Parus major*; Ouyang et al., 2013) and earlier egg laying dates and total numbers of fledglings for the breeding season in free-living house sparrows (*Passer domesticus*; Ouyang et al., 2011). PRL has also been found to be positively correlated with hatching success in wild common terns (*Sterna hirundo*; Riechert et al., 2014), nestling feeding and provisioning rates in house finches (*Carpodacus mexicanus*; Badyaev and Duckworth, 2005; Duckworth et al., 2003), black-legged kittiwakes (*Rissa tridactyla*; Chastel et al., 2005), red-cockaded woodpeckers (*Picoides borealis*; Khan et al., 2001), Florida scrub-jays (*Apheloma e. coerulesens*; Schoech et al., 1996) and Harris' hawks (*Parabuteo unicinctus*; Vleck et al., 1991), and nestling weight in mourning doves (*Zenaida macroura*; Miller et al., 2009). Conversely, low PRL is associated with poor environmental conditions, poor body condition, breeding failure, and nest abandonment (reviewed in Angelier and Chastel, 2009; Angelier et al., 2016). However, it is still unknown whether variation in reproductive success is a result of different PRL concentrations altering parental care behavior, or whether the variation in PRL concentrations observed is a result from

cues from external breeding stimuli.

There is evidence for a bidirectional relationship between elevated PRL and parental behavior, and they likely feedback onto one another reciprocally. For instance, maintenance of elevated PRL during incubation depends on physical contact with the nest and eggs in avian species that hatch precocial young, such as galliformes (poultry) and anseriformes (ducks), (reviewed in Angelier et al., 2016; Buntin, 1996; Sockman et al., 2006). Removal of the nest or eggs during incubation results in a decline in PRL, while replacing the nest or eggs after removal reinstates elevated levels of PRL (reviewed in Buntin, 1996). Likewise, in avian species that hatch altricial young, PRL is highest immediately post-hatch, when the most intensive parental care occurs. Experimentally replacing chicks with younger ones can prolong the period of elevated PRL, while replacing chicks with older ones can truncate the period of elevated PRL (reviewed in Buntin, 1996). Taken together, elevated PRL may be necessary to show parental behavior, but this elevation may depend on breeding stimuli, and possibly other external and internal conditions, such as weather and body condition.

In order to begin analyzing the relationship between PRL, parental behavior, and reproductive success further, we measured plasma PRL concentrations on day two post-hatch and related them to variation in parental behavior and other reproductive success measures for the first time in male and female zebra finches (*Taeniopygia guttata*). We, and others, have shown that plasma PRL concentrations are significantly elevated above the non-breeding baseline during late incubation and during early post-hatch care in male and female zebra finches (Christensen and Vleck, 2008; Smiley and

Adkins-Regan, 2016). Males and females are socially monogamous and contribute roughly equally to nest building, egg incubation, and post-hatch chick care (Zann, 1996). In addition, parental behavior between breeding partners tends to be well synchronized (Mariette and Griffith, 2012; Van Rooij and Griffith, 2013). Thus, we hypothesized that PRL would be correlated with the amount of parental care behavior displayed immediately post-hatch. In addition, since breeding stimuli, such as eggs and chicks, also appear to influence or maintain elevated PRL concentrations, we hypothesized that PRL would be positively related to other reproductive success measures including clutch size, number of chicks hatched, and chick survival to fledging. Results from this study will inform hypotheses and predictions for future manipulation studies which test for a causal role for PRL in parental traits.

2.3. METHODS

Subjects

Subjects were 12 zebra finches (6 males and 6 females) that were bred in the lab (Cornell University, Ithaca, NY). All subjects were reproductively mature adults, but age and reproductive history were unknown for most subjects. All birds were kept on a 14:10 light:dark schedule, in a temperature and humidity controlled room. Birds were identified by a unique sequence of colored leg bands and one silver metal leg band engraved with a unique number. Prior to the start of the experiment, subjects were housed in sex-specific aviaries (0.94 m × 0.76 m × 0.94 m) with seed, grit, cuttlebone, and water available *ad libitum*. All methods and procedures were approved by the Cornell University IACUC.

Study design

Breeding pairs

Four of the 12 subjects (i.e., two pairs) were previously established pairs from our lab's breeding colony. The other eight subjects were pairs that formed in social aviaries that housed four males and four unfamiliar females for one week prior to the study. Daily 30-minute behavioral observations took place during this week to determine which birds were paired (see Smiley et al., 2012 and Vahaba et al., 2013 for methods). Once pairs were determined, each pair (including the two established pairs) were moved to separate testing cages (0.6 m × 0.4 m × 0.35 m), each equipped with a nest box, and nesting material, seed, grit, cuttlebone, and water available *ad libitum*. Testing cages were housed in two different rooms to allow for blood sampling across multiple subjects on the same day if needed. Daily nest checks were performed to look for eggs and chicks in order to monitor the breeding status for each pair. Incubation typically lasts for 14 days (Zann, 1996). Chicks rely on parental brooding for thermoregulation for at least the first seven days of life and rely on parental feedings for 16-18 days post-hatch, which is around the time that they fledge from the nest (Zann, 1996). Offspring continue to rely on parental feeding for some time after fledging, but are fully transitioned to self-feeding by 30-40 days post-hatch (Zann, 1996).

Cabergoline manipulation

The study was originally intended to be a pilot study to look at the effects of orally administered cabergoline, a potent and orally active dopamine D2 receptor

agonist in mammals (Kvernmo et al., 2006), on circulating prolactin levels and parental behavior. Pairs were evenly divided into cabergoline treatment or vehicle groups, but cabergoline treatment did not affect PRL concentrations (see section 2.4. *Cabergoline treatment*), so the two groups were combined for behavioral analysis in this study. Briefly, if females laid one egg each day for four consecutive days, pairs were determined to have reached breeding status. Incubation was recorded as beginning on the first day an egg appeared in the nest. Egg viability was checked throughout the incubation cycle by candling eggs with flashlights. All 6 nests had at least one fertile egg in their nest prior to treatment. Beginning on day 12 of incubation, half of the pairs (n=6 birds) received 0.05 ml of cabergoline (Sigma-Aldrich C0246; dose = 0.25 mg/kg body weight based on Brooks et al., 2005) dissolved in fractionated coconut oil on top of 1.3 g of hardboiled egg. The other half of the pairs (n=6 birds) received 0.05 ml of the vehicle alone (control) on top of 1.3 g of hardboiled egg. Each member of the pair received the same treatment. Treatments were randomly assigned to pairs. All subjects received five daily doses of their assigned treatment, approximately 24 hours apart. Treatments were administered each morning during the last three days of incubation (incubation days 12, 13, 14) and during the first two days of post-hatch care. Seed dishes were removed at this time to encourage egg eating and returned two hours later. All of the egg was typically consumed during this two-hour period. Parental behavior was recorded on days one and two post-hatch in the mornings after the egg was provided (see *Recording and measuring parental behavior* for details). On the last day of treatment (day two post-hatch) a blood sample was taken three hours after birds were given the egg mixture (see *Blood sample collection*

and processing for blood sampling methods).

Recording and measuring parental behavior

To video record behavior inside the nest, web cameras (Logitech C600) were modified by removing the casing and the IR filter and attached to the outside of clear plastic nest boxes. The back of each nest box had a small opening where the lens rested at an angle to view inside the nest. The camera's view was cleared of any obstructing nesting material prior to recording. Cameras were attached to nests several days before recording started so that subjects could habituate to their presence. Cameras were connected to computers (Dell Optiplex 745) and videos were recorded using Eyeline Video Surveillance Software, version 1.18 (NCH Software). Videos were on average 198 minutes long (±s.d. 48 minutes). Video files (saved as MP4 files) were then coded for behavior using the software ELAN, version 4.6.2. (Max Planck Institute for Psycholinguistics, Nijmegen, The Netherlands) in a randomized order by a trained coder who was blind to treatment. Behavior coding was based on previous work scoring parental behavior in zebra finches (Gilby et al., 2011). Brooding behavior (the time spent sitting on top of chicks) and feeding behavior (the time spent regurgitating to chicks) were coded for each individual separately.

Blood sample collection and processing

We captured birds for bleeding by turning the lights off in the room and locating subjects with flashlights. Blood samples were obtained by pricking the alar vein with a 26 G needle and collecting approximately 100 µl of blood in heparinized

microhematocrit capillary tubes. PRL concentrations decline with handling stress (Christensen and Vleck, 2008), hence, blood was collected as quickly as possible, which took no longer than three minutes from turning lights off in the room. To prevent stress and time-of-day effects we only sampled birds from each room once per day and all birds were bled between 1130 and 1300. Collected blood samples were immediately put on ice and then centrifuged for 4-5 min at 5125 *g*. Plasma was extracted using a Hamilton syringe and was stored at -80ºC until assayed for PRL.

Measuring plasma PRL

Plasma PRL samples were analyzed using a validated heterologous competitive enzyme linked immunosorbent assay (ELISA), as in Smiley and Adkins-Regan (2016) with slight modification. Briefly, 96 well ELISA plates (Nunc MaxiSorp) were coated with 0.1 ml of goat anti-rabbit IgG (Jackson Immunoresearch) diluted 1:2000 in 0.05M phosphate buffer (pH = 7.4) and incubated overnight at 4ºC. Twenty-four hours later, wells were blocked by adding 0.1 ml of blocking solution containing 0.15 M PBS (pH = 7.2), 0.4% Casein, and 0.25M EDTA and incubated for two hours at room temperature. After blocking, plates were washed (ELX 405 AutoPlate Washer, Biotek Instruments, Inc.) three times using wash buffer containing 10X PBS diluted 1:50 and 0.05% Tween-20. Fifty microliter samples, either 10 µl of plasma diluted in 40 µl of assay buffer containing 0.15M PBS (pH = 7.2), 0.1% casein, and 0.25M EDTA, or serially diluted chicken PRL standard (Dr. AF Parlow, National Hormone and Peptide Program) in assay buffer were added to wells. We then added 25 µl of biotinylated PRL tracer (kindly provided by Dr.s I. Rozenboim and R.

Heiblum, Hebrew University of Jerusalem) diluted 1:50,000 in assay buffer, followed by 25 µl of rabbit anti-chicken PRL antiserum (Dr. AF Parlow, National Hormone and Peptide Program) diluted 1:45,000 in assay buffer to wells. Plates were incubated overnight at 4ºC. Following incubation, plates were washed and 0.1 ml of streptavidin horseradish peroxidase diluted 1:30,000 in assay buffer was added to each well and incubated for two hours at room temperature. Plates were washed again and 0.1ml of ABTS (2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was dispensed across all wells. The color reaction was read 45 minutes later (450 µm; Synergy HT plate reader, Biotek). All samples were run on one plate. The intra-assay coefficients of variation were 9.42% (high-PRL pool) and 17.21% (low-PRL pool).

Statistical Analysis

All statistical analyses were performed using IBM SPSS software, version 21.0 (Armonk, NY: IBM Corp.). We used generalized linear mixed models (GLMM), which allow for both fixed and random variables to be fitted into each model. We ran a GLMM testing for the effects of cabergoline treatment and sex (fixed factors) on PRL concentrations. We also tested whether PRL concentrations were related to brooding behavior and feeding behavior. Feeding behavior was arc-sin transformed to normalize the data. We also tested whether PRL was related to clutch size, the number of chicks that successfully hatched, and whether or not at least one chick survived to fledging (coded as a binary yes or no response). For each model, a pair ID variable was included as a random factor to control for being paired and housed with another subject and to address the non-independence of the data. Finally, the correlation

between brooding and feeding behavior was analyzed using Pearson's R test.

2.4. RESULTS

Cabergoline treatment

We found no effect of treatment ($F_{1, 3} = 0.229$, $p = 0.665$), sex ($F_{1, 3} = 0.008$, $p = 0.933$), or interaction between treatment and sex ($F_{1, 3} = 3.761$, $p = 0.148$) on plasma PRL concentrations. See figures 2.1.A. and 2.1.B. for PRL means for treatment and sex, respectively.

Figure 2.1. No effect of cabergoline treatment or sex on plasma PRL concentrations

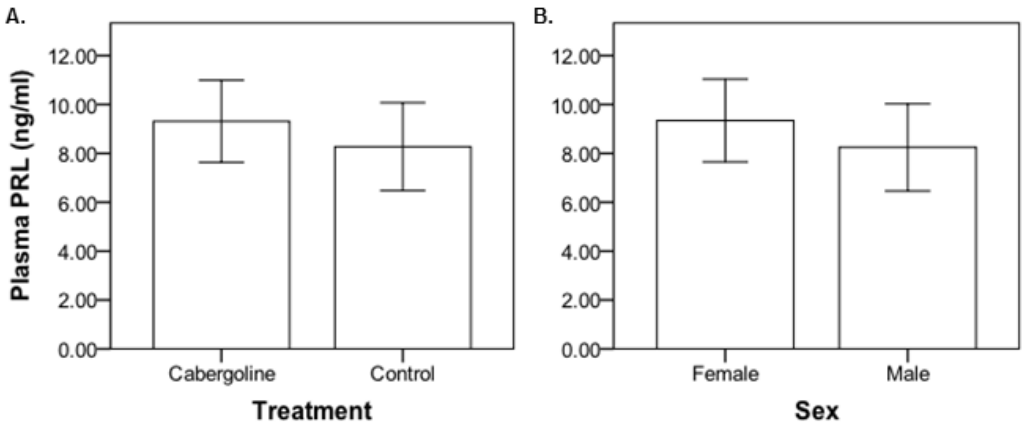

Figure 2.1. (A) No effect of cabergoline treatment on plasma PRL concentrations. Mean ± 1 standard error PRL concentration measured on day 2 post-hatch for pairs treated with cabergoline (N= 6 birds) or vehicle (control, N=6 birds). PRL concentrations are similar to normal breeding levels that have been previously reported in zebra finches in other studies (Christensen and Vleck, 2008; Smiley and Adkins-Regan, 2016). Because there was no effect of cabergoline on plasma PRL concentrations, subjects were pooled together for all other analyses. (B) Plasma PRL does not differ between sexes. Mean ± 1 standard error PRL concentration measured on day 2 post-hatch for males (N= 6) and females (N=6).

Relationship between PRL and parental behaviors

There was a significant, positive relationship between PRL and chick brooding behavior ($F_{1, 9.354} = 16.972$, $p = 0.002$; figure 2.2.A.) and PRL and chick feeding ($F_{1, 10.002} = 8.634$, $p = 0.015$; figure 2.2.B.). Because the number of chicks may influence the amount of behavior displayed, we also ran a GLMM for each behavior while controlling for both the pair ID and number of chicks in the nest during the period when behavior was recorded. Both brooding ($F_{1, 7.953} = 20.038$, $p = 0.002$) and feeding ($F_{1, 6.770} = 15.800$, $p = 0.006$) behavior remained significantly associated with PRL concentrations. Chick brooding and feeding behavior were highly correlated ($r(10) = 0.727$, $p = 0.007$).

Figure 2.2. Chick brooding and chick feeding are positively related to plasma PRL concentrations

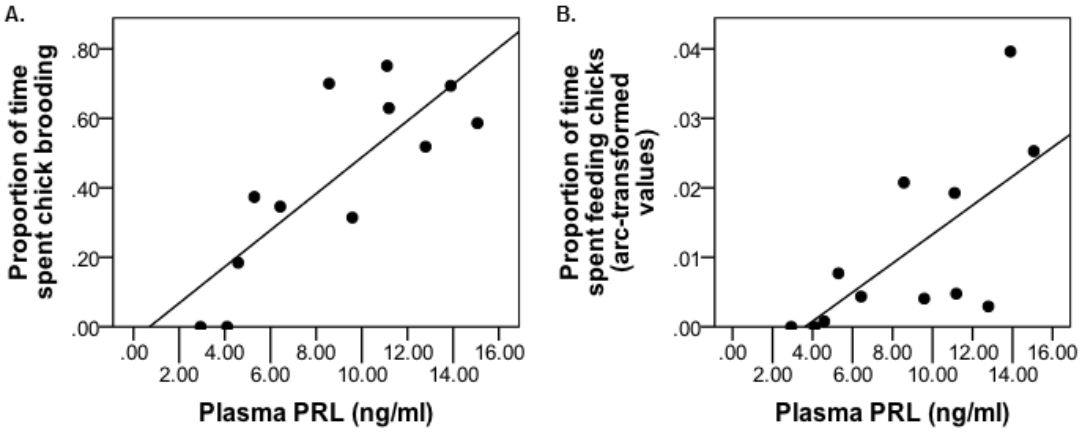

Figure 2.2. (A) Chick brooding behavior increases with plasma PRL concentrations. Chick brooding behavior was recorded inside the nest box on days 1 and 2 post-hatch. Plasma PRL was measured on day 2 post-hatch following the recording. The Y-axis is the proportion of the total recording time that an individual spent brooding chicks. (B) Chick feeding behavior increases with plasma PRL concentrations. Chick feeding behavior was recorded inside the nest box on days 1 and 2 post-hatch. Plasma PRL was measured on day 2 post-hatch following the recording. The Y-axis is the proportion of the total recording time that an individual spent brooding chicks. Arc-transformed values are shown because transformation was required for statistical analysis.

Relationship between PRL and reproductive success

PRL was not related to clutch size ($F_{3, 2} = 1.480$, $p = 0.428$; figure 2.3.A.), but was significantly associated with greater numbers of chicks that hatched ($F_{2, 7} = 8.642$, $p = 0.013$; figure 2.3.B.). In addition, chick survival to fledging was also significantly associated with higher PRL concentrations ($F_{1, 3} = 13.122$, $p = 0.036$; figure 2.3.C.).

Figure 2.3. Relationship between plasma PRL and clutch size, number of hatched chicks, and chick survival to fledging

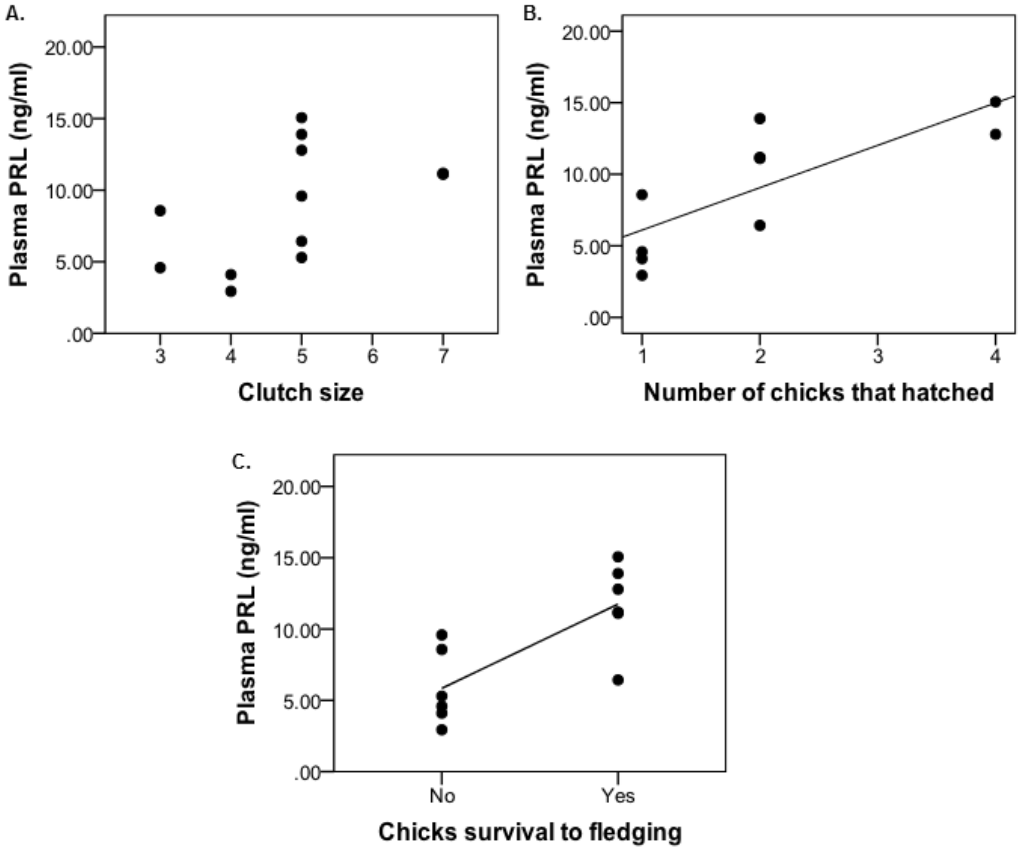

Figure 2.3. (A) No relationship between plasma PRL concentrations and clutch size. Data points represent individual plasma PRL concentrations measured in male and female parents on day 2 post-hatch based on how many eggs were in their clutch. There was no statistical relationship between plasma PRL concentrations and clutch size. (B) The number of chicks that hatch increases with plasma PRL. Data points represent individual plasma PRL concentrations measured in male and female parents on day 2 post-hatch based on how many chicks successfully hatched. (C) Chick survival to fledging is positively related to plasma PRL. Data points represent individual plasma PRL concentrations measured in male and female parents on day 2 post-hatch based on whether at least one chick survived to fledging.

2.5. DISCUSSION

Relationship between PRL, parental behavior, and reproductive success

As predicted, we found significant, positive associations between PRL concentrations and the amounts of chick brooding and feeding behavior in zebra finches. In house finches, suppressing PRL in parental males reduced nestling feeding rates while elevating PRL in non-parental males significantly increased nestling feeding rates (Badyaev and Duckworth, 2005). Likewise, non-breeding female ring doves (*Streptopelia risoria*) treated twice daily with injections of ovine PRL showed more regurgitation feeding, crouching, and sitting behavior in the nest with a foster squab than did controls (Wang and Buntin, 1999). While there is no causal evidence that PRL plays a role in zebra finch post-hatch care yet, these results strongly suggest that PRL may be a candidate mechanism for parental behavior and that variation in this behavior may result from variation in PRL titers.

In support of this, we found that higher PRL titers were significantly related to whether chicks survived to fledging. Even though all breeding pairs in this study hatched chicks, in three of the six nests we studied, all of the chicks died before fledging. For these nests, the last chicks were found dead on post-hatch day 3 (two nests) and post-hatch day 6 (one nest). Interestingly, these parents also had the lowest PRL concentrations and also displayed the least amount of parental behavior during the first two days of post-hatch care. Therefore, one could speculate that the relationship between low PRL and low chick survival was likely caused by a lack of post-hatch parental care. This is consistent with other studies that have found relationships between nest abandonment and low PRL titers (reviewed in Angelier et

al., 2016; Angelier and Chastel, 2009). Manipulation studies that suppress PRL during post-hatch parental care are needed in order to determine if PRL is playing a causal role in the expression of parental behavior.

However, there may be a bidirectional relationship between PRL titers and parental care, such that external breeding stimuli may influence the amount of PRL secreted, which, in turn, may affect parental care. In support of this hypothesis, we found that higher PRL titers were significantly associated with a greater number of hatched chicks. This is consistent with findings in wild common terns that PRL correlated with hatching success (Riechert et al., 2014). Contrary to our prediction, however, PRL concentrations were not associated with clutch size. Egg stimuli are required for maintaining elevated PRL titers in other avian species (reviewed in Angelier et al., 2016; Buntin, 1996; Sockman et al., 2006), therefore one may have predicted that a greater number of eggs could have led to a greater PRL peak at the time of chick hatching. On the other hand, this result may not be surprising as PRL does not appear to influence egg laying or clutch size determination in zebra finches (Ryan et al., 2015, 2014) or American kestrels (*Falco sparverius*; Sockman et al., 2000). In addition, this relationship is observed in both sexes, so this phenomenon cannot be limited to the fact that only females lay eggs. Other endocrine factors such as gonadotropins, growth factors, and sex steroid levels may be a better predictor of clutch size than PRL (Haywood, 1993; Klomp, 1970).

If PRL is influencing parental care behavior in a significant way, such as determining how much energy and investment to put towards provisioning offspring, then PRL concentrations at day two post-hatch, when we sampled birds, may be

reflective of incubation effort and parental investment for the current brood, regardless of brood size. This idea is supported by the positive relationship between PRL titers and parental behavior reported in this study when the number of chicks in the nest is controlled for. However, these hypotheses remain speculative until further manipulation studies have been performed to demonstrate if, and what, PRL's role in parental behavior is. In addition, quantifying the change in plasma PRL in nonbreeding birds after chick exposure is necessary in order to demonstrate that a rise in PRL is causally linked to the onset of parental behavior.

Cabergoline treatment

Although half the pairs were treated with cabergoline, it is unlikely that our treatment influenced the correlations that resulted between PRL and parental care. First, cabergoline treatment had no effect on plasma PRL (see section 2.4.) and behavior did not differ by treatment (data not shown). Our treatment was administered orally and thus, was a noninvasive (i.e., not stressful) method of administration and no adverse effects were seen in subjects after consuming the cabergoline. It is worth noting the ineffectiveness of cabergoline at the dose we used. In mammals, PRL is tonically inhibited by dopamine (DA) (reviewed in Freeman et al., 2000) and when administered orally, cabergoline greatly reduces circulating PRL (e.g., Almond et al., 2006; Brooks et al., 2005; Moro et al., 1999). In birds, PRL is not tonically inhibited by DA as it is in mammals, but rather, is tonically stimulated by vasoactive intestinal peptide (El Halawani, 1997). In turkeys, DA has been shown to have both stimulatory and inhibitory effects on PRL secretion (Youngren et al., 1998, 1996, 1995).

Specifically, DA acting at the D1 receptor at the level of the hypothalamus activates VIP neurons (Youngren et al., 2002, 1996), and hence, enhances PRL secretion, while DA acting at the D2 receptor in the anterior pituitary inhibits PRL by antagonizing the actions of VIP, inhibiting PRL release (El Halawani et al., 1991). Therefore, although DA inhibition is not as potent as it is in mammals, there is still the potential for D2 agonists to inhibit PRL release in other birds. Although cabergoline has been traditionally used in mammals, it is a long-lasting, potent D2 agonist with little affinity for D1 receptors (reviewed in Curran and Perry, 2004). When given chronically (i.e., daily), we hypothesized that it would have the potential to inhibit PRL release in breeding zebra finches. Based on studies with galliform birds, our negative results with cabergoline suggest that either DA does not play a strong role in inhibiting PRL release in zebra finches, our drug manipulation did not effectively target the anterior pituitary, or that this particular DA agonist or dosage was not appropriate for such a manipulation in this species. Another intriguing hypothesis is that the pituitary may become resistant to DA inhibition during the peak times of PRL secretion. In turkeys, DA can inhibit PRL during egg laying (when PRL concentrations are still relatively low) but is ineffective during incubation (when PRL is highest) (El Halawani et al., 1991). In fact, D2 receptors are downregulated in the pituitary during incubation, compared to egg laying times (Macnamee and Sharp, 1989). The regulation of PRL secretion in non-galliform birds has not been well studied, and therefore it is currently unknown whether the inhibitory effects of DA could be diminished during late incubation/early chick care, when PRL is at its peak. Additional work on the hormonal, genetic, and other molecular regulation of PRL in non-galliform species is

needed before we can generalize our findings to other birds.

Conclusions

Hormonally-mediated variation in behavior is important for modifying behaviors according to environmental and social conditions to maximize fitness. As we have shown here, PRL is directly associated with parental behaviors and post-hatch reproductive success in the zebra finch and thus, makes for a strong candidate mechanism to further investigate as a regulator of this variation. However, until the right manipulation studies are conducted, the exact role of PRL during parental care remains speculative.

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CHAPTER 3

INCUBATING ZEBRA FINCHES CAN CARE FOR FOSTER CHICKS IN THE ABSENCE OF HIGH PROLACTIN

Submitted, *under review*

3.1. ABSTRACT

Parental care is an important component of fitness in many diverse taxa. The hormone prolactin (PRL) plays a causal role in mammalian maternal care, but fewer studies have established causality in avian parental care. In most birds with altricial young, circulating PRL levels are low during non-breeding times and significantly increase during late incubation and early post-hatch care. Reproductive experience may increase the sensitivity to PRL, which may affect parental behavior. This study aimed to test the hypothesis that PRL plays a causal role in the priming and onset of parental behavior in interaction with experience using biparental zebra finches. To test this, we injected vasoactive intestinal peptide (VIP), the primary avian PRL-releasing factor, or vehicle for 5 days to mimic the natural peak in PRL observed during the end of incubation and beginning of post-hatch care. Subjects were age-matched, inexperienced or experienced pairs in the early stages of incubation, when circulating PRL concentrations are still low. Two foster chicks were presented during the last two days of treatment and chick brooding and feeding, eating, and drinking were recorded. Contrary to our prediction, VIP did not increase parental behavior. Instead, a substantial number of subjects across treatment and experience groups

provided care to foster chicks, but only if they had eggs in the nest. Unexpectedly, VIP increased the latency to feed chicks and reduced food intake in adults. Experienced but not inexperienced pairs were positively correlated in their chick feeding behavior. Our results suggest that zebra finches are highly sensitive to chick stimuli after egg laying which can stimulate a baseline level of parental care. We propose that high PRL increases the probability that parental care occurs at the time of hatching.

3.2. INTRODUCTION

Parental care is an important component of reproduction, and hence fitness, in many diverse vertebrate taxa. Offspring directed behaviors are often novel for first time parents and require a series of neural, endocrine, and other physiological changes to promote their onset. Prolactin (PRL), a peptide hormone produced in the pituitary, may be a mechanism that has conserved functions in stimulating parental care across vertebrate species. PRL was initially discovered as a hormone involved in milk production in lactating female mammals (reviewed in Bole-Feysot, Goffin, Edery, Binart, & Kelly, 1998) and plays a causal role in the onset of mammalian maternal behaviors (reviewed in Bridges, 2015). Increased PRL is also positively related to paternal care as well, though evidence for a causal relationship between PRL and mammalian paternal care is less conclusive (Bales & Saltzman, 2016; Saltzman & Ziegler, 2014; Wynne-Edwards & Timonin, 2007). PRL's role in parental care likely dates back even further in evolutionary time, however. In fish, pituitary and plasma levels of PRL increase during the late stages of brooding and early egg hatching and this increase in PRL promotes oral egg carrying (mouth brooding), nest building, and

egg fanning behavior in a variety of fish species (reviewed in Whittington and Wilson, 2013). PRL is also important for proliferation of skin mucous cells and mucus production, or discus milk, which is used to provide nutrients to offspring in discus fish (reviewed in Whittington & Wilson, 2013).

Similarly, in virtually all birds that raise altricial young, circulating PRL levels are low during non-breeding times and become significantly elevated during incubation and early chick care (reviewed in Angelier, Wingfield, Tartu, & Chastel, 2016; reviewed in Buntin, 1996; Smiley & Adkins-Regan, 2016a). Because of this pattern, PRL has been suggested to cause the initiation of parental care in birds, but rarely has this hypothesis been tested. The strongest evidence for PRL playing a role in parental care comes from ring doves (*Streptopelia risoria*), where PRL is causally related to squab brooding and feeding (Buntin, Becker, & Ruzycki, 1991; Wang & Buntin, 1999). However, both male and female ring doves feed squabs crop milk which is produced by the epithelial mucosal cells along the wall of the crop sac in response to PRL, an evolved trait unique to pigeons and doves (Buntin, 1996; Patel, 1936). Therefore, because of crop milk production, it is difficult to generalize a role for PRL in chick feeding to other kinds of birds. Few studies have manipulated PRL during parental care in other birds with altricial young. One study used house finches (*Carpodacus mexicanus*), which have two plastic and distinct male phenotypes: one that provides substantial parental care and one that provides little or no parental care (Duckworth, Badyaev, & Parlow, 2003). Artificially increasing PRL in the non-parental male morph increased nestling feeding rates while lowering PRL in the parental morph decreased feeding rates (Badyaev & Duckworth, 2005). However,

reducing PRL in male Adélie penguins (*Pygoscelis adeliae*) during the chick-rearing period did not affect chick growth or survival or food foraging durations (Cottin et al., 2014), indicating males were still capable of providing care, particularly feeding, in the absence of high PRL. Therefore, the role of the PRL peak observed at chick hatching in birds that do not feed chicks with an internally produced food source (i.e., crop milk) remains unclear.

Importantly, in all species mentioned above that provide care (mammals, birds, and fish), an increase in circulating PRL is generally observed in both sexes *before* the arrival of offspring, which might ensure that the mechanisms are in place to show parental behavior as soon as offspring appear. In many species, high PRL is maintained by the presence of sensory stimuli associated with eggs or young. For example, Silverin and Goldsmith (1990) showed that the PRL peak observed at hatching in pied fly catchers (*Ficedula hypoleuca*) could be prolonged for up to 12 days by repeatedly swapping out chicks for younger ones. Conversely, PRL was reduced when newly hatched chicks were swapped out for older ones (Silverin & Goldsmith, 1990), demonstrating that the presence of young chicks can stimulate PRL release. On the other hand, PRL levels remain high during long foraging trips that can last up to 8-11 days in Adélie penguins which are devoid of all egg or chick contact, a pattern observed in many penguins and other sea birds (Vleck et al., 2000). Therefore, it has been suggested that the role of PRL is to maintain the motivation to consume enough food and return to the chicks, as opposed to directly affecting parental care behavior itself. In support of this, while reducing PRL in male

Adélie penguins did not affect foraging trip duration, it did affect diving and foraging effort (Cottin et al., 2014), suggesting males were less motivated to provide food to chicks. In addition, although egg or nest removal is known to drastically reduce PRL in many avian species, treating ring doves with PRL for 10 days following nest removal maintained the interest in resuming incubation, if provided the opportunity to do so (reviewed in Buntin, 1996; reviewed in Sharp, 2009). These studies illustrate the need for more work to decipher what role PRL plays prior to and after the time of hatching.

In addition to hormonal mechanisms, reproductive experience (having raised at least one offspring to independence) may also play an important role in the onset of parental behaviors. In general, most female mammals show increased responsiveness to offspring stimuli with experience, regardless of physiological state (reviewed in Bridges, 2015). For example, experienced, non-breeding female rats show shorter latencies to interact with foster pups compared to adult females that have no experience with pups (reviewed in Bridges, 2015). Permanent neural and/or other physiological changes that occur during an animal's first reproductive bout may increase the sensitivity to, or enhance the effectiveness of, mechanisms that promote parental care. For instance, experienced, non-breeding female rats have lower circulating PRL levels than age-matched virgin females, but have increased central responsiveness to PRL (Anderson, Grattan, van den Ancker, & Bridges, 2006). In contrast to rats, however, circulating PRL levels in breeding zebra finches *increase* after gaining reproductive experience, consistent with a variety of other avian species (reviewed in Smiley & Adkins-Regan, 2016a). However, it is unknown if this

difference in PRL secretion serves any behavioral function. Treating reproductively experienced ring doves with PRL resulted in a greater frequency of regurgitation feedings, greater squab weight gain, and more time spent sitting in the nest than treating inexperienced ring doves with PRL (Wang & Buntin, 1999), suggesting ring doves, like rats, also have an increased sensitivity to PRL as a result of reproductive experience. Therefore, reproductive experience may have important interactions with endocrine mechanisms, by either overriding or enhancing the influences of hormones, and should be taken into consideration when designing experiments that look at the role of hormones in the onset of parental behaviors.

This study sought to test the hypothesis that PRL plays a causal role in the priming and onset of avian parental behavior in interaction with reproductive experience using a socially monogamous, biparental songbird species, the zebra finch (*Taeniopygia guttata*). Both male and female zebra finches show an equally strong positive association between PRL and parental care and reproductively experienced birds showed a greater increase in PRL during this time than when they were first-time breeders (Smiley & Adkins-Regan, 2016a). Additionally, PRL concentrations on day 2 post-hatch were positively correlated with the amount of chick brooding and feeding behavior similarly in both sexes (Smiley & Adkins-Regan, 2016b). In this study, we aimed to mimic the natural peak in PRL that occurs at the end of incubation in non-parenting zebra finch pairs prior to exposing them to foster chick stimuli to test for the effects of PRL on the onset of parental care. Additionally, we used age-matched birds differing in reproductive experience (inexperienced, first-time breeders or experienced breeders) to test for interactions between PRL and reproductive experience in their

effects on parental behavior.

Based on PRL's associations with parental care, we predicted that VIP-treated birds would show greater amounts of parental care (chick brooding and feeding) and have shorter latencies to display parental care towards foster chicks compared to control birds. Likewise, we predicted that experienced birds would be more likely to provide care to chicks than inexperienced birds and would show shorter latencies and greater amounts of parental care. We also predicted that there would be an interaction between treatment and experience such that reproductively experienced, VIP-treated birds would show the highest levels of behavior compared to all other groups. Consistent with PRL's role in promoting nutrient provisioning (e.g., lactation in female mammals, crop milk production in ring doves, mucous cell production for 'discus milk' in fish), PRL also plays a role in food intake and osmoregulation in many vertebrate species (Bole-Feysot et al., 1998), both of which also may be modulated differently during parental care and affected by a PRL manipulation. Therefore, we also measured eating and drinking behavior. We predicted that VIP-treated birds would consume more food and water, relative to controls. Because PRL's relationship to reproductive experience and parental care behavior should be similar in both males and females in a biparental bird, we did not expect to find any sex differences in any of our measures.

3.3. METHODS

Subjects

We started with a total of 66 adult, age-matched zebra finches (i.e., 33 male-female pairs) raised in the lab with a mean age = 3.02 ± 0.4 years. Thirty-two individuals (i.e., 16 male-female pairs) were reproductively inexperienced (INEX), defined as having no interaction with the opposite sex since removal from the natal cage at independence and 34 individuals (i.e., 17 male-female pairs) were reproductively experienced (EXP), defined as having raised at least one chick to fledging prior to the experiment. Before and during the experiment, all birds had access to food (commercial seed mix; Kaytee Fortifinch Diet), water, and grit *ad libitum*. Birds were supplemented with hardboiled egg once per week. All rooms were kept in temperature (22.2° C) and humidity (range 30-70%) controlled rooms on 14:10 light:dark cycles. All methods and procedures were approved by the Cornell University IACUC.

All subjects were housed in sex-specific aviaries (0.94 m x 0.76 m x 0.94 m) which held up to 20 birds prior to the beginning of the experiment. Subjects were randomly assigned to remain inexperienced or to become an experienced breeder before the experiment. Inexperienced birds were given the opportunity to pair with a partner by moving them into a social aviary containing four males and four unrelated, unfamiliar females. Aviaries were observed for 20 minutes a day for five days to determine pairs. Males and females who exclusively clumped, allopreened, and shared a nest box were considered paired (Smiley et al., 2012). (Note that while we had nest boxes in the INEX cages during pair observations, this was not enough time to begin breeding. However, it is possible that subjects may have gained sexual experience in the pairing cage prior to the experiment, but no parenting experience). Experienced

birds were paired in the same way but were allowed to complete one breeding cycle after pairing. Experienced birds remained with their breeding partner for the experiment.

Study Design

Prior to the start of the experiment, pairs were randomly assigned to receive either a VIP or saline control treatment and were also randomly assigned to be tested in one of six cohorts, each balanced for reproductive experience and treatment manipulation. For each cohort, four different testing rooms, each housing 4-6 cages, were used and balanced for treatment and experience conditions. Each individual was weighed to the nearest 0.1 gram (pre-treatment weight) before the start of the experiment. For each cohort, male-female pairs were housed together in testing cages (0.6 m x 0.4 m x 0.35 m) which contained an empty nest box, nesting material (coconut fibers), food, water, and grit available *ad libitum*.

We aimed to test birds with foster chicks in between nest building and egg-laying, when circulating PRL is low (Smiley & Adkins-Regan, 2016a), so that they were still in a non-parental state but had a nest to accommodate foster chicks. Each pair's nest box was checked daily for nest building activity. Out of the 66 initial birds that were put together at the beginning of the study, 18 INEX birds (n=12 saline-INEX; n=6 VIP-INEX) and 20 EXP birds (n=8 saline-EXP; n=12 VIP-EXP) built a nest. Once pairs had built a nest, subjects began to receive one daily intramuscular (IM) injection, alternating between the right and left pectoral muscle, for five consecutive days, of either 0.05 ml of VIP (see *VIP Injection Pilot Study and Dosage*

for details) dissolved in 0.09% physiological saline or 0.05 ml of 0.09% physiological saline alone (vehicle control). Both members of the pair received the same treatment. On the last two days of treatment, two 1-4 day old foster chicks (see *Foster Chick Stimuli* for details) were placed in the nest immediately following injections and subjects' parental behavior (chick brooding and feeding), eating, and drinking behavior were video recorded for two hours post-injection (see *Video Cameras and Behavior Video Coding* for details). This paradigm was used in order to mimic the natural peak in PRL that occurs approximately 3 days prior to hatching and continues through days 1 and 2 post-hatch (Smiley and Adkins-Regan, 2016a). Foster chicks were weighed to the nearest 0.0001 gram immediately before and after the video was taken each day and were returned to their original home nest after video recording to ensure survival for the day 2 test. Adult subjects were weighed again after the video on the last day of treatment (post-treatment weight). Blood samples were not taken during the study so that behavior was not disrupted.

VIP Injection Pilot Study and Dosage

We used vasoactive intestinal peptide (VIP), the main prolactin releasing peptide in birds (Kingsbury, 2015) to pharmacologically increase PRL. VIP is released from neurons in the hypothalamus into the median eminence and acts via its receptors on the lactotroph cells in the anterior pituitary to stimulate PRL gene synthesis and the release of PRL into the circulation (Kahtane, Chaiseha, & Halawani, 2003; Sharp, Dawson, & Lea, 1998; Xu et al., 1996). Peripheral manipulations of VIP produce fast-acting, yet short-term, increases in circulating PRL concentrations in many passerines,

including Mexican jays (*Aphelocoma ultramarina*), blue jays (*Cyanocitta cristata*), white-crowned sparrows (*Zonotrichia leucophrys gambelii*), dark-eyed juncos (*Junco hyemalis*), Florida scrub-jays (*Aphelocoma coerulescens*), western scrub-jays (*A. californica*) and zebra finches (Christensen & Vleck, 2008; Maney et al., 1999; Vleck & Patrick, 1999). In addition, VIP manipulations have yielded significant behavioral effects on incubation and nesting behavior in turkeys and bantam hens (reviewed in Buntin, 1996; Kingsbury, 2015) and on chick feeding in house finches (Badyaev & Duckworth, 2005). Therefore, VIP is a useful and reliable tool to easily manipulate PRL during behavioral studies.

Previous work by Christensen and Vleck (2008) showed that zebra finches responded to intravenous (IV) injections of VIP with increased circulating PRL within three minutes and for up to one hour. We ran a pilot study using a separate group of 20 non-breeding zebra finches of mixed sexes. Reproductive experience was unknown for pilot subjects, but Christensen and Vleck (2008) showed that both experienced and inexperienced non-breeding zebra finches responded similarly to VIP injections. Our goal was to test whether intramuscular (IM) injections increase PRL similarly to IV injections (used in Christensen & Vleck, 2008) and to see if the effects of VIP last longer than one hour. VIP was dissolved in 0.09% physiological saline (dose = 100 ug/kg body weight, volume = 0.05 ml; Sigma-Aldrich V6130) administered via IM injections in the pectoral muscle. Prior to injection, subjects were randomly assigned to have a blood sample taken at 0.5 (n=4), 1 (n=4), 2 (n=3), or 3 (n=5) hours post-injection. Control non-breeding birds were injected with 0.05 ml of saline alone and bled at 1 (n=1), 2 (n=2), or 3 (n=1) hours later. Our blood collection and plasma

storage methods are described in (Smiley & Adkins-Regan, 2016a, 2016b). We assayed PRL in plasma across two plates using an enzyme-linked immunosorbent assay (ELISA), following the methods described in Smiley and Adkins-Regan (2016b). For validation of the assay, see Smiley and Adkins-Regan (2016a). We used a high- and low-pool of PRL to calculate the coefficients of variation (CV) within and between plates. For plate 1, the high-pool intra-CV = 3.33% and low-pool intra-CV = 2.56%. For plate 2, the high-pool intra-CV = 7.52% and low-pool intra-CV = 5.75%. The inter-CV values were 2.78% for the high-pool and 7.36% for the low-pool.

Foster Chick Stimuli

Breeding cages (0.94 m x 0.76 m x 0.94 m) containing eight male-female breeding pairs (separate from the subjects used in this study) and eight nest boxes were set up to produce offspring stimuli for each cohort. Breeding cages were supplied with nesting material (coconut fibers), food, water, and grit available *ad libitum*. Daily nest checks were performed to track incubation and to predict chick-hatching dates (Zann, 1996). Eggs were checked for fertility by candling them one week after laying. If a nest had at least two fertile eggs, then that nest's chicks were randomly assigned to be given as foster chicks to one of the subject pairs. Study cohorts were set up approximately seven days prior to expected chick hatching dates to coordinate chick hatching with the onset of nest building in our study pairs.

Video Cameras and Behavior Video Coding

Video cameras used to record behavior in the nest were identical to the ones

used in Smiley and Adkins-Regan (2016b). In addition to the nest camera, we placed a second, identical camera on the outside of the back of the cage which recorded activity at both the feeder and the water bottle spout. Cameras were set up several days prior to recording so that subjects could habituate to their presence. Behavior was recorded using both cameras on the last two days of treatment for two hours after subjects received chicks in their nest.

Figure 3.1. Representative behavior from video stills

Figure 3.1. Photos are representative still images from videos taken during this study to illustrate the four behaviors that were measured. Note that while only one sex is pictured in each photo, both sexes partake in all behaviors and behaviors were coded separately for each sex. The sexes are easy to

(figure 3.1 continued) visually distinguish as males have bright orange cheek patches and females are all grey. (A) Male zebra finch brooding chicks. (B) Male feeding a chick (regurgitating). (C) Female eating seed from the food dish. (D) Male drinking from waterspout.

Chick brooding (an adult sitting on top of chicks) and chick feeding (an adult regurgitating to a chick) were coded as in Smiley and Adkins-Regan (2016b) using the program ELAN version 4.9.2 (Max Planck Institute for Psycholinguistics, Nijmegen, The Netherlands). Eating was coded as the total time an adult subject spent consuming seed from the feeder and drinking was coded as the total time an adult subject spent consuming water from the water bottle spout. See figure 3.1. for sample video stills of each behavior. Each behavior was coded for males and females separately by a trained coder who was blind to treatment and reproductive experience conditions. Total durations for all behaviors were summed for males and females separately, for each video. The first time point at which a behavior was observed for each video was used as the latency value. This produced eight different response variables for all subjects: brooding duration, brooding latency, chick feeding duration, chick feeding latency, eating duration, eating latency, drinking duration, and drinking latency.

Statistical Analyses

All statistical analyses were performed using IBM SPSS Statistics for Windows, version 24.0. (IBM Corp., Armonk, NY). Our goal for this study was to test birds with foster chicks in between nest building and egg-laying. However, aligning the exact timing of nest building in subjects with the exact time of chick hatching in the foster breeders' cages proved difficult and inevitably some nests had eggs prior to their first exposure to foster chicks. However, this allowed us to categorize birds in the

following way: No nest (excluded from study), nest with no eggs, or nest with at least one egg. No birds that had a nest with no eggs showed any parental behaviors, so for all analyses below, unless otherwise noted, we only analyzed data for birds that had a nest and eggs (see Table 3.1. for sample sizes of birds in each treatment, whether they had eggs in the nest, and whether they provided care). For all models, non-significant terms were removed in a backwards, stepwise manner until only significant terms remained; significance was accepted if p-values were < 0.05. Model assumptions were checked by plotting predicted values vs. residuals and by plotting residuals on a Q-Q plot to visually inspect for normality.

VIP injection pilot study

We ran a one-way ANOVA to test the effect of time of blood sample post-VIP-injection on plasma PRL concentrations. Control birds were pooled together to create one control group to compare to VIP groups. Post-hoc comparisons between groups were performed using Fisher's least significant difference (LSD) test.

Effect of treatment and reproductive experience on the probability on having eggs in the nest when presented with foster chicks

We tested whether the probability of having eggs on the first day foster chicks were placed in the nest was influenced by treatment or reproductive experience by running a generalized estimation equation model (GEE) with a logit-link and binary response, where 0= no eggs and 1= at least one egg. These data were analyzed at the level of the nest, since both males and females would have the same responses. All

nests were included in this analysis. Treatment (VIP or control) and reproductive experience (INEX or EXP) and the 2-way interaction between treatment and experience were included as fixed effects. PairID (a unique number to identify each pair used in the study) was included as a repeated variable.

Effect of treatment, reproductive experience, and sex on probability of displaying parental behavior

We tested whether the probabilities of displaying chick brooding and chick feeding were affected by treatment, reproductive experience, or sex by running a GEE for each behavior with a logit-link and binary response of either 0=no behavior or 1=some behavior on either day of video. There were no differences in probability for either behavior when day 1 and day 2 were analyzed separately; therefore, we used the probability of showing behavior on either day as the response variable in the final model. The probabilities of eating and drinking could not be analyzed in this way because all subjects consumed some food and all but one individual drank water during each video, each day. For each model, treatment, reproductive experience, sex, and all possible 2- and 3-way interactions were included as fixed effects. PairID was included as a repeated variable in each model.

Effect of treatment, reproductive experience, and sex on behavior duration and latency

Each outcome variable (brooding duration, brooding latency, chick feeding duration, chick feeding latency, eating, eating latency, drinking, and drinking latency) was analyzed separately using a generalized linear mixed model (GLMM). Durations

were analyzed as proportions by dividing the total amount of time the behavior was observed by the total length of the video. We plotted the distribution of each variable on a histogram to check for normality and only eating proportion required arc-sin transformed to normalize the data.

We were able to analyze data at the level of the individual by using mixed models that control for pair identity (PairID), with the caveat that an individual's behavior may be dependent on the behavior of their similarly treated partner. For each model the fixed factors included treatment, reproductive experience, sex, day of video and all possible 2-, 3-, and 4-way interactions. We controlled for testing room, cohort, PairID nested within testing room and cohort, and incubation day of the first day of the video by including these as random factors. Note that we initially included incubation day as a fixed factor, but there were no associations found between any behavior duration or latency and incubation day. Nonetheless, we included incubation day as a random factor in all of our behavior GLMMs to control for any possible influence on behavior.

There was no effect of day the video on any outcome variable; therefore, values were averaged by taking the mean across the two days and these values were used in the final model analyses. If there was only one video available due to technical failures of the camera, then only the value from the one day was used in the final model. For parental behaviors, two pairs (i.e., four individuals) only had one day of video. For eating and drinking behavior, three pairs (i.e., six individuals) only had one day of video.

Effect of treatment and reproductive experience on number of eggs in the nest and day of incubation when presented with foster chicks

We tested if there were any differences in the number of eggs in the nest or the day of incubation pairs were on the first day they were recorded in the nest with foster chicks. Each outcome (i.e., number of eggs or day of incubation) was analyzed using a linear mixed model (LMM) which included treatment and reproductive experience and the interaction between the two as fixed factors and testing room and cohort as random factors. These data were analyzed at the level of the nest, since both males and females would have the same responses.

Correlations between behavior durations and behavior latencies

Correlations between all combinations of behavior durations and behavior latencies were analyzed using a Pearson's R test, controlling for PairID.

Effect of treatment and reproductive experience on chick weight change

We subtracted the pre-video weight from the post-video weight for each chick on each day the video was taken. For each day, the weight change for the two chicks was averaged to create one day 1 weight change response variable and one day 2 weight change response variable. There was no difference in weight change when days 1 and 2 were analyzed separately, so we averaged these two values to create one average weight change value per nest. Chick weight change was analyzed at the level of the nest, since male and female subjects would have identical values. We tested if these values were affected by the treatment, reproductive experience or the interaction

between the two for each nest by including these as fixed factors in a LMM, while controlling for testing room and cohort by including those as random factors.

Effect of treatment, reproductive experience, and sex on subjects' weight

We subtracted the pre-treatment weight from the post-treatment weight to produce a weight-change response variable for each individual for all birds that had a nest. We then ran a LMM that tested if these values were affected by the treatment, reproductive experience, sex, or presence of eggs (yes or no), and all possible 2-, and 3- way interactions for each subject by including these as fixed factors, while controlling for testing room, cohort and PairID nested within testing room and cohort.

Correlations between behavior durations and latencies within male-female pairs

Correlations between all behavior durations and behavior latencies were analyzed within pairs using a Pearson's R test, controlling for PairID. Correlations were run between all subjects together, as well as pairs within VIP and saline treatment groups separately and within inexperienced and experienced groups separately. For three out of the 12 pairs that showed parental behavior, only one member of the pair provided care (two pairs = female only; one pair = male only); therefore 9 pairs (18 individuals) were analyzed.

3.4. RESULTS

VIP pilot study

The ANOVA did not reach significance for an effect of time post-VIP-

injection on plasma PRL concentrations ($F_{4,19}$ = 2.65, p=0.07). This was likely due to low sample size. However, VIP injections were able to increase PRL to a similar concentration as previously reported in breeding zebra finches (see Figure 3.2.), which is consistent with results from Christensen and Vleck (2008). LSD post-hoc comparisons revealed that the mean PRL concentration at 0.5 hours was significantly higher than the mean at baseline (p=0.02), 1 hour (p=0.03), 2 hours (p=0.03), and 3 hours (p=0.02).

Figure 3.2. Effect of VIP injections on circulating PRL across time

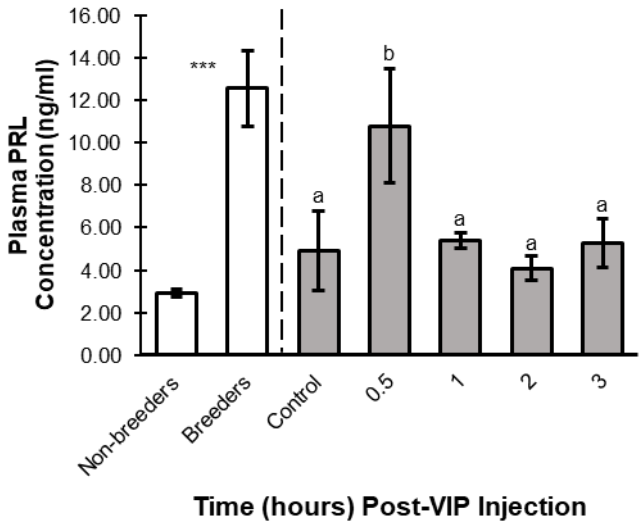

Figure 3.2. Peripheral VIP injections increase circulating PRL in non-breeding zebra finches to breeding concentrations. All bars are means ± 1 standard error of plasma PRL concentrations. The white bars to the left of the dashed line are data from non-manipulated non-breeders (N =10 males and 11 females) housed in sex specific cages and non-manipulated breeders on day 1 post-hatch (N= 5 males and 3 females) collected in a previous study in our lab (Smiley and Adkins-Regan, 2016a). *** indicates p<0.001. Grey bars to the right of the dashed line are PRL concentrations in non-breeding zebra finches measured 0.5, 1, 2, and 3 hours post VIP injection. Controls were injected with saline vehicle and bled 1, 2, or 3 hours post-injection (all times were pooled into one control group for analysis). Differing letters above the bars represent significant differences between groups as determined by LSD post-hoc analysis. These results replicate those found in Christensen and Vleck (2008).

Effect of treatment and reproductive experience on probability of having eggs in the nest when presented with foster chicks

There was no effect of treatment or reproductive experience on the probability of having eggs at the time foster chicks were placed in the nest for behavioral recording (all *p*-values > 0.05; see Table 3.1.F. and 3.1.G. for counts). Across all four groups, 63% of pairs had eggs, and the mean number of eggs at the time of the first video was 2.6 ± 1.6 (standard deviation; sd).

Effect of treatment, reproductive experience, and sex on probability of displaying parental behavior

There was no effect of treatment, reproductive experience, or sex on the probability of performing chick brooding or feeding (see Table 3.1.D. and 3.1.E. for counts). Across all four groups, 87.5% of subjects brooded chicks and 54.2% of subjects fed chicks.

Effect of treatment, reproductive experience, and sex on behavior duration and latency

There were no significant effects of treatment, reproductive experience, or sex on either the proportion of time spent chick brooding duration or latency to begin chick brooding. For all subjects, the mean time spent brooding was 1990.9 s ± sd 1297.2 (27.7 % ± 18.0 % of total video time), with a range of 0 – 4082.9 s. The mean latency to brood was 1398.2 s ± sd 1282.8 with a range of 36.0 – 4183.3 s.
There were also no effects of treatment, reproductive experience, or sex or significant interactions on the proportion of time spent chick feeding. The mean time spent

Table 3.1. Sample sizes and numbers of birds in different categories and which cared for foster chicks.

(A) The initial sample size before the experiment commenced. (B) Occurred prior to the beginning of treatment. (C) Occurred prior to placing the foster chick in the nest. (D) and (E) occurred during treatment when tested with foster chicks. (F) and (G) are means ± standard deviations and refer to the first day that behavior was recorded in the nest with foster chicks. There were no effects of treatment or experience on the probabilities of B, C, D, or E.

		Reproductive experience			
		INEXPERIENCED		EXPERIENCED	
		Treatment			
		SALINE	VIP	SALINE	VIP
A. Original N=		16	16	16	18
B. Built nest?	No	4	10	8	6
	Yes	12	6	8	12
C. Laid eggs in nest?	No	6	2	0	6
	Yes	6	4	8	6
D. If eggs, brooded chicks?	No	0	2	1	0
	Yes	6	2	7	6
E. If eggs, fed chicks?	No	5	2	3	1
	Yes	1	2	5	5
F. If eggs, how many eggs in nest?		2 ± 1	3 ± 1	3 ± 2	3 ± 0
G. If eggs, what day of incubation when behavior was first recorded?		5 ± 1	6 ± 2	6 ± 2	6 ± 3

feeding was 127.8 s ± sd 240.5 (1.8 % ± 3.3 % of total video time), with a range of 0 – 838.7 s. There was, however, a significant main effect of treatment on the latency to chick feed ($F_{1,4.85}$ = 19.31, p=0.01; Fig. 3.3.A) such that VIP-treated birds had, on average, longer latencies to begin feeding chicks than did saline-treated birds. There was no effect of sex or any significant interactions between variables.

For proportion of time spent eating, there was a significant main effect of treatment ($F_{1,19.38}$ = 6.46, p=0.02; Fig. 3.3.B) such that VIP-treated birds ate for less time than saline-control birds. There was no effect of experience or sex or any significant interactions between variables. For eating latency, there was a significant main effect of experience ($F_{1,19.40}$ = 8.05, p=0.01) such that experienced birds had longer latencies (mean = 2634.7 s ± sd 1635.2, range 98.1 – 5211.9 s) to begin eating than did inexperienced birds (mean = 1255.2 s ± sd 886.1, range 174.3 – 2697.6 s). There was no effect of sex or any significant interactions between variables.

There were no significant main effects or interactions of treatment, reproductive experience, or sex on the proportion of time spent drinking duration (mean = 5.0 s ± sd 4.6, range 0 – 19.4 s. There was a significant main effect of experience on drinking latency ($F_{1,20.63}$ = 4.59, p=0.04) such that experienced birds had longer latencies (mean = 2751.26 s ± sd 1550.65, range 157.3 – 5338.7 s) to begin drinking than did inexperienced birds (mean = 1994.2 s ± sd 1703.1, range 188.2 – 4346.8 s). There was no effect of sex or any significant interactions between variables.

Figure 3.3. Effects of VIP treatment on eating duration and chick feeding latency

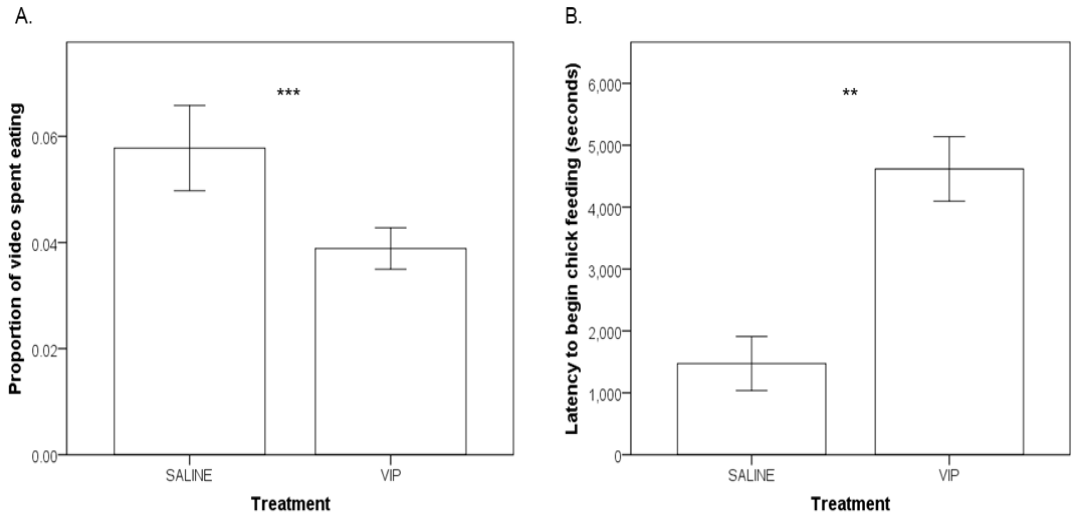

Figure 3.3. Data shown here are means ± 1 standard error. (A) VIP treatment significantly reduced the amount of time zebra finches spent eating seeds at the feeder; *** represents p <0.001. (B) VIP-treated birds took significantly longer to begin chick feeding than did saline-treated control birds; ** indicates p<0.05.

Correlations between behavior durations and behavior latencies

As would be expected, significant negative correlations were found between chick feeding duration and latency to begin chick feeding (r^2= -0.76, df=7, p=0.02), between eating duration and latency to begin eating (r^2= -0.75, df=7, p=0.02), between drinking duration and latency to begin drinking (r^2= -0.89, df=7, p<0.01), and between latency to begin eating and latency to begin drinking (r^2= -0.84, df=7, p<0.01).

Effect of treatment, reproductive experience, and sex on subjects' body weight change

There was a significant interaction between sex and presence of eggs ($F_{2,32}$ = 12.26, p<0.01; Fig. 3.4.) such that females lost weight if there were eggs in the nest, whereas males showed no significant difference in weight change. There were no

effects of treatment or experience.

Figure 3.4. Adult weight change depends on sex and if eggs were in the nest

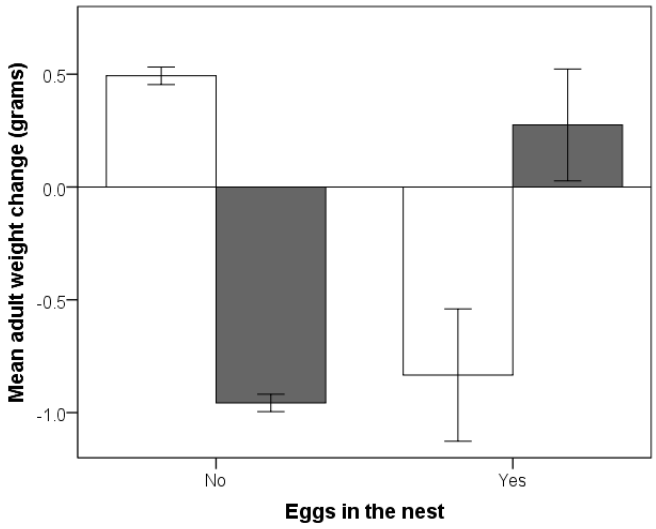

Figure 3.4. Figure shows mean weight change ± 1 standard error in female (white bars) and male (dark bars) subjects. The solid line indicates no change in weight; values above signify weight gain whereas values below signify weight loss. There was a significant interaction (p<0.01) between sex and eggs in the nest such that females lost a significant amount of weigh from the beginning to the end of the experiment weight if there were eggs in the nest, whereas there was no significant change in males.

Effect of treatment and reproductive experience on number of eggs in the nest and day of incubation when presented with foster chicks

There were no significant effects of treatment or reproductive experience on the average number of eggs in the nest or the day of incubation on which the subjects were first recorded with foster chicks.

Effect of treatment and reproductive experience on chick weight change

There were no significant main effects or interactions between effects of

treatment or experience on chick weight change. The average chick weight change was -0.02 ± sd 0.16, with a range of -0.10 to 0.10.

Correlations within male-female pairs between behavior durations and behavior latencies

Across all pairs, there were significant within-pair correlations between male latency to eat and female latency to brood (r^2= -0.99, df=1, p=0.03) and male latency to eat and female drinking duration (r^2= -0.99, df=1, p<0.01).

For inexperienced pairs only there were significant correlations between male latency to eat and female latency to brood (r^2= 0.96, df=1, p=0.04), male drinking duration and female eating duration (r^2= -0.92, df=1, p=0.03), and male latency to drink and female eating duration (r^2= 0.93, df=1, p=0.02),

For experienced pairs only there were significant correlations between male chick feeding duration and female chick feeding duration (r^2= 0.93, df=1, p<0.01), male brooding duration and female chick feeding duration (r^2= -0.92, df=1, p=0.03), male latency to brood and female chick feeding latency (r^2= 0.98, df=1, p<0.01), male latency to eat and female chick feeding latency (r^2= 0.96, df=1, p=0.04), male latency to eat and female brooding (r^2= 0.88, df=1, p=0.02), male eating duration and female eating duration (r^2= -0.84, df=1, p=0.04), male drinking duration and female drinking duration (r^2= 0.97, df=1, p<0.01), and male latency to drink and female drinking duration (r^2= -0.85, df=1, p=0.03).

For saline pairs only there were significant correlations between male chick feeding duration and female chick feeding duration (r^2= 0.80, df=1, p=0.03), male

drinking latency and female latency to chick feed (r^2= -0.99, df=1, p<0.01), male drinking duration and female eating duration (r^2= -0.77, df=1, p=0.04), male brooding duration and female latency to eat (r^2= 0.79, df=1, p=0.04), and male chick feeding latency and female drinking duration (r^2= -0.99, df=1, p=0.04).

For VIP pairs only there were significant correlations between male latency to brood and female latency to chick feed (r^2= 0.98, df=1, p=0.01), male latency to eat and female latency to chick feed (r^2= 0.99, df=1, p=0.04), male eating duration and female brooding duration (r^2= -0.957, df=1, p=0.04), male latency to brood and female latency to eat (r^2= 0.99, df=1, p=0.02), male drinking duration and female latency to eat (r^2= -0.97, df=1, p=0.03), male latency to drink and female latency to eat (r^2= 0.99, df=1, p=0.01), and male drinking duration and female drinking duration (r^2= 0.96, df=1, p=0.04).

3.5. DISCUSSION

This study is the first to test for an effect of PRL on the priming and onset of parental care behavior in nesting male and female zebra finch pairs provided with foster chicks. By administering VIP, the primary avian PRL-releasing factor, for five days, we aimed to mimic the natural peak in PRL observed at the end of incubation (days 12-14) and beginning of post hatch care (days 1-2), essentially fast-forwarding birds from a nest-building state to a chick-rearing state.

There were no differences in the probability of providing parental care to foster chicks or duration of parental behavior due to treatment. Instead, we observed a substantial and roughly equal number of saline-control and VIP-treated birds,

especially experienced birds, brooding and feeding chicks, indicating that zebra finches can provide care to foster chicks in the absence of the high PRL which is normally observed at the end of incubation and beginning of post-hatch care. Although plasma PRL is not significantly different from non-breeding baseline concentrations until day 12 of incubation, PRL gradually raises during incubation, which suggests, that at least for some birds, there is conceivably a lower PRL threshold needed in order to stimulate parental care. However, a recent study from our lab has shown that decreasing PRL in normally breeding zebra finches resulted in the elimination of or severely reduced parental care on days 1 and 2 post-hatch (Smiley and Adkins-Regan, submitted). So, although incubating birds *can* provide parental care during times of low PRL, the key difference between incubating birds and birds that have recently hatched chicks is the percentage of birds which perform parental behavior. In this study, while roughly 88% of incubating birds brooded chicks as a motor act, brooding does not appear to be different behaviorally from egg incubation and therefore, chick feeding, which is a more novel, offspring-directed behavior, may be a stronger indicator of parental behavior initiation. In this study only 54% of incubating subjects fed the chicks whereas 86 % of control, normally breeding birds fed chicks during the first two days post-hatch in our other study (Smiley and Adkins-Regan, submitted).

A similar phenomenon is also observed in mice which can show spontaneous maternal behavior towards foster pups in the absence of the hormonal changes that occur with pregnancy and parturition (Stolzenberg & Rissman, 2011). Virgin mice (C57BL⁄6J (B6) strain) display similar levels of pup-retrieval, licking and grooming, and crouching behavior as post-partum females; however, they are slower to retrieve

and crouch over pups and show less maternal motivation (Stolzenberg & Rissman, 2011). This responsiveness increases over time with increased interaction with pups, suggesting exposure to sensory stimuli from pups can induce maternal behavior. Taken together, our results suggest that the role of PRL is not to stimulate parental care *per se*, but rather, it is to increase the probability that parental care occurs at the time of chick hatching in response to chick stimuli. In other words, similar to the phenomenon described in rodents and other mammals, PRL likely peaks *before* hatching to ensure that parental behavior can begin immediately upon chick arrival. One mechanism by which this occurs may be through the modification of motivational processes related to the processing of chick stimuli salience, but this hypothesis has yet to be tested. Despite PRL's hypothesized role in increasing the likelihood of parental behavior occurring, our PRL manipulation via VIP did not increase parental behavior relative to saline controls. There are several possible explanations for why we did not see any treatment effects in this study.

One possibility is that the presence of eggs and/or chicks stimulates PRL release and influences chick care behavior, and this occurred in both treatment groups. It is well established in bantam hens, turkey hens, and female domestic mallard ducks that contact with the eggs and nest is necessary to maintain the high levels of PRL observed during egg brooding (reviewed in Buntin, 1996). In yellow-eyed penguins (*Megadyptes antipodes*) PRL levels significantly rose in females after placing an artificial egg in the nest prior to natural egg-laying (Massaro, Setiawan, & Davis, 2007). In chickens, tactical stimuli from the eggs is transmitted to VIP neurons in the hypothalamus via neural pathways stemming from the brood patch, which stimulates

PRL release (reviewed in Sharp, 2009). We confirmed that there was no effect of treatment on the probability of laying eggs (treatment was assigned before the experiment started) and that there was no difference between treatment or experience groups in the average day of incubation birds were on at the time of the first video, meaning all subjects were exposed to roughly the same breeding stimuli, for a similar amount of time, before exposure to the chicks. In addition to egg stimuli, PRL release can be stimulated by newly hatched offspring exposure in both male and female ring doves and female pied flycatchers (*Ficedula hypoleuca*; reviewed in Buntin, 1996) while circulating PRL decreases following chick removal immediately after hatching in female native Thai chickens (*Gallus domesticus*; Chaiyachet et al., 2013). Zebra finches may be particularly sensitive to stimuli that elicit parental behavior as they are short lived and must respond to unpredictable breeding conditions by reproducing quickly (Adkins-Regan & Tomaszycki, 2007). While we did not measure plasma PRL after interaction with foster chicks in this study, if PRL is released in response to egg or chick stimuli in zebra finches, then this may explain why both control and VIP-treated birds were able to show care. This remains a hypothesis, however, until further testing.

It is also possible that the timing and duration of our manipulation was not enough to significantly alter behavior above the baseline levels displayed in this study. Although VIP increases circulating PRL to breeding levels quickly (within 3 minutes; Christensen and Vleck, 2008), these effects do not last more than one hour. Perhaps birds would need to experience sustained high levels of PRL throughout the day, which would be better achieved with hormone implants, rather than five daily spikes

of increased PRL, to effect parental care. While peripheral injections of PRL produced behavioral effects 5-10 mins after testing in ring doves (Wang and Buntin, 1999), it is unknown how long it would take PRL to have direct effects on behavior in zebra finches.

Lastly, other mechanisms involved in nest building and incubation, such as mesotocin (avian homologue of oxytocin), vasotocin (avian homologue of vasopressin), oestrogens, and progesterone (Buntin, 1996; Klatt & Goodson, 2013; Lynn, 2016), may also prime the birds to be in a parental state before the rise in PRL. The fact that both sexes were equally capable of caring for foster chicks suggests that the priming or onset of parental care in females is not a direct result of egg laying. Further exploration into other mechanisms that promote parental care will be important in order to test future hypotheses about potential interactions of hormones that promote the onset of parental care.

Reproductive experience is also known to increase the sensitivity to chick stimuli, and/or sensitivity to endocrine mechanisms that promote parental care, which can influence the quality, intensity, and/or latency to perform parental care. Surprisingly, of the subjects that had eggs in their nest, there were no significant effects of experience on the probability of providing parental care or the duration of parental behavior, indicating that both inexperienced birds, who have never been exposed to chicks other than in their natal nest, are equally capable of caring for foster chicks as experienced birds. This also indicates that even though experienced birds have the advantage of already performing these novel parental behaviors, a high circulating PRL signal is still required at the time of hatching to ensure that parental

care occurs immediately and at the required levels for chick survival. Although there were no effects of experience in the ability to provide parental care, experienced pairs showed a strong, positive correlation in chick brooding and feeding duration, whereas inexperienced pairs did not, suggesting one benefit of experience is increased behavioral coordination. Indeed, behavioral coordination is critical for successful reproduction in biparental species (Royle, Schuett, & Dall, 2010) and greater behavioral coordination leads to greater reproductive success (Sánchez-Macouzet, Rodríguez, & Drummond, 2014).

Contrary to our prediction, VIP injections increased the latency to feed chicks. However, this did not affect the ability to feed chicks because, despite a significant negative correlation between the chick feeding duration and latency to feed chicks, there were no differences in the overall duration of chick feeding between treatment groups. In addition, there was no significant effect of treatment on weight change in the chicks, indicating that chicks were fed roughly the same amounts. This effect, instead, may be a consequence of VIP treated birds spending less time eating overall. In domestic chicken chicks, intracerebroventricular (ICV) injections of VIP inhibited food intake, regardless if chicks were food deprived or had access to food *ad libitum* (Khan, Cline, Aramaki, Ueda, & Tachibana, 2013), whereas ICV injections of anti-chicken VIP antiserum stimulated feeding (Tachibana et al., 2003). While we cannot know if our VIP manipulation targeted the neural regions with VIP receptors that control eating, it is likely that VIP also reduces food intake in zebra finches and this would have potentially competing effects with PRL or the ability to feed chicks, but these hypotheses remain open to testing. The effects of VIP on behavior are still not

well-understood, especially peripheral effects. This study may provide novel insight to the role of VIP in food intake in this species,

Females lost weight if there were eggs in the nest, whereas males did not, which may be related to egg laying and incubation. There are energetic costs associated with egg laying and incubation (Williams, 1996; Williams, 2012) and although chick brooding duration was not significantly different between the sexes, female zebra finches have been reported to incubate eggs more than males in other studies (Delesalle, 1986; El-Wailly, 1966; Zann & Rossetto, 1991), which was likely happening prior to placing chicks inside the nest. This is also supported by the fact that there was no significant weight loss in female subjects that did not have eggs in the nest. Although this weight loss could simply be due to losing the weight of the eggs, we measured the pre-weight before subjects were placed into the experiment, so the weight loss is likely a combination of egg weight loss and incubation.

Finally, one potential concern is that VIP affected egg laying since egg laying began during the five days of treatment. However, this seems unlikely, as there were no differences in the average number of eggs in the nest between treatment groups and no difference in the probability of having eggs in the nest based on treatment. Furthermore, previous studies have found no effect of PRL on clutch size determination in zebra finches (Ryan, Dawson, Sharp, Meddle, & Williams, 2014) or in American kestrels (*Falco sparverius*; Sockman, Schwabl, & Sharp, 2000).

Conclusions

In conclusion, this is the first study to manipulate VIP, and hence PRL, in non-

parenting zebra finches to test for a role of PRL in the priming and onset of parental behaviors. While PRL has been suggested to be involved in the initiation of parental care in birds, we found that zebra finches are capable of providing parental care in the absence of high PRL. Although incubating zebra finches can provide care to foster chicks, there was a lower percentage of birds that displayed chick feeding, a strong indicator of parental care onset, relative to the percentage of normally breeding birds which fed chicks in another study from the same lab. We also found that both male and female zebra finches will only provide care *after* egg laying, drawing attention to the importance of external sensory stimuli in promoting the onset of parental care. How stimuli from the nest, eggs, and chicks feed back into the brain in normally breeding birds is still largely unknown, but tactile egg stimulation and PRL release via hypothalamic VIP neurons may be a candidate mechanism in this process as it is in chickens (Sharp, 2009). We found reproductively inexperienced and experienced zebra finches are equally likely and capable of providing care to foster chicks, however, experienced pairs appear to be more behaviorally coordinated. Lastly, counter to our predictions, peripheral VIP injections increased the latency to feed chicks, which was likely because VIP reduced food intake. VIP's effects on behavior are still not well understood, especially peripheral effects, and investigation into the role of VIP in food intake regulation would help clarify these results and inform future hypotheses of the role of PRL in avian parental care.

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CHAPTER 4

LOWERING PROLACTIN REDUCES POST-HATCH PARENTAL CARE IN MALE AND FEMALE ZEBRA FINCHES

Submitted, *under review*.

4.1. ABSTRACT

Parental care is a widespread phenomenon observed in many diverse taxa. Neuroendocrine systems have long been thought to play an important role in stimulating the onset of parental behavior. In most birds with altricial young, circulating prolactin (PRL) levels are low during non-breeding times and significantly increase during late incubation and early post-hatch chick care. Because of this pattern, PRL has been suggested to be involved in the initiation of parental care in birds, but rarely has this hypothesis been causally tested. To begin testing the hypothesis, we inhibited the release of endogenous PRL with bromocriptine (BR) on the 3 days prior to hatching in incubating parents and the first 2 days of post-hatch care, when PRL was found to be highest in zebra finches. Nest temperatures were recorded during all 5 days and parental behavior was recorded on days 1-2 post-hatch. In addition to hormonal systems, reproductive experience may also influence parental care; therefore, we tested age-matched inexperienced and experienced pairs in each group. BR either eliminated or drastically reduced chick brooding and feeding behavior, resulting in decreased nest temperatures on days 1 and 2 post-hatch. Experienced birds brooded and fed chicks more than inexperienced birds and females

brooded more than males. Male-female chick feeding behavior was positively correlated in control pairs, but not in BR pairs. This is one of the few causal studies to demonstrate that PRL is necessary for post-hatch care in a biparental songbird, and is the first to show this effect in zebra finches.

4.2. INTRODUCTION

Parental care is undoubtedly important for offspring survival, offspring quality, and future offspring reproductive success (Klug and Bonsall, 2014), and hence, is an important component of fitness for parents. Birds are the largest parental vertebrate clade, and 98% of avian species provide some variation of parental care, with over 80% providing biparental care (Cockburn, 2006). Despite this, we still know surprisingly little about the endocrine regulation of avian parental care. Endocrine systems have long been thought to play an important role in the onset of parental care as they are known to regulate various aspects of both physiology and behavior. In particular, the hormone prolactin (PRL) has an important association with parental care in many vertebrate species, including birds. PRL is generally low during non-breeding times, but significantly increases during late incubation and early post-hatch care in birds that hatch altricial young (reviewed in Angelier et al., 2016; Smiley and Adkins-Regan, 2016a; reviewed in Wingfield and Farner, 1993). High PRL concentrations in breeding birds have been positively correlated with increased parental care behaviors (Chastel et al., 2005; Khan et al., 2001; Miller et al., 2009; Schoech et al., 1996; Smiley and Adkins-Regan, 2016b; Vleck et al., 1991) and increased reproductive success (Ouyang et al., 2013, 2011; Riechert et al., 2014;

Smiley and Adkins-Regan, 2016b) while low PRL concentrations have been related to poor body condition, nest abandonment, low rates of parental care, and offspring mortality (reviewed in Angelier et al., 2016; reviewed in Angelier and Chastel, 2009; Smiley and Adkins-Regan, 2016b). Because of these relationships, there has been a growing interest in PRL's role in regulating life history stages and trade-offs, environmental change produced stress during breeding, and control of breeding decisions (reviewed in Angelier et al., 2016; reviewed in Angelier and Chastel, 2009). However, most studies on PRL in birds to date have been primarily correlational, describing patterns of PRL secretion during the breeding cycle and its relationship with parental behavior. There remains a striking lack of causal experiments that demonstrate PRL's role in regulating various aspects of avian post-hatch parental care.

Moreover, the causal studies that have manipulated PRL during post-hatch parental care have produced mixed results. The most prominent work on the role of PRL during post-hatch care has been conducted in ring doves (*Streptopelia risoria*), in which PRL has been shown to be causally related to squab feeding and brooding (Buntin et al., 1991; Wang and Buntin, 1999). However, both male and female ring doves feed squabs crop milk which is produced by the epithelial mucosal cells along the wall of the crop sac in response to PRL, an evolved trait unique to pigeons and doves (Buntin, 1996; Patel, 1936), making these findings difficult to generalize to other kinds of birds. Another study in house finches (*Carpodacus mexicanus*) showed that pharmacologically increasing or decreasing PRL respectively increased and decreased nestling feeding rates (Badyaev and Duckworth, 2005). Additionally, reducing PRL in black-legged kittiwakes (*Rissa tridactyla*) caring for young reduced

nest attendance and motivation to return to the nest following a disturbance (Angelier et al., 2009). On the other hand, while reducing PRL in male Adélie penguins (*Pygoscelis adeliae*) during the chick-rearing period reduced their diving efficiency, it did not affect chick growth or survival or food foraging durations (Cottin et al., 2014), suggesting that parental behavior, particularly feeding, was not disrupted by inhibiting PRL. Most recently, Smiley and Adkins-Regan (submitted) have shown that zebra finches (*Taeniopygia guttata*) in the early stage of incubation, when PRL is still low, will care for foster chicks, demonstrating that high PRL is not necessary to display parental behavior. Treating incubating zebra finches with VIP, the primary avian PRL releasing peptide (Christensen and Vleck, 2008), had no effect on parental behavior (Smiley and Adkins-Regan, submitted), whereas injections of mammalian PRL in willow ptarmigans (*Lagopus l. lagopus*) during incubation resulted in hatching twice as many chicks as controls and increased post-hatch parental behavior (Pedersen, 1989) and induced squab feeding in ring doves (Buntin et al., 1991). Therefore, although PRL is clearly *related* to parental care, it is currently unclear whether it has a direct role in parental care behavior or, alternatively, is involved in some other physiological process that coincides with the time of parental care and breeding. Testing this hypothesis in a wider range of birds would help generalize the role of PRL and would help clarify what the PRL-parental care relationship is.

In addition, birds with different levels of reproductive experience may have different sensitivities to PRL. Circulating PRL is greater in experienced breeding birds, compared to inexperienced breeding birds, in zebra finches (Smiley and Adkins-Regan, 2016a), pigeons (*Columba livia*; Dong et al., 2013), wandering albatrosses

(*Diomedea exulans*; Angelier et al., 2006), common terns (*Sterna hirundo*; Riechert et al., 2012), black-browed albatrosses (*Thallasarche melanophris*; Angelier et al., 2007) and dark-eyed juncos (*Junco hyemalis*; Deviche et al., 2000), suggesting experienced birds may have a higher sensitivity to PRL, relative to inexperienced birds. Changes in central nervous system sensitivity to PRL may explain the differences in circulating PRL. Christensen and Vleck (2015) found that reproductively experienced zebra finches had nearly 50% more PRL producing cells in the anterior pituitary gland than did age-matched, inexperienced zebra finches. To our knowledge, it is currently unknown whether PRL receptors change (e.g., are upregulated) as a function of reproductive experience, but this may also influence an individual's sensitivity to the effects of PRL. Notably, treating reproductively experienced ring doves with PRL resulted in a greater frequency of regurgitation feedings, greater squab weight gain, and more time spent sitting in the nest than treating inexperienced ring doves with PRL (Wang and Buntin, 1999), suggesting ring doves have an increased sensitivity to PRL as a result of reproductive experience. Aside from ring doves, however, it is currently unknown whether the increase in PRL or PRL sensitivity that comes with experience also regulates any behavior changes that accompany reproductive experience, such as increased chick feeding. For that reason, there are two different predictions one could make in respect to the interaction between PRL and experience. (1) If experienced birds require greater levels of hormones (e.g., PRL) to achieve the same level of behavior as inexperienced breeders, then the behavioral deficits will be greater in experienced birds if PRL is inhibited. (2) Alternatively, if experienced birds are more sensitive to PRL, then they may still be able to perform that same amount of

parental behavior with lower amounts of hormone, and therefore, would not be as greatly affected by a hormonal manipulation as an inexperienced bird would be. In addition, experience may act as a buffer against hormonal perturbations by way of learning and memory which could allow them to compensate for behavior in the absence of hormones. In rats, experience with pups immediately post-partum forms a 'maternal memory' which can later be activated in the presence of pups and in the absence of the hormonal milieu that accompanies birth (Cohen and Bridges, 1981; Scanlan et al., 2006). Therefore, PRL may not be as important for experienced birds, if they can rely on learning and memory from previous reproductive cycles, and their behavior may be less hormonally dependent.

To begin testing the hypothesis that PRL is causally related to parental care behavior, and to test whether this relationship is differentially affected by reproductive experience, we inhibited the release of endogenous PRL on the 3 days prior to hatching in incubating parents and the first 2 days of post-hatch care, when PRL was found to be highest in zebra finches (Smiley and Adkins-Regan, 2016a). Zebra finches are socially monogamous and biparental, which allows both males and females to be tested. PRL was lowered by using bromocriptine (BR), a proven PRL inhibitor in a variety of birds [e.g., emperor penguins (*Aptenodytes forsteri*; Angelier et al., 2006); house finches (Badyaev and Duckworth, 2005); Adélie penguins (Cottin et al., 2014; Thierry et al., 2013); domestic chicken hens (*Gallus gallus domesticus*; Reddy et al., 2007)].

There are two potential sources of PRL's influence on parental care that we aimed to test in this experiment: (1) PRL plays a preparatory or stimulatory role in

parental behavior itself and/or (2) PRL plays a role in other physiological or behavioral changes that co-occur with the onset of parental care. To test the first outcome, we measured chick brooding and chick feeding behavior in male-female pairs that either received BR or vehicle control treatments. Age-matched inexperienced and experienced birds were tested in each treatment group. We predicted that BR treatment would reduce parental care and that these deficits would be greater in inexperienced pairs, relative to experienced pairs, as experienced birds likely have increased sensitivity to PRL and may rely on learning and memory from their previous reproductive bout. In the control condition, we predicted that experienced pairs would show more behavior than inexperienced pairs. To measure if PRL was affecting other processes related to parental care, we measured eating and drinking behavior. The reasons for this were two-fold. First, PRL has well documented effects in feeding (hyperphgia) (Buntin, 1989; Woodside, 2007) and osmoregulation (Bole-Feysot et al., 1998), and eating and drinking are two behaviors that are most likely to increase with parental care *and* be affected by PRL. Second, these two behaviors also served as a non-social behavior such that these data could help interpret whether the effect(s) on parental behavior were due to lowered PRL or due to an unintended side-effect of the treatment. We predicted that control birds would eat more than BR treated birds and that these behaviors would not be affected by experience.

4.3. METHODS

Subjects

This study used a total of 100 age-matched zebra finches (n= 50 males, n= 50 females) raised in the lab. The exact hatch date was unknown for most subjects, but all subjects came from the same cohort of birds that were hatched during February 2015 – August 2015 and would have been approximately 1 ± 0.25 years old at the time of the experiment. Roughly half of the subjects (n = 24 males, n= 24 females) were inexperienced (INEX) breeders, which had no interaction with the opposite sex since removal from the natal cage at independence (~day 40 post-hatch). The other half (n = 26 males, n= 26 females) were reproductively experienced (EXP), defined as having raised at least one chick to fledging prior to the experiment. Before and during the experiment, all birds had access to food (commercial seed mix; Kaytee Fortifinch Diet), water, and grit *ad libitum.* Birds were supplemented with hardboiled egg once per week. All rooms were kept in temperature (22.2° C) and humidity (range 30-70%) controlled rooms on 14:10 light:dark cycles. All methods and procedures were approved by the Cornell University IACUC.

All subjects were housed in sex-specific aviaries (0.94 m x 0.76 m x 0.94 m) which held up to 20 birds prior to the beginning of the experiment. Subjects were randomly assigned to remain inexperienced or to become an experienced breeder before the experiment. Inexperienced birds were given the opportunity to pair with a partner by moving them into a social aviary containing four males and four unrelated, unfamiliar females. Aviaries were observed for 20 minutes a day for five days to determine pairs. Males and females who exclusively clumped, allopreened, and shared a nest box were considered paired (Smiley et al., 2012). (Note that while we had nest boxes in the INEX cages during pair observations, this was not enough time to begin

breeding. However, it is possible that subjects may have gained sexual experience in the pairing cage prior to the experiment, but no parenting experience). Experienced birds were paired in the same way but were allowed to complete one breeding cycle after pairing. Experienced birds remained with their breeding partner for the experiment.

Study design

Prior to the start of the experiment, subject pairs were randomly assigned to receive either a bromocriptine (BR) or vehicle-control (CON) treatment and were also randomly assigned to be tested in one of six cohorts, each balanced for reproductive experience and treatment manipulation. For each cohort, four different testing rooms, each housing four to six cages, were used. Each room was balanced for treatment and experience conditions. All subjects were weighed to the nearest 0.1 gram and tarsi were measured to the nearest 0.01 mm using electronic calibers (average of four measurements) on the day before they were put into the experiment.

For each cohort, male-female pairs were housed together in smaller testing cages (0.6 m x 0.4 m x 0.35 m) that contained an empty nest box and nesting material (coconut fibers) available *ad libitum*. Daily nest checks were performed to monitor each pair's breeding status. The first day an egg was observed in a nest was counted as "day 1" of incubation. The median incubation cycle for zebra finches is 14 days (Zann, 1996). Eggs were candled on day 6 or 7 of incubation to check for fertility. We only used pairs that had at least one fertile egg in their clutch. The average clutch size was 4.3 ± 0.9 (standard deviation; sd). Pairs that did not lay eggs or produced only

infertile eggs were not tested and were randomly reassigned to a new cohort to attempt breeding again. The final sample sizes were as follows: BR-EXP n=28 (n=14 male-female pairs); BR-INEX n=28 (n=14 male-female pairs); CON-EXP n=24 (n =12 male-female pairs); CON-INEX n=20 (n=10 male-female pairs).

Circulating PRL concentrations peak during the end of incubation and beginning of post-hatch care in zebra finches (Smiley and Adkins-Regan, 2016a) and our goal was to block this peak and test the effects on post-hatch parental care. Therefore, subjects received their assigned BR or CON treatment (see section 2.3. for drug details) at the same time each morning on days 12, 13, and 14 of incubation and days 1 and 2 post-hatch. All treatments were administered between 0900 and 1100. Video cameras were attached to each study pairs' nest box and cage on day 11 incubation so that subjects could habituate to their presence. Camera equipment and recording procedures are described in Smiley and Adkins-Regan (2016b). We added a second, identical camera to the back of each cage to measure eating and drinking behavior. In addition, temperature data loggers (Thermochron ibuttons, model DS1921G-F5#, Maxim Integrated, San Jose, CA) were placed just under the nest material where the eggs sat on day 11 to record the nest temperature throughout the five-day experimental period at a sample rate of every 10 minutes as a proxy to monitor nest attendance. In addition to monitoring nest occupancy, we wanted to test whether BR treatment would affect parental investment (i.e., brooding at lower temperatures as in Thierry et al., 2013). Furthermore, PRL may play a role in regulating temperature as it is involved in energy metabolism (reviewed in Bole-Feysot et al., 1998).

On day 1 post-hatch, immediately after subjects received treatment, all eggs were removed and two 1-4 day old foster chicks (see *Foster chick stimuli* for details) were placed in the nest in order to standardize the hatching dates/times and chick rearing load for all pairs. No pairs had hatched chicks of their own at the time of foster chick placement. Behavior was videotaped on both days 1 and 2 post-hatch for four consecutive hours immediately following treatment administration, which should accurately reflect the overall amount of parental care that birds would provide throughout the day (Morvai et al., 2016). Foster chicks were weighed to the nearest 0.0001 gram immediately before and after the video each day and remained in the nest overnight. After the last video on day 2, each adult subject was weighed again to the nearest 0.1 gram. Subjects were sacrificed the following day for another project and foster chicks were returned to their natal nests.

Parental behavior inside the nest was coded as in Smiley and Adkins-Regan (2016b) using the program ELAN, version 4.9.2. Briefly, chick brooding was coded as the total amount of time a subject spent sitting on top of the chicks (to provide heat for chick thermoregulation). Chick feeding was coded as the total time a subject spent regurgitating into either of the chick's mouths. In addition, we also coded eating and drinking behavior in subjects as in Smiley and Adkins-Regan (submitted). Eating was coded as the total time an adult subject spent consuming seed from the feeder and drinking was coded as the total time an adult subject spent consuming water from the water bottle spout. A trained research assistant who was blind to treatment and reproductive experience conditions coded each behavior for males and females separately. Total durations for all behaviors were summed for males and females

separately, for each video. The first time point at which each behavior was observed for each subject was used as the latency value. This produced eight different response variables for all subjects, for each day of video: brooding duration, brooding latency, chick feeding duration, chick feeding latency, eating duration, eating latency, drinking duration, and drinking latency.

A total of 50 videos were taken for each behavior (i.e., one per nest); however, some videos had to be excluded from the behavioral coding because the video quality was either too poor to accurately code the behavior or there was a technical failure of the camera/computer equipment to record or save the video to the computer. The total number of videos used to code each behavior were as follows: brooding n=45; chick feeding n=40; eating n=38; drinking n=39.

Bromocriptine dosage and pilot study

Bromocriptine (2-Bromo-α-ergocryptine methanesulfonate salt, Sigma-Aldrich B2134) was suspended in peanut oil at a dose of 10 mg/kg body weight. This solution was thoroughly vortexed before being taken up by a pipette. Subjects were fed either 50 µl of the BR solution or 50 µl of peanut oil alone (control). To orally administer the treatment, birds were captured by turning off the lights in the study room and were located using flashlights. Treatments were slowly pipetted into the subject's mouth and were readily consumed. Subjects were immediately placed back into their testing cage and lights were turned back on.

The effectiveness of this treatment to lower PRL was validated in a pilot study that used a separate cohort of 17 adult, breeding zebra finches (n=9 males; n=8

females) of mixed reproductive experiences. Pilot subjects were randomly assigned to either receive a BR or control treatment during days 12, 13, and 14 of incubation and day 1 and 2 post-hatch. Blood samples were taken on day 2 post-hatch. BR subjects were randomly assigned to have a blood sample taken at 1 (n=3), 2 (n=4), 3 (n=3), or 4 (n=3) hours after the treatment; control subjects (n=4) were bled at 3 hours post treatment. Our blood sampling and plasma collection and storage procedures are described in Smiley and Adkins-Regan (2016a, 2016b). Circulating PRL concentrations were assayed in plasma across two plates using an enzyme-linked immunosorbent assay (ELISA), following the methods described in Smiley and Adkins-Regan (2016b); for validation of the assay, see Smiley and Adkins-Regan (2016a). We used a high- and low-pool of PRL to calculate the coefficients of variation (CV) within and between plates. For plate 1, the high-pool intra-CV = 3.33% and low-pool intra-CV = 2.56%. For plate 2, the high-pool intra-CV = 7.52% and low-pool intra-CV = 5.75%. The inter-CV values were 2.78% for the high-pool and 7.36% for the low-pool. One pilot subject (BR bled at 1 hour) was not analyzed because the assay failed to read the sample.

Foster chick stimuli

Breeding cages (0.94 m x 0.76 m x 0.94 m) containing eight male-female breeding pairs (separate from the subjects used in this study) and eight nest boxes were set up to produce offspring stimuli for each cohort. Breeding cages were supplied with nesting material (coconut fibers), food, water, and grit available *ad libitum* and were provided weekly hard-boiled egg supplement. Daily nest checks were performed

to track incubation and to predict chick-hatching dates. Eggs were checked for fertility by candling them one week after laying. Foster chicks were returned to their natal nest when the study concluded.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows, version 24.0 (IBM Corp, Armonk, NY) unless otherwise noted. All data were analyzed at the level of the individual, unless otherwise noted. The use of mixed models addresses the non-independence of the data by including a random variable that control for pair identity (referred to as "PairID"), with the caveat that subjects' behavior may be dependent on the fact their partner received a similar drug treatment. For all mixed models that were used, non-significant terms were removed in a backwards, stepwise manner until only significant terms remained. Assumptions were checked for all mixed models by plotting predicted values vs. residuals and by plotting residuals on a Q-Q plot to visually inspect for normality. In all analyses, significance was accepted if p-values were < 0.05.

Effect of bromocriptine on plasma prolactin concentrations: pilot study

A linear mixed model (LMM) was used to analyze the effects of time post-treatment on circulating PRL concentrations. Time of blood sample post-treatment was the fixed factor and NestID, a unique number to identify which nest subjects occupied (which also accounts for PairID), and SubjectID, a unique number to identify each individual subject, nested with NestID were included as random factors.

Analyzing each time point separately did not yield an adequate sample size to detect effects between groups, therefore hours 1 and 2 post-bromocriptine administration were combined and hours 3 and 4 were combined to create two BR-treated groups to compare to the control group. Least significant difference (LSD) post-hoc tests were used to determine significant differences between these three groups.

Effect of treatment, experience, sex, and day of video on probability of showing parental behavior

We tested whether the probability of displaying either parental behavior (chick brooding or feeding) was affected by treatment, experience, sex, or day of video and all possible 2-, 3-, and 4-way interactions (fixed factors) by running two separate generalized linear mixed models (GLMM) with a binomial distribution and logit-link, where 0 = no behavior and 1 = some behavior. CohortID (a unique number to denote which cohort birds were used in) and PairID nested within CohortID were included as random factors for both models. These analyses were performed in SAS, University Edition for Windows 10.

Effect of treatment, experience, sex, and day of video on behavior durations and latencies

For each behavior duration and latency, a LMM was used to test the effects of treatment, experience, sex, day of video, and all possible 2-, 3-, and 4- way interactions. For each of these models SubjectID nested within PairID, PairID nested within CohortID, PairID nested within StudyRoomID (a unique number to denote

which room birds were tested in) *CohortID, and StudyRoomID nested within CohortID, were included as random factors. Durations were analyzed as proportions by dividing the total amount of time the behavior was observed by the total length of the video. Our parental behavior (chick brooding and feeding) data distributions were highly skewed due to many zeros in the data set, therefore, we analyzed these behaviors in two separate ways. First, we analyzed both behaviors with all subjects' data included. To see if these effects were driven by the zero's in the data, however, we analyzed these two behaviors again, but with all zeros removed from the data set. These data were log-transformed to normalize the data. Eating and drinking durations were also analyzed as proportions. Eating durations were log-transformed (all subjects were observed eating on both days) and drinking durations were log transformed with an added 0.001 constant because three 0's existed in the drinking data set (CON-EXP n=2; CON-INEX n=1). All latency values were log-transformed.

Effect of treatment, experience, and day on nest temperature

Nest temperatures were averaged during the 4-hour period following treatment administration, referred to as treatment "on," and over the 20-hour period in between treatments, referred to as treatment "off," for each nest. This was done for all 5 days resulting in 10 outcome variables: Day 12 incubation treatment on, Day 12 incubation treatment off…Day 2 post-hatch treatment on, and Day 2 post-hatch treatment off. Five out the 25 total nests only had temperature data for days 1 and 2 post-hatch (BR-INEX n=1; BR-EXP n=2; CON-EXP n= 1; CON-INEX n=1).

We used a LMM to analyze the effects of treatment, experience, day of

treatment (e.g., day 12 incubation through day 2 post-hatch), treatment on/off, and all possible 2-, 3-, and 4- way interactions. CohortID, day of treatment nested within Cohort ID, and NestID nested within CohortID, were included as random factors. These data were analyzed at the level of the nest since both members of the pair would have the same values. Pairwise comparisons were assessed using LSD post-hoc tests for all significant fixed effects.

Effect of treatment, experience, and sex on change in adult body condition

Body condition (BC) was calculated by taking the standardized residuals from the linear regression between body weight and tarsus length (Fokidis et al. 2011). Since body weight was taken before (pre-weight) and after (post-weight) the experiment, we were able to calculate a pre-BC score and a post-BC score. We subtracted the pre-BC score from the post-BC score to create a "change in BC" score for each individual. A LMM was used to test the effects of treatment, experience, sex, and all possible 2- and 3-way interactions on the change in BC score. PairID nested within StudyRoomID * CohortID, StudyRoomID, and CohortID were included as random factors.

Effect of treatment, experience, and day of video on chick survival probability

We tested whether the probability of chick survival during the two days of videotaping was affected by treatment, experience, or an interaction between the two by running two separate GLMMs with a binomial distribution and logit-link, where 0 = no chicks died on either day 1 or 2 and 1 = at least one chick died on either day 1 or

2. These data were analyzed at the level of the nest since both members of the pair would have the same values. CohortID was the random variable for each model. These analyses were performed in SAS, University Edition for Windows 10.

Effect of treatment, experience, and day of video on chick weight change

We subtracted the pre-video weight from the post-video weight for each chick on each day the video was taken. For each day, the weight change for the two chicks was averaged to create one day 1 average weight change response variable and one day 2 average weight change response variable. Chick weight change was analyzed at the level of the nest, since male and female subjects would have identical values. We tested if these values were affected by the treatment, reproductive experience, day of video and all possible 2- and 3-way interactions for each nest by including these as fixed factors in a LMM, while including CohortID and NestID nested within CohortID as random factors.

Correlations between behavior durations and latencies within male-female pairs

Correlations between all behavior durations and behavior latencies were analyzed within pairs using a Pearson's R test, controlling for PairID. Correlations were run with all subjects together, as well as pairs within BR and control treatment groups separately and pairs within inexperienced and experienced groups separately. The number of pairs in which only one member performed behavior are as follows: chick brooding (BR-EXP n=5; BR-INEX n=2); chick feeding (BR-EXP n=3; BR-INEX n=1; CON-EXP n=2); eating (CON-EXP n=1); drinking (CON-EXP n=1;

CON-INEX n=1).

4.4. RESULTS

Bromocriptine pilot study results

There was an overall effect of time of blood sample post-treatment on PRL concentrations ($F_{2,13} = 4.02$, $p = 0.04$). LSD post-hoc tests revealed that PRL concentrations during hours 1-2 post-treatment were significantly lower than hours 3-4 ($p = 0.04$) and controls ($p = 0.02$). Means and standard errors (SE) are presented in Figure 4.1.

Figure 4.1. Effects of bromocriptine on plasma prolactin concentrations: A pilot study

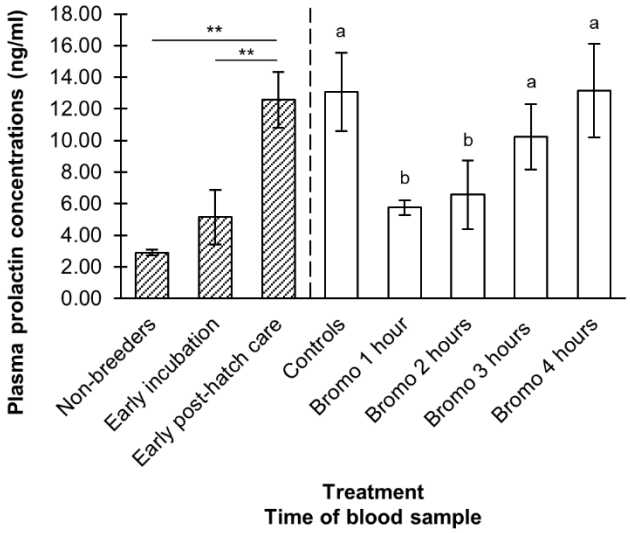

Figure 4.1. The effect of treatment (bromocriptine (bromo) or control) on mean ± standard error plasma concentrations of prolactin following oral administration. Hatched bars to the left of the dashed line are data from non-manipulated non-breeders (N =10 males and 11 females), early incubators (day 4 incubation; N= 3 males and 3 females), and early post-hatch care (day 1 post-hatch; N= 5 males and 3 females) collected in a previous study in our lab (Smiley and Adkins-Regan, 2016a). ** indicate significant difference ($p < 0.05$). Open bars to the right of the dash lined are data from the current pilot study. Differing letters indicate a significant difference. Control birds were fed vehicle (peanut oil) only and were bled 3 hours post-treatment. For statistical analysis, bromo subjects bled 1 and 2 hours post-

(figure 4.1 continued) treatment and birds that were bled 3 and 4 hours post-treatment were pooled together. All pilot subjects were on day 2 post-hatch when blood samples were taken. Plasma prolactin concentrations at bromo hours 1 and 2 are similar to early incubators, which are not significantly different from non-breeders.

Effect of treatment, experience, sex, and day of video on probability of showing parental behavior

There was a significant effect of treatment on the probability of displaying chick brooding ($F_{1,67} = 11.93$, $p < 0.01$) such that control subjects were more likely to brood (least square mean (LSM) = 0.94 ± SE 0.05) than BR subjects (LSM = 0.28 ± SE 0.12), regardless of sex or experience level. There was also a significant effect of treatment on the probability of displaying chick feeding behavior ($F_{1,59} = 10.19$, $p < 0.01$) such that control subjects were more likely to feed (LSM = 0.91 ± SE 0.07) than BR subjects (LSM = 0.24 ± SE 0.12), regardless of sex or experience level.

Effect of treatment, experience, sex, and day of video on behavior durations and latencies

For all analyses in this section, refer to Table 4.1. for statistical data. For a visual representation of the data, see Figures 4.2. and 4.3. For sample sizes and proportions of birds that were observed for each parental behavior, each day, refer to Table 4.2.

Chick brooding

When all data were included in the analysis, there was a significant 3-way interaction between experience, treatment, and day. Pairwise comparisons revealed

several significant pairwise comparisons across each factor level. First, in all four treatment-experience groups, for each day, controls brooded more than BR subjects. Second, CON-EXP birds brooded more than CON-INEX birds, but only on day 2. Lastly, CON-EXP subjects increased their total amount of time spent brooding from day 1 to day 2, but brooding durations for CON-INEX, BR-INEX and BR-EXP groups were not different between days. There was a second significant interaction between sex and day such that, regardless of treatment or experience, females brooded more than males on day 2.

When all the zeros were removed from the data set, the 3-way interaction between experience, treatment, and day remained significant. However, the pairwise comparisons were slightly different. First, and differently, CON birds brooded more than BR birds only on day 2 only for EXP birds and on day 1 only for INEX birds. Second, CON-EXP birds continued to brood more than CON-INEX birds on day 2. Lastly, and differently, both CON-EXP birds *and* BR-INEX birds increased their total brooding time from day 1 to day 2. There was one outlier (>2 sd above the mean) in the day 2 BR-INEX condition, however this effect remains whether the outlier is included or removed from the analysis. The significant interaction between sex and day remained such that females brooded more than males on day 2. However, a third, new interaction emerged between experience and sex such that females brooded more than males, but only in the EXP condition.

There was a significant effect of sex on latency to begin brooding such that females were quicker to initiate brooding (mean = 933.6 s ± SE 352.7) compared to males (mean = 1395.7 s ± SE 215.9).

Chick feeding

When all subjects were analyzed, there was a significant interaction between treatment and experience such that CON birds fed chicks more than BR birds in the EXP condition only. Experienced birds fed for a significantly longer proportion of time (mean=0.02 ± SE 0.004) than inexperienced birds (mean=0.01 ± SE 0.004). There was also a near significant interaction ($p = 0.06$) between treatment and sex such that females tended to feed chicks more than males in the CON group, but not the BR group. There were no significant effects on chick feeding when all zeros were removed, however, indicating that the above effects are likely driven by the large number of zeros in the BR and INEX groups.

There was an effect of day on chick feeding latency such that birds were quicker to initiate feeding on day 1 (mean = 1740.4 s ± SE 478.1) compared to day 2 (mean = 3454.3 s ± SE 681.7).

Eating

There was a significant effect of treatment such that BR birds spent more time eating (mean = 1396.1 s ± SE 169.0) compared to CON birds (mean = 679.3 s ± SE 64.8). There was also an effect of day on eating latency such that subjects were quicker to eat on day 2 (mean = 1886.0 s ± SE 377.0), compared to day 1 (mean = 2293.1 s ± SE 365.2).

Drinking

There were no effects of treatment, experience, sex, or day of video on

drinking behavior duration or latency.

Table 4.1. Effect of treatment, experience, sex, and day of video on behavior durations and latencies.

Statistical data corresponding to results in section 3.3. *Effect of treatment, experience, sex, and day of video on behavior durations and latencies.* Each behavior duration or latency was run as a LMM. Fixed factors included treatment, reproductive experience (ReproExp), sex, and day post-hatch the video was recorded (Day1or2), and all initial 2-, 3- , and 4-way interactions. Final models reported here have only significant factors remaining. Duration data was analyzed in two separate ways: (1) with all data included and (2) with all zeros in the data set removed. N is the total number of subjects included in each respective model.

Behavior	Parameter	Numerator df	Denominator df	F	*p*-value	N =
Brooding duration						90
	Treatment	1	39.39	36.89	<0.01	
	Day1or2	1	38.18	20.36	<0.01	
	Treatment * Day1or2	1	38.18	14.15	<0.01	
	Sex * Day1or2	2	38.80	5.32	0.01	
	ReproExp * Treatment * Day1or2	4	38.73	3.09	0.03	
Brooding duration						50
(zeros removed)	Treatment	1	26.73	17.92	<0.01	
	Day1or2	1	22.75	14.46	<0.01	
	ReproExp * Day1or2	1	22.75	4.82	0.04	
	ReproExp * Sex	1	26.01	5.34	0.03	
	Sex * Day1or2	1	19.24	8.59	0.01	
	ReproExp * Treatment * Day1or2	3	31.76	6.08	0.00	
Brooding latency						52
	Treatment	1	16.42	4.48	0.05	
	Sex	1	39.67	7.12	0.01	
Chick Feeding duration						80
	ReproExp	1	74.48	8.43	0.01	
	Treatment	1	74.48	12.52	<0.01	
	ReproExp * Treatment	1	74.48	4.99	0.03	
	Treatment * Sex	2	74.48	2.97	0.06	
Chick feeding duration						44
(zeros removed)	Sex * Day1or2	2	32.00	3.97	0.03	
	ReproExp * Treatment * Sex	4	32.00	2.56	0.06	
	Treatment * Sex * Day1or2	2	32.00	3.33	0.05	

Table 4.1. continued

Behavior	Parameter	Numerator df	Denominator df	F	*p*-value	N =
Chick feeding latency	Day1or2	1	32.80	8.91	0.01	43
Eating duration	Treatment	1	16.51	6.37	0.02	74
Eating latency	Day1or2	1	33.86	4.39	0.04	73
Drinking duration	No effects					77
Drinking latency	No effects					75

Figure 4.2. Parental behavior data across days, treatments, experience levels, and sex

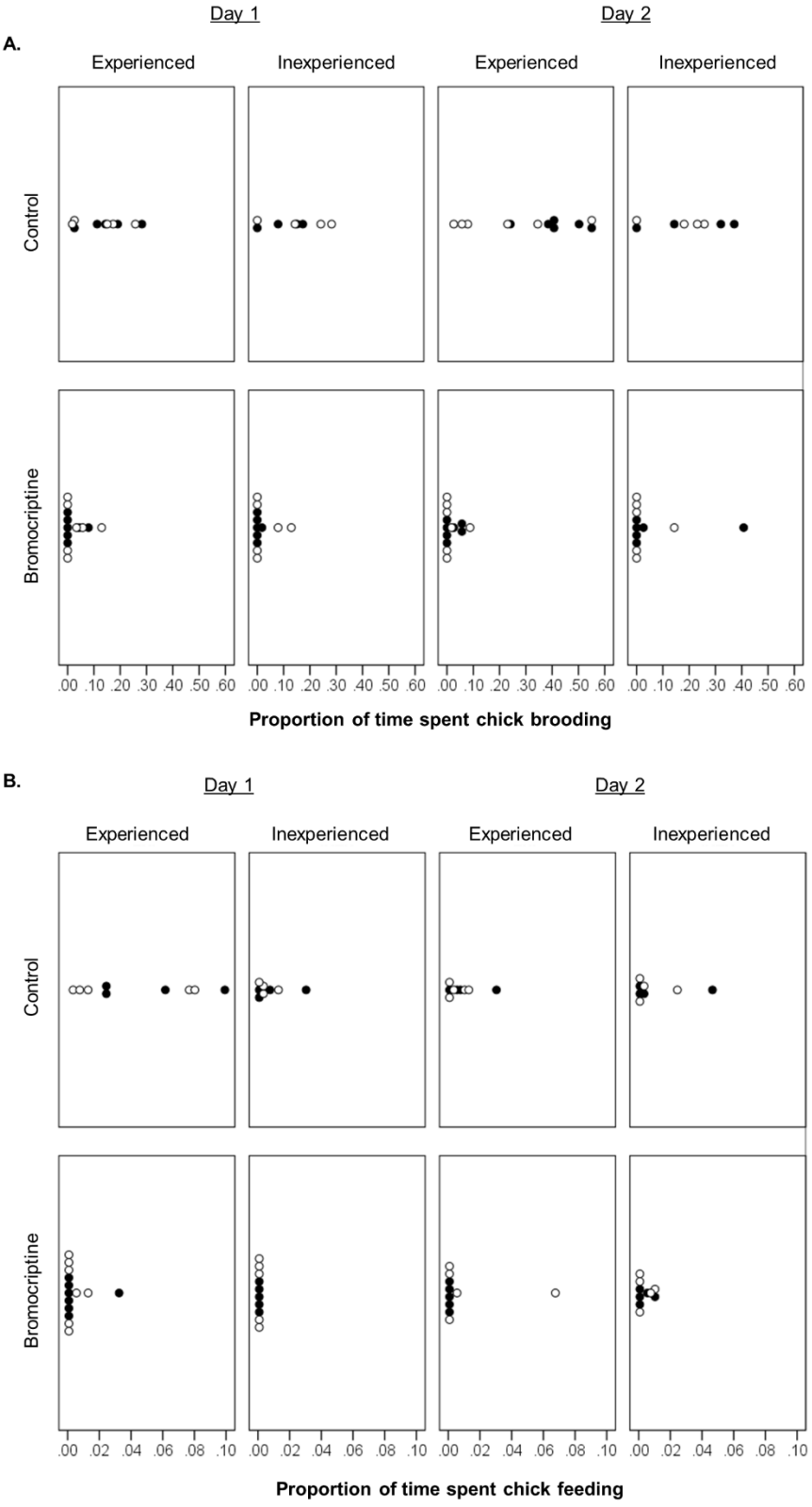

Figure 4.2. Parental behavior data. Dot plots show (A) chick brooding and (B) chick feeding duration data from individuals plotted across days 1 and 2 post-hatch, divided by treatment (Y-axis) and reproductive experience groups (top X-axis). Bottom X-axis is the proportion of time during each video that subjects were observed displaying each behavior. Open dots represent male subjects; closed dots represent females.

Figure 4.3. Eating and drinking data across days, treatments, experience levels, and sex

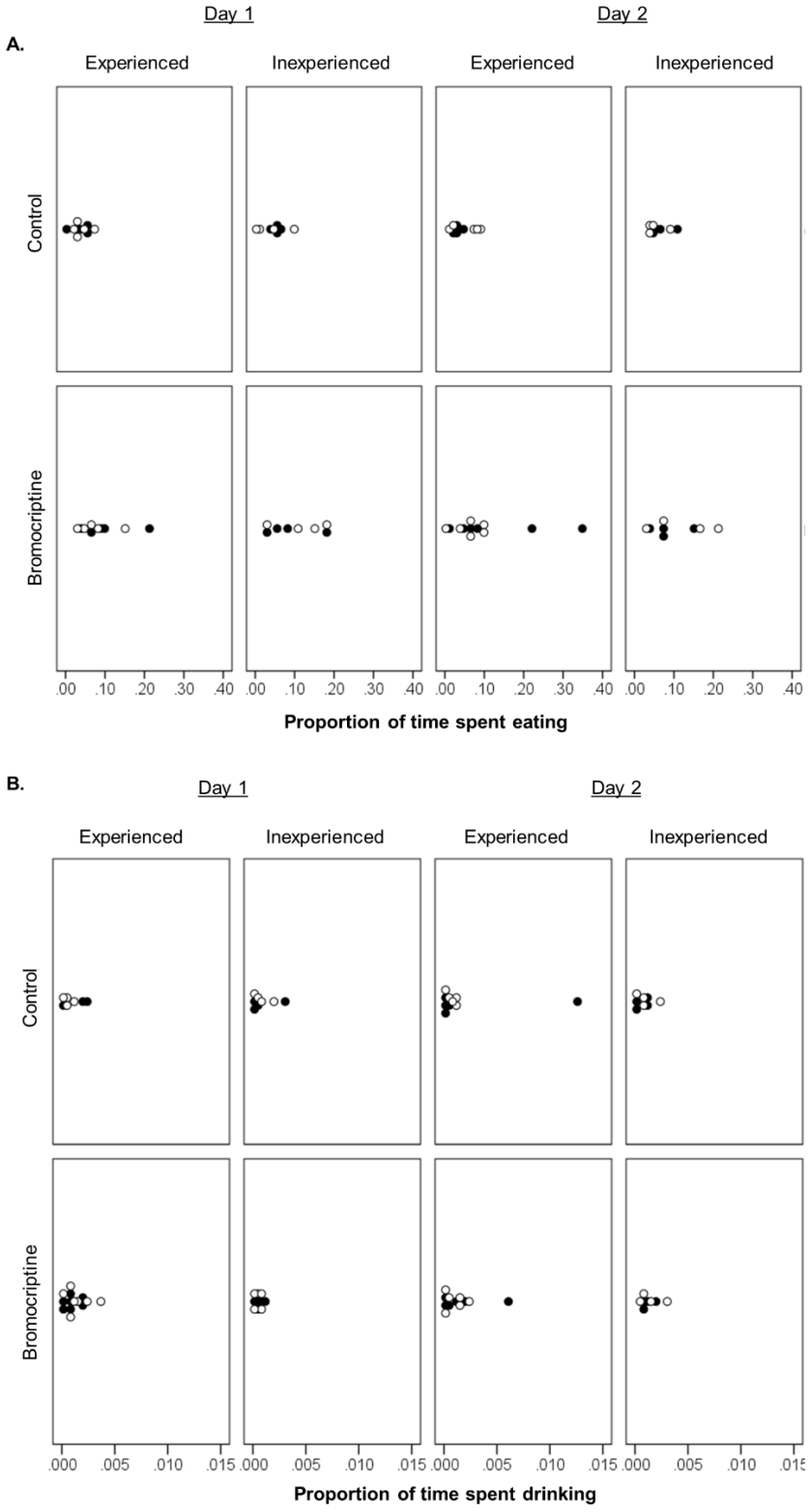

Figure 4.3. Dot plots show (A) eating and (B) drinking duration data from individuals plotted across days 1 and 2 post-hatch, divided by treatment (Y-axis) and reproductive experience groups (top X-axis). Bottom X-axis is the proportion of time during each video that subjects were observed displaying each behavior. Open dots represent male subjects; closed dots represent females.

Table 4.2. Percentage of bromocriptine and control subjects observed performing parental behaviors.

The number and percentage of subjects, separated by treatment groups, that were observed chick brooding and chick feeding across days 1 and 2 post-hatch. Both reproductive experience groups and both sexes are included in each treatment group. N is the total number of subjects for which there was data available.

		Control				*Bromocriptine*			
		N =	**N behavior observed**	**N behavior not observed**	**% behavior observed**	**N =**	**N behavior observed**	**N behavior not observed**	**% behavior observed**
Day 1	**Chick brooding**	18	16	2	88.9	26	8	18	40.0
	Chick feeding	18	16	2	88.9	24	2	22	8.3
Day 2	**Chick brooding**	20	18	2	90.0	26	8	18	30.8
	Chick feeding	18	15	3	83.3	20	2	18	10.0
Average Days 1 and 2	**Chick brooding**	-	-	-	89.4	-	-	-	35.4
	Chick feeding	-	-	-	86.1	-	-	-	9.2

Effect of treatment, experience, and day on nest temperature

We found a significant interaction between treatment and day of treatment ($F_{4,168.79} = 5.26$, $p < 0.01$). For BR treated birds, there were significant nest temperature differences between day 12 incubation and day 1 post-hatch ($p = 0.01$), between day 12 incubation and day 2 post-hatch ($p < 0.01$), between day 13 incubation and between day 14 incubation ($p < 0.01$), between day 13 incubation and day 1 post-hatch ($p < 0.01$), between day 13 incubation and day 2 post-hatch ($p < 0.01$), and

between day 14 incubation and day 2 post-hatch ($p = 0.02$); see Figure 4.4. for means and SE. There were no differences in nest temperature between days for CON treated birds.

There was also a significant interaction between treatment and treatment on/off ($F_{1,160.83} = 13.42$, $p < 0.01$). When the treatment was "on" there was a significant difference between BR and CON birds on day 1 post-hatch ($p = 0.01$) and on day 2 post-hatch ($p < 0.01$). When the treatment was "off" there was a near significant difference between BR and CON on day 2 post-hatch ($p = 0.06$).

Figure 4.4. Nest temperatures across five-day experimental period

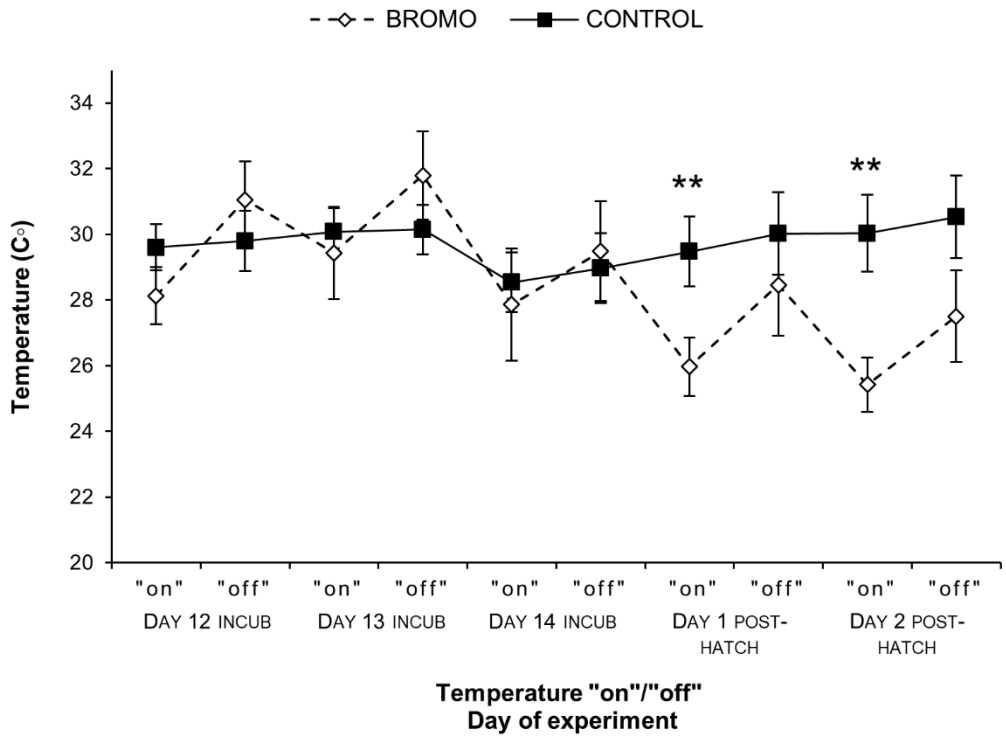

Figure 4.4. The average temperature across the entire experimental period, between treatment groups (bromocriptine (bromo) and control). Temperatures were recorded every 10 minutes beginning after the first treatment on day 12 incubation (INCUB) and were recorded for 24 hours after the last treatment on day 2 post-hatch. Treatment "on" refers to the average temperature during first 4 hours after treatment was administered. Treatment "off" represents the average temperature during the 20 hours in between

(figure 4.4. continued) treatments (treatments were administered 24 hours apart). Bromo and control nests only differed in temperature significantly on days 1 and 2 post-hatch during the treatment "on" period as indicated by ** $p < 0.01$.

Effect of treatment, experience, and sex on change in adult body condition

There was a significant 3-way interaction between sex, experience, and treatment on change in adult body condition ($F_{6,30.78} = 2.59$, $p = 0.04$). Pairwise comparisons revealed several differences among groups, across each factor level. First, BR-INEX females had a nearly significant higher BC than CON-INEX females (p=0.05). Second, BR-INEX females had higher BC than BR-EXP females whereas BR-INEX males had lower BC than BR-EXP males. There was no difference in BC due to experience for either male or females in the CON condition. Lastly, females in the BR-EXP, CON-EXP (p=0.05), and CON-INEX condition all had lower BC than males. There was no difference in BC between males and females in the BR-INEX condition. For visual representation of the data, see Figure 4.5.

Effect of treatment, experience, and day of video on chick survival

Across all groups, 88% of chicks survived both days of treatment. There were no effects of treatment, experience, sex, and day of video on the probability of a chick surviving during days 1 and 2 post-hatch. The number of nests that had at least one chick die during days 1 or 2 post-hatch are as follows: BR-EXP n=1; BR-INEX n=2; CON-EXP n=3; CON-INEX n=1.

Figure 4.5. Change in body condition from beginning of breeding to end of experiment

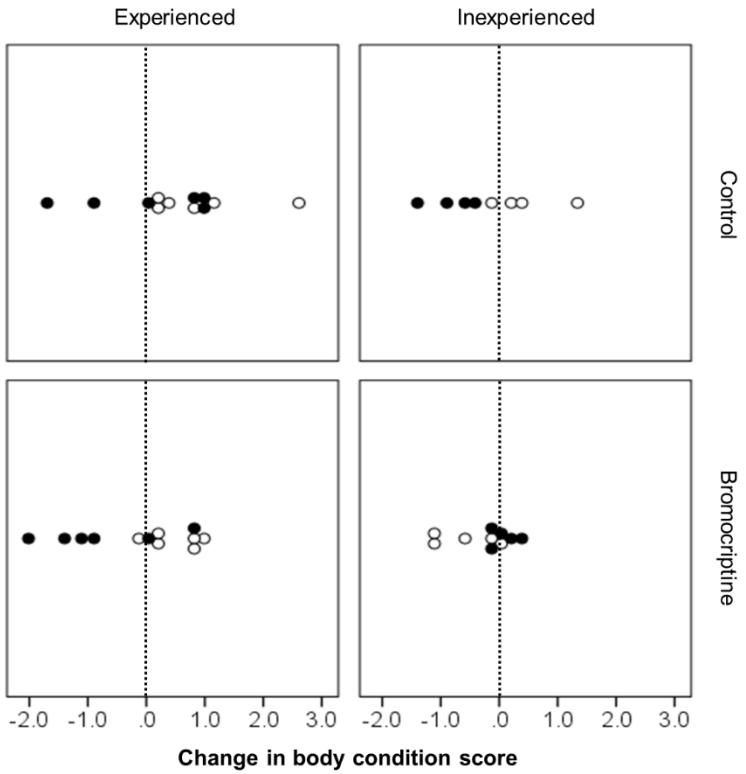

Figure 4.5. Dot plot panels represents change in body condition (BC) score from the beginning of breeding to the end of the experiment for individuals. Panels are divided by treatment (Y-axis) and reproductive experience (top X-axis) conditions. Open dots represent male subjects; closed dots represent females. Bottom X-axis represents a change in BC score. Dotted lines in each panel indicate no change during the experiment. Individuals to the left of the dotted line ended the experiment in worse BC; individuals to the right of the dotted line ended the experiment in better condition.

Effect of treatment, experience, and day of video on chick weight change

There was a significant effect of treatment on chick weight change ($F_{1,42.90}$ = 15.22, $p < 0.01$) such that chicks from BR-treated nests weighed less at the end of the video (mean = -0.01 g ± SE 0.004) compared to chicks from CON-treated nests (mean = 0.07 g ± SE 0.03).

Within pair correlations of behavior and latencies

All Person's r, p-values, and degrees of freedom (df), and a visual correlation matrix are reported in Figure 4.6. Overall, there was a strong, positive correlation between male and female chick feeding durations between pairs. There were also positive correlations between female chick brooding latency and male chick brooding latency, male chick feeding latency, and male eating latency. Female chick feeding latency was positively correlated with male chick brooding and feeding latencies. Female eating and drinking latencies were positively associated with male brooding duration and male drinking latency. Female drinking duration was positively related to male eating duration.

There were no significant correlations when only BR subjects were analyzed. Male and female chick brooding and feeding latencies could not be included in the correlation analysis because there were too few data points to analyze. However, in CON birds, the correlation between male and female chick feeding duration was nearly perfectly positively linear. There were positive correlations between female chick brooding latency and male chick brooding latency, male chick feeding latency, male eating latency, and male drinking duration. Female chick feeding latency was positively correlated with male chick brooding, male feeding latencies, and male eating latency. Female eating and drinking latencies were positively associated with male's drinking latency. Finally, female drinking duration was positively related to male eating duration, while female eating duration was negatively correlated with male drinking duration.

In both INEX and EXP groups, the correlation between male and female chick

feeding duration was strongly positive. For INEX birds, negative relationships existed between female chick feeding duration with male brooding latency and female chick feeding latency with male brooding duration. Both female chick brooding and feeding latencies were positively correlated with male chick brooding and feeding latencies. For EXP birds, male chick brooding duration was positively related to female eating latencies. Male chick brooding latency was negatively correlated with female eating duration. Finally, male drinking latency was positively correlated with both female eating and drinking latencies.

Figure 4.6. Pair behavior and latency correlation matrices

A.

		MALE							
		Chick brooding duration	Chick feeding duration	Eating duration	Drinking duration	Chick brooding latency	Chick feeding latency	Eating latency	Drinking latency
FEMALE	Chick brooding duration	0.39	-0.35	0.23	-0.04	-0.17	-0.07	-0.25	-0.09
	Chick feeding duration	-0.18	0.78	0.09	0.17	0.02	-0.17	0.11	-0.03
	Eating duration	-0.15	0.37	0.32	-0.24	-0.48	-0.34	-0.44	-0.14
	Drinking duration	-0.09	-0.06	0.69	0.37	0.17	0.29	0.10	-0.34
	Chick brooding latency	0.03	-0.11	-0.02	0.54	0.81	0.83	0.73	0.13
	Chick feeding latency	-0.24	-0.36	0.07	0.29	0.61	0.74	0.50	-0.09
	Eating latency	0.68	-0.23	-0.11	0.32	0.11	0.19	-0.08	0.69
	Drinking latency	0.67	-0.15	-0.41	-0.11	-0.12	-0.07	0.02	0.87

Figure 4.6 continued

B.

FEMALE	Chick brooding duration	-0.13	0.32	-0.08	0.49	---	---	-0.41	-0.26
	Chick feeding duration	-0.05	0.05	0.23	0.34	---	---	-0.16	-0.19
	Eating duration	0.36	0.36	-0.16	-0.14	---	---	0.28	0.26
	Drinking duration	0.12	0.30	0.21	-0.11	---	---	0.07	-0.15
	Chick brooding latency	---	---	---	---	---	---	---	---
	Chick feeding latency	---	---	---	---	---	---	---	---
	Eating latency	-0.19	0.14	0.19	0.30	---	---	-0.19	-0.22
	Drinking latency	0.32	0.06	0.01	0.08	---	---	0.17	0.22

C.

FEMALE	Chick brooding duration	0.14	-0.48	0.38	-0.15	-0.30	-0.08	-0.46	-0.30
	Chick feeding duration	-0.44	0.97	0.10	0.12	0.00	-0.18	0.08	-0.14
	Eating duration	0.05	-0.10	-0.11	-0.65	-0.52	-0.54	-0.29	-0.25
	Drinking duration	-0.28	-0.29	0.66	0.32	0.29	0.37	0.21	-0.48
	Chick brooding latency	0.03	0.11	0.20	0.65	0.78	0.85	0.69	0.17
	Chick feeding latency	0.13	-0.23	0.37	0.62	0.76	0.93	0.64	0.14
	Eating latency	0.60	-0.55	-0.31	0.25	0.21	0.29	-0.04	0.67
	Drinking latency	0.59	-0.26	-0.55	-0.21	-0.14	-0.05	0.00	0.86

D.

FEMALE	Chick brooding duration	0.38	-0.10	0.65	0.80	0.25	0.53	0.45	0.01
	Chick feeding duration	-0.18	0.81	0.04	-0.02	0.14	-0.35	0.35	-0.11
	Eating duration	0.21	0.10	0.13	0.16	-0.87	-0.32	-0.75	-0.05
	Drinking duration	0.01	-0.27	0.75	0.58	0.21	0.57	0.44	-0.40
	Chick brooding latency	-0.22	0.51	0.18	0.11	0.48	0.18	0.40	-0.01
	Chick feeding latency	-0.46	-0.45	0.13	-0.24	0.06	0.43	-0.26	-0.32
	Eating latency	0.89	-0.30	-0.21	0.50	-0.01	0.24	-0.26	0.92
	Drinking latency	0.76	-0.08	-0.40	0.28	-0.01	0.05	-0.31	0.95

Figure 4.6 continued

E.

FEMALE	Chick brooding duration	-0.25	-0.12	0.65	-0.06	-0.04	0.04	-0.54	-0.41
	Chick feeding duration	0.82	0.99	-0.54	0.19	-0.90	-0.84	-0.13	0.39
	Eating duration	0.40	-0.03	-0.13	-0.74	-0.34	-0.42	-0.16	0.10
	Drinking duration	0.46	0.79	0.17	0.53	-0.73	-0.67	-0.76	-0.29
	Chick brooding latency	-0.82	-0.76	0.27	0.19	0.93	0.93	0.45	-0.14
	Chick feeding latency	-0.89	-0.85	0.34	0.04	0.96	0.98	0.47	-0.13
	Eating latency	-0.39	-0.42	0.33	0.50	0.55	0.40	-0.19	-0.60
	Drinking latency	0.15	0.03	-0.39	-0.74	-0.19	-0.07	0.53	0.72

Figure 4.6. Numbers inside each cell are Pearson's r statistic. Darker green cells are closer to positive 1 correlation whereas darker red colors are closer to negative 1 correlation. Yellow cells indicate no relationship. Cells outlined in a thick black line are significantly correlated ($p < 0.05$). (A) All subjects included, df=10. (B) Only subjects that received the bromocriptine treatment are included, df=10. Cells with a dashed line (---) could not be analyzed because there were too few data points. (C) Only subjects that received the control treatment are included, df=8. (D) Only reproductively experienced subjects are included. For all correlations, df=4. (E) Only reproductively experienced subjects are included. For all correlations, df=3.

4.5. DISCUSSION

Lowering PRL using bromocriptine (BR) either eliminated or drastically reduced parental care in both male and female zebra finches, regardless of reproductive experience, which greatly reduced the probability of displaying either chick brooding or chick feeding behavior. These results are in agreement with other studies in house finches (Badyaev and Duckworth, 2005) and black-legged kittiwakes (Angelier et al., 2009) which found that lowering PRL reduced post-hatch parental care and nest attendance, respectively. In this study, the behavioral effects of BR appear to be specific to the time chicks were present as BR nest temperatures were only significantly lower than control nests during days 1 and 2 post-hatch, immediately following treatment (Fig. 4.4). The lack of feeding during this time was validated as BR chicks lost weight, while control chicks gained weight, after each video. This lack of feeding, however, was not enough to affect chick survival. Previous work from our lab has shown negative correlations between naturally occurring low PRL concentrations on day 2 post-hatch and increased pre-fledging mortality in zebra finches (Smiley and Adkins-Regan, 2016b), suggesting if we had continued the PRL manipulation and measured behavior for more days, we would have found an effect on offspring mortality. We did not detect any effects of treatment on chick feeding or brooding latency, but that may be because there were so few points to compare in the BR group.

While these results strongly support our prediction that PRL plays a preparatory or stimulatory role in the onset of parental care, it is important to consider these results along with findings from another study which found that that early

incubating zebra finches, which have low PRL, will care for foster chicks (Smiley and Adkins-Regan, submitted). Furthermore, treating incubating zebra finches with VIP, the primary PRL releasing factor which increases PRL to breeding levels (Christensen and Vleck, 2008), did not increase the amount of foster chick care above controls, indicating that high PRL may not necessary for parental behavior. Although these findings appear contradictory to the results in this study, there are several explanations which may reconcile these two studies.

One key finding in the Smiley and Adkins-Regan (submitted) study was that while a substantial number of subjects across treatment (VIP/control) and reproductive experience (INEX/EXP) groups provided care to foster chicks, subjects only provided care if they already had eggs in the nest. Pairs with no nest or pairs with a nest without eggs did not provide any foster chick care. On average, the birds that provided care had been incubating for 5.75 (±2) days before they received foster chicks in their nest. Exposure to egg stimuli for several days may have been enough to sensitize the birds into parent care, independent of PRL – just as continuous exposure to pups can induce maternal responsiveness in nonbreeding rodents, independent of the hormonal changes that occur with pregnancy (Rosenblatt, 1967; Stolzenberg and Rissman, 2011). Alternatively, although circulating PRL levels were not statistically significantly higher than non-breeding baseline levels at the time incubating birds were tested with foster chicks, PRL does begin to gradually rise after incubation begins. The results from Smiley and Adkins-Regan (submitted) may indicate that there is a lower threshold of PRL that is required to stimulate parental care than the peak levels observed at the end of incubation and the beginning of post-hatch care. Lastly, another

possibility is that contact with the foster chicks, in addition to the eggs, may have stimulated a premature increase in circulating PRL, which may have facilitated chick care in subjects across treatment and experience groups. There is evidence that contact with the eggs and chicks alone can stimulate PRL release in other birds (e.g., Massaro et al., 2007; Silverin and Goldsmith, 1990), though this has not been directly tested in zebra finches. Blood samples were not taken following the interaction with foster chicks, so this explanation remains a hypothesis. In addition to PRL, other mechanisms involved in nest building and incubation, such as mesotocin (avian homologue of oxytocin), vasotocin (avian homologue of vasopressin), estrogens, and progesterone (Buntin, 1996; Klatt and Goodson, 2013; Lynn, 2016), may also have primed the birds to be in a parental state before the rise in PRL.

Although the Smiley and Adkins-Regan (submitted) study showed that incubating zebra finches, which have low plasma PRL levels, *can* show parental care towards foster chicks, only 54% of subjects in that study fed foster chicks, which is arguably the strongest behavioral indicator of parental care, and is a considerably lower number of birds that would normally feed chicks after hatching, when PRL is high (e.g., roughly 86% of control birds fed chicks in the current study). This variability in whether subjects fed chicks or not may be attributed to the variability in circulating PRL concentrations, the individual threshold of PRL, or the central sensitivity to PRL at this point in the incubation cycle. In addition to increasing levels of circulating PRL, it is also likely that an increase in sensitivity to PRL via an upregulation of central PRL receptors is required to for PRL to have an effect on parental care. In rats, central PRL receptors upregulate during pregnancy and lactation,

when circulating PRL levels are highest (Sakaguchi et al., 1996; Sugiyama et al., 1996). Similar to rats, it is plausible that an upregulation in central PRL receptors may occur in zebra finches that are near the end of the incubation cycle, when circulating levels of PRL are highest. Although this hypothesis has not yet been directly tested in zebra finches, a central specific binding study using radio-active labeled PRL in Wilson's Phalarope (*Phalaropus tricolor*), suggested that incubating males have increased sensitivity to central PRL binding, relative to non-incubating males, in the preoptic area and lateral septum (Buntin et al., 1998), two brain regions known to be involved in avian and mammalian parental care (reviewed in: Bridges, 2015; Buntin, 1996; Swain et al., 2014). Although the birds in the Smiley and Adkins-Regan (submitted) study had been incubating for approximately 5-6 days before exposure to foster chicks, this may not have been a sufficient time for a complete upregulation in central PRL receptors to occur in all birds, and may explain why only some birds were able to provide care in response to the low levels of circulating PRL, and/or why the increase in PRL from the VIP injections were not effective in stimulating an increase in parental behavior above baseline. Studies which quantify central PRL distribution, in relationship to circulating PRL levels, across the breeding cycle will be a critical first step before testing this hypothesis.

The question remains, however, as to why PRL increases at the end of incubation, and why did blocking PRL release during this time have such an impact on parental care? In rodents, since there appears to be a basic level of maternal care that can be given independent of hormonal stimulation from pregnancy, the role of hormonal stimulation near the end of pregnancy has been hypothesized to increase the

motivation and reduce the latency to care for pups to ensure offspring survival. In birds, we propose that the role of sustained high levels of PRL, which are normally observed during the end of incubation and the beginning of post-hatch care, is to *ensure* that chick care will occur immediately at chick hatching, when it is most appropriate and critical for offspring survival – immediately after hatching. One way in which this could occur is for PRL to increase the interest in the nest by increasing the positive saliency of egg and chick sensory cues. That way, birds would remain in constant and close proximity to the nest so that parental care can be elicited by offspring sensory cues. In the current study, inhibiting PRL during the time when the circulating PRL levels are highest likely deceased the interest and/or motivation to be near the nest, resulting in minimal or no parental behavior (Fig. 4.2.). Furthermore, the absence of egg, chick, and nest stimuli during the treatment "on" times may have further attenuated low levels of PRL beyond the 2 hour BR treatment effect (Fig. 4.1.), which may explain why we saw extreme behavioral differences and significantly lower nest temperatures between treatment groups during all 4 hours of the video. However, it is likely that after the BR wore off, PRL levels were eventually restored to normally high breeding levels and parental care resumed at some point, as the BR nest temperatures were no different from controls during treatment "off" times on days 1 and 2 post-hatch. Indeed, while removing the nest, eggs, or chicks reduces plasma PRL in ring doves and chickens, PRL can be restored to high levels if the nest and eggs are returned and incubation resumes (Buntin, 1996; Sharp, 2009). Taken together, although it appears that zebra finches can care for young before the natural peak in circulating PRL occurs, the role of increased PRL is likely to increase the

saliency of the nest, eggs, and chicks, in order to increase the motivation and reduce the latency of parental care occurring immediately upon hatching. This signal likely increases over the incubation cycle through a combination of increased contact with the nest and eggs and an upregulation in central PRL receptors – which may also be influenced by increasing circulating PRL levels and/or increase contact with the eggs/nest. Studies which can demonstrate that circulating PRL and/or central PRLR increase with egg/chick contact are necessary before testing this hypothesis.

Contrary to our prediction, reproductive experience did not buffer subjects against the BR treatment, as experienced birds were unable to compensate for the effect of decreased PRL. This is somewhat surprising as maternal memory in multiparous rats can be activated in the absence of hormonal cues from pregnancy, and therefore, rats can perform maternal care behaviors without hormonal stimulation after gaining experience (Fleming et al., 1996). However, this maternal memory phenomenon has not been well studied in birds, so it is unclear whether the same principles of experience that have been discovered in rodents would also apply to avian systems. There were, however, clear effects of reproductive experience on parental behaviors in the control group. Experienced birds increased their brooding durations from day 1 to day 2 and brooded more than inexperienced birds on day 2. Experienced birds also fed chicks more than inexperienced birds. In most birds, including zebra finches, reproductive experience is positively associated with greater reproductive success (e.g., Baran and Adkins-Regan, 2014; Cichoñ, 2003; Lv et al., 2016; Riechert et al., 2012), which may be the result of increased parental behavior and investment into the second brood. Indeed, Desalle (1986) found that experienced

zebra finches increased their parental care relative to their first breeding bout and produced more offspring, resulting in greater reproductive success.

Although experience appears to positively affect parental behavior, the questions remains: does PRL regulate this increase in parental behavior and reproductive success in experienced birds? Reproductively experienced zebra finches showed a greater increase in PRL beginning in late incubation, compared to when they were first-time breeders (Smiley and Adkins-Regan, 2016a). While it is conceivable that the increase in PRL that is observed during late incubation and chick hatching in experienced breeders may increase parental behavior, Christensen and Vleck (2008) also found that non-breeding baseline PRL concentrations were higher in experienced zebra finches, compared to non-breeding inexperienced birds. Non-breeding baseline levels of PRL positively predict parental behavior and reproductive success in free-living house sparrows (*Passer domesticus*; Ouyang et al., 2011) and mourning doves (*Zenaida macroura*; Miller et al., 2009) which may indicate that PRL sets up the amount of care or motivation to care to be given ahead of time, before breeding begins. Zebra finches are short lived and breeding opportunities are unpredictable (Zann, 1996), and thus, may require increased PRL in order to increase parental investment if they are granted a second opportunity to breed during their lifetime. However, as described above, PRL and parental behavior also appear to have a bidirectional relationship such that stimuli from the nest, eggs, and chicks stimulate PRL release in birds (reviewed in Buntin, 1996). One may predict that the increase in PRL observed in experienced breeders causes them to spend more time in the nest, resulting in increased amounts of parental care to young, which continues to increase

or maintain extra high levels of PRL. An intriguing hypothesis worth testing would be to see if there is greater PRL release when exposed to egg or chick stimuli in reproductively experienced birds and if that influences subsequent parental behaviors. In addition, behavioral changes and the improvement in reproductive success that occur with experience may also depend on factors such as improvement of self-maintenance, access to better resources (nest sites, feeding areas, territories, etc.), and increase in foraging and feeding efficiency (reviewed in Curio, 1983), which may be independent of PRL. These factors are less detectable in lab settings and would require experiments in free living birds to assess.

Another possibility is that PRL influences parental behavior coordination between breeding pairs. Reproductive success in biparental species depends on the pair's behavior coordination (reviewed in Royle et al., 2010) and pairs that are more behaviorally similar have higher reproductive success (e.g., Gabriel and Black, 2012; Mariette and Griffith, 2015; Spoon et al., 2006). In addition, pairs of free living great tits (*Parus major*) that are more hormonally similar during breeding had greater reproductive success (Ouyang et al., 2014). However, it is unknown whether this hormone similarity is beneficial because it modifies the parents' behavior such that they provide similar amounts of care behavior and investment or because it plays a role in coordinating or synchronizing the pairs' behaviors together, or both. Control pairs in this study had a near perfect positive linear relationship in their chick feeding durations, as well as other strong, positive relationships between brooding and feeding behaviors, yet these correlations were nonexistent in BR treated birds (Fig. 4.6.). Chick feeding durations could not be included in the correlational analyses for BR

birds because so few subjects were observed feeding. However, while control males and females both increased feeding from day 1 to 2, BR pairs adjusted their feeding independent of one another, in different directions (BR-treated males increased while female decreased the amount they fed chicks on day 2). Furthermore, the majority of groups in which only one member of the pair performed parental behavior were in the BR treated group, which together suggests that their behavior was not coordinated. While we did not measure circulating PRL in subjects during this experiment, Crino et al. (2017) found that breeding zebra finch pairs had correlated PRL concentrations. Reducing PRL may decrease the ability to synchronize behavior with the breeding partner and this reduction in coordination may lead to lower reproductive success. Additionally, pair coordination may increase through experience with a partner. Pairs that stay together for longer have the opportunity to increase partner familiarity and coordination and have higher reproductive success (Sánchez-Macouzet et al., 2014). Indeed, experienced pairs in this study were more positively correlated in their behaviors than inexperienced pairs, which were often negatively correlated (Fig. 4.6.). Zebra finches are socially monogamous and remain with the same partner throughout their life, unless a partner dies (Zann, 1996). Because they are also short lived, zebra finches adopt a 'fast-track' reproductive life history trajectory (Adkins-Regan and Tomaszycki, 2007). Remaining with the same breeding partner speeds up the initiation of reproduction (Adkins-Regan and Tomaszycki, 2007) and increases parental care and reproductive success (Baran and Adkins-Regan, 2014; Delesalle, 1986), whereas forced repairing lowers reproductive success by causing slower clutch initiation (Adkins-Regan and Tomaszycki, 2007; Baran and Adkins-Regan, 2014; Crino et al.,

2017), lowered offspring mass (Schweitzer et al., 2014), and reduced parental care (Desalle,1986). Thus, an additional benefit of remaining with a partner may be the increase in parental care by way of increased behavioral coordination. The increase in PRL is observed in experienced breeders may facilitate the increased pair behavior coordination during the post-hatch care period.

Females brooded more than males on day 2 and were quicker to initiate brooding than males across all conditions. Consistent with the Delesalle (1986) findings, control females tended to fed chicks more than control males, although this effect nearly missed significance (p=0.06). Previous works have reported female zebra finches invest more into incubation (Delesalle, 1986; El-Wailly, 1966; Zann and Rossetto, 1991), while reports of female-biased investment in post-hatch have been mixed [female biased: (Delesalle, 1986); no sex differences (Gilby et al., 2011; Royle et al., 2006)]. The studies that did not find sex differences in post-hatch care measured parental behavior on days 5-10 post-hatch (Gilby et al., 2011) and on days 8-13 post-hatch (Royle et al., 2006), which are both later in the chick rearing period from when we measured post-hatch care. Delesalle (1986), on the other hand, found that females fed chicks more than males when the entire post-hatch rearing period was measured, which includes the time point at which we measured parental care. One potential explanation for these discrepancies is that the birds used in the Gilby et al. and Royle et al. studies were inexperienced and so the female bias would not be observable yet (since many of the sex effects were only observed in experienced birds in this study), but initial breeding experience levels were not reported in either study. Another potential explanation is that males and females start off providing different amounts of

care, but this difference disappears after several days of post-hatch care, perhaps through increased pair coordination, and so it would not have been captured during the time when Gilby et al. and Royle et al. quantified parental care. A detailed quantification of zebra finch parental behavior across the entire post-hatch period is needed to clarify the possible sex effects in parental investment.

Bromocriptine was previously found not to be effective in lowering PRL in zebra finches (Ryan et al., 2014), even though its use has been successful in other avian species such as house finches (Badyaev and Duckworth, 2005), domestic chicken hens (*Gallus gallus domesticus*; Reddy et al., 2007), emperor penguins (*Aptenodytes forsteri*; Angelier et al., 2006) and Adélie penguins (Cottin et al., 2014; Thierry et al., 2013). Our treatment was suspended in peanut oil and orally administered in order to accommodate a much higher dose than Ryan et al. (2014) used, which is likely the reason our treatment regime was successful. While the exact time course for PRL action to take effect in zebra finches is unknown, our pilot data suggest BR is fast acting on circulating PRL and PRL-mediated behavioral effects could be quickly detected 5-10 minutes post peripheral injection of PRL (Wang and Buntin, 1999) and 1.5 – 2.5 hours post intracranial injection of PRL in ring doves (Buntin et al., 1991); both time points would have been captured during the four hour video window during this study. Importantly, we did not see any adverse effects on eating, drinking, or body condition in BR-treated animals. Bromocriptine is a dopamine (DA) agonist so its actions on PRL are indirect via DA-pathways that inhibit PRL secretion (birds: Chaiseha et al., 1997; El Halawani et al., 1991; mammals: reviewed in Freeman et al., 2000) Therefore, there is a possibility that the

reduction in parental care behavior is via other downstream effects, other than PRL, in this study. In particular, decreasing DA using BR reduces locomotor activity in rodents but, to our knowledge, the effects of BR on locomotor activity have not been examined in birds. We do not believe this was responsible for our parental behavior deficit as we did not observe any adverse effects in our birds during the pilot testing period. Additionally, eating and drinking behaviors were not disrupted, indicating that birds could move freely between the feeder and drinking spout (they were almost always on opposite sides of the cage), and BR subjects ended up in better body condition than controls. Finally, while no avian studies to date have used a remove and replace paradigm with BR, maternal behavior is rescued by administering PRL in rodents treated with BR (reviewed in Bridges, 2015) indicating PRL is the mechanism of action for maternal care.

Conclusions

In sum, this was the first experimental manipulation study in zebra finches to demonstrate a causal role for PRL in post-hatch parental care in both males and females. However, considering Smiley and Adkins-Regan's (submitted) finding that incubating zebra finches can care for foster chicks in the absence of high PRL, we propose the role of PRL is to increase the likelihood of maintaining proximity to the nest so that the chicks can stimulate the appropriate level of parental care required for chick survival. More information is needed, however, to understand the potential bidirectional relationship between PRL and chick stimuli in the zebra finch to fully test this hypothesis. In addition, it is unknown whether central PRL sensitivity changes

over the course of the breeding the cycle and whether this affects the ability to provide parental care in response to increasing circulating PRL levels. Future studies which look at central PRL receptor distribution across different time points in the breeding cycle will be valuable for determining the mechanism for which PRL affects parental care behavior. Although BR treatment reduced parental care in both inexperienced and experienced parents, reproductive experience increased parental behavior in normally breeding zebra finches (controls). However, whether the increase in PRL observed in experienced breeders facilitates this increase in parental behavior, or whether the increased interactions with offspring cause an increase in PRL in experienced breeders is still unclear. PRL and reproductive experience may also affect parental behavior coordination in biparental pairs, which is an important component of reproductive success in biparental species. It is important to continue causal studies using PRL in a variety of avian species in order to generalize its role in parental behavior and to understand the evolution of PRL-mediated behaviors.

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4.6. REFERENCES

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CHAPTER 5

CENTRAL PROLACTIN RECEPTOR DISTRIBUTION IN BREEDING AND NON-BREEDING ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

In prep for submission.

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5.1. ABSTRACT

Parental care is a widespread phenomenon observed in many diverse taxa and is an important component of fitness. Neuroendocrine systems have long been thought to play an important role in coordinating and stimulating the onset of parental behavior. In particular, the hormone PRL has a well-established role in mediating maternal behavior in mammals through its actions on central prolactin receptors (PRLR). PRL also plays a causal role in stimulating avian post-hatch parental care in ring doves, house finches, and zebra finches, however, ring doves are the only avian species with altricial young in which the neural mechanisms of parental care have been extensively studied. Ring doves are unique to birds in that both males and females feed squabs with a PRL dependent crop milk, making these findings difficult to generalize to other avian species. Studies which manipulate central PRL in birds

that do not produce crop milk are necessary to understand PRL's role in avian parental care more generally. However, there is a considerable lack of information on the distribution of PRLRs in the avian CNS to test the hypothesis that post-hatch parental care is mediated through central PRLRs. Furthermore, one of the ways PRL affects parental behavior may involve an upregulation in central PRL abundance. Recent evidence (Ch. 3-4) suggests that zebra finches may become increasingly sensitive to PRL during the breeding cycle, as they approach chick hatching. In order to advance the research on the role of central PRL in post-hatch parental care in the zebra finch, we developed a novel IHC protocol to visualize the distribution of central PRLRs. Additionally, we compared brains from breeding and non-breeding zebra finches to test the hypothesis that PRLR are upregulated in breeders. This is the first detailed description of PRLR in a songbird, and we provide the first evidence that PRLRs are upregulated in breeding birds in several brain regions relevant to parental care. This work is an essential first step to facilitate future research that uses central PRL targets to manipulate parental behavior, and/or other physiological or behavioral functions of PRL.

5.2. INTRODUCTION

Parental care is a widespread phenomenon observed in many diverse taxa and is an important component of fitness. Parental care often requires a novel and complex series of behaviors, and additional energetic demands for the individual parent. Neuroendocrine systems have long been thought to play an important role in coordinating and stimulating the onset of parental behavior. In particular, the hormone

PRL has a well-established role in mediating maternal behavior in mammals through its actions on central prolactin receptors (PRLR) in brain regions that promote the onset of maternal care (Bridges, 2015). The PRLR is a membrane-bound receptor that is part of the class 1 cytokine receptor superfamily and is widely expressed in various tissues of vertebrates, including the brain (reviewed in Bole-Feysot et al., 1998; reviewed in Ben-Jonathan et al., 2008). Activation of PRLR by PRL binding can initiate the intracellular signaling cascades, including the activation of the intracellular JAK2- STAT5 (JAnus Kinase–Signal Transducer and Activator of Transcription) signaling pathway which can transmit information from extracellular chemical signals to the nucleus resulting in DNA transcription and expression of genes (Aaronson and Horvath, 2002; Bole-Feysot et al., 1998).

PRL also plays a causal role in stimulating avian post-hatch parental care in male and female ring doves (*Streptopelia risoria*; Buntin et al., 1991; Wang and Buntin, 1999), male house finches (*Carpodacus mexicanus*; Badyaev and Duckworth, 2005), and male and female zebra finches (*Taeniopygia guttata*; Smiley and Adkins-Regan, submitted; Ch. 4). However, the manipulations used in house finches and zebra finches have only altered peripheral levels of PRL. To date, ring doves are the only avian species with altricial young in which the neural mechanisms of parental care have been extensively studied (reviewed in Buntin, 1996). Although limited, there is strikingly similar evidence that PRL stimulates the onset of parental behavior through its actions on central PRLRs in birds.

In most birds with altricial young, circulating PRL levels are low during non-breeding times and significantly increase during late incubation and early post-hatch

chick care (Angelier et al., 2016; Buntin, 1996; Smiley and Adkins-Regan, 2016). PRL crosses the blood brain barrier through a specialized protein transport and uptake system at the choroid plexus (reviewed in Buntin, 1996). Lesions to the POA, LHy, or VMH disrupt the onset of incubation and the rise in plasma PRL that accompanies incubation in turkeys (Youngren et al., 1989). Both systemic and central injections of PRL induce parental behavior in male and female ring doves (reviewed in Buntin, 1996). Specifically, ICV injections of PRL into the POA, VMH, or TU stimulate regurgitation and hyperphagia (increased eating) in ring doves (reviewed in Buntin, 1996; refer to table 5.1 for abbreviation definitions). Ring doves are unique, however, in that both male and female ring doves feed squabs with crop milk which is produced by the epithelial mucosal cells along the wall of the crop sac in response to PRL, an evolved trait unique to pigeons and doves (Buntin, 1996; Patel, 1936). Studies which manipulate central PRL in birds other than ring doves, which do not produce crop milk, are necessary to understand PRL's role in avian parental care more generally. However, there is a considerable lack of information on the distribution of PRLRs in the avian CNS to test the hypothesis that parental care is mediated through central PRLRs in species that have post-hatch parental care, but do not require the production of crop milk.

Central specific binding sites for PRL have been detected in pigeons (*Columba livia domestica*), Wilson's phalarope (*Phalaropus tricolor*), redwing blackbird (*Agelaius phoeniceus*), European starlings (*Sturnus vulgaris*), and dark-eyed juncos (*Junco hyemalis*) (reviewed in Buntin, 1996), but a detailed mapping and characterization of PRLRs is most complete only in ring doves (Buntin et al., 1993;

Buntin and Ruzycki, 1987), chickens (Ohkubo et al., 1998b), and turkeys (Zhou et al., 1996). In ring doves, the greatest amount of specific binding for PRLRs using autoradiography were found in the POA, LHy, TU, VMH, and PVM, as well as several extrahypothalamic brain regions such as the SL, and AC for both males and females (Buntin et al., 1993; Buntin and Ruzycki, 1987). Buntin and Buntin (2014) showed that PRLR activation, as measured by pSTAT5-ir (one of the transcription factors that is expressed when the PRLR is activated), is greatest in the POA, LHy, PVM, TU, VMH, BNST, and the SL in ring doves that were in the late incubation and early post-hatch care stage of breeding, relative to non-breeding or incubating birds. A detailed description and mapping of the PRLR in birds that do not produce crop milk, but care for altricial young, is necessary to make informed hypotheses and predictions in future studies that will systematically manipulate PRL to test for the effects on parental care in other avian species.

In addition to describing the PRLR distribution in the avian brain, recent evidence from Smiley and Adkins-Regan (submitted, submitted; Ch. 3 and Ch. 4) suggests that zebra finches may become increasingly sensitive to PRL during the breeding cycle, as circulating PRL increases. Part of the mechanism of how PRL affects behavior may involve changes in central PRL abundance. In rats, central PRLR mRNA increases over the pregnancy cycle, as circulating PRL increases (Anderson et al., 2006). In addition, peripheral injections of PRL increased central PRL receptor mRNA expression in the POA and arcuate nucleus, areas known to be involved in maternal care and PRL secretion, respectively (Anderson et al., 2006). Similar to rats, it is plausible that an upregulation in central PRL receptors may occur in zebra finches

that are near the end of the incubation cycle, when circulating levels of PRL are highest. Although this hypothesis has not yet been directly tested, a central specific binding study using radio-active labeled PRL in Wilson's Phalarope suggests that incubating males have increased sensitivity to central PRL binding in the POA and LS (Buntin et al., 1998). However, in domestic turkeys (*Meleagris gallopavo*) and bantam chickens (*Gallus domesticus*), increasing blood levels of PRL during incubation were associated with *decreased* levels of PRLR mRNA in the hypothalamus, but increasing levels in the pituitary gland (Ohkubo et al., 1998a; Zhou et al., 1996). However, these studies were performed with a ribonuclease protection assay for PRLR mRNA, which does not differentiate distinct nuclei within the hypothalamus, nor measure the final protein expression of PRLR. At this point, it is unclear whether there would be an up or down regulation of PRLR, or no change, in other avian species that hatch altricial young. In addition to breeding cycle stages, it is also possible that permanent changes in the PRLR distribution occur as a result of reproductive experience. Not only does PRL increase over the breeding cycle, but experienced zebra finches have higher circulating PRL during the breeding cycle relative to when they were inexperienced breeders (Smiley and Adkins-Regan, 2016). Changes in PRLR abundance may help explain why plasma levels of PRL increase with experience.

In order to advance the research on the role of central PRL in post-hatch parental care, we developed and validated a novel immunohistochemistry protocol to map the central PRLR distribution in zebra finches. For this chapter, the analysis is focused on several key brain areas that have been implicated in parental care and other social behaviors (see Table 5.1.). Furthermore, to test whether breeding status or

reproductive experience affects the central PRLR distribution, we compared brains from breeding (~ day 2 post-hatch) and non-breeding (non-paired birds) males and females that are either inexperienced (first-time) or experienced breeders. We predicted that the zebra finch PRL distribution would be similar to the ring dove distribution and that there will not be significant sex differences. We also predicted that there would be an upregulation in PRLR in breeders, relative to non-breeders, especially in areas known to be important for parental care, such as the POA. If this upregulation is permanent, then we would expect a greater amount of PRLR staining in experienced non-breeders, relative to inexperienced non-breeders.

This study is the first to describe the central PRL receptor distribution in the zebra finch, a monogamous and biparental songbird species which PRL has been shown to play a causal role in parental care (Smiley and Adkins-Regan, submitted; Ch. 4). This is a necessary first step to facilitate future research that uses central PRL targets to manipulate parental behavior. Although our focus is on parental care, this study will be of value to many researchers from many fields that are interested in the physiological and behavioral functions of PRL.

5.3. METHODS

Subjects

This study used seven reproductively experienced breeding (n= 4 females; n=3 males) and eight non-breeding (n= 4 females; n=4 males) adult zebra finches of mixed reproductive experience levels [n=4 inexperienced (2 males/2females); n=4 experienced (2 males/2females)] raised in the lab. Breeding birds were housed only

with their partner and were either on day 1 (n=1), day 2 (n=4), or day 3 (n=2) post-hatch. Non-breeding subjects were non-paired birds that were housed in sex-specific cages prior to brain collection.

All birds had access to food (commercial seed mix; Kaytee Fortifinch Diet), water, and grit *ad libitum*. Birds were supplemented with hardboiled egg once per week. All rooms were kept in a temperature (22.2° C) and humidity (range 30-70%) controlled rooms on 14:10 light:dark cycles. Male-female breeding pairs were housed together in small breeding cages (0.6 m x 0.4 m x 0.35 m) that contained an empty nest box and nesting material (coconut fibers) available *ad libitum*. Daily nest checks were performed to monitor each pair's breeding status. Non-breeding birds were housed in sex specific aviaries (0.94 m x 0.76 m x 0.94 m) that held up to 20 birds. All methods and procedures were approved by the Cornell University IACUC.

Brain collection and sectioning procedures

Briefly, animals were deeply anesthetized with isoflurane and perfused transcradially with phosphate-buffered solution followed by 4 % paraformaldehyde. Brains were removed and post-fixed in 4 % paraformaldehyde for approximately 24 hours before they were transferred to 70 % ethyl alcohol. Brains were then embedded into a paraffin block and sectioned at 6 μm, taking every other section, at the Histology/Cytology Core Facility at Cornell University. Slides were divided into four series and one series (every 4^{th} slide) was stained for PRLR using immunohistochemistry.

Immunohistochemistry

Six μm thick sections of paraffin-embedded sections were used for immunohistochemical analysis and were processed by L.D. at the Immunopathology Research and Development Laboratory (Cornell University). After deparaffinization in xylene and rehydration in graded ethanol, the sections were subjected to antigen retrieval by steaming in citrate buffer (10 mM, pH6.0). The endogenous peroxidase activity was quenched by 0.3% hydrogen peroxide in distilled water for 10 minutes. Nonspecific staining was blocked with a mixture of 10% goat serum and 2X casein for 30 minutes at room temperature. The primary antibody, rabbit polyclonal anti-dove prolactin serum, was used at 1:3,000 and incubated for 1.5 hr at room temperature. The antiserum was custom generated by Affinity Bioreagents against the cytoplasmic domain of the dove prolactin receptor using sequence data that was obtained by cloning the dove prolactin receptor (cloning done by S.R.) and was generously provided by the Buntin lab (U. Wisconsin, Madison). After washing, the sections were further incubated with biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) for 30 min and followed by streptavidin-horseradish peroxidase (Invitrogen) for 15 min at room temperature. Negative controls were run in parallel by replacing the primary antibody with rabbit serum at the same dilution. For easy handling, slides of multiple cases were loaded into MicroProbe System (Fisher Scientific) and PBST (0.05% Tween 20) was used for washing throughout the procedure. Nova Red (Vector Laboratories) was used as chromogen to visualize antigen localization, and the sections were lightly counterstained with hematoxylin. IHC results were examined by Olympus AX 70 compound microscope equipped with

MicroFire camera and PictureFrame for image processing and capture (Optronics).

Quantification of PRLR immunostaining

All stained slides were digitized using an Aperio Scanscope (Model CS2, Leica Biosytems) and images were analyzed in the program ImageScope (Aperio, version 12.3.2.8013, Leica Biosytems) using a customized positive pixel count algorithm. Specifically, the algorithm counted the number of pixels in a specified area that matched the nova red color spectrum used to visualize the PRLR ('positive pixels') based on various, custom color parameters that we defined (e.g., hue width, hue value, and color saturation threshold). The algorithm also counted the number of pixels which matched the hematoxylin counterstain color spectrum ('negative pixels') using customized color parameters that we set in the program.

The brain regions that were analyzed are listed in table 5.1. and were identified using the canary (Stokes et al., 1974) and zebra finch (Nixdorf-Bergweiler et al., 2007) brain atlases, using the updated nomenclature from Reiner et al. (2004). These areas were chosen based on previous findings in the ring dove PRLR distribution (Buntin et al., 1993) and PRLR activation during post-care care in ring doves (Buntin and Buntin, 2014). In addition, we looked at the TV as PRL may be involved in the motivation and saliency processing of chick stimuli in breeding birds (Smiley and Adkins-Regan, submitted; Ch. 4). The cerebellum (CB) was chosen as a control region, as we did not expect any PRLR staining there. Areas were analyzed at the same brain level for all animals in the breeding and non-breeding conditions. Representative images of immunostaining can be found in Appendix A. All scanned

slide images will be available online through the Cornell eCommons data archive and can be viewed up to 20X on any computer using the program ImageScope (free download from http://www.leicabiosystems.com).

Table 5.1. Abbreviations for brain regions

Below are the 12 brain regions that were analyzed in breeding and non-breeding brains in this study, using the updated nomenclature from Reiner et al. (2004).

Abbreviation	Brain Region
AC	Nucleus accumbens
CB	Cerebellum
IN	Tractus infundibularis
LHy	Nucleus lateralis hypothalami
nST	Nucleus stria terminalis
POM	Nucleus preopticus medialis
PVM	Nucleus paraventricularis magnocellularis
SL	Nucleus septalis lateralis
TnA	Nucleus taeniae of the amygdala
TU	Nucleus tuberis
TV	Nucleus tegmenti ventralis
VMH	Ventromedial hypothalamus [defined as the Nucleus lateralis hypothalami posterioris (PLH) and the Nucleus medialis hypothalami posterioris (PMH)]

Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics for Windows, version 24.0 (IBM Corp, Armonk, NY). For each brain region, two measures were calculated. The first measure was the proportion of cells that expressed positive staining for PRLR or the 'positivity score' which was the total number of positive pixels for the PRLR stain divided by the total number of pixels in the entire image (total number of positive pixels + total number of negative pixels). The second

measure was the 'positive density score' which was the total number of positive pixels for the PRLR stain divided by the area (μm^2) of the region measured. The positivity score was used to show the relative number of PRLR in each brain region, while the positive density was used to determine actual number differences in PRLR across brain regions. Breeder and non-breeder data were first analyzed separately to examine the differences in positivity and positive density scores within each data set. Breeders and non-breeder data were then combined to test for an effect of brain region, breeding status, and brain region*breeding status interaction on positivity score and positive density scores.

For the breeder data, the effect of brain region, sex, and brain region*sex interaction on positivity score and positive density score were tested using two separate linear mixed models (LMM). In both models, a PairID variables were included as random factors to address the non-independence of the data. Positivity scores were arc-sin transformed and positive density scores were log-transformed to normalize the data. These two LMMs were repeated using the non-breeder data. For the non-breeder data, only positive density scores had to be normalized for the non-breeder data using a log +0.001 constant transformation because some zeros existed in the data set. Although this study intended to test the effects of sex and reproductive experience on PRLR staining in non-breeders, the non-breeding brains were unknowingly rearranged in the paraffin block when submitted for sectioning, so it was impossible to tell which brains belonged to which subject, so only brain region differences could be analyzed.

When the breeder and non-breeder data were combined, the LMMs to analyze

the effect of brain region on positivity score and positive density score included brain region, breeding status (breeding or non-breeding), and a brain region*breeding status interaction term as fixed factors and PairID as a random factor. Positivity scores were arc-sin transformed and positive density scores were normalized using a log +0.001 constant transformation.

For all LMMs, significant pairwise comparisons were assessed using least significant difference (LSD) post-hoc tests and a Bonferroni correction to account for multiple comparisons. Assumptions were checked for all mixed models by plotting predicted values vs. residuals and by plotting residuals on a Q-Q plot to visually inspect for normality. In all analyses, significance was accepted if p-values were < 0.05.

In addition to the LMMs, for all three groups (breeders only, non-breeders only, and breeders and non-breeders together) correlations between the positivity scores and positive density scores were analyzed using a Person's R correlation test.

5.4. RESULTS

Breeders only: Effect of brain region and sex on positivity scores and positive density scores

There was a significant effect of brain region on positivity scores ($F_{11, 49.34}$ = 21.12, $p < 0.01$). The highest amount of positive stain was found in the IN, TnA, POM, TU, TV, and VMH, with moderate staining found in the AC, LHy, nST, PVM, and SL, and either very minimal or no amount of staining in the CB (control region), which is consistent with findings in ring doves (Buntin et al., 1993). Significant

pairwise comparisons are shown in Figure 5.1.A.

There was also a significant effect of brain region on positive density scores ($F_{11, 49.88} = 21.81$, $p < 0.01$). The brain regions with the highest density were the IN, TnA, TU, and TV, moderate density in the POM, and VMH, and low density in the AC, LHy, nST, PVM, and SL, and either very minimal or no amount of density in the CB (control region). Significant pairwise comparisons are shown in Figure 5.1.B. There were no significant effects of sex or sex*region interaction on either positivity scores or positive density scores.

Non-breeders only: Effect of brain region on positivity scores and positive density scores

There was a significant effect of brain region on positivity scores ($F_{11, 50.00} = 4.99$, $p < 0.01$). The pattern was slightly different from breeders in that the highest amount of positive stain was found in the AC, LHY, POM, TU, and VMH with moderate staining found in the PVM, SL, and a lesser amount of staining in the IN, TN, nST, and TV, and either very minimal or no amount of staining in the CB (control region). Significant pairwise comparisons are shown in Figure 5.2.A.

There was a significant effect of brain region on positive density scores ($F_{11, 45.74} = 6.27$, $p < 0.01$), which was very different from breeders. In non-breeders, a high density of PRLR were only found in the AC and TU, and all other regions measured had a very low density of PRLR. Significant pairwise comparisons are shown in Figure 5.2.B.

Breeders and non-breeders: Effect of brain region and breeding status on positivity scores and positive density scores

There was a significant interaction between brain region and breeding status on positivity scores ($F_{11, 50.00} = 95.43$, $p < 0.01$) such that breeders had a greater positivity score than non-breeders in the IN, TnA, TU, and TV; see Figure 5.3.A. for significant pairwise comparisons.

There was a significant effect of brain region on positive density scores ($F_{11, 96.06} = 2.80$, $p < 0.01$), such that breeders had a greater density of PRLR in all brain regions measured, relative to non-breeders. Significant pairwise comparisons are shown in Figure 5.3.B.

Correlations between the positivity scores and positive density scores

There were significant, positive correlations between positivity scores and positive density scores for breeders: r(67)= 0.70, $p < 0.01$, non-breeders: r(62)= 0.72, $p < 0.01$, and for combined breeders and non-breeders: r(129)= 0.62, $p < 0.01$. Data shown in Figure 5.4.

Figure 5.1. Breeders only: Effect of brain region on positivity scores and positive density scores

A.

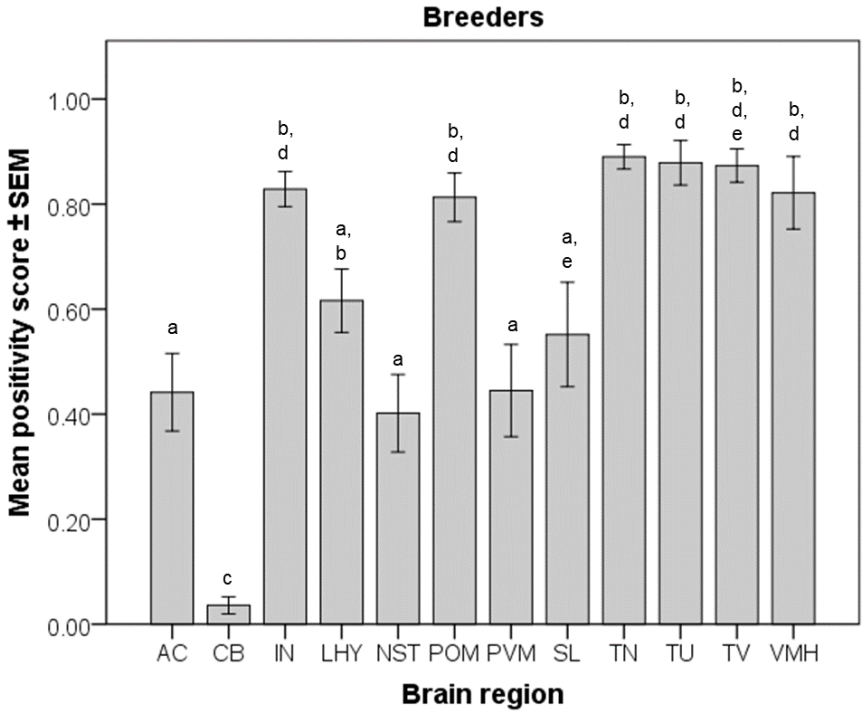


Figure 5.1. continued

B.

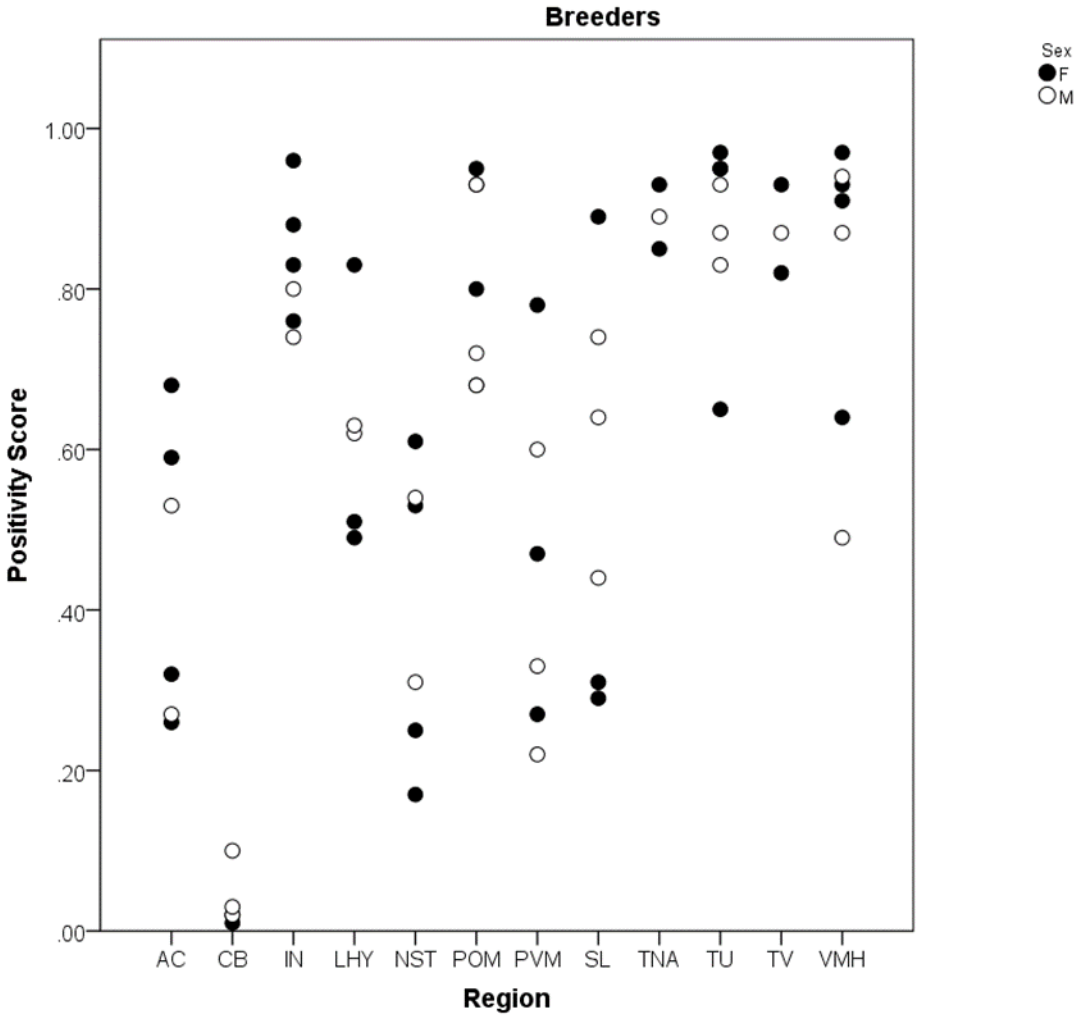


Figure 5.1. continued

C.

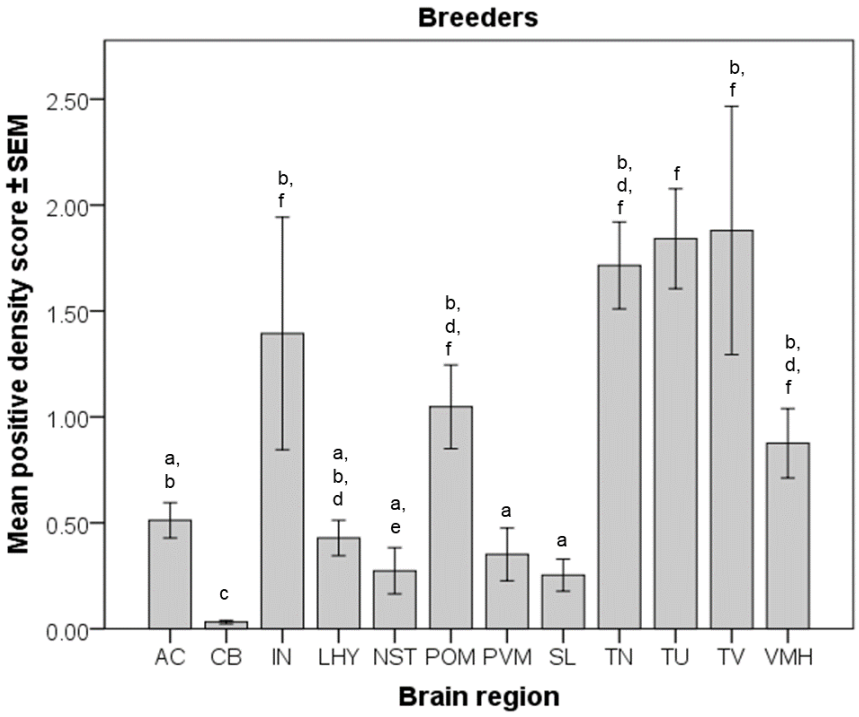


Figure 5.1. continued

D.

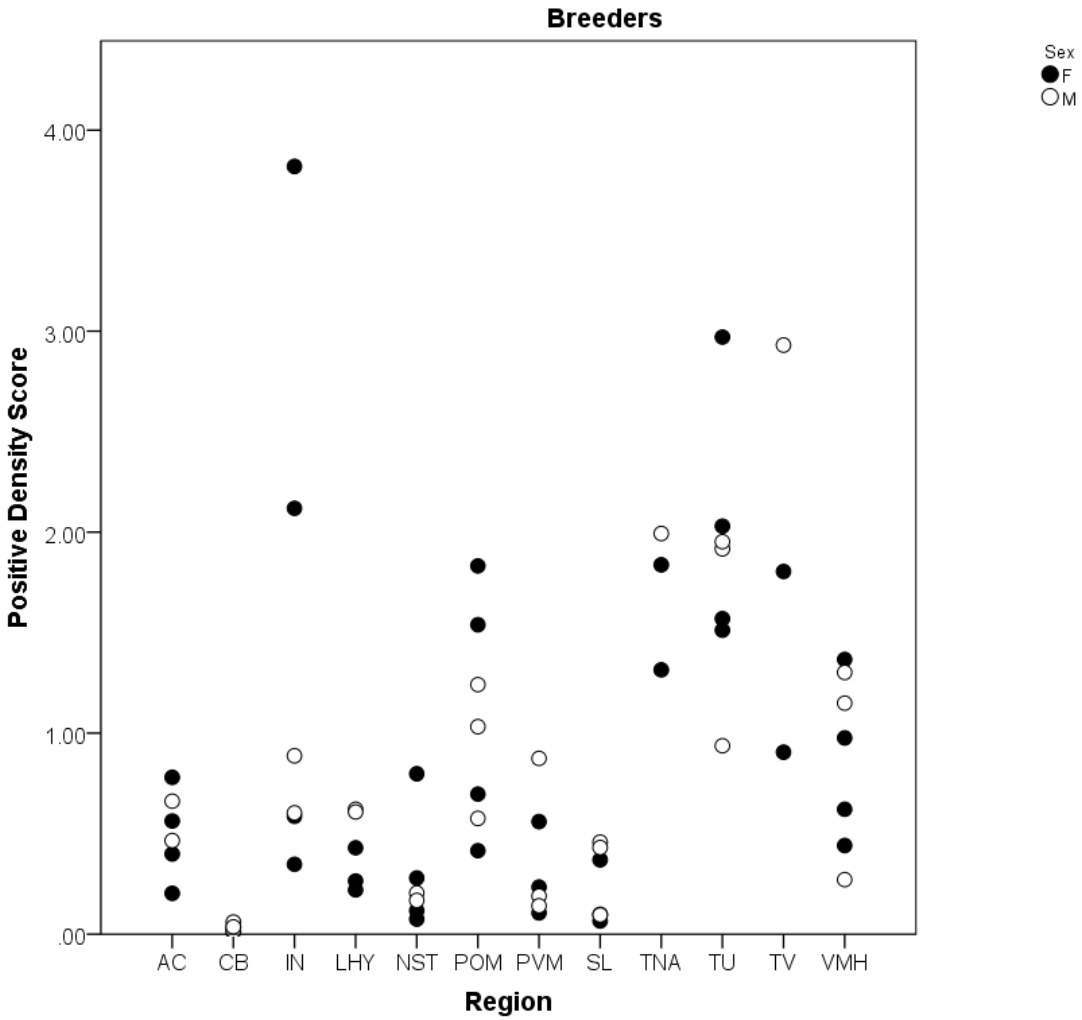


Figure 5.1. (A) Mean positivity scores, or the ratio of positive PRLR color pixels to total pixels (PRLR stain+ nuclear counterstain) ± 1 standard error (SEM) across the 12 regions (abbreviations defined in table 5.1.) for breeding birds on days 1-3 post-hatch. Bars that share at least one similar letter are not significantly different. Bars that have differing letters are significantly different (p<0.05). (B) Distribution of positivity scores across the 12 brain regions. (C) Mean positive density scores, or the total number of positive PRLR color pixels divided by the area measured (pixels/μm^2) ± 1 SEM across the 12 regions for breeding birds on days 1-3 post-hatch. Bars that share at least one similar letter are not significantly different. Bars that have differing letters are statistically significantly different (p<0.05). (D) Distribution of positive density scores across the 12 brain regions.

Figure 5.2. Non-breeders only: Effect of brain region on positivity scores and positive density scores

A.

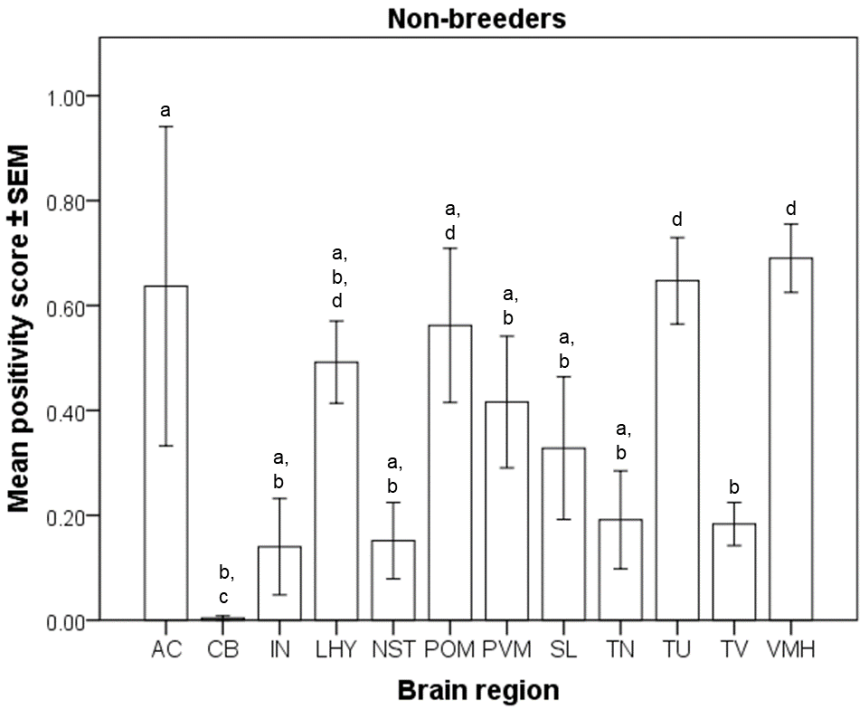


Figure 5.2. continued

B.

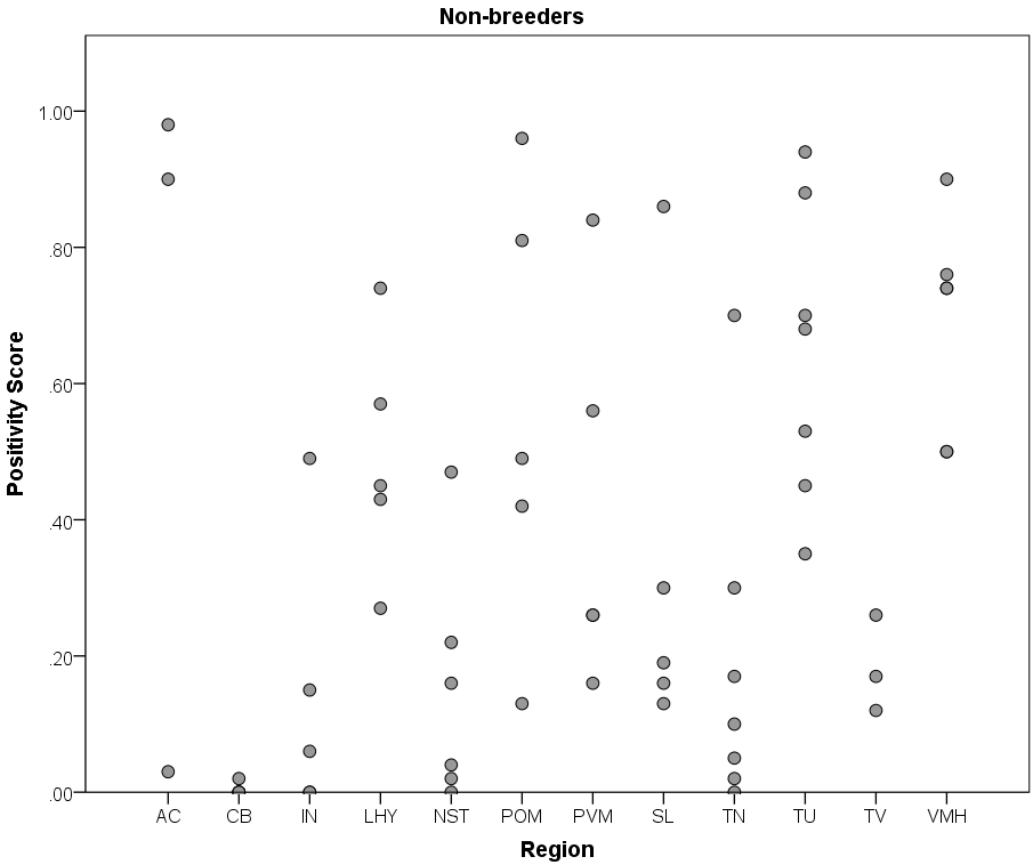


Figure 5.2. continued

C.

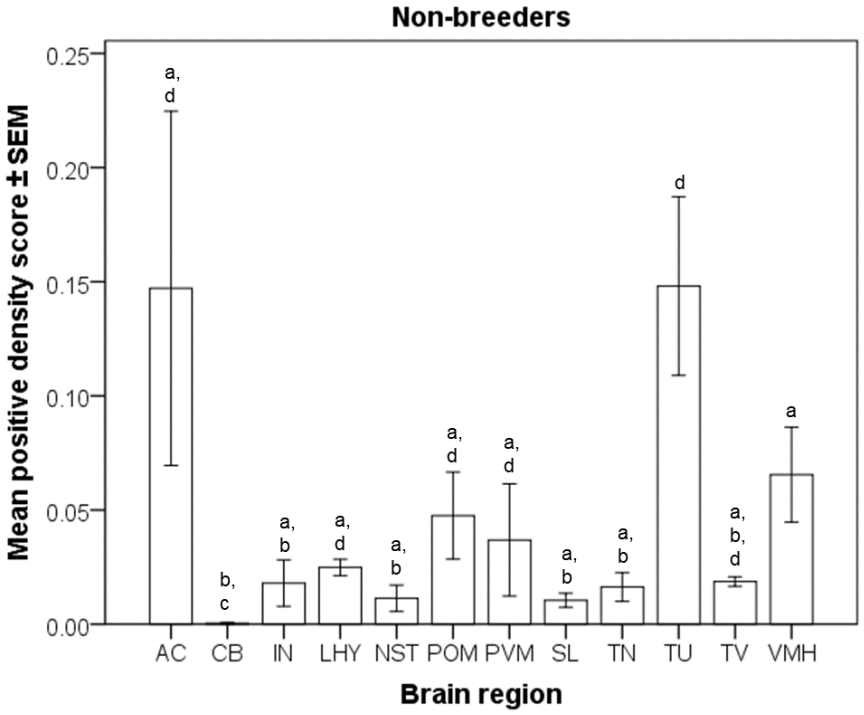


Figure 5.2. continued

D.

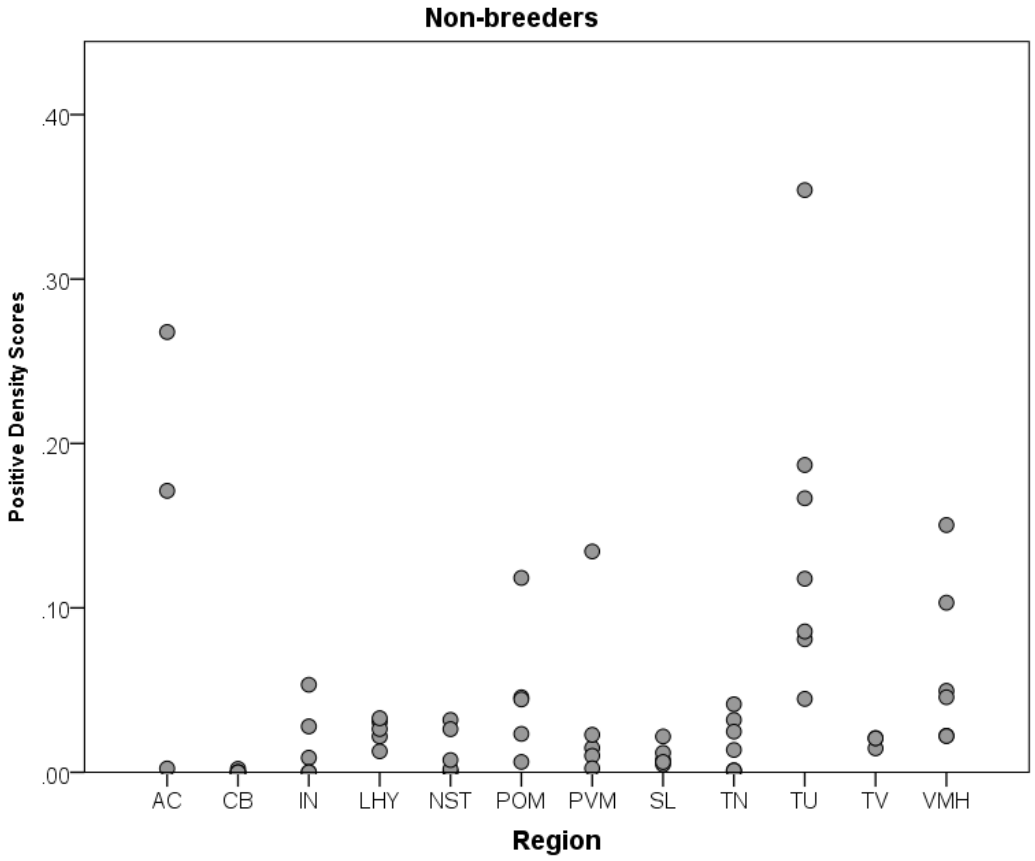


Figure 5.2. (A) Mean positivity scores, or the ratio of positive PRLR color pixels to total pixels (PRLR stain+ nuclear counterstain) ± 1 standard error (SEM) across the 12 regions (abbreviations in table 5.1.) for non-breeding birds. Bars that share at least one similar letter are not significantly different. Bars that have differing letters are statistically significantly different (p<0.05). (B) Distribution of positivity scores across the 12 brain regions. (C) Mean positive density scores, or the total number of positive PRLR color pixels divided by the area (pixels/μm^2) ± 1 SEM across the 12 regions for non-breeding birds. Bars that share at least one similar letter are not significantly different. Bars that have differing letters are statistically significantly different (p<0.05). (D) Distribution of positive density scores across the 12 brain regions.

Figure 5.3. Breeders and non-breeders: Effect of brain region and breeding status on positivity scores and positive density scores

A.

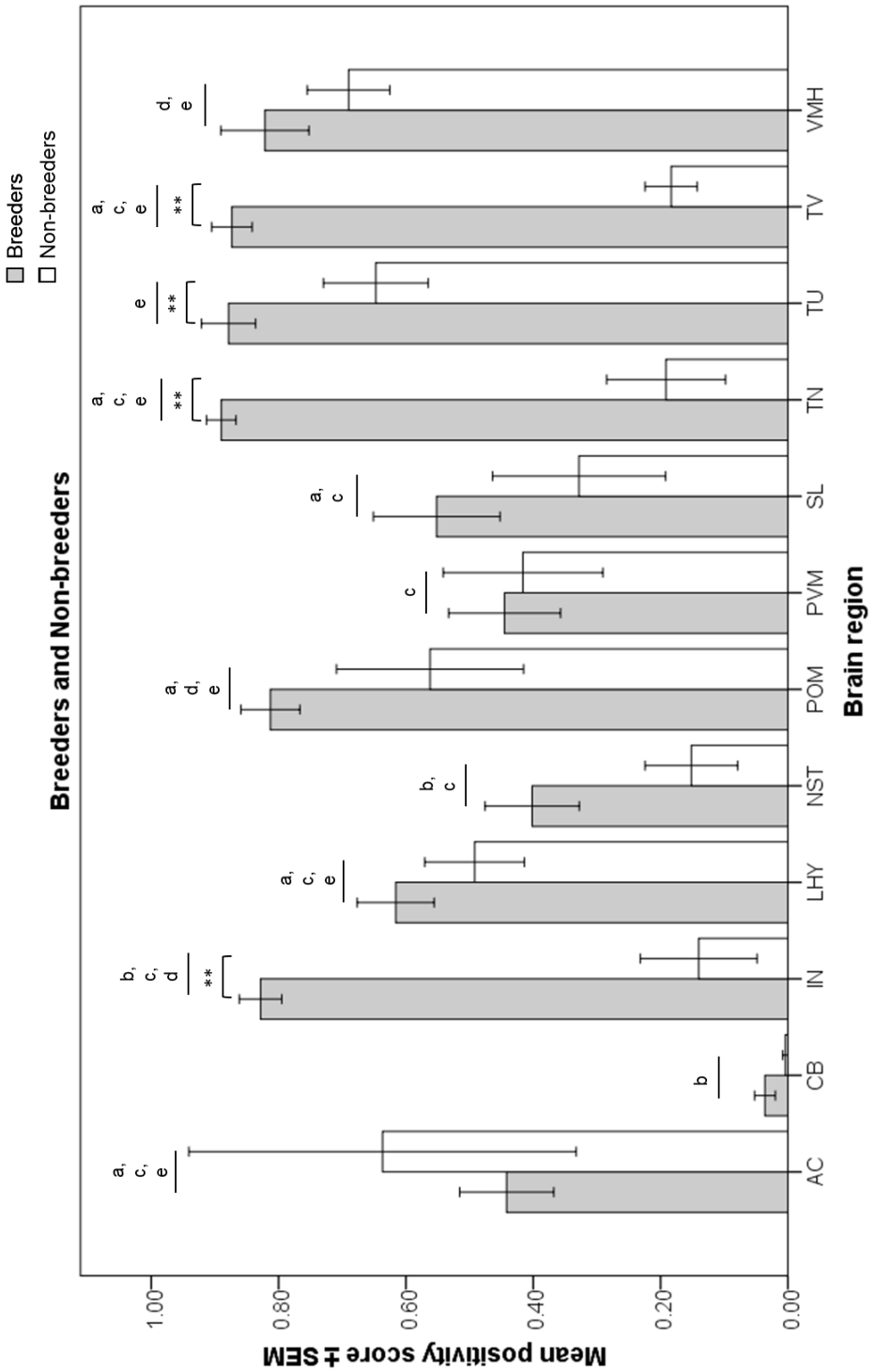


Figure 5.3 continued

B.

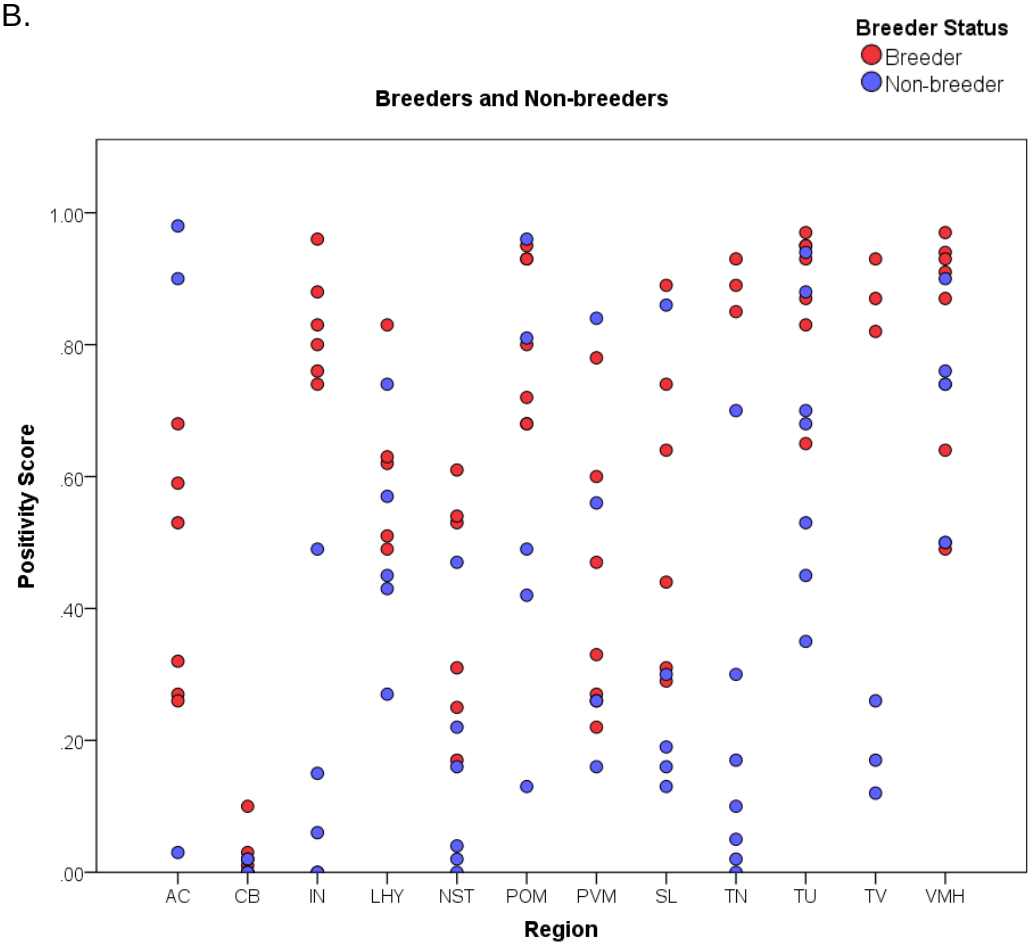


Figure 5.3 continued

C.

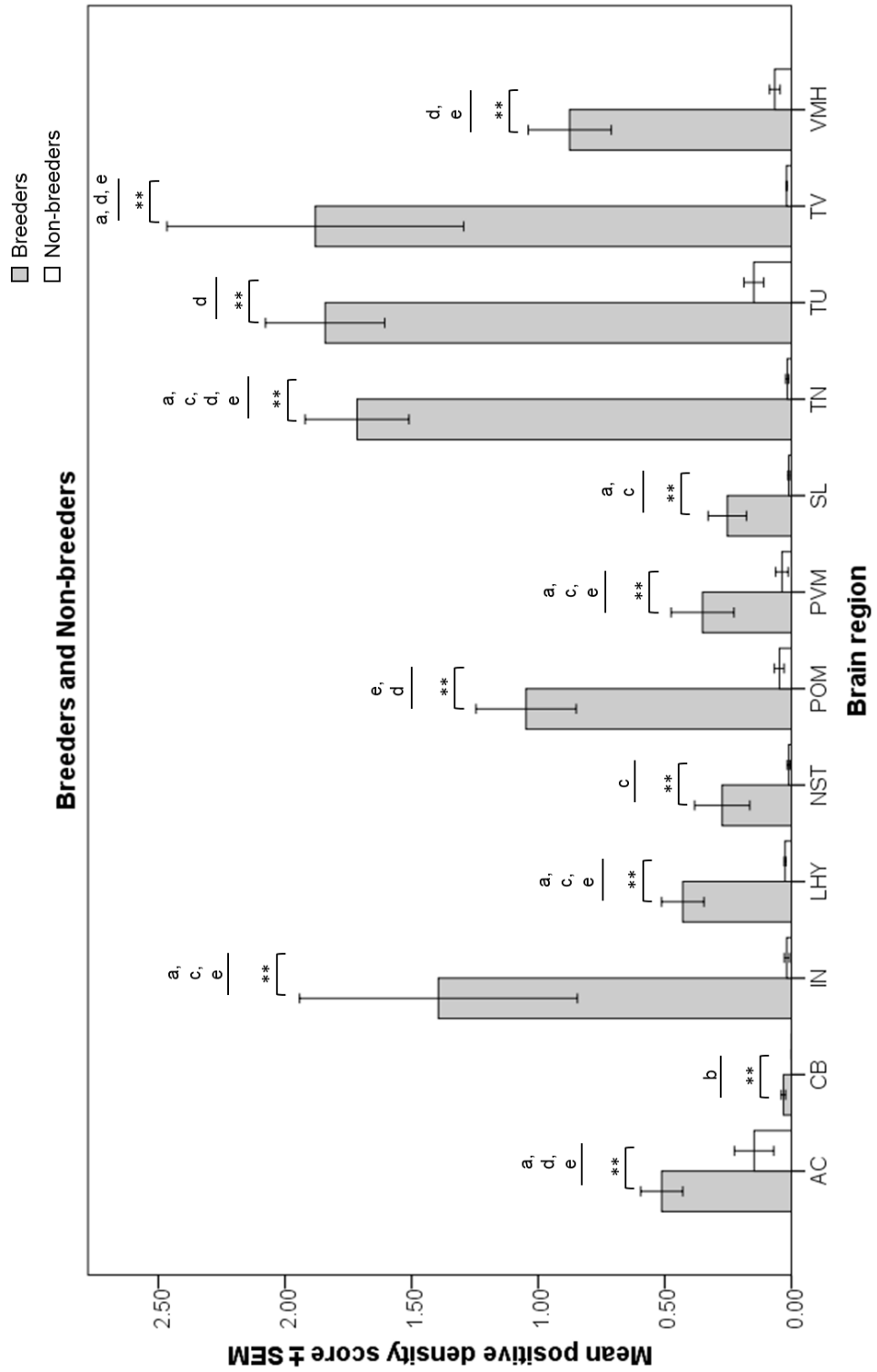


Figure 5.3 continued

D.

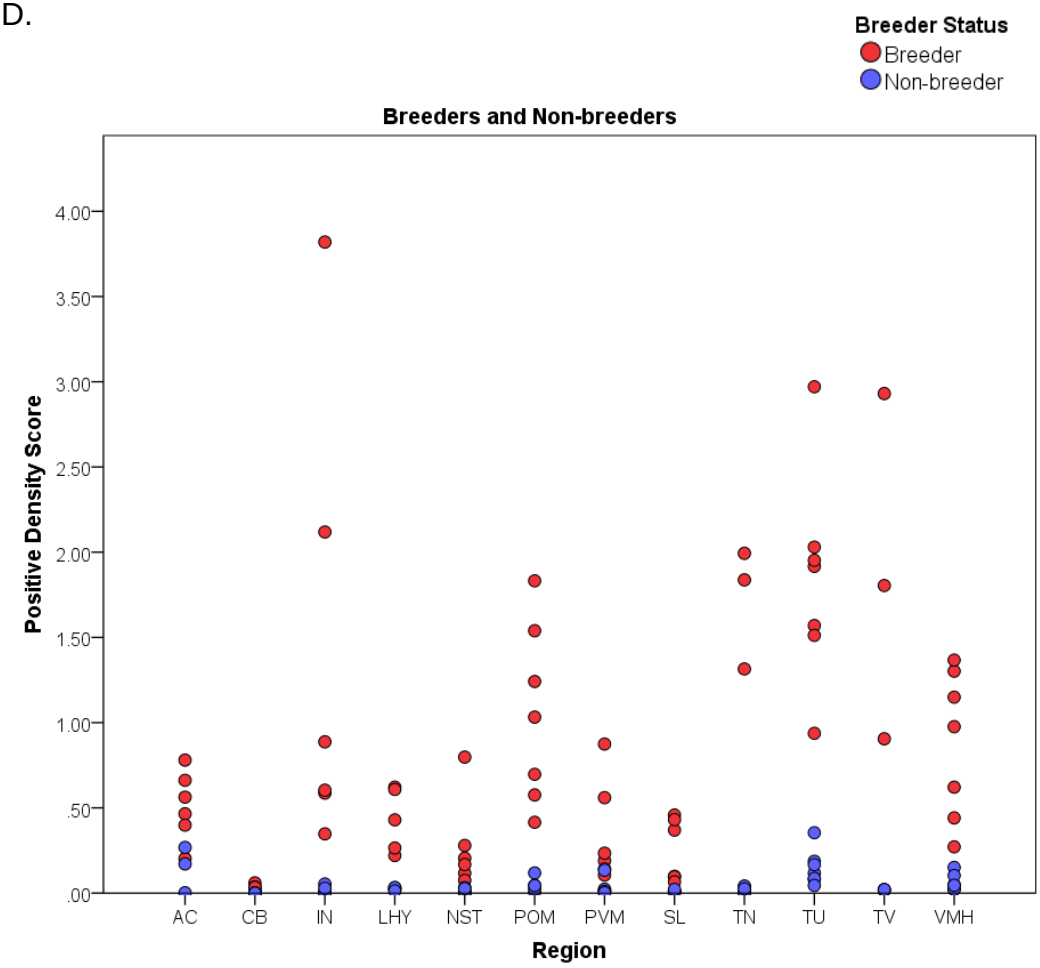
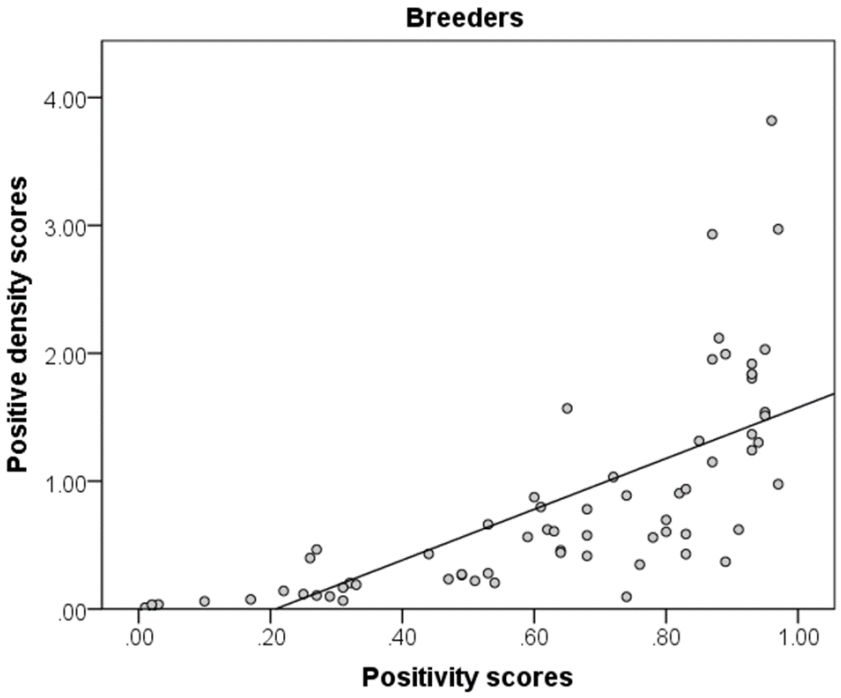


Figure 5.3. (A) Mean positivity scores ± 1 standard error (SEM) across the 12 regions (abbreviations in table 5.1.) compared between breeding birds (grey bars) and non-breeding birds (white bars). Bars with ** indicate a significant difference between breeders and non-breeders (p<0.01). Letters indicate significant differences between positivity scores between brain regions. Bars that share at least one similar letter are not significantly different. Bars that have differing letters are statistically significantly different (p<0.05). (B) Distribution of positivity scores across the 12 brain regions. (C) Mean positive density scores ± 1 SEM across the 12 regions compared between breeding birds (grey bars) and non-breeding birds (white bars). Breeding birds, overall, had higher positive density scores relative to non-breeders in all regions analyzed, as indicated by ** = p<0.01. Letters indicate significant differences between positivity scores between brain regions (breeder and non-breeder data combined). Bars that share at least one similar letter are not significantly different. Bars that have differing letters are statistically significantly different (p<0.05). (D) Distribution of positive density scores across the 12 brain regions.

Figure 5.4. Correlations between the positivity scores and positive density scores

A.



B.

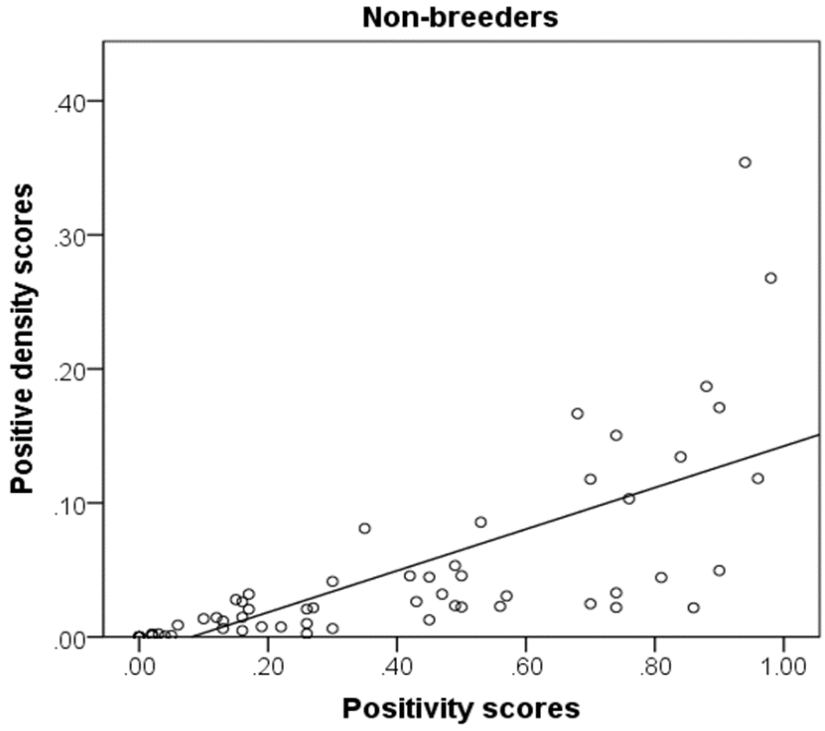


Figure 5.4 continued

C.

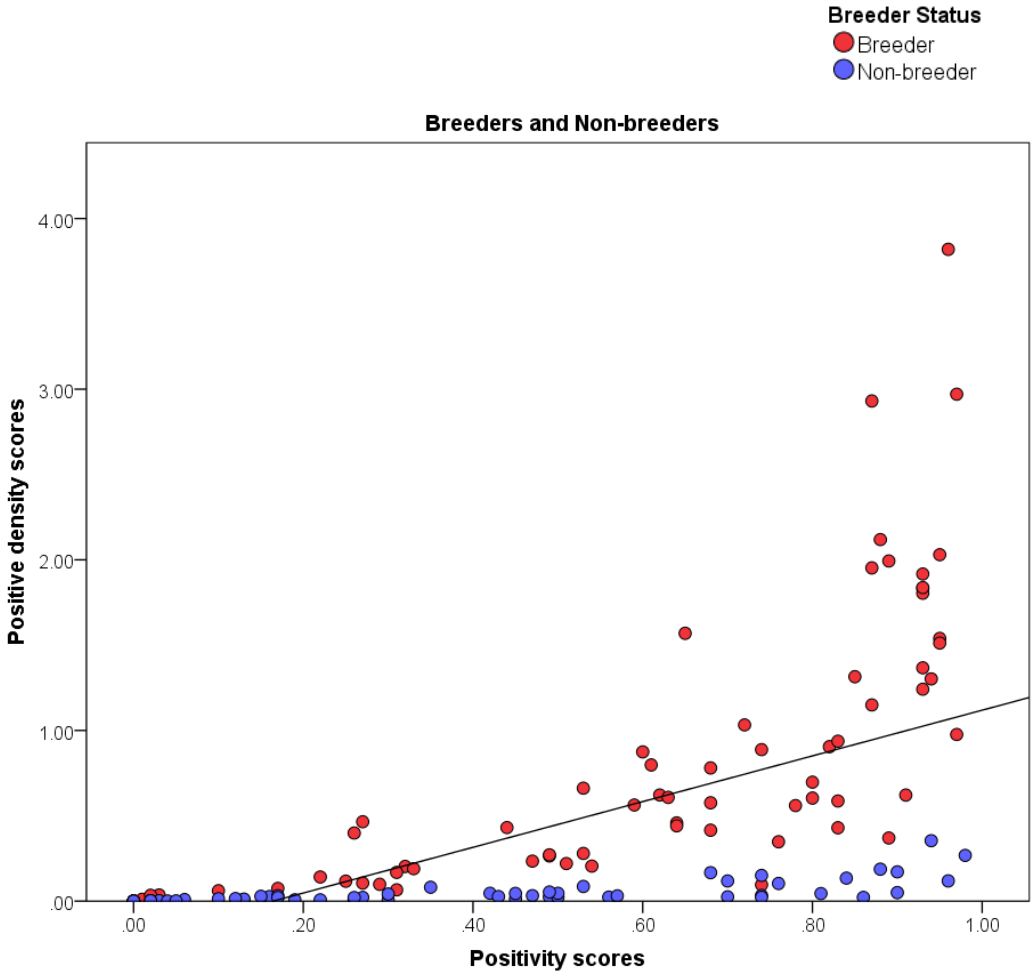


Figure 5.4. (A) Positive density scores plotted against the positivity scores for breeding birds on days 1-3 post-hatch. There is a positive correlation such that density of positive pixels increases as the positivity score increases ($p<0.05$). (B) Positive density scores plotted against the positivity scores for non-breeding birds. There is a positive correlation such that density of positive pixels increases as the positivity score increases ($p<0.05$). (C) Positive density scores plotted against the positivity scores for breeding (grey dots) and non-breeding birds (white dots). There is a positive correlation such that density of positive pixels increases as the positivity score increases ($p<0.05$).

5.5. DISCUSSION

There was significant immunoreactivity (-ir) staining for PRLR in areas previously implicated in mammalian and avian parental care. Although sex differences could only be tested in the breeding group, there were no significant sex differences found in either the positivity or positive density scores within the brain regions tested. For breeders, the highest amount of positive immunostaining was found in the IN, TnA, POM, TU, TV, and VMH, which is consistent with findings in ring doves (Buntin et al., 1993). Moderate staining was found in the AC, LHy, nST, PVM, and SL, and either very minimal or no amount of staining in the CB (control region). This distribution was relatively the same in non-breeder brains with several key exceptions: IN, TU, TnA, and TV. In all four of these regions, breeders had significantly higher positivity scores than non-breeders. Remarkably, even though the distribution of positive staining was similar between breeders and non-breeders (with the exception of IN, TU, TnA, and TV), non-breeders had a dramatically lower density of positive PRLR staining across all regions, indicating that PRLR significantly increase during breeding, which may be in response to increasing circulating PRL concentrations in breeding birds. While our results do not specify whether the actual number of receptors per neuron increases, or if the number of PRL-sensitive neurons increases, these results do suggest that, overall, breeding birds have an increased central sensitivity to PRL, especially in early post-hatch care, when PRL has been found to have a causal role in parental behavior (Ch. 4). Because we only tested breeders and non-breeders at one time point, it is unknown whether PRLRs slowly increase over

incubation, in parallel with increasing plasma PRL levels, or if they are quickly upregulated at the end of incubation or at hatching.

Although the effects of reproductive experience could not be tested in the non-breeder group, there do not appear to be any systemic patterns in the distributions of non-breeder positivity or density scores that would suggest two distinct groups (i.e., inexperienced and experienced birds). We only tested a small number of birds, so while this is a cautious interpretation that is still up for the debate, the data nonetheless show that the PRLR density are plastic and only upregulate during breeding times, when birds are hypothesized to be the most sensitive to the effects of PRL. The specific effects and implications of these findings for each of these areas are discussed below.

IN and TU

For breeders, two of the areas with the highest amount of positive staining were the infundibular nucleus (IN; avian homologue of the arcuate nucleus) and the tuberal hypothalamic region (TU). The IN contains neurons that project to the TU that regulate PRL secretion. In mammals, PRL is tonically inhibited by dopamine (DA) released from tuberoinfundibular dopamine (TIDA) neurons in the arcuate nucleus, which release DA into the pituitary gland, where it acts on D_2 receptors on lactotroph cells to inhibit PRL secretion (Grattan, 2015; Lyons and Broberger, 2014). PRLRs are located on the TIDA neurons, creating a short negative feedback loop such that PRL controls its own secretion (Grattan, 2015; Lyons and Broberger, 2014). During late pregnancy and lactation, however, there is a loss of sensitivity to PRL on the TIDA

neurons as hypothalamic DA neurons stop releasing DA in response to PRL even though PRLR expression remains.

In the birds, PRL is not tonically inhibited by DA as it is in mammals, but rather, is tonically stimulated by vasoactive intestinal peptide (VIP) during breeding (Sharp, 2009; Sharp et al., 1998). DA can have either stimulating or inhibiting effects on PRL based on the dose and which receptor it acts at (Youngren et al., 1998, 1996, 1995), but VIP is considered to be the primary PRL releasing factor. VIP is released from neurons in the hypothalamus into the median eminence and acts via its receptors on the lactotroph cells in the anterior pituitary to stimulate PRL gene synthesis and the release of PRL into the circulation (Kahtane et al., 2003; Sharp et al., 1998; Xu et al., 1996). The distribution of VIP cells in the hypothalamus mirrors the pattern of PRL secretion during the breeding cycle. Consistent with plasma PRL profiles, VIP neuron counts in the nucleus inferioris hypothalami and IN are low during non-laying times and significantly increase during incubation in native Thai hens and turkeys, when circulating PRL is highest (Kosonsiriluk et al., 2008). VIP neuron counts return to low, non-laying counts once chicks hatch, which mimics the drop in circulating PRL (Kosonsiriluk et al., 2008). In addition, cells in the ventral IN increase in size and the intensity of VIP staining as ring doves transition from a non-breeding state to the end of incubation/early post-hatch care (Cloues et al., 1990). VIP-ir staining in the IN is higher in ring doves that are caring for two squabs, compared to one squab (Cloues et al., 1990), suggesting that this increase may be stimulus driven. Interestingly, inexperienced birds show a prolonged period of increased VIP-ir expression,

compared to experienced birds, as inexperienced birds take longer to wean their chicks and begin a new clutch (Cloues et al., 1990).

Given the striking difference in positivity and positive density between breeders and non-breeders in this study in the IN and TU, and the plasticity of VIP cells in these regions, one possibility worth investigating is that PRLR in the IN/TU are on VIP-neurons, rather than DA neurons as they are in mammals. If this the case, then this also raises the possibility that PRL regulates its own secretion, in a short-loop positive feedback mechanism, which is opposite from mammals. In rats, there is a permanent upregulation of PRL in the TIDA neurons, and subsequently, experienced rats have lower serum PRL levels compared to virgin females (Anderson et al., 2006). If there was a permanent upregulation of PRLR on VIP neurons in the IN, then that could potentially be a mechanism to explain why experienced breeders have increased plasma levels of PRL during breeding (Smiley and Adkins-Regan, 2016; Ch. 1). Indeed, Christensen and Vleck (2015) also found that reproductively experienced zebra finches had more PRL producing cells in the anterior pituitary than inexperienced birds, which would also contribute to the increase in PRL secretion in experienced birds. While it remains a hypothesis that PRLR are located on VIP cells in the avian hypothalamus, this may be an exciting new advancement in understanding of avian PRL regulation.

TV and AC

One of the most novel findings of this study is the presence of PRLR in the TV and its significant upregulation during breeding. To our knowledge, this is the first

report of PRLRs in the avian TV. There was moderate staining for PRLR in the AC, which is consistent with the PRLR distribution in ring doves (Buntin et al., 1993) and the Chaiseha et al. (2012) finding of PRL mRNA in the AC of domestic turkeys. These findings suggest that PRL may play a role in the reward processing aspects of parental care.

Parents find offspring stimuli rewarding, which stimulates parental motivation, a critical component of parental care. The TV (VTA in mammals) releases dopamine (DA) into the AC (NAcc in mammals) in response to rewarding stimuli, including rewarding social stimuli, as part of the mesolimbic reward circuitry to evaluate the saliency and rewarding properties of stimuli which promote motivated behavior (reviewed in O'Connell and Hofmann, 2011). Reinforcement from rewarding stimuli encourages approach, contact, and parental behavior towards the offspring (reviewed in D'Cunha et al., 2011) and is highly conserved across species (reviewed in O'Connell and Hofmann, 2011). DA is released in the NAcc following pup exposure in rat mothers (Afonso et al., 2013) and the amount of extracelluar DA in the NAcc shell is positively correlated with the amount of pup licking and grooming (Champagne et al., 2004). In addition, there is a negative dose-dependent response to the number of pups retrieved when a DA receptor antagonist is injected into the NAcc in day 7-postpartum rats (Keer and Stern, 1999). However, NAcc lesions alone did not disrupt maternal motivation (Lee et al., 1999) and had only minor or no effects on maternal care behavior (Numan et al., 2005), indicating that there are likely multiple and redundant mechanisms which influence maternal motivation and behavior. Although other neuropeptides that are important for social behavior, such as oxytocin,

have been shown to regulate reward aspects of parental care in prairie voles in the NAcc (Keebaugh et al., 2015; Olazábal and Young, 2006), a role of PRL in parental reward and motivation has not been researched.

While it is very likely the VT-AC circuit plays a similar role in evaluating offspring stimuli as rewarding during avian parental behavior, there is a surprising lack of studies which have manipulated these areas during parental care. The fact that there is an upregulation of PRLR in the TV supports the conclusions from Smiley and Adkins-Regan (submitted; Ch. 4) that PRL may play a role in the increase in the saliency of egg/chick stimuli, which motivates parental care behavior. One could hypothesize that the mechanism for this is that PRLR located on DA neurons in the TV and PRL regulate DA release into the AC during late incubation and early post-hatch care, which increases the interest in the nest, eggs, and chicks. Therefore, even though a substantial number of incubating birds took care of foster chicks in Ch. 3, it is likely that PRLRs were not fully upregulated yet in the TV, and the motivation to care for chicks would continue to increase as birds approach the time of chick hatching, resulting in increased chick care. It is possible that since birds only cared for chicks after egg laying, eggs are the sensory cue to upregulate PRLR to breeder levels. Although it is unknown whether PRLRs are upregulated gradually during incubation, in concert with the rising plasma PRL levels, or if they quickly increase right at the end of incubation, a lack in motivation to care for chicks may explain why 86% of normally breeding, control birds fed chicks in Ch. 4 and only 54% of incubating birds (with low PRL) fed chicks in Ch. 3. To formally test this hypothesis, one would first need to establish that TV and/or AC lesions affect parental care and motivation in a

behavioral paradigm similar to that used in Ch 4. Although motivation is slightly more challenging to access in birds, if birds were trained in an operant chamber paradigm where, for example, they had to peck a key to access the nest and to feed the chicks, then that would be a strong indicator that parental care is a motivated behavior. Next, one could see if the neurons which express PRLR are dopamergic in the TV. Injections of a PRLR antagonist in the VT could then test whether this interferes with parental care and parental motivation and to see if PRL activity in these neurons is regulating dopamine release into the AC.

TnA

There was an unexpectedly high concentration of PRLR in the TnA and the positivity of PRLR in the TnA was significantly upregulated in breeders. The TnA has been proposed to be the homologue of the mammalian medial amygdala (reviewed in O'Connell and Hofmann, 2011) and has been critically understudied regarding avian parental care. Therefore, it is currently unknown what the role of the TnA in avian parental care is. In general though, the amygdala plays a role in emotional processing, fear and arousal, and salience processing (reviewed in Kaldewaij et al., 2017). In rodents, sexually naïve males and females often find pup odors aversive or not interesting and either ignore or display aggressive, fearful, and/or anxiety-like responses towards young (Fleming et al., 2008; Tachikawa et al., 2013). The MeA receives direct input from the accessory olfactory bulb (AOB) and mediates the avoidance and suppression of maternal care in virgin female rats by processing offspring odor cues as aversive (Numan and Insel, 2003). Sexually naïve male mice

show higher levels of c-fos expression in the vomeronasal organ (VNO), AOB, posterior MeA, as well as several other hypothalamic regions compared to fathers that have had pup exposure (Tachikawa et al., 2013), which suggests the downregulation of the VNO-AOB-MeA pathway is important for the transition into parenthood. Importantly, the amygdala appears critical for the motivation to approach (appetitive response) towards pups, likely through the suppression of the fear response. Temporary lesions of the basolateral nucleus of the amygdala (BLA) or basomedial nucleus of the amygdala (BMA) via muscimol injections produced major deficits in retrieval behavior in postpartum rats (Numan et al., 2010). Complete BLA and BMA lesions reduced maternal motivation in female rats as assessed by lower bar-presses for pups in an operant responding paradigm (Lee et al., 1999) and increased the latency to retrieve pups in both male and female California mice (Lee and Brown, 2007). While it is currently unknown whether PRL plays a role in this process at the amygdala, high concentrations of PRL-ir fibers and receptors have been reported in the amygdala (Sanford et al., 1998). Oxytocin (OT), which is released during parturition, acting at both the olfactory bulb and the amygdala, appears to play a part in switching pup stimuli from aversive to promoting approach in parturient females (reviewed in Numan and Young, 2016). It is plausible that PRL plays a similar role in the amygdala in both males and females. A PRLR antagonist study would be useful to tease apart the role(s) of PRL in the amygdala during parental care. Additionally, it will be important to carefully investigate which area of the amygdala is critical for parental care, as it appears different nuclei within the amygdala could potentially regulate different aspects of parental care.

Although birds lack a VNO, the TnA has been shown to be crucial for the appetitive and approach response in male Japanese quail (*Coturnix japonica*) sexual behavior (Absil et al., 2002; Thompson et al., 1998). TnA lesions in male zebra finches also led to a decrease in pairing with a female as males showed less sexually motivated behavior (Ikebuchi et al., 2009). The effects of the TnA on sexually motivated behavior are mediated by sex steroids so it possible that PRL acting in the TnA plays a similar role in promoting motivational responses, but in the context of parental behavior, not sexual behavior. The basolateral amygdala can modulate VTA DA firing to the Nacc, which integrates sensory information during emotional behaviors (reviewed in O'Connell and Hofmann, 2011), so it is possible that the TnA and TV work in concert together, perhaps via PRL signaling, to promote the onset of approach behavior towards chicks during parental care. Unlike mammals, where olfactory cues are most prominent in stimulating or avoiding parental care, birds respond more to tactile, visual, and auditory cues from chicks. Furthermore, zebra finches do not show a fearful response towards chicks, as they will care for foster chicks (Smiley and Adkins-Regan, submitted; Ch. 3). However, not *all* incubating birds will care for foster chicks and birds spend less time caring for foster chicks, compared to their own chicks (Ch. 3 and 4). This may indicate that there still needs to be some regulation of the TnA and/or TV near the end of incubation to influence the approach response in parental birds and to increase the saliency of chick cues. Studies which first lesion the TnA will be important to establish the behavioral effects during parental care. Then, blocking PRL activity in the TnA will be critical to figure what the role of PRL in the TnA, if any, there is. Alternatively, PRL could play a role in the

TnA for many other different behaviors or processes, as the amygdala is involved in many functions.

POM

Another surprising result is that there was *not* an upregulation in positivity score in the POM. The POM is a critical brain region for both mammalian and avian parental care and is a well-established hotspot for PRLRs (Bridges, 2015; Buntin, 1996). In rats, there is a permanent upregulation in PRLRs in the MPOA after gaining reproductive experience, and PRLR continue to increase in response to increased plasma PRL levels during pregnancy and lactation (Anderson et al., 2006). In addition, the number of cell bodies in the MPOA increases significantly over pregnancy, peaking near late pregnancy in rats (Keyser-Marcus et al., 2001).

Although the positive density of PRLR is greater in breeders relative to non-breeders, there is still an abundance of PRLR positive cells in the POM of non-breeders. If the POM is involved in post-hatch parental care in the zebra finch, as it is in ring doves and other birds (Buntin, 1996), then this may explain why some incubating birds were able to provide parental care to foster chicks in Ch 3. The POM also plays a role incubation behavior in galliform species (Buntin, 1996), so the fact that POM PRLR positivity is not different in breeders and non-breeders may also suggest that the POM plays a role in incubation and post-hatch chick care. However, the positivity score was much more variable in non-breeders then it was in breeders, suggesting that there may need to be some upregulation in PRLR in the POM for post-hatch care behaviors. The variability in the amount of foster care provided by

incubating parents (Ch. 3), or whether incubating birds provided care at all, may also be potentially explained by the variability in PRLR in the POM during that time. Although there was no effect of incubation day on the amount of parental care in the Ch. 3 study, if PRLR are upregulated gradually, just as plasma PRL is, and we had tested birds during multiple time points throughout incubation, one could hypothesize that the POM PRLR positivity scores would positively correlate with the amount of foster care given. However, this remains a hypothesis until it can be can be formally tested.

In addition to variability in the POM PRLR distribution, another critical component, as described earlier, is the increase in saliency and motivation to care for chicks. So even though non-breeding birds may have the "machinery" in the POM to provide parental care, without the increase in motivation to do so, not all birds will show parental care, or will not show care at the levels of a normally breeding birds. Importantly, the POM has significant projections to the VTA (Numan and Insel, 2003). PRL acting at the POM could be responsible for modulating VTA activity, such as increased DA into the AC. In support of this, Lee and others (1999) demonstrated that in addition to disrupting maternal behavior, rat mothers with MPOA lesions also found pups to be less rewarding, as demonstrated by reduced bar pressing to gain access to pups in an operant conditioning paradigm compared to sham controls. In mammals, the MPOA coordinates the processing of pup odors cues as either aversive or rewarding via input from the MeA and VTA. Since the TnA also showed upregulation in PRLR, it is possible that a similar phenomenon occurs in birds, but perhaps through tactile or visual cues.

LHy and VMH

Mammals and birds each have their own unique energy expenditures to cope with during reproduction and parental care. Parents often need to increase food intake before and after offspring arrive to prepare for and meet the increasing energy expenditure of caring for young. This often results in altered feeding habits or foraging strategies to meet these higher metabolic needs. For example, avian parents usually decrease feeding or even fast during incubation because they cannot leave the eggs unattended for long periods of time (Buntin et al., 2008; Sherry et al., 1980). However, parents increase foraging activity significantly after hatching to meet the needs of feeding chicks as well as themselves (Buntin et al., 2008). To meet these energetic needs, reproductively active birds generally need to eat 3-5 times more than nonreproductive birds (Farmer, 2000).

The lateral hypothalamus (LHy in birds), or the lateral hypothalamic area (LHA in mammals), and the ventromedial hypothalamus (VMH) are both important for the regulation of food intake, thirst, and weight gain. Stimulation of the LHA increases eating, drinking, and physical activity (reviewed in Brown et al., 2015), while lesions to the LHA/LHy decrease food intake or induce aphagia (the inability to eat) and adipisa (reduced thrist) in both mammals and birds (Kuenzel, 1994; Kuenzel et al., 1999). These effects, however, usually only last several days and are not permanent (Kuenzel, 1994; Kuenzel et al., 1999). The LHA/LHy coordinates peripheral cues of energy status via peripheral hormonal signals such as insulin, leptin, and ghrelin to alter behaviors that affect weight gain (reviewed in Brown et al., 2015).

Lesions to the VMH increase food intake, which results in over-eating in both mammals and birds, and increased weight gain in food restricted rats (Kuenzel, 1994). Importantly, both areas are important for the *motivation* to eat. Rats will work for an electrical stimulation of the LHA, which increases feeding and reinforces learning about instrumental responses which yield food reward (Stuber and Wise, 2016), whereas VMH lesions lead to a decrease in motivation to work for food without affecting the motor ability to eat (King, 2006).

There was moderate staining for PRLR in the LHy and a very high concentration of PRLRs in the VMH of zebra finches, which is consistent with the ring dove PRLR distribution (Buntin et al., 1993). PRL plays an important role in food consumption in ring doves. Food intake increases in a dose-dependent manner in response to either systemic or central ICV injections of PRL, but that are below the level to stimulate crop sac growth and crop milk production (Buntin, 1989; Buntin and Figge, 1988). Specifically, microinjections of PRL into the VMH were the most effective in stimulating food intake, although injections into the POA or TU also stimulated increase food take (Buntin et al., 1991; Hnasko and Buntin, 1993). However, ring doves with a lesioned VMH are still susceptible to the effects of increased food intake via PRL injections (Buntin et al., 1999), confirming there are multiple and redundant mechanisms which control food intake that PRL can affect. The fact that zebra finches also show a rich concentration of PRLR in the VMH suggests that this effect of PRL on food intake is not unique to ring doves or crop sac feedings. Therefore, one could hypothesize that PRL also plays a role in increased feeding during post-hatch care in other birds. Although parents may have fewer

opportunities to leave the nest to forage during offspring care, perhaps PRL influences the amount of food consumed during each feeding bout or increases the motivation to continue eating and foraging. In support of this, while reducing PRL in parental male Adélie penguins did not affect foraging trip duration, it did affect diving and foraging effort (Cottin et al., 2014), suggesting males were less motivated to provide food to chicks. These types of effects are not easily detectable in lab setting where food is typically freely available and so this hypothesis would be better tested in semi-natural or free-living birds.

Although the role of PRL in the LHy is not well understood in birds, Buntin et al. (2006) found increased c-fos (immediate early gene) activity in the LHy following an interaction with chicks and feeding chicks. P-STAT5 activity, a transcription factor expressed after activation of the PRLR, was also found to be significantly higher in the LHy of parents during post-hatch care, relative to birds in the pre-lay, early incubation, or non-breeding stages (Buntin and Buntin, 2014). While it still not clear what the role of PRL in the LHy is, given the results of PRL in the VMH, perhaps PRL plays a greater role in the disinhibition of satiety, rather than the stimulation of feeding during parental care. A similar phenomenon has been described in rats where central PRL administration induces leptin resistance (which normally signals satiety) and increased food intake in female rats (Woodside et al., 2008).

SL and nST

The onset of parental care also includes the suppression of circuits that influence fear, anxiety, and defensive behavior towards offspring. Similar to the MeA,

the lateral septum (LS in mammals), and bed nucleus of the stria terminalis (BNST) are involved in defensive behavior and inhibiting maternal care in rats (Numan and Insel, 2003). Immediate early gene studies using c-fos indicate that the ventral LS (LSv), BNST, MeA, and several other medial hypothalamic sites show increased activation in virgin female rats exposed to pups, compared to maternal rats, when defensive behavior towards pups is highest (Sheehan et al., 2000). The hippocampus projects to the LS, which has been shown to play a role in evaluating novel stimuli, social recognition, and social memory (reviewed in O'Connell and Hofmann, 2011). Because virgin rats actively avoid or attack offspring, it is thought that brain regions involved in processing aggressive, threatening, and stressful responses are involved in the inhibition of maternal behavior. However, once a rat becomes maternal, her aggression must be redirected from pups towards intruders. Decreasing the fear response is important for both positive pup interactions and for the increase in maternal aggression during this time (Numan and Insel, 2003). LS lesions in lactating rats increase anxiety behaviors and disrupt maternal care (Numan and Insel, 2003) and complete septal lesions reduce aggression towards male intruders and increase defensive behaviors (Flannelly et al., 1986). In contrast to the LSv, the dorsal lateral septum (LSd) and intermediate subdivision of the lateral septum (LSi) increase in c-fos activity in maternal rats, relative to virgin pup-exposed rats (Sheehan et al., 2000), indicating that the LS is likely involved in the regulation of aggressive behaviors towards both pups (in virgins) and intruders (in postpartum rats), and that the specific nuclei within the LS are differentially responsible for these actions. PRLR expression slightly increased in the BNST in pregnant rats just before birth and has also been

implicated in male intruder-induced and other predator evoked maternal aggression (Cabrera-Reyes et al., 2017).

Both the LS and BNST have been primarily studied in the context of the nonapeptide system and the effects of oxytocin (OT) and vasopressin (AVP) on social behavior, including paternal behavior (Wang et al., 1994). OT centrally infused into the central amygdaloid nucleus or the BNST decreased the frequency of male intruder attacks (Consiglio et al., 2005). Does PRL regulate any of these aggressive interactions? One study found that while PRLR mRNA was upregulated in the MPOA and BNST from diestrus to the post-partum period in rats, it was not upregulated in the LSv (Mann and Bridges, 2002), suggesting that PRL is unlikely to play a role in pup-directed aggression. PRL may, however, play a role in the disinhibition of these circuits during the transition into maternal care, but until PRL or PRLRs are manipulated in the LS (specifically, the LSd/LSi), it is unknown whether PRL is causally involved in maternal aggression or the switch between pup and intruder aggression. Alternatively, PRLR in the LS may also be regulating other behaviors or physiological process including feeding in female rats, thermoregulation, analgesia, and lactation (Mann and Bridges, 2002).

There was moderate staining for PRLR in the lateral septum (SL in birds) and a slightly lower amount of staining in the nST, which is consistent with the ring dove distribution (Buntin et al., 1993) and with the finding that PRL-ir neurons are found in the SL of turkeys and ring doves (Ramesh et al., 2000). Again, the SL has been primarily studied in the context of MT/AVT and social behavior such as gregariousness, territoriality, flocking behavior, aggression, and pair-bonding in birds

(Goodson, 2013). Given that isotocin (fish homologue of oxytocin) in the SL is also important for parental care in cichlid fish (O'Connell et al., 2012), it is likely that the SL has a conserved role in parental care, although this may or may not be regulated by PRL. Jurkevich and Grossman (2003) found sexually dimorphic AVT-ir fibers in the SL of male chickens such that males show an increased AVT innervation into the nST and SL, relative to females. A similar finding has been reported in Japanese quail where males show a population of AVT-ir response cells that are not visible in the female (Panzica et al., 1997). Since male chickens and quail do not provide parental care, it is possible that the SL and nST are important for aggression towards offspring or the inhibition of parental care in those species, which is different from zebra finches. One possibility is that these areas are involved in the increased nest defense behaviors that are observed in parental zebra finches. Another possibility is that since the SL receives heavy hippocampal input, it may play a role in tuning into specific social stimuli, and PRL may play a role in shifting the positive salience toward offspring, as has been proposed in other regions, and has been discussed elsewhere. Specific lesions or PRLR antagonist targets into these subareas of the SL and nST will be critical in separating out the role of the SL and nST in avian parental care.

PVM

In mammals, circulating PRL levels have been shown to both increase and decrease in response to acute and chronic stressors (Freeman et al., 2000; Martí and Armario, 1998). While generally acute stress increases and chronic stress decreases PRL, the opposite effects have also been found, or sometimes no change at all in

response to stress. PRL nonetheless "reacts" to stress by either increasing or decreasing, and this reaction appears highly dependent on the type, intensity, and timing of the stressor (Freeman et al., 2000; Martí and Armario, 1998). PRL is synthesized and released in the PVN in mammals (Torner and Neumann, 2002), however, outside of pregnancy, PRLR mRNA is non-detectable in the PVN (Kokay et al., 2006). Specifically, during pregnancy and lactation, PRLR mRNA increases in OT neurons in the PVN and PRL reduces stress induced OT release (Kokay et al., 2006) and inhibits the stress response by attenuating the ACTH response (Torner and Neumann, 2002). Attenuated HPA (hypothalamic-pituitary-adrenal) activity and stress behavior responses have been well documented during lactation, which may be adaptive to reduce the new mother's fearfulness towards pups and increase aggression to protect offspring (Torner and Neumann, 2002). Therefore, PRL is thought to be a protective factor during pregnancy and lactation against stress-induced biological modifications such as gastric uclers or hypothermia (Drago et al., 1989).

In this study, there was moderate staining for PRLR in the PVM, which is consistent with moderate PRL binding sites in the PVM in ring doves (Buntin et al., 1993), and PRL-ir in perikarya and fibers in the turkey and ring dove (Ramesh et al., 2000). PRL also reacts to stress in birds (Angelier and Chastel, 2009). In contrast to mammals, PRL generally decreases in response to both acute (e.g., standard capture and restraint protocol) or chronic stress (e.g., energy constraints; fasting) (Angelier and Chastel, 2009). However, this change in PRL is non-linear as PRL has been found to increase within the first several minutes after the stressor initiation, followed by a significant decrease in PRL (Angelier and Chastel, 2009). Other possible variables

which influence this pattern are the secretion, synthesis, and clearance rates of PRL (Angelier and Chastel, 2009). Similar to mammals, the PRL stress response changes over the breeding cycle in birds. In response to handling stress, black-legged kittiwake (*Rissa tridactyla*) parents caring for young had an attenuated PRL stress response (9% decline in PRL) compared to failed breeders, which had a 41% decline in PRL (Chastel et al., 2005). In Manx shearwaters (*Puffinus puffinus*), plasma PRL decreases in response to an acute capture and restraint stressor during incubation and post-hatch care, but in birds which showed little or no parental care (pre-breeders, failed breeders, and animals in the late post-hatch care phase) an increase in PRL is observed in response to acute stress (Riou et al., 2010). This suggests that the stress response in general is attenuated during parental care, just as it is during pregnancy and lactation in mammals. Given PRL's influence on OT neurons in mammals, it is plausible that PRL plays a similar role in birds as mesotocin (avian homologue of OT) neurons reside in the avian PVM as well. While this has not yet been directly tested, evidence from turkeys and native Thai hens (*Gallus domesticus*) show an increase in the number of MT-ir neurons in the PVM during late incubation, when circulating PRLR density increases (Chokchaloemwong et al., 2013; Thayananuphat et al., 2011), and MT neurons in the PVM decrease when hens are chick deprived (Chokchaloemwong et al., 2013). Studies which investigate PRL's influence on MT neurons and MT release over the breeding cycle would be fruitful in understanding the regulation of PRL, MT, and the stress response during breeding times.

Conclusions

In conclusion, this is the first study to describe the PRLR distribution in the zebra finch brain. The highest amount of positive stain was found in the IN, TnA, POM, TU, TV, and VMH, with moderate staining found in the AC, LHy, nST, PVM, and SL, and either very minimal or no amount of staining in the CB (control region), which is consistent with findings in ring doves (Buntin et al., 1993). In order to establish that the effects of PRL on parental behavior are mediated by PRLRs, PRL or PRLR manipulation targeting these areas will need to be performed. Given the roles of PRL in these regions of other birds and mammals, such as PRL secretion regulation, reward processing, parental behavior, and feeding, one can hypothesize that PRL plays a role in each of these components during parental care. Instead of PRL having "one" role during parental care, it seems more likely that PRL acts at multiple regions, altering multiple biological functions and synchronizing multiple aspects of parental care together. These processes may include, but are not limited to: increasing the positive saliency of egg/chick stimuli via actions in the POM, TV, AC, or TnA, regulating its own secretion in the IN/TU, altering feeding patterns in the VMH/LHy, and attenuating the stress response in the PVN/PVM. All of these functions, together, are adaptive for maintaining a bird in a parental state to care for itself and its young.

The fact that breeders have an overall higher density of PRLR is consistent with the fact that circulating PRL is only high near the end of incubation and the beginning of post-hatch care, when PRL was shown to have the strongest effect in parental care behavior (Ch. 4). These results make a strong case that, overall, the brain is more sensitive to PRL when birds are in a breeding state, relative to a non-breeding state. It is still unknown, however, whether these changes are permanent as we were

unable to test differences between experienced and inexperienced birds in our non-breeding groups. Although there appears to be no systemic patterns in the distributions of non-breeder positivity or density scores that would suggest two distinct groups (i.e., inexperienced and experienced birds), a formal test of whether experienced birds have a greater central PRLR density is worthy of pursuing in order to further understand the interaction between PRL and reproductive experience in parental care behavior.

Studies which manipulate central PRL in birds other than ring doves, which do not produce crop milk, are necessary understand PRL's central role in avian parental care more generally. This study is the necessary first step to facilitate future research that uses central PRL targets to manipulate parental behavior. Although our focus is on parental care, this study will be of value to many researchers from many fields that are interested in the physiological and behavioral functions of PRL.

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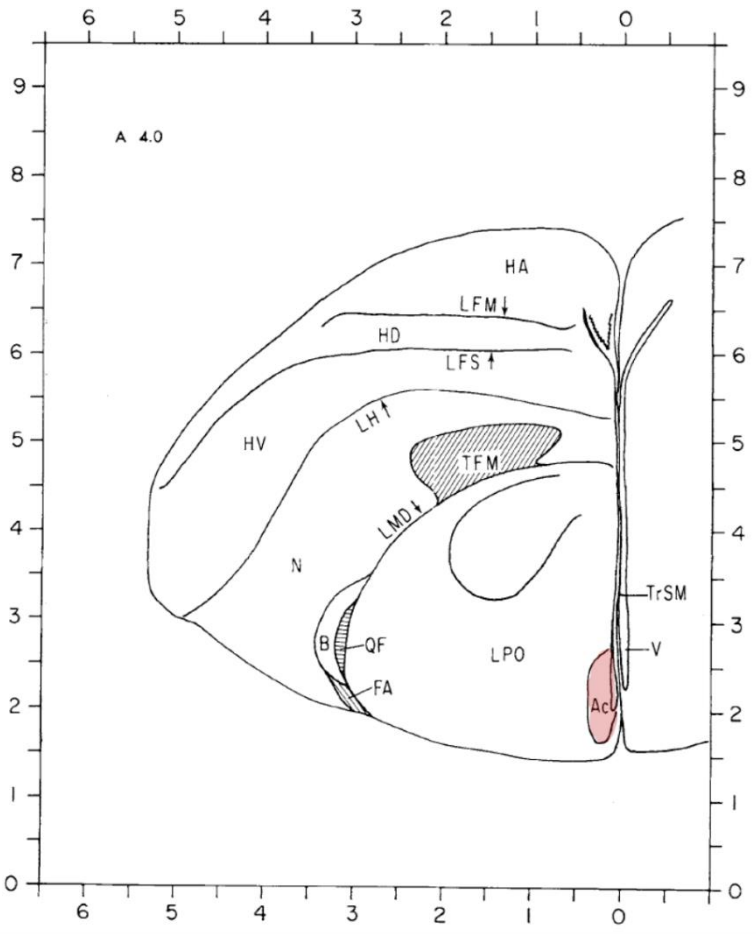
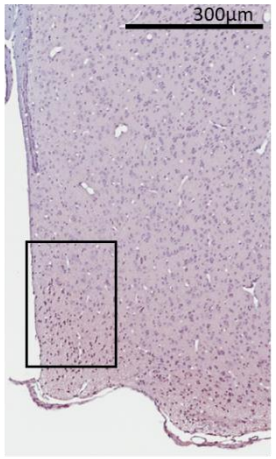
APPENDIX A.

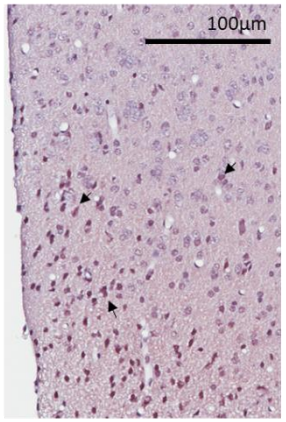
REPRESENTATIVE IMAGES OF PROLACTIN RECEPTOR IMMUNOSTAINING IN BREEDING AND NON-BREEDING ZEBRA FINCH BRAINS

Atlas images are from the canary brain atlas (Stokes et al., 1974). For each brain region, an image taken at 7x and 20x magnification are presented from one breeder and one non-breeder brain. Small black boxes on the 7x images indicate the area which is zoomed into 20x. Small black arrows point out examples of PRLR immunostaining. Brain regions abbreviations are defined in table 5.1.

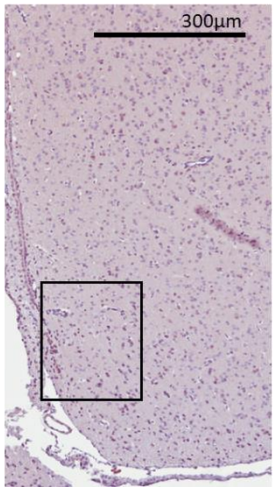


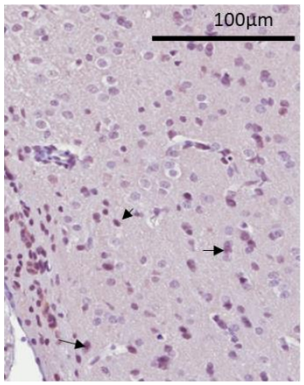
AC: Breeder

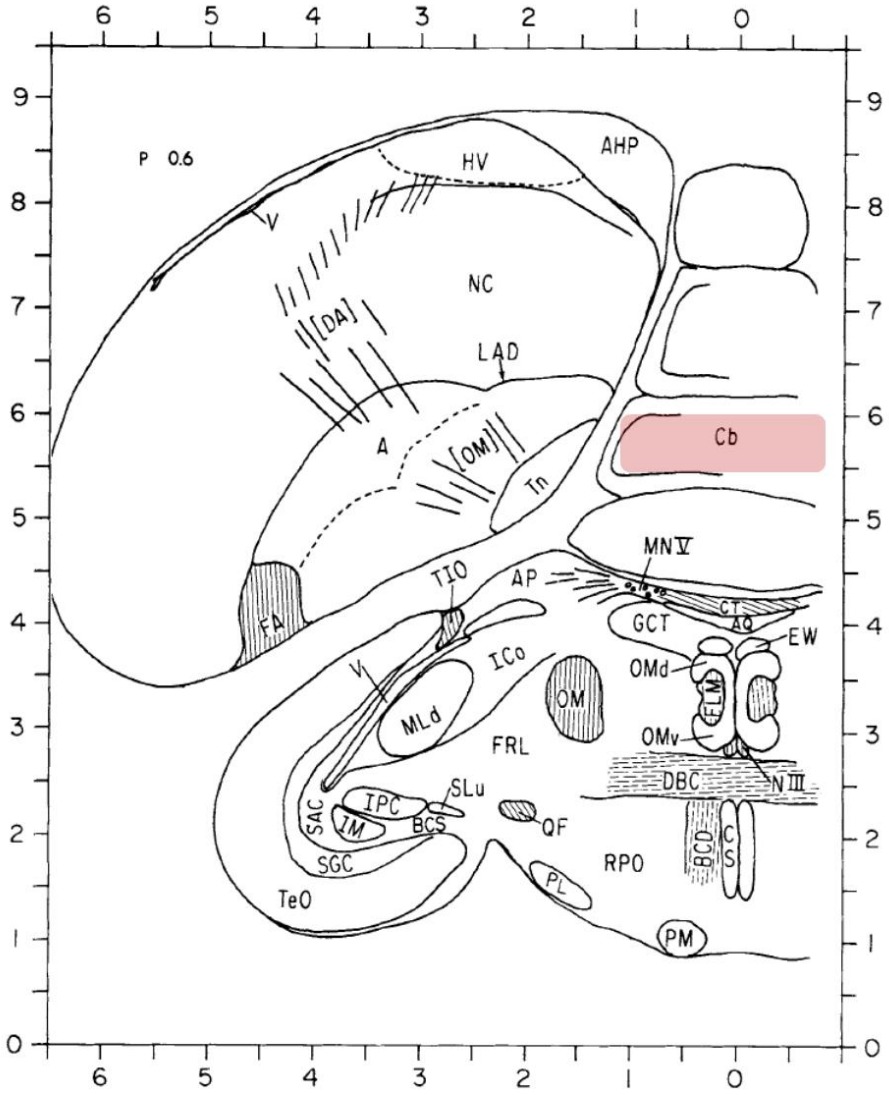




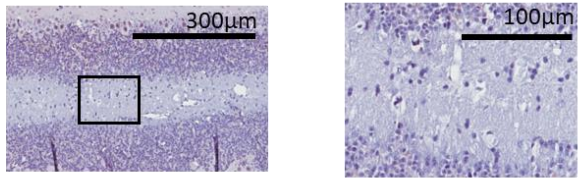
AC: Non-breeder



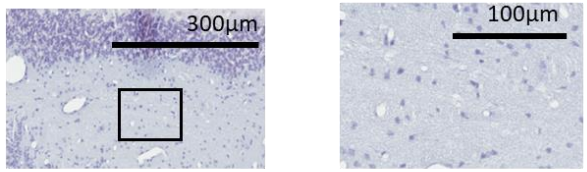


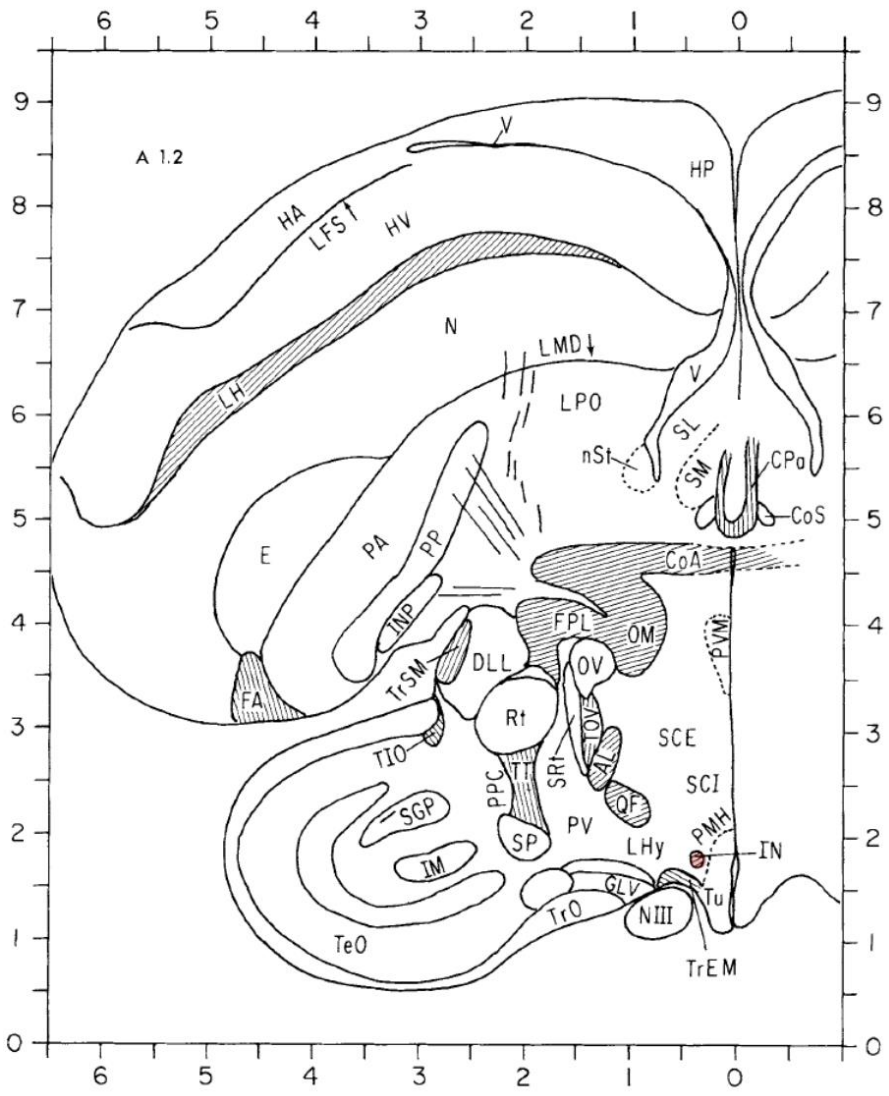


Cb: Breeder

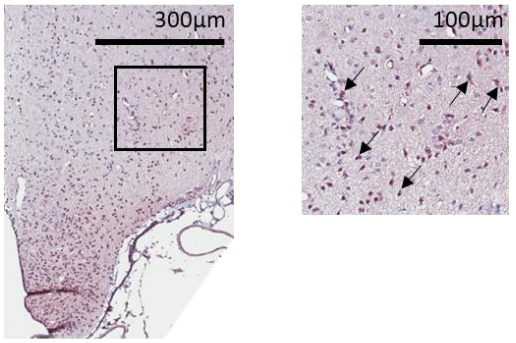


Cb: Non-breeder





IN: Breeder



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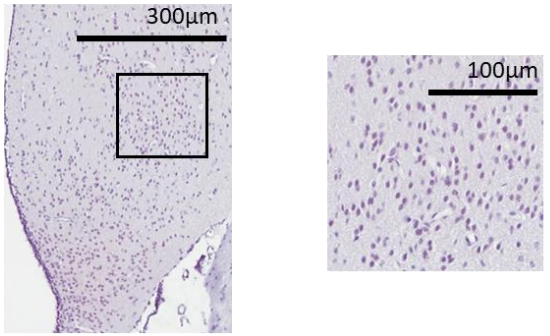
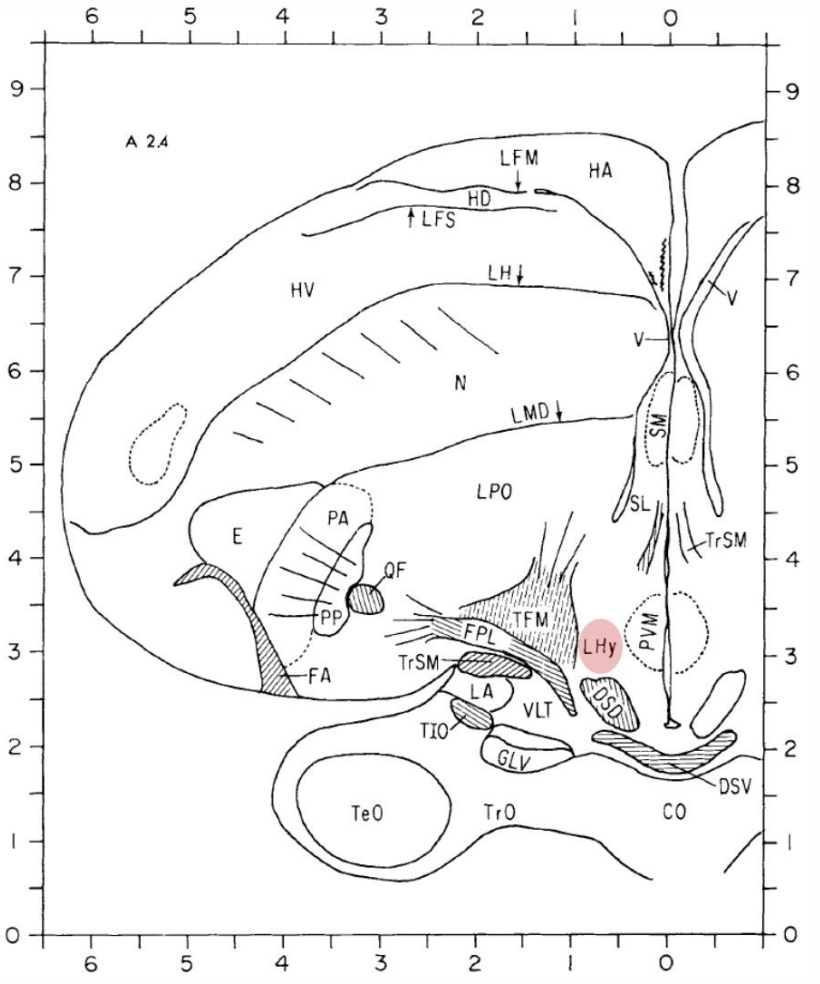
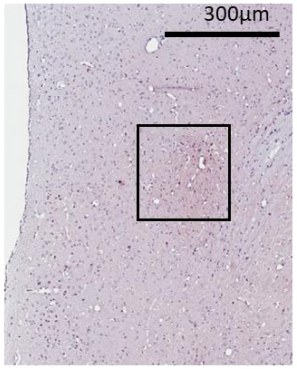
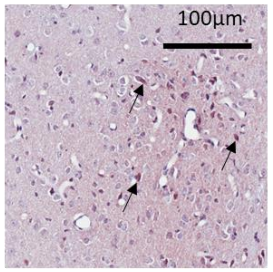


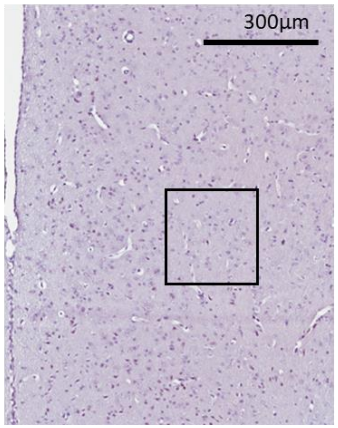


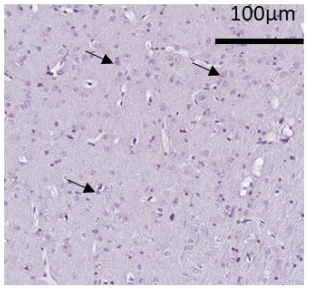
LHy: Breeder

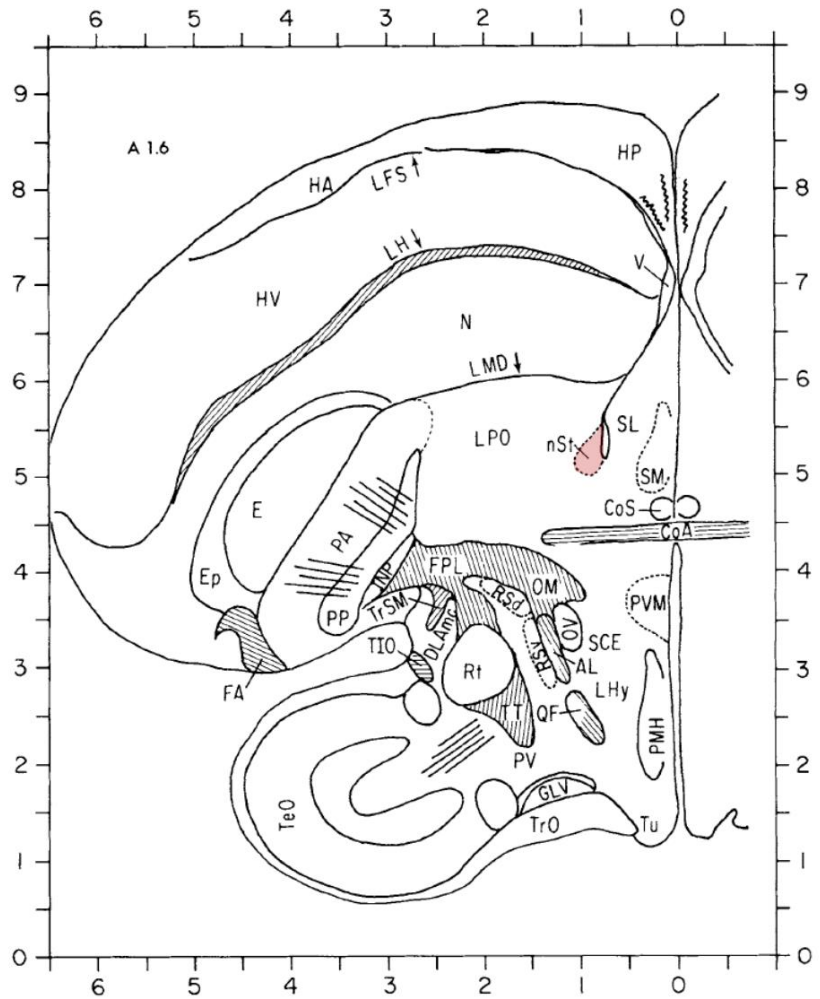




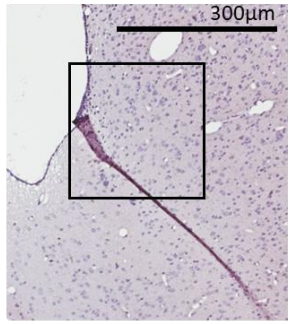
LHy: Non-breeder

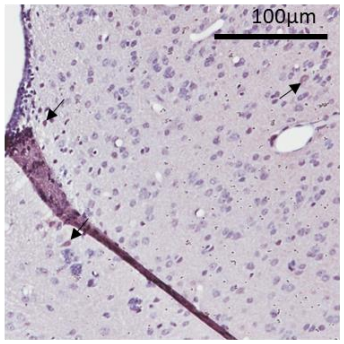




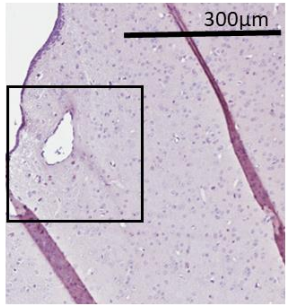


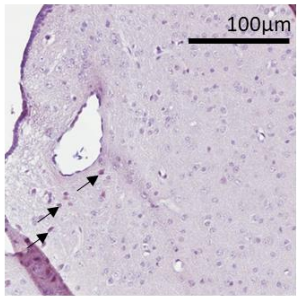
NST: Breeder

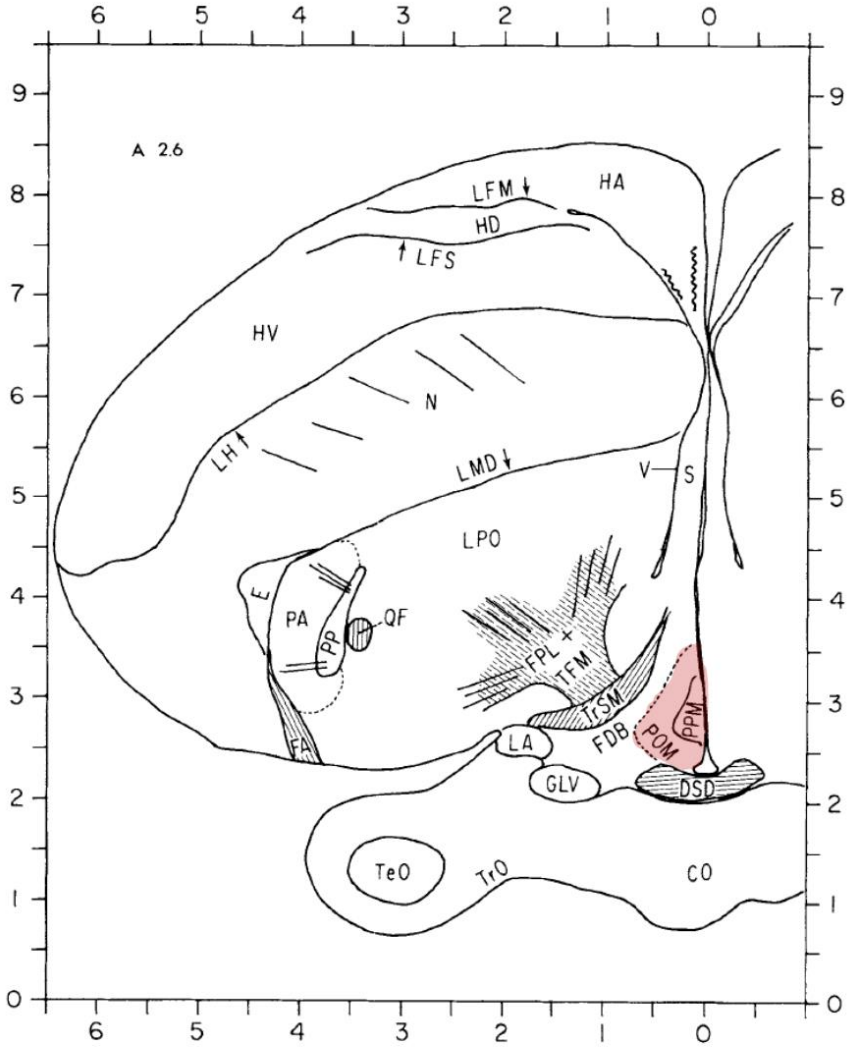




NST: Non-breeder







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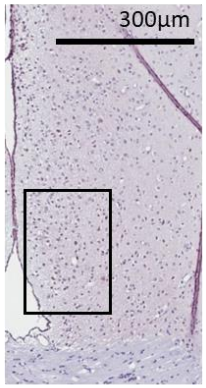

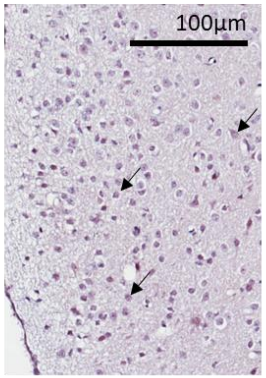

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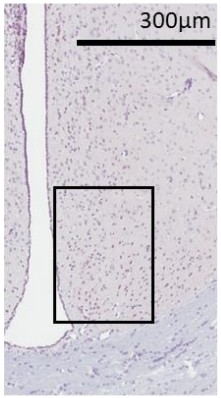
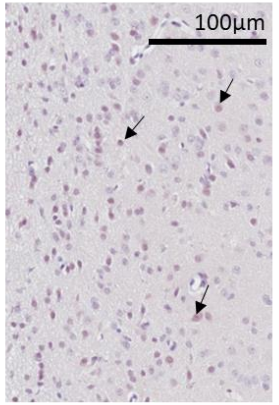
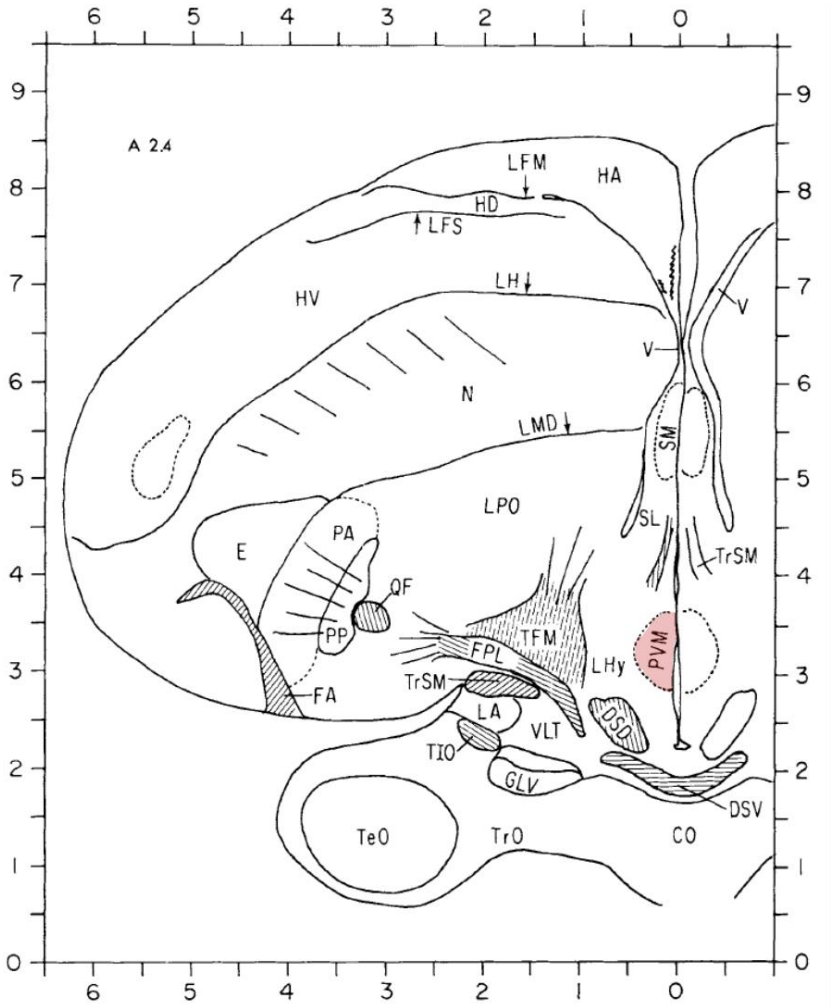
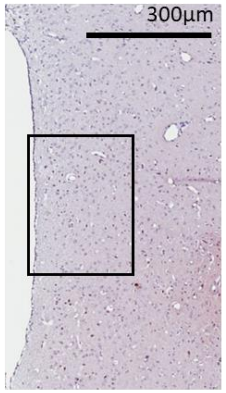
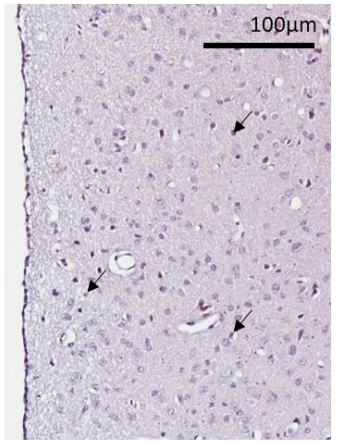






PVM: Breeder





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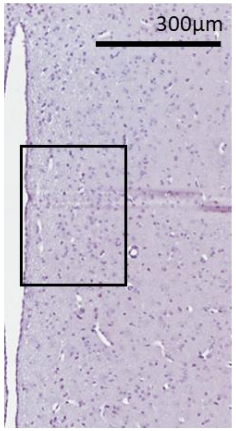
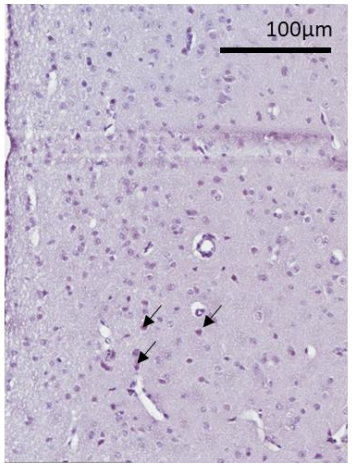
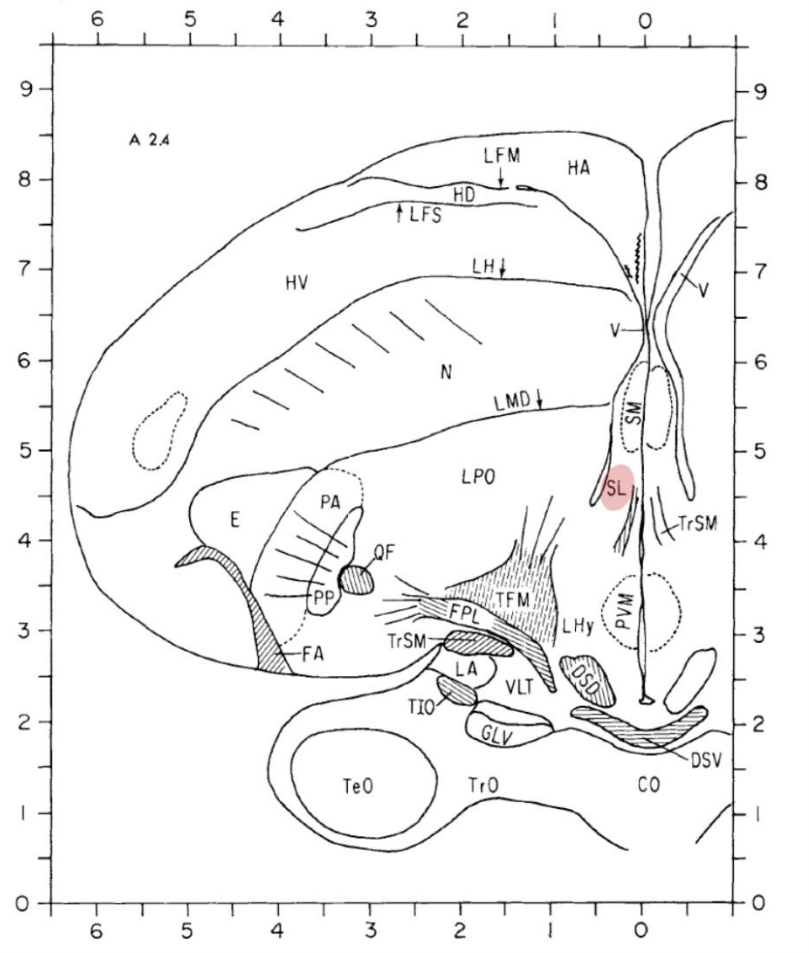
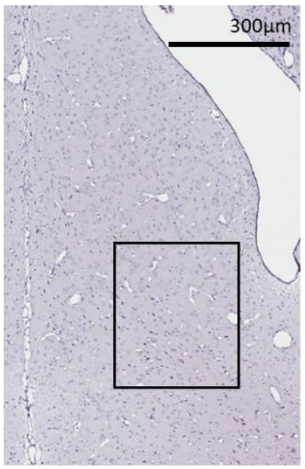
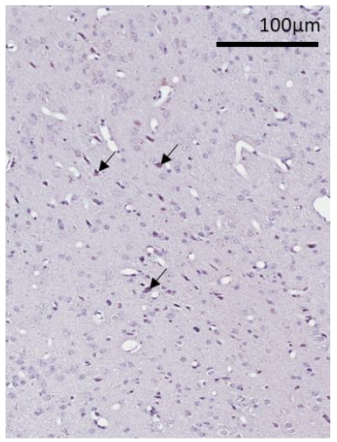






SL: Breeder





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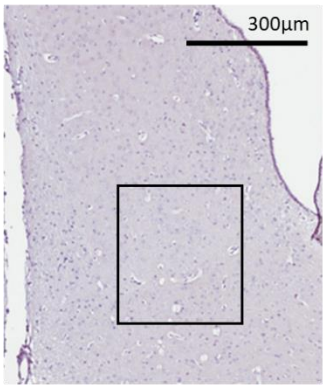
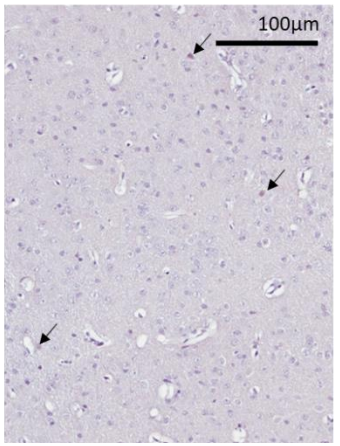
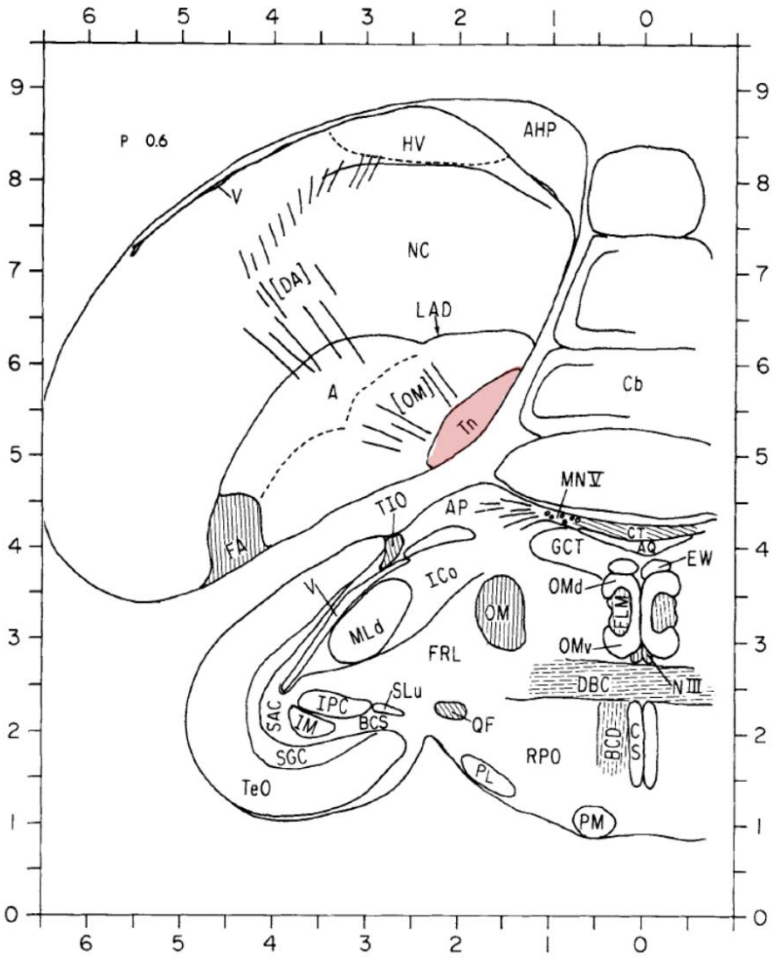
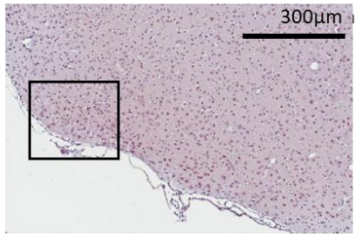
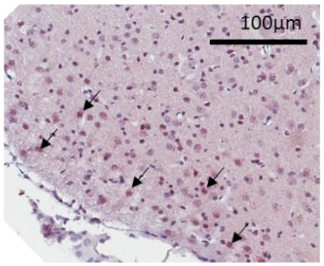




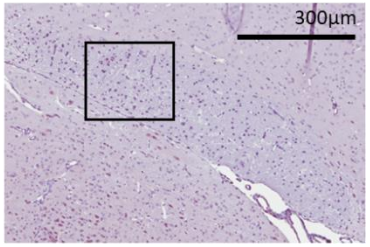


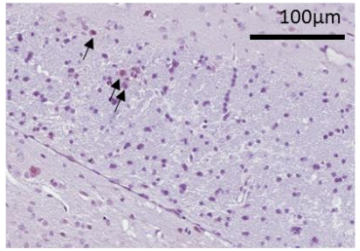
TnA: Breeder

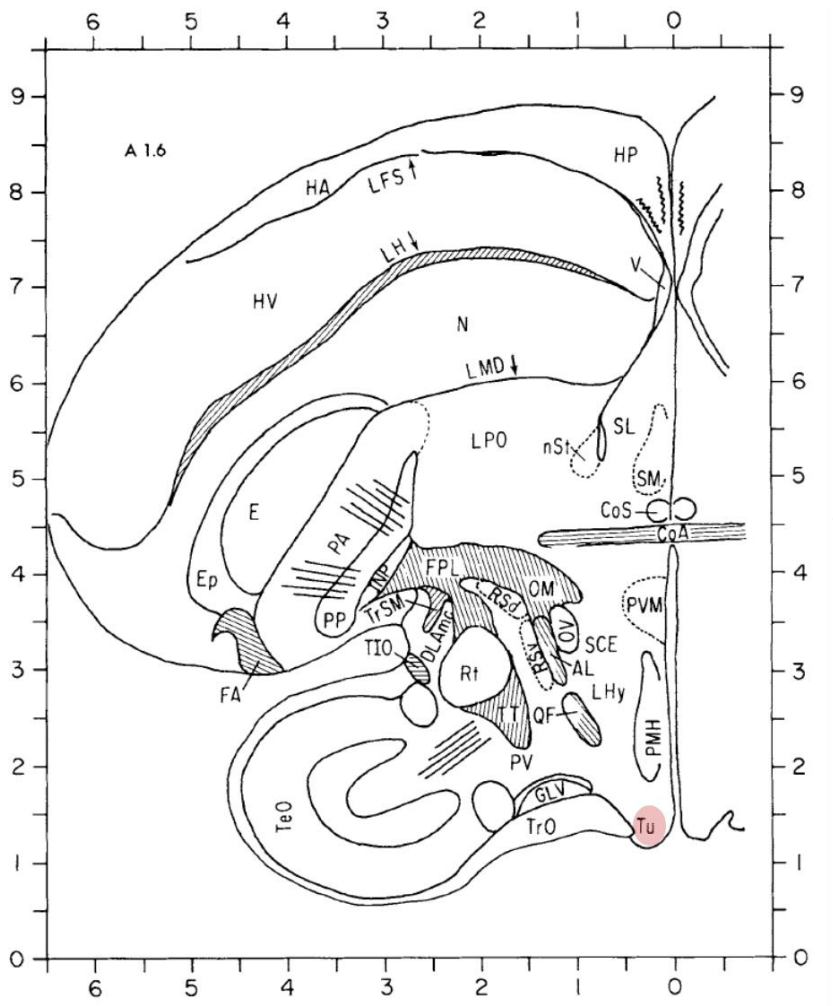




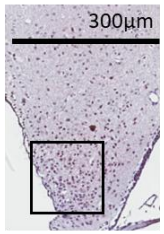
TnA: Non-breeder

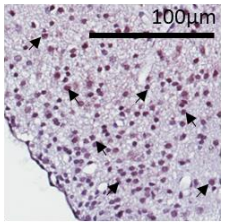


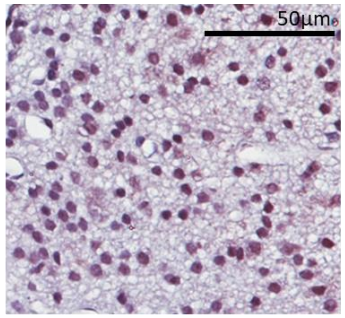




TU: Breeder







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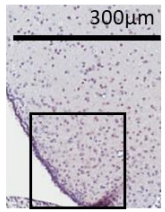
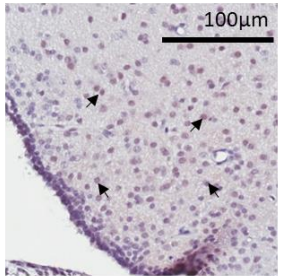
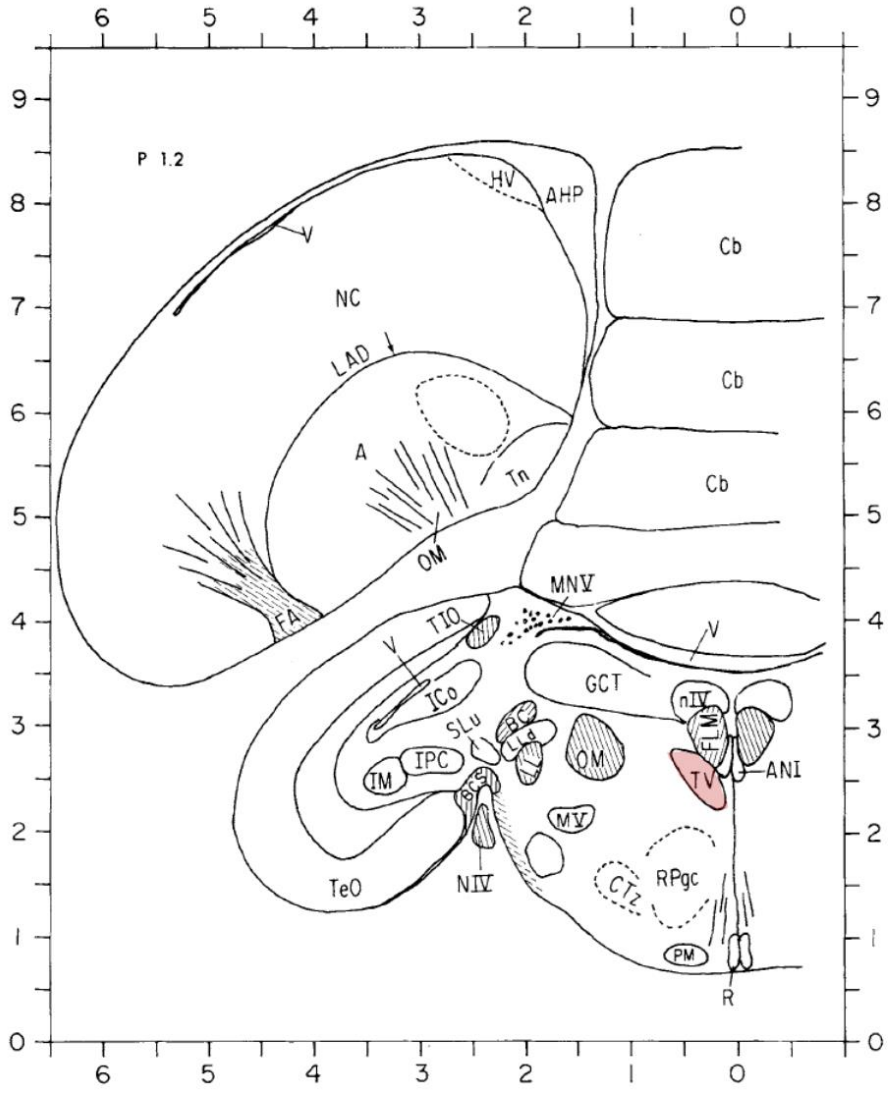
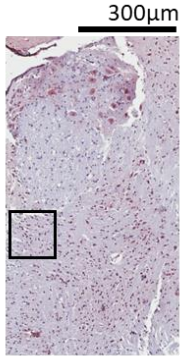
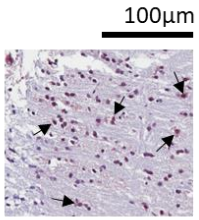






TV: Breeder





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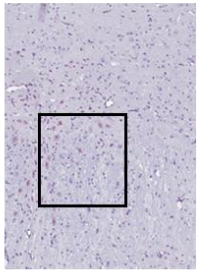
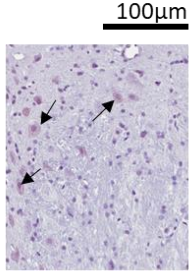
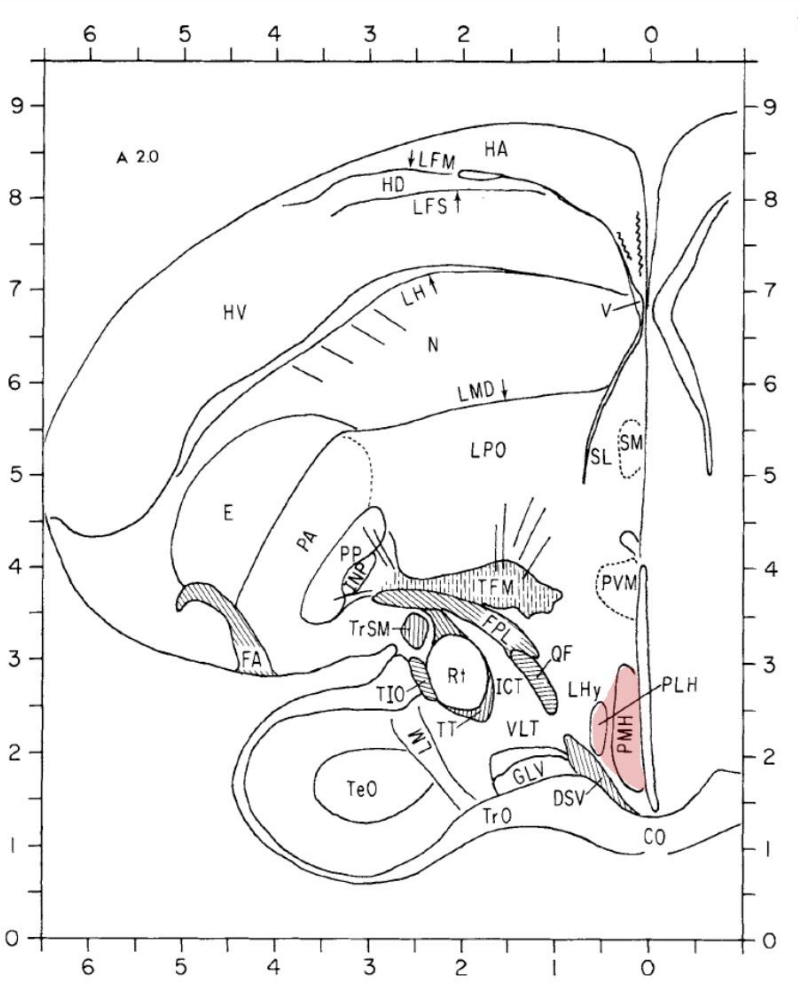
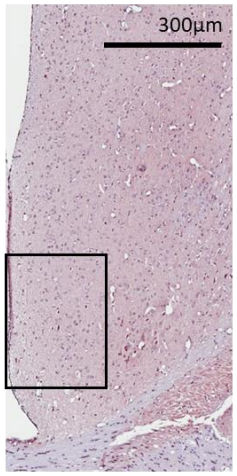
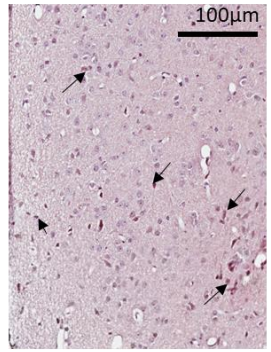




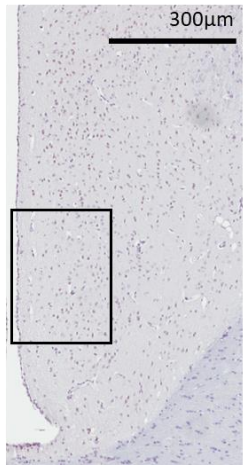


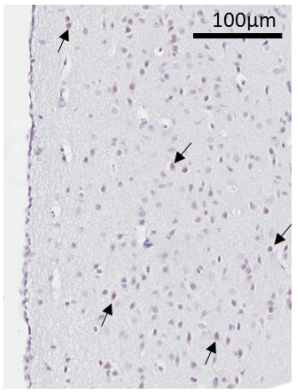
VMH: Breeder





VMH: Non-breeder





GENERAL CONCLUSIONS

Summary of dissertation conclusions

This dissertation provides the first causal evidence for a role of PRL in post-hatch parental care behavior in the zebra finch, and is the first to show that central changes in PRLR density result from breeding. We showed that while incubating birds with low PRL can provide some level of baseline parental care, high PRL is required to provide full post-hatch parental care in normally breeding birds. Reproductive experience enhances PRL secretion, and increases parental care in normally breeding birds. We also provide the first description of the central prolactin receptor (PRLR) distribution and are the first to show that PRLRs upregulate in breeding zebra finches.

Specifically, the conclusions from each chapter are:

1) In zebra finches, plasma PRL concentrations gradually increase from low, non-breeding levels after egg laying, throughout incubation, peaking just before egg hatching. PRL remains high throughout the first week of post-hatch care, then slowly declines back to non-breeding levels by the time chicks are ready to fledge from the nest. This occurs in both males and females and there is no sex difference. This entire PRL pattern is increased in zebra finches after gaining reproductive experience; this is especially apparent at the peak in PRL just before and after hatching.
2) Plasma PRL concentrations at day 2 post-hatch positively correlates with the amount of chick brooding and chick feeding parents provide, as well as the

number of chicks in the nest and the likelihood of at least one chick surviving to fledging, but not with egg clutch size.

3) Early incubating zebra finches that have relatively low circulating PRL levels can show parental care towards foster chicks. This is true in both males and females that are either inexperienced or experienced breeders. However, birds will only provide care to foster chicks after egg laying. Pairs without a nest or with a nest without eggs will not care for foster young, suggesting that eggs are an important cue for the onset of parental care. Incubating experienced pairs showed a strong, positive correlation in chick brooding and feeding duration, whereas inexperienced pairs did not.
4) Inhibiting PRL secretion with bromocriptine (BR) during the last three days of incubation and the first two days of post-hatch care significantly reduces or eliminates post-hatch parental care, in both sexes and of both experience levels. Reproductive experience enhances the amount of parental care provided to chicks in normally breeding control birds. Control breeders had a near perfect positive linear relationship in their chick feeding durations, whereas BR pairs adjusted their feeding independent of one another.
5) For breeders, the highest amount of positive immunostaining for the PRLR was found in the IN, TnA, POM, TU, TV, and VMH, with moderate staining in the AC, LHy, nST, PVM, and SL, and either very minimal or no amount of staining in the CB (control region). This distribution was relatively the same in non-breeder brains with several key exceptions: IN, TU, TnA, and TV. In all four of these regions, breeders had significantly higher positivity scores than

non-breeders. While breeding and non-breeding birds have a similar distribution of PRLR (apart from IN, TU, TnA, and TV), overall, breeders have a much higher density of PRLR than non-breeders.

New hypotheses for the role of prolactin and reproductive experience in parental care

Synthesizing the results from this dissertation together, here we present the current running hypothesis of how PRL influences parental behavior. After mating and egg laying by the female, the presence of eggs in the nest likely triggers the onset of PRL release in both males and females and the beginning of the upregulation of central PRLRs. There appears to be a strong bidirectional relationship with sensory cues from the nest and eggs and the increase in circulating PRL. Tactile contact with the eggs likely drives the increase in PRL and the increase in PRL is reinforced by the increasing time spent on the eggs, as it does in other birds (reviewed in Buntin, 1996). The continuous increase in circulating PRL likely begins the initiation of the upregulation of central PRLRs. The rate of increase or the total amount of PRL release maybe be predetermined (before egg laying) as higher pre-breeding baseline PRL concentrations correlated positively with earlier laying dates in free-living great tits (*Parus major*; Ouyang et al., 2013), or increasing PRL may be more directly influenced by environmental cues – both internal (e.g., body condition) and external (e.g., weather/food availability (for reviews in other birds, see: Angelier et al., 2016; Angelier and Chastel, 2009) and/or directly from sensory cues (e.g., tactile contact with eggs). The increase in circulating PRL occurs slowly, over the course of incubation as time spent on the eggs increases. As PRL increases, parental investment

in the current clutch also increases, so PRL may signal whether to keep incubating or to abandon the clutch (e.g., in the case of severe weather). In addition, increasing PRL may begin to synchronize the pairs' behavior to maximize reproductive success, which is enhanced with reproductive experience.

The increase in PRL production and release is probably driven, in part, by the increase in PRLR in the IN/TU which may enhance the secretion of plasma PRL via a positive feedback on VIP-neurons (which stimulate PRL release from the pituitary). Because PRLR expression in the POM remains relatively high in both breeding in non-breeding birds, PRL action at the POM could also be driving incubation behavior, as it does in chickens and turkeys (reviewed in Buntin, 1996). As incubation behavior and circulating PRL increases, PRLR upregulation in areas such as the TV and TnA also increase, which likely begins to increase the positive saliency of chick stimuli. This occurs *before* the expected hatching date so that parents are prepared to provide chick care immediately when the chicks hatch. By the time PRL peaks, around day 12 incubation, PRLR are also likely fully upregulated in the hypothalamus, and possibly other extrahypothalamic regions involved in parental care, which may begin, or prepare the brain, for other PRL-driven functions, such as increased feeding and attenuated HPA activity.

Once the chicks hatch, the parents are very attracted to chick sensory cues, such as tactile contact, auditory begging calls, olfactory information, and visual cues from the chicks. Therefore, one of the roles of PRL is likely to shift the birds into a state where the eggs, chicks, and nest stimuli becomes increasingly positively salient. PRL may also play a role in stimulating motivated behaviors towards the chicks,

which leads them to spending a greater amount of time in close proximity to the nest, which in turn, increase the chances for offspring to elicit parent care behaviors from the parents when needed. In addition to affecting the motivation to care for chicks, PRL also likely affects other aspects of parental care such as increased feeding and attenuating the stress response. These effects likely occur through increasing the central PRL sensitivity at brain regions responsible for altering these states. Increased PRL may also play a role in increasing parental behavior coordination between breeding pairs. Reproductive experience enhances this entire process by increasing circulating PRL, increasing reproductive output (i.e., number of eggs), which may provide increased sensory feedback to parents, and stimulates more care to chicks, perhaps via increased behavioral coordination between parents. While reproductive experience affects PRL and vice versa, other influences such as learning and memory also likely influence the reproductive experience effects.

Sensory information from young chicks elicits parental care and maintains PRL and PRLR at peak levels, which lasts for several days during the first week of post-hatch care. As chicks grows older, their sensory cues begin to elicit different types of parental care, and parental care is no longer dependent on the increased sensitivity to PRL. Because of the changing chick stimuli, and the decrease in dependence of PRL to stimulate parental behavior, PRL and PRLR begin to decrease and downregulate. Again, this is a gradual process that is also likely stimulus driven. By the time chicks are ready to fledge from the nest, PRL and PRLR have returned to low, baseline concentrations, and parental care begins to wane as chicks reach parental independence. In addition, if the conditions are right for breeding, zebra finches will

begin to lay another clutch soon after fledging. Reproductive experience enhances this entire process by increasing PRL production and secretion. While we were not able to directly test this, it is possible that experienced birds have either a permanent upregulation or a greater upregulation in PRLR, relative to first-time, inexperienced breeders. Reproductive experienced also speeds up time to incubation and reproductive output, which also provides more stimulus input to cause an increase in circulating PRL, contributing to the bidirectional relationship between breeding stimuli and the increase/upregulation of PRL/PRLR.

Future directions and recommendations

While this dissertation has provided new and essential information on the role of PRL, PRLR, and reproductive experience in parental care of the zebra finch, there remains many unanswered questions and new hypotheses to be tested. The first regards the bidirectional relationship between PRL and breeding stimuli. It has been assumed that the presence of eggs and chicks stimulates PRL release in zebra finches based on prior evidence in other avian species (reviewed in Buntin, 1996). Studies which measure plasma PRL before and after egg/chick exposure in non-breeding or early incubating zebra finches are needed to confirm that indeed, there is a bidirectional relationship. To see if this is affected by reproductive experience, one could test if there is a greater PRL release in reproductively experienced birds, compared to inexperienced birds, in response to egg and/or chick stimuli. In addition, *how* stimuli from the nest, eggs, and chicks feed back into the brain in normally breeding birds is still largely unknown, but tactile egg stimulation and PRL release via

hypothalamic VIP neurons may be a candidate mechanism in this process as it is in chickens (Sharp, 2009).

Second, we proposed that one of the primary roles of PRL is to affect the motivation to provide care and/or to increase the positive saliency of the chicks. To formally test this hypothesis, one would need to first establish that parental care is indeed a motivated behavior in this species. If parents could be trained in an operant conditioning paradigm in order to work for access to young chicks, this would indicate that parents are motivated to provide parental care. Next, to test that PRL influences that motivation, one would need to manipulate PRL during an experimental paradigm that measures parental motivation. One could repeat the experiments in chapters 3 and 4, but instead of simply measuring parental behaviors, the subjects could be tested in a motivational paradigm, such as an operant chamber.

We found that inhibiting PRL also interfered with pair behavior coordination and that reproductively experienced birds were more behaviorally correlated than inexperienced birds. In addition, pairs of free living great tits (*Parus major*) that were more hormonally similar during breeding had greater reproductive success (Ouyang et al., 2014). However, it is unknown whether this hormone similarity is beneficial because it modifies the parents' behavior such that they provide similar amounts of care behavior and investment or because it plays a role in coordinating or synchronizing the pairs' behaviors together, or both. A study which directly measures circulating PRL within pairs at different time points along with behavioral similarity will be important to formally test the hypothesis that PRL influences behavior synchrony in zebra finches. Additionally, one would also predict that PRL

concentrations would be more strongly correlated in more reproductively successful male-female pairs, compared with less successful breeding pairs. Lastly, manipulating PRL in only one partner can test whether partners compensate when the synchrony is disrupted.

Lastly, there is much to do on the central front of PRL. The next steps will be to causally demonstrate that PRL affects parental care through its actions on central PRLRs. Because some of the regions we analyzed for PRLR, such as the TnA, have rarely been studied in the context of avian parental care, perhaps lesion studies would be the first step in order to predict the possible influences of PRL on parental care in those regions. To establish causality of the PRLR in these behaviors, one would need to inject a PRLR antagonist into a brain region of interest and measure parental behavior, or parental motivation. In a complimentary experiment, injecting PRL directly into the brain in both breeding and non-breeding birds will be integral in establishing causality and whether the upregulation of PRLR is required for the behavior. Specific predictions on PRL's role in feeding via PRL action in the VMH, or LHy, for example could be tested in this way as well. In addition, further describing the PRLR distribution in additional areas will further elucidate whether PRL plays a role in other aspects of parental care or in other physiological and/or behaviors outside of parental care. Finally, specific predictions about the role of PRL in other brain regions will be advanced by furthering characterizing the types of neurons PRLR reside on. For instance, one could test that hypothesis that PRLR are on DA neurons in the VTA, on VIP neurons in the IN/TU, and on MT neurons in the PVM.

Broader implications and conclusions

The conclusions drawn from this dissertation suggest a great level of conservation in the role of PRL in parental care in vertebrate species (see *General Introduction*) and how parental care evolves in vertebrate species. In general, it appears that the hormonal mechanisms which drive parental care serve the purpose of reducing fear and increasing motivation/saliency to sensory cues which elicit parental care. For these hormones to elicit specific effects, at certain times, the brain becomes increasing sensitive to these hormones, in particular regions which must be altered, or regulated differently to put the animal in a certain behavioral state.

Mechanisms which coordinate the onset of parental behavior with the arrival of offspring have been selected for in species that show parental care. It is important to emphasize that parental care is stimulus driven by offspring (e.g., cries in babies; begging calls in birds). Therefore, hormonal mechanisms that promote parental care do not activate those motor parental behaviors *per se*, but rather, put the parent in a state in which they pay attention to offspring cues, are more attracted to offspring cues, and are selective about offspring cues, which in turn elicit parental care behaviors. Selection has also favored mechanisms for the maintenance of parental care in which parents respond to sensory input from young, dependent offspring and not to sensory input from older, independent offspring. The cues from offspring that elicit care from parents gradually fade away as the offspring age and approach independence, as caring for offspring longer than necessary would preclude subsequent breeding opportunities and lower the lifetime reproductive success of parents.

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