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Prolactin is related to individual differences in parental behavior and reproductive success in a biparental passerine, the zebra finch (*Taeniopygia guttata*)

Kristina O. Smiley [a,*], Elizabeth Adkins-Regan [a,b]

[a] Department of Psychology, Cornell University, Ithaca, NY 14853, USA
[b] Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14853, USA

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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.

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1. 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 provi-

* Corresponding author.
E-mail addresses: kos24@cornell.edu (K.O. Smiley), er12@cornell.edu (E. Adkins-Regan).

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sioning 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 (*Aphelocoma coerulescens*; 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. Methods

2.1. 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*.

2.2. Study design

2.2.1. 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-min behavioral observations took place during this week to determine which birds were paired (see Smiley et al., 2012 and Vahaba et al., 2013 for Section 2). 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 (see Section 2.2.4. for details). 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).

2.2.2. 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 3.1.), 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 h 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 Section 2.2.3. 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 Section 2.2.4. for blood sampling methods).

2.2.3. 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 min long (±s.d. 48 min). 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.

2.2.4. 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 −80 °C until assayed for PRL.

2.2.5. 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 0.05 M phosphate buffer (pH = 7.4) and incubated overnight at 4 °C. 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 0.25 M 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 0.15 M PBS (pH = 7.2), 0.1% casein, and 0.25 M 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 Drs. 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 4 °C. 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 0.1 ml of ABTS (2,2′-azino-bis(3-ethylbenz thiazoline-6-sulfonic acid) was dispensed across all wells. The color reaction was read 45 min 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).

2.2.6. 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.

3. Results

3.1. 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 Fig. 1A and B. for PRL means for treatment and sex, respectively.

3.2. Relationship between PRL and parental behaviors

There was a significant, positive relationship between PRL and chick brooding behavior ($F_{1,9.354} = 16.972$, $p = 0.002$; Fig. 2A) and PRL and chick feeding ($F_{1,10.002} = 8.634$, $p = 0.015$; Fig. 2B). 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 ($r(10) = 0.727$, $p = 0.007$).

3.3. Relationship between PRL and reproductive success

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

4. Discussion

4.1. 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,

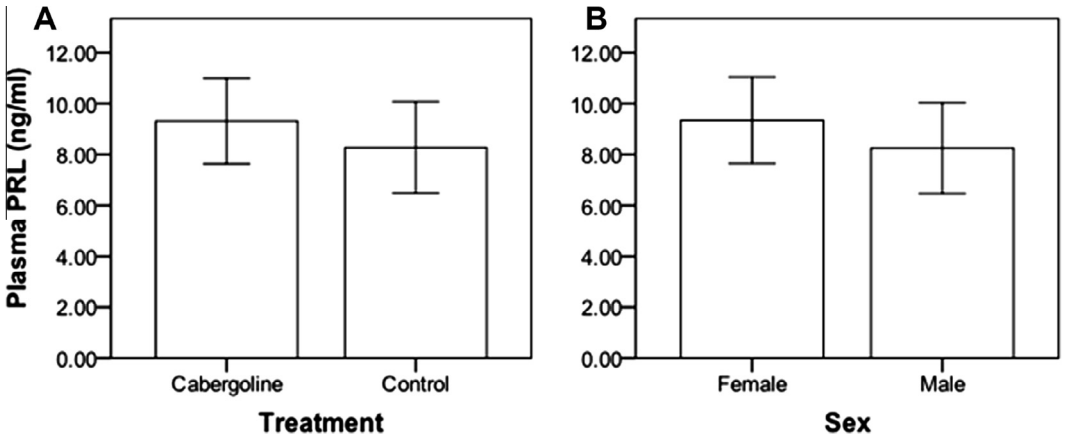

Fig. 1. (A) No effect of cabergoline treatment on plasma PRL concentrations. Mean ± 1 standard error PRL concentration measured on day 2 post-hatch for pairs treated with cabergoline (N = 6 birds) or vehicle (control, N = 6 birds). PRL concentrations are similar to normal breeding levels that have been 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).

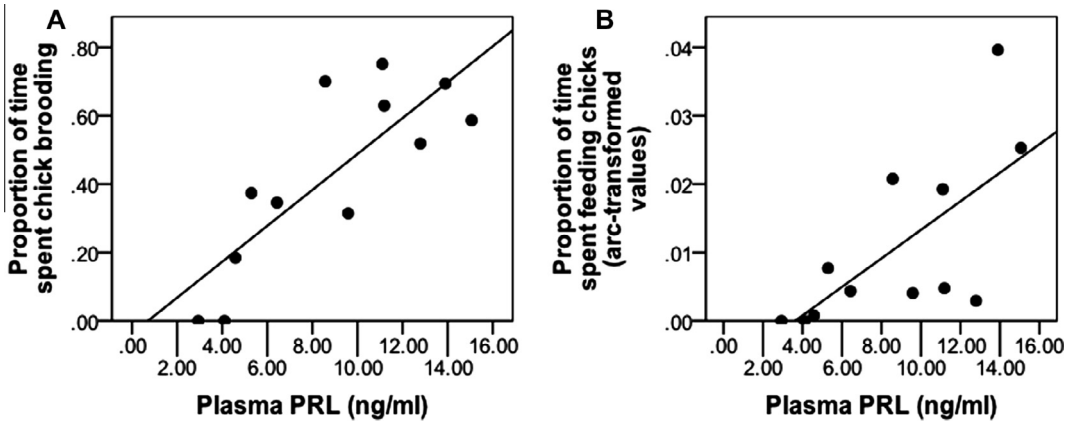

Fig. 2. (A) Chick brooding behavior increases with plasma PRL concentrations. Chick brooding behavior was recorded inside the nest box on days 1 and 2 post-hatch. Plasma PRL was measured on day 2 post-hatch following the recording. The Y-axis is the proportion of the total recording time that an individual 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.

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

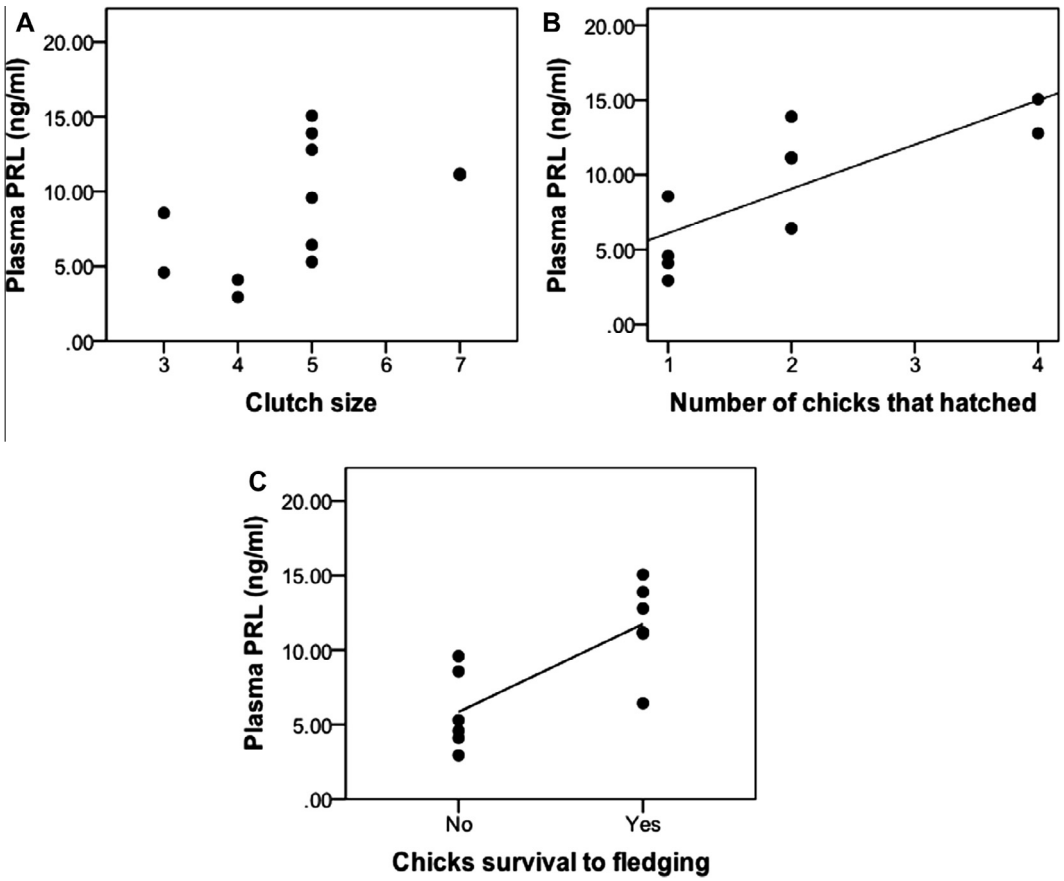

Fig. 3. (A) No relationship between plasma PRL concentrations and clutch size. Data points represent individual plasma PRL concentrations measured in male and female parents on day 2 post-hatch based on how many eggs were in their clutch. There was no statistical relationship between plasma PRL concentrations 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 or not at least one chick survived to fledging.

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.

4.2. 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 3.1.) 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 et al., 1997). In turkeys, DA has been shown to have both stimulatory and inhibitory effects on PRL secretion (Youngren et al., 1995, 1996, 1998). Specifically, DA acting at the D1 receptor at the level of the hypothalamus activates VIP neurons (Youngren et al., 1996, 2002), 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.

4.3. 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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