General and Comparative Endocrinology 232 (2016) 17–24

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Relationship between prolactin, reproductive experience, and parental care in a biparental songbird, 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

ARTICLE INFO

Article history:
Received 19 August 2015
Revised 14 November 2015
Accepted 17 November 2015
Available online 18 November 2015

Keywords:
Prolactin
Songbird
Parental care
Reproductive experience
Zebra finch

ABSTRACT

Hormonal systems have long been thought to play an important role in stimulating the onset of parental behavior, a critical component of reproductive success in a variety of taxa. Elevations in the peptide hormone prolactin (PRL) have been repeatedly positively correlated with the onset and maintenance of parental care across vertebrate species. A causal role for PRL in parental care has been established in several mammalian species, but less evidence for a causal role of PRL and parental care exists in birds. 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. 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 ($N = 28$) across two breeding cycles, using a repeated measures design. PRL was measured in the birds' first, reproductively inexperienced, breeding cycle beginning at courtship and extending through chick fledging. PRL was measured again during the birds' second, reproductively experienced, breeding cycle, beginning with egg laying until chick fledging. We found that plasma PRL is significantly elevated from non-breeding concentrations during late incubation and early post-hatch care and that this elevation is greater in the reproductively experienced cycle compared to the inexperienced cycle. Findings of this study will be used to inform hypotheses and predictions for future experimental manipulations of PRL during parental care.

1. 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.

PRL's role in female mammalian parental care has been well established. In female mammals, including humans, circulating PRL is significantly elevated during pregnancy and remains high during lactation (Ben-Jonathan et al., 2008; Freeman et al., 2000; González-Mariscal and Poindron, 2002). 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. Elevated circulating PRL levels have also been associated with paternal care in several mammalian species (Saltzman and Ziegler, 2014; Schradin and Anzenberger, 1999; Wynne-Edwards, 2001). However, evidence for PRL playing a causal role in male parental care still remains inconclusive (Bales and Saltzman, 2015; Saltzman and Ziegler, 2014; Wynne-Edwards and Timonin, 2007).

Similar to mammals, there are an impressive number and variety of bird species that show a close association between PRL and

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

http://dx.doi.org/10.1016/j.ygcen.2015.11.012
0016-6480/

parental care (see Angelier et al., 2015; Buntin, 1996; Sockman et al., 2006 for extensive reviews). In virtually all birds studied to date, PRL is low during non-breeding times and significantly elevated near the end of egg incubation and during early post-hatch care in both males and females that hatch altricial young (reviewed in Angelier et al., 2015; Buntin, 1996; Sockman et al., 2006). However, experimental studies that provide strong evidence that PRL plays a causal role in chick-directed parental behavior are limited. In 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 which provides a food source for the offspring, analogous to lactation in mammals (Buntin, 1996; Wang and Buntin, 1999). In house finches (*Carpodacus mexicanus*), elevating PRL in non-parental males during nestling provisioning increased nestling feeding, while blocking PRL in parental males during nestling provisioning decreased nestling feeding (Badyaev and Duckworth, 2005). Although these studies provide strong evidence that PRL affects parental care of altricial young in those two species, they may not necessarily generalize to other avian species. Therefore, it would be beneficial to have more information about PRL release during the breeding cycle in other experimentally tractable avian species in order to design experiments in which PRL is manipulated to determine if it is playing a causal role in behavior in other types of birds.

In addition to understanding the pattern of PRL release over the breeding cycle, it is important to know whether this pattern is affected by reproductive experience. In many birds, reproductive success generally improves with experience as determined by fitness measures such as earlier laying dates (e.g., Adkins-Regan and Tomaszycki, 2007; Baran and Adkins-Regan, 2014; Ouyang et al., 2013), increased hatching success (e.g., Riechert et al., 2014), and increased nestling weights (e.g., Miller et al., 2009). While the pattern of PRL secretion during the breeding cycle has been established in many species of birds, fewer researchers have systematically compared circulating PRL levels in both reproductively inexperienced and experienced birds at similar time points during the breeding cycle. 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 correspond to breeding stage, is greater in experienced male dark-eyed juncos (*Junco hyemalis*), compared to first time breeding males (Deviche et al., 2000).

High PRL titers have been correlated with increased reproductive success generally, and thus, may be a candidate mechanism for the improvement in reproductive success that tends to comes with reproductive experience. 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). 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, and egg predation (reviewed in Angelier et al., 2013, 2015; Angelier and Chastel, 2009). What is unknown, however, is if PRL is altering these fitness measures by modifying parental behavior. In ring doves, Wang and Buntin (1999) found that reproductively experienced females treated with PRL showed a greater frequency of regurgitation feedings, greater squab weight gain, and spent more time sitting in the nest than PRL-treated inexperienced ring doves, suggesting there is increased sensitivity to PRL as a result of reproductive experience, which may indeed affect parental behaviors. Evidence for a causal role of PRL in parental behavior and its interactions with reproductive experience are needed in a greater variety of avian species, however, before we can conclude that PRL plays a role in avian parental care generally.

Songbirds, part of the passerine clade, are the most speciose group of birds, the majority of which display intensive parental care. Zebra finches, a socially monogamous, biparental songbird species, are an excellent model for studying PRL secretion and its potential relationship to parental behavior. Zebra finches are opportunistic breeders and 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 late-incubating zebra finches compared to paired but non-breeding zebra finches, regardless of sex. Our study sought to determine the pattern of circulating PRL in zebra finches across the different stages of the breeding cycle and whether this pattern differed with reproductive experience. We predicted that PRL would remain low throughout pairing and nest building and would show a steady rise over the course of incubation, peaking at chick hatching, as previously established in other avian species that hatch altricial young (reviewed in Angelier et al., 2015; Buntin, 1996; Sockman et al., 2006). 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 future causal experiments. Additionally, Christensen and Vleck (2008) found that non-breeding zebra finches with reproductive experience had higher non-breeding baseline PRL concentrations than inexperienced birds. Thus, we predicted that experienced zebra finches would show higher levels of PRL throughout the breeding cycle compared to inexperienced breeders. Lastly, we predicted that there would be no sex differences in PRL concentrations because both sexes provide equal care to offspring and Christensen and Vleck (2008) did not find sex differences in PRL concentrations in either breeding or non-breeding zebra finches.

2. Methods and materials

2.1. Subjects

Subjects included 16 male and 16 female zebra finches (*Taeniopygia guttata*) that were bred in the lab. All birds were kept on 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 engrained with a unique number. Subjects were on average 146 days old (s.d. ± 21.7 days) at the beginning of the study. All subjects had been housed in same sex aviaries since they were separated from their parents at day 40

post-hatch (independence) and had no pairing or reproductive experience prior to the study.

2.2. Study design

All methods and procedures were approved by the Cornell University IACUC.

2.2.1. Housing

Prior to the start of the study, subjects were randomly selected and assigned to 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.

2.2.2. Determining breeding pairs and reproductive status

Beginning on the first day an aviary was set up, courtship and pairing behavior was observed and recorded for 1 h/day for 10 consecutive days to determine breeding pairs and nest box occupancy (see Smiley et al., 2012 and Vahaba et al., 2013 for methods). 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 (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. If birds did not show pairing behavior with one partner exclusively by 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). Daily nest checks were performed thereafter to monitor the breeding status for each pair for the rest of the study.

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.

2.2.3. 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 a minimum number of subjects (see Table 2 for final sample sizes). All four bleeding schedules were represented in each aviary, with the exception of 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 study, 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 their 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 study, 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. Latencies to form a pair bond and begin nest building are reported in Table 1. 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 the first chick 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. Plasma samples were assayed as they were collected (see Section 2.4. for details).

2.3. 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 100 µl 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 −80 °C until assayed for PRL.

2.4. Hormone assay and validation

2.4.1. ELISA validation

There is a history of using a RIA to measure PRL in the zebra finch (Christensen and Vleck, 2008; 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

Table 1
Reproductive outcomes based on 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.

			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	*W*=	–	–	0	16	16
		N=	–	–	12	12	12
		p-Value	–	–	0.003**	0.776	0.952

** Indicates significance at $p < 0.01$.

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 by Vleck and Patrick (1999).

2.4.2. 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 0.05 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 three times (ELX 405 AutoPlate Washer, Biotek Instruments, Inc.) using wash buffer containing 10× 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. 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 4 °C. Following incubation, plates were washed three times 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 0.1 ml of ABTS reagent was dispensed across all wells. The color reaction was read 30 min later (450 µm; Synergy HT plate reader, Biotek). All samples were run in duplicate across five plates.

2.5. Statistical analysis

All statistical analyses were performed using IBM SPSS software, version 21.0 (Armonk, NY: IBM Corp.).

2.5.1. Relationship between PRL, reproductive experience, and breeding cycle stage

To analyze the effect of sample day, reproductive experience, and sex on plasma PRL throughout the breeding cycle, we used a generalized linear mixed model (GLMM), 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.

2.5.2. Relationship between reproductive experience and reproductive success

We compared three reproductive success measures, clutch size, number of chicks that hatched, and number of chicks that survived to fledging, between breeding cycle 1 and breeding cycle 2 (inexperienced VS. experienced) for the 12 pairs that successfully completed both cycles using Wilcoxon signed-ranks tests.

3. Results

3.1. ELISA validation

The serial dilution was parallel to the standard curve for chicken PRL (Fig. 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%.

3.2. Positive 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 Fig. 2. 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

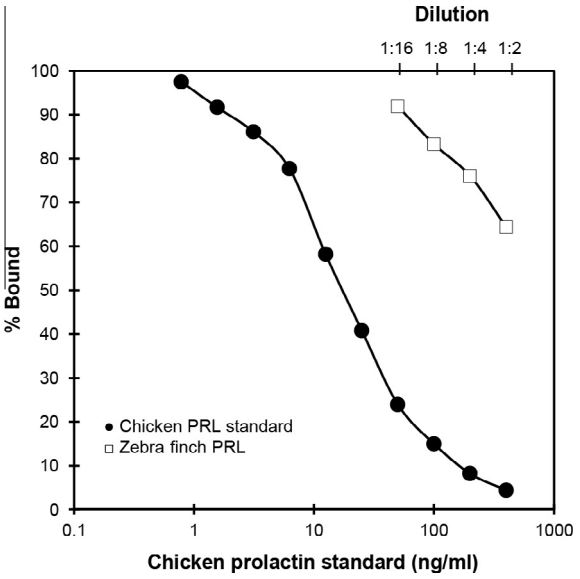

Fig. 1. Standard curve for chicken prolactin standard and zebra finch prolactin dilution. The zebra finch prolactin dilution curve is relatively parallel to the chicken prolactin standard reference hormone, showing that this heterologous ELISA can be used to assay relative levels of plasma prolactin in zebra finches.

when they were inexperienced, but followed the same general pattern as when inexperienced (Fig. 3). There was no significant main 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. Sample sizes, divided by sex, for Figs. 2 and 3 are presented in Table 2.

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

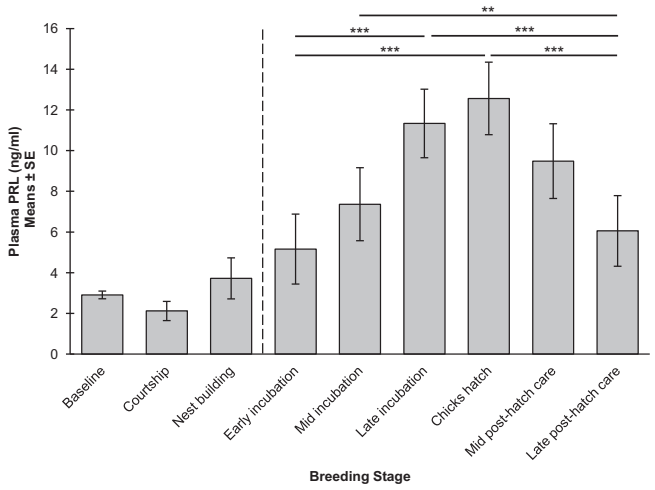

Fig. 2. PRL concentrations across different breeding cycle stages in the zebra finch. Shaded bars represent average PRL concentrations (y-axis) measured at various times in breeding cycle (indicated on the x-axis). Error bars represent standard error (SE). Solid bars with asterisks denote significant 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 2.

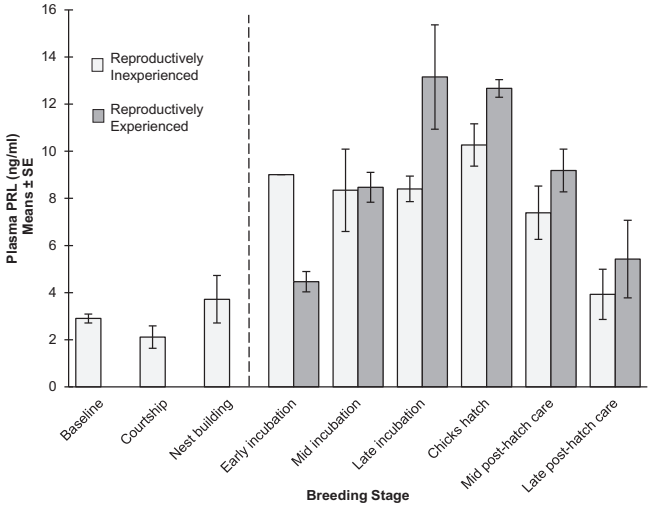

Fig. 3. PRL concentrations by reproductive experience across the zebra finch breeding cycle. Lightly shaded bars represent average PRL concentrations (y-axis) measured in reproductively inexperienced zebra finches at various times in breeding cycle (indicated on the x-axis). Darkly shaded bars represent 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 2.

correction value to adjust each plate's value accordingly. We then re-ran the same statistical model as described in Section 2.5.1. 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 ($p = 0.045$).

3.3. Positive relationship between reproductive experience and reproductive success

We found that the clutch size was significantly greater in reproductively experienced birds, compared to inexperienced birds. There was no difference, however, between the number of chicks that hatched or the number of chicks that survived to fledging. See Table 1A for reproductive outcome data and Table 1B for the results from the Wilcoxon signed-ranks tests.

4. Discussion

4.1. 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 (reviewed in Angelier et al., 2015; Buntin, 1996; Sockman et al., 2006). Our findings show that PRL remains at low levels during courtship and nest building 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. We found no sex differences in PRL concentrations during the breeding cycle. This was expected as this pattern of PRL has been observed in both

Table 2
Sample sizes for main effect of sample size. Samples sizes, broken down by sex, correspond with Figs. 2 (total N) and 3 (cycle 1 and cycle 2).

		Sample day								
		Baseline	Courtship	Nest building	Early incubation	Mid incubation	Late incubation	Chicks hatch	Mid post-hatch care	Late post-hatch care
Cycle 1: Reproductively inexperienced	Female	10	3	2	1	1	2	2	2	3
	Male	11	3	1	0	1	3	3	3	3
	n=	21	6	3	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
	Total *N*=	21	6	3	6	8	8	8	9	10

sexes in other biparental species that provide post-hatch care (reviewed in Angelier et al., 2015; Buntin, 1996; Sockman et al., 2006). In contrast, in birds that hatch 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 (reviewed in Angelier et al., 2015; Buntin, 1996; Sockman et al., 2006). 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 (reviewed in Angelier et al., 2015; Buntin, 1996; Sharp et al., 1998; Sockman et al., 2006), 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. Understanding which environmental, social, or other external or internal cues trigger the release of PRL during the breeding cycle may differ across species and will be important to determine.

4.2. Experience effects

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. These findings are similar to other avian species (see citations in the introduction), suggesting that this phenomenon is not limited to zebra finches or songbirds necessarily. In support of this finding, 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, which may be a potential mechanism for the elevated secretion of PRL observed during subsequent breeding cycles in this study. 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 non-breeding reproductively experienced zebra finches had higher PRL concentrations than did inexperienced non-breeders, so conceivably circulating PRL levels could also have been higher during courtship and nest building in experienced birds as well, had we measured those time points twice.

In many studies, reproductive experience is confounded with age. The majority of studies on PRL and reproductive experience have been conducted on free-living avian species, so it is often difficult 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). However, Christensen and Vleck (2015) found no effect of age on plasma PRL concentrations in zebra finches. Furthermore, Angelier et al. (2007) found that reproductive experience was a better predictor of PRL concentrations than age in black-browed albatrosses (*T. melanophris*). Additional laboratory experiments that measure hormones and behavior in age-matched individuals varying in reproductive experience would likely separate the effects of age and reproductive experience on PRL secretion and parental behavior.

In addition to age, PRL may also depend on experience with a pair-bonded partner. 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 breeding pairs, compared with less successful breeding pairs.

Lastly, in addition to the possibility that PRL affects reproductive output and behavior, nest, egg, or chick stimuli may influence PRL secretion (reviewed in Angelier et al., 2015; Buntin, 1996). In this study we found that reproductively experienced breeders laid significantly more eggs compared to inexperienced breeders, but that there was no difference in the number of chicks hatched or chicks fledged. Interestingly, though, the experience effects on PRL concentrations were most prominent during late incubation and throughout the post-hatch period. If birds are indeed more sensitive to egg stimuli as experienced breeders, one could hypothesize that this may cause PRL to reach higher concentrations at chick hatching, which may, in turn, influence parental care behaviors. Therefore, even though the number of hatched or fledged chicks didn't change, perhaps the intensity, quality, or synchrony of parental care behaviors would be influenced by the greater increase in PRL. However, this hypothesis remains speculative until experimental manipulations of PRL in age-matched birds that vary in reproductive experience are conducted. In addition, measuring changes in PRL in response to egg and chick stimuli would be beneficial in understanding the possible cyclic relationship of PRL and reproductive outcomes.

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 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 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 in Christensen and Vleck, 2008). Because our data remain significant when standardized (i.e., corrected for the high inter-plate CV), we are confident we are capturing real effects and thus report results from our original (non-standardized) data. However, given our low sample size, we may be lacking the statistical power to pick up interactions between variables. Nonetheless, zebra finches appear to exhibit the same pattern of PRL release and this, and other studies, indicate that reproductive experience should be taken into consideration when designing experiments that test the role of hormones in parental behavior.

5. Conclusions

In sum, 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. However, whether there is a causal role for PRL during parental care is still largely untested in passerines and most other avian groups, despite the extensive correlational evidence that suggest this relationship. In addition, there is a positive association between reproductive experience and PRL. However, it is unclear what functional role, if any, this has in the increased reproductive success that tends to come with experience. Furthermore, exploration of the possible bidirectional nature of PRL and behavior should also be investigated in greater detail in order to understand the direction of causality, if any. Overall, understanding the role of PRL in a greater number of avian species will provide fruitful insights into the evolution of prolactin-mediated behavior and parental care generally.

Acknowledgments

We are immensely grateful to Dr. Ned Place and Betty Hansen for help with the ELISA validation, Dr. Israel Rozenboim and Dr. Rachel Heiblum for kindly providing the biotinylated PRL used in the ELISA, and Dr. A.F. Parlow for providing the chicken PRL antibodies and reference hormone. In addition we would like to thank our many undergraduate research assistants, Kevin Kim, Dmitriy Podlog, Eun (Jackie) Jung Na, Cory Horowitz, and Christina Ellison, for assistance with blood collection. Lastly, we thank our animal care staff, Percy Smith and Linda Vann, our animal facility managers, Tim Van Deusen and Stephanie Martin, and veterinarian Luce Guanzini for all of their assistance. This research was supported by NSF (United States) grant IOS-1146891.

References

Adkins-Regan, E., Tomaszycki, M., 2007. Monogamy on the fast track. Biol. Lett. 3, 617–619. http://dx.doi.org/10.1098/rsbl.2007.0388.

Angelier, F., Chastel, O., 2009. Stress, prolactin and parental investment in birds: A review. Gen. Comp. Endocrinol. 163, 142–148. http://dx.doi.org/10.1016/j.ygcen.2009.03.028.

Angelier, F., Shaffer, S.A., Weimerskirch, H., Chastel, O., 2006. Effect of age, breeding experience and senescence on corticosterone and prolactin levels in a long-lived seabird: The wandering albatross. Gen. Comp. Endocrinol. 149, 1–9. http://dx.doi.org/10.1016/j.ygcen.2006.04.006.

Angelier, F., Weimerskirch, H., Dano, S., Chastel, O., 2007. Age, experience and reproductive performance in a long-lived bird: A hormonal perspective. Behav. Ecol. Sociobiol. 61, 611–621. http://dx.doi.org/10.1007/s00265-006-0290-1.

Angelier, F., Wingfield, J.C., Tartu, S., Chastel, O., 2015. Does prolactin mediate parental and life-history decisions in response to environmental conditions in birds? A review. Horm. Behav. http://dx.doi.org/10.1016/j.yhbeh.2015.07.014.

Angelier, F., Wingfield, J.C., Trouvé, C., de Grissac, S., Chastel, O., 2013. Modulation of the prolactin and the corticosterone stress responses: Do they tell the same story in a long-lived bird, the Cape petrel? Gen. Comp. Endocrinol. 182, 7–15. http://dx.doi.org/10.1016/j.ygcen.2012.10.008.

Badyaev, A.V., Duckworth, R.A., 2005. Evolution of plasticity in hormonally integrated parental tactics. In: Dawson, A., Sharp, P.J. (Eds.), Functional Avian Endocrinology. Narosa Publishing House, New Delhi, pp. 375–386.

Bales, K.L., Saltzman, W., 2015. Fathering in rodents: Neurobiological substrates and consequences for offspring. Horm. Behav. http://dx.doi.org/10.1016/j.yhbeh.2015.05.021.

Baran, N.M., Adkins-Regan, E., 2014. Breeding experience, alternative reproductive strategies and reproductive success in a captive colony of zebra finches (*Taeniopygia guttata*). PLoS ONE 9, e89808. http://dx.doi.org/10.1371/journal.pone.0089808.

Ben-Jonathan, N., Khurana, S., Hnasko, R., 2002. Brain prolactin. Horm. Brain Behav. NY Elsevier Sci., 97–120

Ben-Jonathan, N., LaPensee, C.R., LaPensee, E.W., 2008. What can we learn from rodents about prolactin in humans? Endocr. Rev. 29, 1–41. http://dx.doi.org/10.1210/er.2007-0017.

Bentley, G.E., Spar, B.D., MacDougall-Shackleton, S.A., Hahn, T.P., Ball, G.F., 2000. Photoperiodic regulation of the reproductive axis in male zebra finches, *Taeniopygia guttata*. Gen. Comp. Endocrinol. 117, 449–455. http://dx.doi.org/10.1006/gcen.1999.7430.

Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., Kelly, P.A., 1998. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr. Rev. 19, 225–268. http://dx.doi.org/10.1210/edrv.19.3.0334.

Bridges, R.S., 2015. Neuroendocrine regulation of maternal behavior. Front. Neuroendocrinol. 36, 178–196. http://dx.doi.org/10.1016/j.yfrne.2014.11.007.

Buntin, J.D., 1996. Neural and hormonal control of parental behavior in birds. In: Rosenblatt, Jay S., Snowdon, Charles T. (Eds.), Advances in the Study of Behavior, Parental Care: Evolution, Mechanisms, and Adaptive Significance. Academic Press, 161, p. 213.

Christensen, D., Vleck, C.M., 2008. Prolactin release and response to vasoactive intestinal peptide in an opportunistic breeder, the zebra finch (*Taeniopygia guttata*). Gen. Comp. Endocrinol. 157, 91–98. http://dx.doi.org/10.1016/j.ygcen.2008.04.013.

Christensen, D., Vleck, C.M., 2015. Effects of age and reproductive experience on the distribution of prolactin and growth hormone secreting cells in the anterior pituitary of a passerine. Gen. Comp. Endocrinol. 222, 54–61. http://dx.doi.org/10.1016/j.ygcen.2015.05.018.

Deviche, P., Wingfield, J.C., Sharp, P.J., 2000. Year-class differences in the reproductive system, plasma prolactin and corticosterone concentrations, and onset of prebasic molt in male dark-eyed juncos (*Junco hyemalis*) during the breeding period. Gen. Comp. Endocrinol. 118, 425–435. http://dx.doi.org/10.1006/gcen.2000.7478.

Dong, X.Y., Zhang, M., Jia, Y.X., Zou, X.T., 2013. Physiological and hormonal aspects in female domestic pigeons (*Columba livia*) associated with breeding stage and experience. J. Anim. Physiol. Anim. Nutr. 97, 861–867. http://dx.doi.org/10.1111/j.1439-0396.2012.01331.x.

Eales, L.A., 1985. Song learning in zebra finches: some effects of song model availability on what is learnt and when. Anim. Behav. 33, 1293–1300. http://dx.doi.org/10.1016/S0003-3472(85)80189-5.

Freeman, M.E., Kanyicska, B., Lerant, A., Nagy, G., 2000. Prolactin: structure, function, and regulation of secretion. Physiol. Rev. 80, 1523–1631.

González-Mariscal, G., Poindron, P., 2002. Parental care in mammals: immediate internal and sensory factors of control. In: Pfaff, D.W., Arnold, A.P., Fahrbach, S.E., Etgen, A.M., Rubin, R.T. (Eds.), Hormones, Brain and Behavior. Academic Press, San Diego, pp. 215–298.

Goodson, J.L., Kingsbury, M.A., 2011. Nonapeptides and the evolution of social group sizes in birds. Front. Neuroanat. 5, 13. http://dx.doi.org/10.3389/fnana.2011.00013.

Mariette, M.M., Griffith, S.C., 2015. The adaptive significance of provisioning and foraging coordination between breeding partners. Am. Nat. 185, 270–280. http://dx.doi.org/10.1086/679441.

Mariette, M.M., Griffith, S.C., 2012. Nest visit synchrony is high and correlates with reproductive success in the wild Zebra finch *Taeniopygia guttata*. J. Avian Biol. 43, 131–140. http://dx.doi.org/10.1111/j.1600-048X.2012.05555.x.

Miller, D.A., Vleck, C.M., Otis, D.L., 2009. Individual variation in baseline and stress-induced corticosterone and prolactin levels predicts parental effort by nesting mourning doves. Horm. Behav. 56, 457–464. http://dx.doi.org/10.1016/j.yhbeh.2009.08.001.

Ouyang, J.Q., Sharp, P.J., Dawson, A., Quetting, M., Hau, M., 2011. Hormone levels predict individual differences in reproductive success in a passerine bird. Proc. R. Soc. B Biol. Sci. 278, 2537–2545. http://dx.doi.org/10.1098/rspb.2010.2490.

Ouyang, J.Q., Sharp, P., Quetting, M., Hau, M., 2013. Endocrine phenotype, reproductive success and survival in the great tit, *Parus major*. J. Evol. Biol. 26, 1988–1998. http://dx.doi.org/10.1111/jeb.12202.

Perfito, N., 2010. The reproductive and stress physiology of zebra finches in context: integrating field and laboratory studies. Emu 110, 199–208.

Perfito, N., Zann, R.A., Bentley, G.E., Hau, M., 2007. Opportunism at work: habitat predictability affects reproductive readiness in free-living zebra finches. Funct. Ecol. 21, 291–301. http://dx.doi.org/10.1111/j.1365-2435.2006.01237.x.

Préault, M., Chastel, O., Cézilly, F., Faivre, B., 2005. Male bill colour and age are associated with parental abilities and breeding performance in blackbirds. Behav. Ecol. Sociobiol. 58, 497–505.

Riechert, J., Becker, P.H., Chastel, O., 2014. Predicting reproductive success from hormone concentrations in the common tern (*Sterna hirundo*) while considering food abundance. Oecologia 176, 715–727. http://dx.doi.org/10.1007/s00442-014-3040-5.

Riechert, J., Chastel, O., Becker, P.H., 2012. Why do experienced birds reproduce better? Possible endocrine mechanisms in a long-lived seabird, the common tern. Gen. Comp. Endocrinol. 178, 391–399. http://dx.doi.org/10.1016/j.ygcen.2012.06.022.

Rochester, J.R., Heiblum, R., Rozenboim, I., Millam, J.R., 2008. Post-hatch oral estrogen exposure reduces oviduct and egg mass and alters nest-building behavior in adult zebra finches (*Taeniopygia guttata*). Physiol. Behav. 95, 370–380. http://dx.doi.org/10.1016/j.physbeh.2008.07.008.

Royle, N.J., Smiseth, P.T., Kölliker, M., 2012. The evolution of parental care. OUP Oxford.

Saito, A., 2015. The marmoset as a model for the study of primate parental behavior. Neurosci. Res. Marmoset Neurosci. 93, 99–109. http://dx.doi.org/10.1016/j.neures.2014.12.011.

Saltzman, W., Ziegler, T.E., 2014. Functional significance of hormonal changes in mammalian fathers. J. Neuroendocrinol. 26, 685–696. http://dx.doi.org/10.1111/jne.12176.

Schradin, C., Anzenberger, G., 1999. Prolactin, the hormone of paternity. Physiology 14, 223–231.

Sharp, P.J., Dawson, A., Lea, R.W., 1998. Control of luteinizing hormone and prolactin secretion in birds. Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 119, 275–282. http://dx.doi.org/10.1016/S0742-8413(98)00016-4.

Smiley, K.O., Vahaba, D.M., Tomaszycki, M.L., 2012. Behavioral effects of progesterone on pair bonding and partner preference in the female zebra finch (*Taeniopygia guttata*). Behav. Processes 90, 210–216. http://dx.doi.org/10.1016/j.beproc.2012.01.008.

Sockman, K.W., Sharp, P.J., Schwabl, H., 2006. Orchestration of avian reproductive effort: an integration of the ultimate and proximate bases for flexibility in clutch size, incubation behaviour, and yolk androgen deposition. Biol. Rev. 81, 629–666. http://dx.doi.org/10.1111/j.1469-185X.2006.tb00221.x.

Vahaba, D.M., Lacey, W.H., Tomaszycki, M.L., 2013. DSP-4, a noradrenergic neurotoxin, produces sex-specific effects on pairing and courtship behavior in zebra finches. Behav. Brain Res. 252, 164–175. http://dx.doi.org/10.1016/j.bbr.2013.05.056.

van Rooij, E.P., Griffith, S.C., 2013. Synchronised provisioning at the nest: parental coordination over care in a socially monogamous species. PeerJ 1, e232. http://dx.doi.org/10.7717/peerj.232.

Vleck, C.M., Patrick, D.J., 1999. Effects of vasoactive intestinal peptide on prolactin secretion in three species of passerine birds. Gen. Comp. Endocrinol. 113, 146–154. http://dx.doi.org/10.1006/gcen.1998.7191.

Wang, Q., Buntin, J.D., 1999. The roles of stimuli from young, previous breeding experience, and prolactin in regulating parental behavior in ring doves (*Streptopelia risoria*). Horm. Behav. 35, 241–253. http://dx.doi.org/10.1006/hbeh.1999.1517.

Whittington, C.M., Wilson, A.B., 2013. The role of prolactin in fish reproduction. Gen. Comp. Endocrinol. 191, 123–136. http://dx.doi.org/10.1016/j.ygcen.2013.05.027.

Wynne-Edwards, K.E., 2001. Hormonal changes in mammalian fathers. Horm. Behav. 40, 139–145. http://dx.doi.org/10.1006/hbeh.2001.1699.

Wynne-Edwards, K.E., Timonin, M.E., 2007. Paternal care in rodents: weakening support for hormonal regulation of the transition to behavioral fatherhood in rodent animal models of biparental care. Horm. Behav. 52, 114–121. http://dx.doi.org/10.1016/j.yhbeh.2007.03.018.

Zann, R.A., 1996. The zebra finch: a synthesis of field and laboratory studies. Oxford University Press, Oxford, New York.