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Circulating breeding and pre-breeding prolactin and LH are not associated with clutch size in the Zebra Finch (*Taeniopygia guttata*)

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Abstract

Clutch size is a fundamental predictor of avian fitness, widely-studied from evolutionary and ecological perspectives, but surprisingly little is known about the physiological mechanisms regulating clutch size variation. The only formal mechanistic hypothesis for avian clutch-size determination predicts an anti-gonadal effect of circulating prolactin (PRL) via the inhibition of luteinizing hormone (LH), and has become widely-accepted despite little experimental support. Here we investigated the relationship between pre-breeding and breeding plasma PRL and LH and clutch-size in captive-breeding female zebra finches (*Taeniopygia guttata*). Using a repeated-measures design, we followed individual females from pre-breeding, through multiple breeding attempts, and attempted to decrease PRL using the D_2-receptor agonist, bromocriptine. Clutch size was independent of variation in pre-breeding PRL or LH, although pre-breeding LH was negatively correlated with the time between pairing and the onset of laying. Clutch size was independent of variation in plasma PRL on all days of egg-laying. Bromocriptine treatment had no effect on plasma PRL, but in this breeding attempt clutch size was also independent of plasma PRL. Finally, we found no evidence for an inverse relationship between plasma PRL and LH levels, as predicted if PRL had inhibitory effects via LH. Thus, our data fail to provide any support for the involvement of circulating PRL in clutch size determination. These findings suggest that alternative models for hormonal control of avian clutch size need to be considered, perhaps involving downstream regulation of plasma PRL at the level of the ovary, or other hormones that have not been considered to date.

Keywords: prolactin; clutch size; luteinizing hormone; life history; avian reproduction; plasticity

1. Introduction

Clutch size is among the most important contributors to avian lifetime reproductive success, and sets the upper limit on the number of young that can be successfully fledged in any given reproductive event (Charmantier et al., 2006; McCleery et al., 2004; Rockwell et al., 1987). Explaining the patterns and variability in clutch size has been a major goal for both evolutionary biologists and ecologists (Godfray et al., 1991; Klomp, 1970; Lack, 1947; Ricklefs, 2010; Williams, 1966). These studies have focused largely on how evolutionary forces constrain and shape optimal clutch size (Charnov and Krebs, 1974; e.g. Lack, 1947; Martin et al., 2006; Nager et al., 2000; Pettifor et al., 1988; Ricklefs, 2010; Rowe et al., 1994; Williams, 1966), and the social and ecological cues involved in individually fine-tuning that investment under varying conditions (Bolton et al., 1993; Decker et al., 2012; e.g. Lack, 1947; Travers et al., 2010; Williams and Miller, 2003; Zanette et al., 2011). However, understanding the physiological mechanisms that coordinate life history traits like clutch size can elucidate ecological and evolutionary drivers and constraints (Ricklefs and Wikelski, 2002; Williams, 2012a). Nonetheless, the fundamental physiological and hormonal mechanisms that coordinate clutch size and many other important life history traits remain poorly understood (Haywood, 2013; Klomp, 1970; Sockman et al., 2006; Williams, 2012b).

The only physiological or mechanistic hypothesis to explain avian clutch size determination involves prolactin (PRL), an anterior pituitary peptide hormone that is associated with incubation behavior (Delehanty et al., 1997; Lea and Sharp, 1989; March et al., 1994) and chick rearing (Angelier and Chastel, 2009; Miller et al., 2009; O'Dwyer et al., 2006). This mechanistic model was formulated based on several well-supported observations, namely that: a) incubation behavior, tactile stimulation from the eggs, and plasma PRL levels reinforce each other in a positive feedback loop (El Halawani et al., 1984; Hall and Goldsmith, 1983); b) rapid increases in PRL are temporally correlated with the onset of peak incubation behavior and the cessation of egg laying (Haftorn, 1981; Lea et al., 1981), and; c) seasonal increases in the rate of incubation onset and plasma PRL are accompanied by seasonal declines in clutch size (Dawson and Goldsmith, 1985; Flint et al., 2006; Haftorn, 1981; Meijer et al., 1990;

Müller et al., 2004). Potential anti-gonadal effects of PRL via inhibition of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) have also been demonstrated in *in vitro* assays (El Halawani et al., 1984; Rozenboim et al., 1993; You et al., 1995), and are supported by evidence for anti-gonadal effects of PRL *in vivo* in some species (Bailey, 1950; Meier, 1969; Reddy et al., 2007), but not others (Buntin et al., 1999; Meier and Dusseau, 1968; Small et al., 2007). Much of the data used to support the PRL-based mechanistic model for clutch size determination however, is based on broad temporal correlations rather than direct experimental evidence, and this model has rarely been investigated in species laying discrete clutches (i.e. retaining cyclic reproduction characteristic of wild birds). There remains little support for a direct association between clutch size and plasma PRL during the temporal window when follicular inhibition of clutch size determination is thought to occur (2-4 days after the first egg is laid in several species), or for an anti-gonadal effect of PRL sufficient to cause follicular inhibition and the cessation of laying. Indeed, the only experimental work to examine variation in circulating PRL and clutch size determination directly in a non-domesticated, cyclically-laying species was carried out by Sockman et al. (2000) in the American Kestrel, *Falco sparverius*. This study found weak support for a negative association between clutch size and PRL around the time when follicular inhibition putatively occurs. However, PRL manipulations using ovine-PRL osmotic minipumps were not associated with changes in clutch size (Sockman et al., 2000). Based on these results, the authors themselves emphasized in a later review that "*a role for prolactin in regulating clutch size in any species is not firmly established*", and that further work in this area is necessary (Sockman et al., 2006). Despite the prudent conclusions of Sockman and colleagues, the PRL-based mechanistic model for clutch size determination has since received little attention (Williams, 2012a).

The PRL-based model of clutch size determination generally focuses on variation in circulating PRL levels 2-4 days after the first egg is laid (Haywood, 1993; Meijer et al., 1990). However, several recent studies have suggested that pre-breeding hormone levels might also influence, or potentially predict, subsequent reproductive performance (Chastel et al., 2003; Crossin et al., 2012; Greives et al., 2012). For example, in a study

of free-living house sparrows (*Passer domesticus*), pre-laying PRL levels were correlated with fledging success, although this effect was largely dependent on the effect of lay date (Ouyang et al., 2011). Alternatively, Schaper et al. (2012) suggested that pre-breeding PRL levels may be an indicator of seasonal 'reproductive readiness' (Perfito, 2010) rather than an accurate proxy for breeding investment in the form of clutch size. Whether or not pre-breeding PRL levels are predictive of subsequent reproductive performance (in particular, clutch size) after controlling for environmental and photoperiodic cues has, to our knowledge, not been examined.

Here we investigate individual variability in plasma PRL and LH in pre-breeding and breeding females in relation to individual variation in clutch size in the Zebra Finch, *Taeniopygia guttata,* to test predictions from the PRL-based mechanism of clutch size determination (Haftorn, 1981; Haywood, 1993; Meijer et al., 1990). We used a repeated-measures design to follow individuals of known age and reproductive history through pre-breeding, and multiple breeding attempts under controlled environmental and photoperiodic conditions. Our specific objectives were to determine: 1) the relationships between measures of condition (e.g. mass, hematocrit), plasma PRL and LH in pre-breeding and breeding states in individual females; 2) the relationship between pre-breeding PRL and LH and subsequent clutch size, and; 3) the relationship between plasma PRL, LH and clutch size during egg-laying, in birds sampled at the putative time of clutch size determination for zebra finches (six hours after dawn on the day the third egg is laid; Haywood, 1993; Haywood, 2013) as well as on days 2 and 4 of egg-laying. We also attempted to experimentally decrease plasma PRL levels using the dopamine receptor agonist bromocriptine (Angelier et al., 2006; Badyaev and Duckworth, 2005; Reddy et al., 2007), thereby disrupting the putative endogenous relationship between PRL and clutch size. Based on the PRL-based model of clutch size determination described above, we predicted: a) a negative correlation between circulating PRL and LH; b) a negative association between breeding plasma PRL levels and clutch size, and; c) an increase in clutch size associated with a decrease in PRL in bromocriptine-treated females.

2. Material and Methods

2.1. Animal care and breeding protocol

Zebra finches were maintained in controlled environmental conditions (temperature 19–23°C; humidity 35–55%; constant light schedule, 14 L: 10 D, lights on at 07.00). All birds were provided with a mixed seed diet (*Panicum* and white millet, 1:3, 11.7% protein, 0.6% lipid and 84.3% carbohydrate by dry mass), water, grit and cuttlefish bone (calcium) *ad libitum*, and received a multi-vitamin supplement in the drinking water once per week. Breeding pairs were also provided with 6 g/pair/day of egg food supplement (20.3% protein, 6.6% lipid) between pairing and clutch completion.

Before the experiment, all birds were housed in same-sex cages (61cm x 46cm x 41cm) but were not visually or acoustically isolated from the opposite sex. Individual females used in experiments were 4-8 months of age (12-16 months of age for the follow-up study), had been successfully bred at least once, and were always paired with the same male to minimize variation in investment based on perceived mate quality. Breeding pairs were housed individually in single cages (61cm x 46 cm x 41 cm), each with an external nest-box (11.5cm x 11.5cm x 11.5cm). Females were weighed (± 0.1 g, initial mass) at the time of pairing, just prior to blood sampling, and at clutch completion. During breeding, nest-boxes were checked daily between 09.30 and 11.30 and all new eggs were weighed (to 0.001 g) and numbered, to obtain data on egg size, clutch size and laying interval (the time between pairing and laying of the first egg). A clutch was considered complete when no additional eggs were produced over two consecutive days. At clutch completion, eggs were removed and individuals were returned to same-sex holding cages for a resting period of at least three weeks. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 901B 94), in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

2.2. Blood sampling and hormone analysis

Females were blood sampled (≤ 200 µL, max. 1% body weight, from the brachial vein) prior to breeding while in same-sex holding cages ('pre-breeding', n = 78), and following

pairing (females paired 13-17 days later), in the first experiment, on the day the third egg was laid ('breeding'; n = 39). Egg day three was selected based on experimental work which links the physiological mechanism for clutch size determination in zebra finches with the timing of the third laid egg (Haywood, 1993). Blood samples for the bromocriptine experiment (n = 38) were also taken on the day the third egg was laid. In addition, in a follow-up study (~8 months following the bromocriptine experiment), females were bred and blood sampled for PRL measurement (but not LH) either on the day the second (n = 28) or fourth eggs (n = 27) were laid (days 2 and 4). Blood sampling was always carried out between 11:30 and 13:30 to minimize daily fluctuations in hormone levels. Birds were generally sampled within 1.5 - 5 minutes from the time of capture, and PRL and LH were not associated with estimated handling times. Blood samples were centrifuged at 5,000 *g* for five minutes, and plasma was stored at –20°C until required for hormone assays.

Plasma immunoreactive prolactin (PRL) was determined using a radio-immunoassay for recombinant-derived European Starling (*Sturnus vulgaris)* PRL described by Bentley et al. (1997). Other than two blood samples for which there was insufficient plasma, all samples were measured in duplicate. Day 3 samples were measured in a single assay, diluted 1 in 3, and subsequently day 2 and 4 samples were measured in a single assay, undiluted. The sensitivity of the assay, determined to be the estimated concentration two standard deviations above the mean counts per minute of the lowest standard, was 7.8 $ng \cdot mL^{-1}$. The intra-assay coefficient of variation of this assay was 6.5%, and serial dilution of individual samples ran parallel along the standard curve within the dilution range assayed. Luteinizing hormone (LH) was measured using a micro-modified version of a previously described radioimmunoassay (Sharp et al., 1987). Samples (day 3 only) were run in a single assay, in duplicate when sample volume permitted (>90% of all samples), diluted 1 in 2.3 in radioimmunoassay (RIA) buffer. Assay sensitivity was determined as described above, with a lower limit of $0.087 ng \cdot mL^{-1}$. Samples that fell below the detection limit of the assays were given the median between the cut-off and the lowest measured value, and analyses using these data yielded qualitatively similar results as when they were excluded. The intra-assay

coefficient of variation for the LH assay was 6.4% for a high value pool and 8.1% for a low value pool, and a curve generated by serial dilution of zebra finch plasma ran parallel to the standard curve within the dilution range assayed.

2.3. Bromocriptine treatment

Manipulating PRL in birds for a sustained length of time through active or passive immunization, or through exogenous PRL administration, has proven challenging, (Sockman et al., 2000; A. Dawson and P. Sharp, unpublished data). Similarly, injection of vasointestinal peptide (VIP) provides only short-term changes in circulating PRL levels, and only in *non-breeding* birds (Christensen and Vleck, 2008). Therefore, we used the dopamine (D_2 and D_3) receptor agonist, bromocriptine (2-bromo-α-ergocriptine mesylate; Enzo, PA, USA) to manipulate plasma PRL levels. Bromocriptine binds to the inhibitory D_2 receptor on secretory lactotroph cells in the pituitary, and has been widely used to lower PRL in mammals, but less commonly in birds (see references below). Females were randomly assigned to either one of two doses of bromocriptine (low, n = 13, 333µg/kg body weight or high, n = 14, 3333µg/kg body weight w/v in DMSO (dimethylsulfoxide; Sigma-Aldrich, MO, USA), or vehicle only control (n = 11, 35-45 µL DMSO based on mass, as for bromocriptine). Doses were based on previous work in mammals (Bales et al., 2002; Bridges and Ronsheim, 1990; Roberts et al., 2001) and birds (Angelier et al., 2006; Jouventin and Mauget, 1996). Bromocriptine was administered by intra-muscular injection into the pectoral muscle, daily between 1100 and 1300 hours beginning the day the first egg was laid and terminating at clutch completion (see section 2.1). The timing of the first bromocriptine injection was chosen to limit undue stress from injections and to prevent premature decreases in PRL, both of which could have prevented gonadal development and the initiation of laying (Angelier and Chastel, 2009; Maney et al. 1999; Small et al. 2007). On egg day three of the bromocriptine experiment, injections were carried out immediately after blood sampling (see section 2.2).

2.4. Data analysis

Data were first examined for normality, outliers, collinearity and interactions between explanatory variables. Both hormones showed deviations from normality, which was improved with log transformation. Log transformed data are described using median and interquartile range; otherwise data are stated as mean ± standard error. Repeatability was calculated using previously described methods (Lessells and Boag, 1987). Since there were no statistical differences in the results found using mass alone or the residuals of a regression of mass by tarsus, mass alone was used as the measure of condition in all relevant analyses. For hormone analyses, only clutches equal or greater to the day the blood sample was taken were included (≥3 eggs day 3 and experimental breeding, ≥2 eggs for day 2, ≥4 eggs for day 4). Several females laid clutches larger than those normally observed in the wild (2-7 eggs; Zann, 1996). Since clutch sizes larger than 7 are 'atypical' under normal breeding conditions, analyses were run including and excluding these data. Results from both datasets are presented when the model outcomes differed, otherwise results include larger than normal clutch sizes. For the bromocriptine experiment we predicted individual increases in clutch size in response to the treatment, specifically those greater than the range observed in free-living birds.

Pre-breeding and simple breeding comparisons (excluding clutch size; see below) were conducted using ANOVA or ordinary least squares regression. To examine females through treatment and time (i.e. between pre-breeding and breeding; between control breeding and bromocriptine breeding), we used linear mixed effects models for repeated measures with individual female as a random factor, carried out in the statistical package 'nlme' in R 2.12.2 (Pinheiro et al., 2011; R Core Development Team, 2011). This experimental and statistical design allowed us to make intra-individual comparisons of the effects of treatment, so that treated females were compared to themselves under the untreated breeding conditions (in addition to retaining a vehicle only control group for bromocriptine, see section 2.3). For each stage, a small subset of females did not provide sufficient plasma for both hormone assays, failed to breed, or

laid less than 3 eggs (i.e. no hormone values for egg day three). As a result, model degrees of freedom vary, based on the maximum number of available data points.

Since clutch size is a discrete count variable, all analyses of this trait were conducted using generalized linear or generalized linear mixed effects models, with quasipoisson family to account for underdispersion (R package "glmmPQL"; Fox and Weisberg, 2011). Analyses of egg mass was conducted on mean egg mass within a clutch, and yielded similar results to models incorporating all eggs, laying order and individual female as a random factor. All analyses were followed with standard model validation procedures to test the assumptions of the test employed. Data points with high leverage and Cook's distance (> 4/n) were considered influential, and outputs are presented for models including and excluding these points for transparency. Where multiple explanatory variables were found to affect a dependent variable, p-values are given for the full model including all significant variables (ANCOVA).

3. Results

3.1. Relationship between pre-breeding LH, PRL and measures of body condition

There were no significant relationships between pre-breeding mass or hematocrit, i.e. measures of body condition, and pre-breeding LH ($F_{1,66}$ = 0.288, P = 0.594 and $F_{1,66}$ = 0.128, P = 0.722, respectively), or pre-breeding PRL ($F_{1,75}$ = 0.427, P = 0.516; $F_{1,75}$ = 3.729, P = 0.057, respectively; Table 1). However, pre-breeding PRL was weakly, but significantly and positively correlated with pre-breeding LH ($F_{1,65}$ = 4.272, r^2 = 0.05; P = 0.043), including after removing values at the detection limits of the assay ($F_{1,55}$ = 3.46, r^2 = 0.091; P = 0.013; Fig. 1).

3.2. Relationships between pre-breeding LH and PRL, and breeding hormone levels and reproductive traits

Compared to pre-breeding levels, LH was significantly higher during the 3-egg stage in breeding females (estimate for effect of breeding stage on LH ± S.E.: 0.098 ± 0.051 ng/mL, *df* = 31, *t* = 2.38, *P* = 0.024; Intercept: 0.265 ± 0.039, *df* = 40, *t* = -9.77, *P* < 0.001; Table 1). Furthermore, individual variation in LH was repeatable between pre-

breeding and breeding stages (R = 0.51; 95% CI = 0.25, 0.77; $P < 0.002$). Pre-breeding LH was negatively correlated with laying interval after controlling for the time elapsed between pre-breeding blood sampling and subsequent pairing - females with higher pre-breeding LH had shorter intervals between pairing and laying of the first egg ($F_{2,31} = 15.52$, $P < 0.001$; Fig. 2). However, pre-breeding LH was not significantly correlated with either mean egg mass ($F_{2,30} = 1.66$, $P = 0.207$) or clutch size (Likelihood-ratio test: $\chi^2 = 0.011$, $df = 1$, $P = 0.915$) of the subsequent breeding attempt.

Breeding PRL levels at the 3-egg stage were markedly and significantly higher than pre-breeding levels (Estimate for effect of breeding stage on PRL $\pm$ S.E.: 180 $\pm$ 24ng/mL, $df = 38$, $t = 19.17$, $P < 0.001$; Intercept: 23.07 $\pm$ 2.03, $df = 41$, $t = 37.21$, $P < 0.001$; Table 1). However, in contrast to LH, individual PRL levels were not repeatable between pre-breeding and breeding stages ($P > 0.90$). Log laying interval, egg mass, and clutch size were all independent of pre-breeding PRL levels (P > 0.10 in all cases).

3.3. Relationships between breeding LH, PRL and reproductive traits

Mean egg mass was significantly and positively correlated with body mass at pairing ($F_{1,39} = 5.72$, $P = 0.022$), but not laying interval ($F_{1,39} = 1.29$, $P = 0.264$). In contrast, clutch size was independent of mass at pairing (Likelihood-ratio test: $\chi^2 = 0.873$, $df = 1$, $P = 0.350$), but negatively correlated with laying interval (Likelihood-ratio test: $\chi^2 = 9.234$, $df = 1$, $P = 0.002$). Neither egg mass or clutch size was significantly correlated with individual variation in breeding plasma LH ($P > 0.15$ for both). Breeding plasma PRL on egg day 3 was significantly correlated with variation in mean egg mass ($F_{1,37} = 5.38$, $P = 0.026$) and clutch size ($\chi^2 = 9.17$, $df = 1$, $P = 0.002$; Fig. 3A), but these effects were inconsistent and skewed by several influential data points (i.e. high leverage points from clutch sizes outside the range normally observed in the wild [>7 eggs; Zann 1996]). Within the normal range of clutch sizes, both mean egg mass and clutch size were independent of variation in breeding PRL at day 3 of egg-laying ($F_{1,34} = 0.004$, $P = 0.950$ and $\chi^2 = 0.227$, $df = 1$, $P = 0.634$, respectively; Fig. 3A). Similarly, in the follow-up study, variation in clutch size was independent of variation in breeding

PRL on day 2 (χ^2 = 0.115, *df* = 1, *P* = 0.735) and day 4 (χ^2 = 2.69, *df* = 1, *P* = 0.101) of egg-laying.

3.4. LH, PRL, and reproductive traits for bromocriptine treatment breeding

Luteinizing hormone levels decreased significantly between the control and bromocriptine breeding attempts (estimate for effect of breeding attempt on LH ± S.E.: -0.133 ± 0.029 ng/mL, *df* = 31, *t* = -3.20, *P* = 0.003; Intercept: 0.32 ± 0.045, *df* = 34, *t* = 7.46, *P* < 0.001; Table 1), but this effect was not different for the control group or either treatment (Breeding attempt *Treatment; Likelihood-ratio test: χ^2 = 1.56, *df* = 2, *P* = 0.460). Similarly, hematocrit dropped significantly for the bromocriptine breeding attempt (estimate for effect of Breeding attempt on hematocrit ± S.E.: -0.03 ± 0.01, *df* = 31, *t* = -5.30, *P* < 0.001; Intercept: 0.481 ± 0.001, *df* = 34, *t* = 75.86, *P* < 0.001; Table 1), a change that also did not differ between control or treatment groups (Breeding attempt *Treatment; Likelihood-ratio test: χ^2 = 0.51, *df* = 2, *P* = 0.776).

Prolactin levels were not significantly different between the control and bromocriptine breeding attempts (estimate for effect of breeding attempt on PRL ± S.E.: -1.64 ± 3.65 ng/mL, *df* = 31, *t* = -0.22, *P* = 0.824; Intercept: 197.26 ± 7.31, *df* = 34, *t* = 139.82, *P* < 0.001; Table 1). There were no differences in PRL by treatment group (Treatment; Likelihood-ratio test: χ^2 = 2.93, *df* = 2, *P* = 0.230), nor any interaction between breeding attempt and treatment (Breeding attempt *Treatment; Likelihood-ratio test: χ^2 = 1.12, *df* = 2, *P* = 0.571). In fact, individual PRL levels between the control and bromocriptine treatment breeding attempts were repeatable (R = 0.54; 95% CI = 0.28-0.79; *P* < 0.001).

Clutch size also was not significantly different between the control and bromocriptine breeding attempts (estimate for effect of breeding attempt on clutch size ± S.E.: -0.32 ± 0.90 eggs, *df* = 32, *t* = -1.90, *P* = 0.07; Intercept: 5.69 ± 0.20, *df* = 35, *t* = 48.27, *P* < 0.001; Table 1), and there were no interactions between breeding attempt and treatment (Breeding attempt *Treatment; Likelihood-ratio test: χ^2 = 5.27, *df* = 2, *P* =

0.072). Like PRL, clutch size showed individual repeatability between the control and bromocriptine breeding attempts (R = 0.66; 95% CI = 0.46-0.86; $P < 0.001$).

3.5. Changes in PRL, LH, and clutch size between control and experimental breeding attempts

Since there was no effect of treatment on PRL or clutch size between the control and bromocriptine breeding attempts, we pooled treatment groups from the experimental breeding for further analyses. As in the control breeding attempt, clutch size was independent of plasma PRL for the experimental breeding ($\chi^2 = 0.519$, $df = 1$, $P = 0.471$; Fig. 3B), including with clutch sizes larger than the range typically observed in the wild ($\chi^2 = 0.135$, $df = 1$, $P = 0.713$). However, individual *changes* in PRL levels between a female's control and experimental breeding attempts were significantly, negatively correlated with individual *changes* in clutch size. This relationship remained significant including ($\chi^2 = 4.116$, $df = 1$, $P = 0.043$) or excluding ($\chi^2 = 4.425$, $df = 1$, $P = 0.035$) two influential data points for which we had only a single observation for a given change in clutch size. No such relationship was found for changes in PRL and changes in egg mass ($F_{1,34} = 2.051$, $P = 0.163$), changes in PRL and changes in LH ($F_{1,30} = 0.215$, $P = 0.647$), or changes in LH and clutch size ($\chi^2 < 0.001$, $df = 1$, $P = 0.979$) or egg mass ($F_{1,30} = 0.345$, $P = 0.561$) between the control and experimental breeding attempts.

4. Discussion

In this study we investigated individual variation in pre-breeding and breeding hormone (PRL and LH) levels in relation to variation in reproductive traits (timing of laying, egg mass, clutch size), specifically to test the hypothesis that variation in circulating PRL levels mediates clutch size variation via the inhibition of LH (Haywood, 1993; Lea et al., 1981; Meijer et al., 1990; Sockman et al., 2006). Clutch size was independent of variation in pre-breeding PRL or LH, although pre-breeding LH was negatively correlated with the time between pairing and the onset of egg-laying. We also found no evidence for any inverse relationships between plasma PRL and plasma LH levels

which would have been consistent with an inhibitory effect of PRL on LH. In contrast to previous studies (Badyaev and Duckworth, 2005; Reddy et al., 2007) we observed no effect of bromocriptine on circulating PRL. Nonetheless, and most importantly, we found no evidence to support a causal relationship between individual variation in breeding plasma PRL levels and variation in clutch size in multiple different breeding attempts and for PRL measured on either days 2, 3 or 4 of egg-laying, i.e. during the temporal window when follicular inhibition and clutch size determination is thought to occur. The only evidence we found to support a link between PRL and clutch size was a negative relationship between individual *change* in PRL between the control and experimental breeding and individual *change* in clutch size. While we think this result is interesting we acknowledge this may not be reflective of a causal relationship. Thus our data, from multiple different breeding attempts, fail to provide any support for the involvement of circulating PRL early in egg-laying on clutch size determination.

We first examined variation in pre-breeding PRL and LH and condition-related traits (e.g. body mass, hematocrit) to test the hypothesis that individual variability in these characteristics could be predictive of subsequent reproductive performance (Chastel et al., 2003; Crossin et al., 2012; Ouyang et al., 2011). We observed no relationship between pre-breeding hematocrit or body mass and pre-breeding PRL or LH. We also found no effect of pre-breeding mass, hematocrit, PRL or LH on subsequent clutch size. These results do not support the hypothesis that plasma PRL or LH prior to breeding provide an early 'window' into subsequent reproductive performance, at least for clutch size (but see "reproductive readiness", below). In addition, plasma PRL and LH were significantly, positively correlated in pre-breeding female zebra finches which contrasts with results from other studies, mostly in breeding poultry, which have demonstrated an inhibitory effect of PRL on LH hormone titres or LH mRNA expression (Rozenboim et al., 1993; You et al., 1995). Although the correlation between these two traits in our study was not particularly strong, our results are consistent with growing evidence that PRL can have both inhibitory *and* stimulatory effects on gonadal function, depending on reproductive state and PRL concentration (Hrabia et al., 2004; Li et al., 2011; Maney et al., 1999; Small et al., 2007). The origin of

the positive correlation between PRL and LH is not obvious; LH activates the reproductive axis and steroidogenesis, and steroid hormones can stimulate PRL secretion (El Halawani et al., 1983; Mauro et al., 1992). However, since non-photoperiodic cues (e.g. social stimuli) likely contribute to variation in pre-breeding LH levels in opportunistically breeding species like the zebra finch (e.g. Maney et al., 1999; Perfito et al., 2007; Small et al., 2007), pre-breeding LH and PRL may reflect individual differences in the relative activation of the reproductive axis prior to actual onset of egg-laying, i.e. individual 'reproductive readiness'.

Individual differences in reproductive readiness are supported in our study by the positive correlation between pre-breeding LH levels and the interval between pairing and laying - females with relatively high pre-breeding LH were the quickest to initiate laying. Presumably, variability in pre-breeding LH is indicative of the differences in the developmental state of the ovary and nascent follicles, a suggestion supported by other work in captive pre-breeding zebra finches (see Fig. 4 in Perfito, 2010). The finding that not all females are in a homogeneous pre-breeding state is of critical importance to laboratory studies of reproductive behaviour, particularly those involving the timing of breeding or response to mating stimuli (Perfito, 2010). In contrast to LH, pre-breeding PRL was not predictive of the interval between pairing and laying, contrary to previous work in free-living House Sparrows (*Passer domesticus*), in which females with high PRL prior to breeding, prior to controlling for lay date, laid their first egg sooner (Ouyang et al., 2011). However, as in our study, Schaper et al. (2012) also failed to detect any relationship between pre-breeding PRL and readiness to lay under controlled laboratory conditions in *Parus major*, suggesting an independent role for photoperiod on PRL and activation of the reproductive-axis, possibly via independent control of PRL and LH secretion.

A key component of the PRL-based model for clutch size determination is that PRL exerts anti-gonadal effects indirectly via the inhibition of LH expression at the level of the pituitary (Lea et al., 1981; Sockman et al., 2006). This component of the model predicts an inverse relationship between these hormones, at least at the time of clutch size determination. We were able to examine the relationship between these two

hormones, and how they changed over time, by tracking individual hormonal profiles through the transition between pre-breeding and breeding states. Breeding LH levels were moderately though significantly higher than pre-breeding levels, and were repeatable between pre-breeding and breeding states. In contrast, plasma PRL levels increased dramatically (as high as 27 fold) between pre-breeding and egg day 3, and PRL levels on egg day 3 were independent of pre-breeding PRL. Although LH levels on day three were probably beginning to decline (based on rapid decreases in estradiol around this time; Williams et al., 2005), our data still suggest an uncoupling of the positive correlation between PRL and LH that we observed in pre-breeding females. An uncoupling of these two hormones over time does not support the idea of a systemic inhibitory effect of PRL on LH, since in our study both hormones increase with breeding, yet vary independently between pre-breeding and breeding states. Accordingly, we also found no significant relationship between breeding levels of PRL and LH. Furthermore, while experimental bromocriptine treatment had no effect on circulating PRL (discussed below), we again found no evidence for an inhibitory effect of PRL on LH in our experimental breeding. Though correlational, the lack of empirical support for an inhibitory effect of PRL on LH in this study, as well as in other passerines (Buntin et al., 1999; Meier and Dusseau, 1968; Small et al., 2007), raises questions about the universality of the PRL-dependent control of LH in the current mechanistic hypothesis, and its applicability in this taxon.

In contrast to previous studies on mammals (Bridges and Ronsheim, 1990; Palestine et al., 1987) and some avian species (Angelier et al., 2006; Jouventin and Mauget, 1996; Reddy et al., 2007) we found that bromocriptine treatment had no effect on circulating PRL levels in zebra finches for either the low or high dose groups, nor did we observe a treatment effect on clutch size between the control and experimental breeding. While a range of bromocriptine doses have been employed in birds, from as low as 14 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (Reddy et al., 2007) to as high as 10,000 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (Badyaev and Duckworth, 2005), our doses (low: 333 $\mu g \cdot kg^{-1} \cdot day^{-1}$; high: 3,333 $\mu g \cdot kg^{-1} \cdot day^{-1}$) are comparable to those successfully employed in other avian species (Angelier et al., 2006: 1,500 µg·kg-1·day-1; Jouventin and Mauget, 1996: 4,167 µg·kg-1·day-1) and

commonly used in mammals (Bridges and Ronsheim, 1990: 4,000 µg·kg-1·day-1; Palestine et al., 1987: 1,800 µg·kg-1·day-1). In addition, several studies using injections of bromocriptine reported significant decreases in PRL within 3 days (Roberts et al. (2001; Angelier et al. 2006) approximately the targeted time-frame in our study. Thus, the reason for the failure of bromocriptine to effect PRL levels in our study is not clear, though this is not restricted to *T. guttata* (e.g. bromocriptine had no effect on PRL in *Rissa tridactyla*; F. Angelier, pers. comm.). In contrast, the decrease in both LH and hematocrit we did observe is best explained by injection treatments that all birds, including controls, received, since this effect did not differ by treatment group.

The PRL-based mechanism for clutch size determination predicts a clear negative relationship between plasma PRL and clutch size, i.e. females with higher circulating PRL early during laying should lay smaller clutches, due to the earlier and/or greater inhibitory effect of elevated plasma PRL (Sockman et al., 2000). We found that variation in PRL levels during what is believed to be the critical period for clutch size determination in the zebra finch (day 3 of egg-laying) were not associated with differences in clutch size (cf Sockman et al., 2000). Furthermore, in our follow-up study variation in plasma PRL on day 2 and day 4 of egg-laying, bracketing the putative time window for clutch size determination, was also unrelated to clutch size. Thus, although the current model for clutch size determination has focused on an inhibitory role for *circulating* plasma PRL early in laying (Haywood, 1993; Sockman et al., 2000), our results suggest that individual variation in absolute plasma PRL is not involved in clutch size determination. Furthermore, we found no evidence for an inhibitory effect of PRL on LH. Given our sample sizes and the range of clutch sizes, as well as the tightly controlled diet, photoperiod, age and reproductive history of the individuals included in the study, we believe our study provides a robust test of the PRL-based model for clutch size determination, which posits a regulatory role for *circulating* PRL during early egg-laying (Meijer et al., 1990). Nevertheless, alternative mechanisms, still involving PRL, are worth considering, e.g. differential PRL receptor expression, polymorphisms in gene and receptor, or tissue specific-receptor expression among individuals, could all affect

the biological activity and effects of a given plasma concentration of PRL (Zadworny et al., 2002).

While any PRL-based mechanism for clutch size determination does not appear to involve an absolute inhibitory threshold at the scale of the population, individual differences in either the rate of increase or in the inhibitory threshold (relative PRL level for inhibition for a given breeding attempt) remain plausible alternatives to, or modifications of, the mechanistic model in its current form (Meijer et al., 1990; Williams, 2012b, p. 186). The only evidence we found to support a link between PRL and clutch size was a negative relationship between individual *changes* in PRL between the control and experimental breeding and individual *changes* in clutch size. If this finding is robust, the fact that changes in PRL between breeding attempts were not associated with changes in LH, nor were changes in LH associated changes in clutch size, may imply downstream regulatory effects of PRL (e.g. at the level of the ovary). Although speculative, this hypothesis is supported by work demonstrating the presence of PRL receptors in ovarian follicles (Ohkubo et al., 1998), which can directly inhibit the effects of follicle-stimulating hormone (FSH) and LH on, as well as estrogen and progesterone secretion from, the avian ovary (Hrabia et al., 2004; Li and Yang, 1995).

Studying avian clutch size determination by looking at individual co-variation in PRL and egg number may suggest more biologically-relevant alternatives to the mechanistic hypothesis in its current form (Haftorn, 1981; Haywood, 1993; Meijer et al., 1990), a hypothesis we found no support for in this study. Further experimental work successfully uncoupling PRL from clutch size is necessary to reinforce this conclusion. If the hormonal regulatory control of clutch size is superimposed upon individual variation in downstream effectors (e.g. receptor expression in the ovary), repeated measurements of individuals through time, as conducted in this study, have the benefit of eliminating at least a portion of these potentially confounding effects, which might bring questions about the endocrine control of this key life history trait into greater focus. At present though, it seems most parsimonious to assume that the putative relationship between circulating PRL early in egg-laying and clutch size simply reflects a temporal coincidence, and that the increase in PRL at this time is functionally associated with

onset or maintenance of incubation - a link that is better supported by experimental data (Lea and Sharp, 1989; Williams 2012 and references therein).

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Tables

Table 1. Reproductive and condition-related parameters for pre-breeding and breeding female zebra finches.

	Mass[a] (g)	Hematocrit[a] (%)	LH[b] (ng· mL^{-1})	PRL[b] (ng· mL^{-1})	Mean Egg Mass[a] (g)	Clutch Size[a]
Pre-Breeding	15.0 ± 0.2	53.2 ± 0.4	0.24 (0.14-0.40)	21.0 (13.9-33.5)	na	na
Control Breeding[c]	15.5 ± 0.2	48.6 ± 0.6	0.43 (0.22-0.66)	201.6 (184.6-221.2)	1.08 ± 0.01	5.98 ± 0.25
Bromocriptine Breeding[c]						
DMSO	15.8 ± 0.4	45.3 ± 1.3	0.19 (0.10-0.35)	193.5 (162.5-201.8)	1.02 ± 0.02	5.82 ± 0.54
High	15.8 ± 0.2	44.8 ± 1.0	0.21 (0.14-0.39)	211.0 (169.2-225.9)	1.04 ± 0.03	5.27 ± 0.33
Low	15.7 ± 0.3	43.6 ± 1.2	0.19 (0.06-0.40)	207.6 (201.1-232.0)	1.07 ± 0.03	5.33 ± 0.19
Follow-up Breeding[c]						
Day 2	16.3 ± 0.2	48.0 ± 0.6	na	104.7 (95.73-159.4)	1.12 ± 0.02	5.63 ± 0.26
Day 4	15.8 ± 0.2	46.5 ± 1.0	na	131.6 (104.4-153.9)	1.08 ± 0.02	5.61 ± 0.21

[a]Mass, hematocrit, mean egg mass and clutch size values are mean ± standard error
[b]Luteinizing hormone (LH) and prolactin (PRL) given as median and interquartile range
[c]Control breeding and Bromocriptine breeding blood samples were taken on the day the 3rd egg was laid (Day 3); Follow-up breeding blood samples were taken on the days the 2nd (Day 2) or 4th (Day 4) eggs were laid; see text for additional information

Circulating breeding and pre-breeding prolactin and LH are not associated with clutch size in the Zebra Finch (*Taeniopygia guttata*)

Figure Captions:

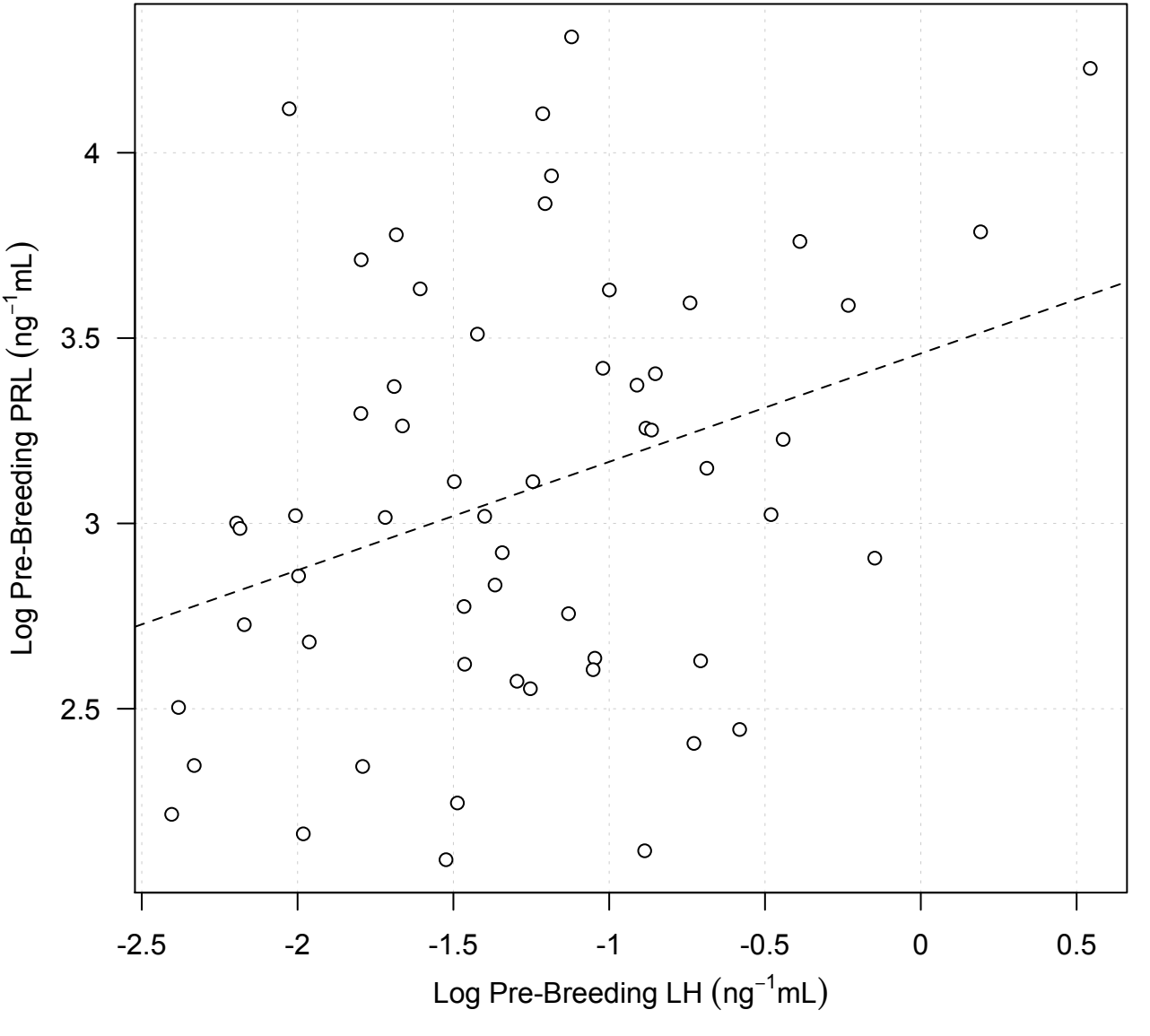
Figure 1. Relationship between pre-breeding log prolactin (PRL) and pre-breeding log luteinizing hormone. Correlation between these two traits was significant, including after removing values at the detection limits of the assay ($F_{1,55} = 3.46$, $r^2 = 0.091$; $P = 0.013$).

Figure 2. Relationship between pre-breeding luteinizing hormone (LH) and the interval between pairing and the first egg in subsequent pairing. Females were paired roughly two weeks following pre-breeding blood sampling, and the relationship between log LH and log laying interval was significant ($F_{2,31} = 15.52$, $P < 0.001$), controlling for the time between blood sampling and pairing.

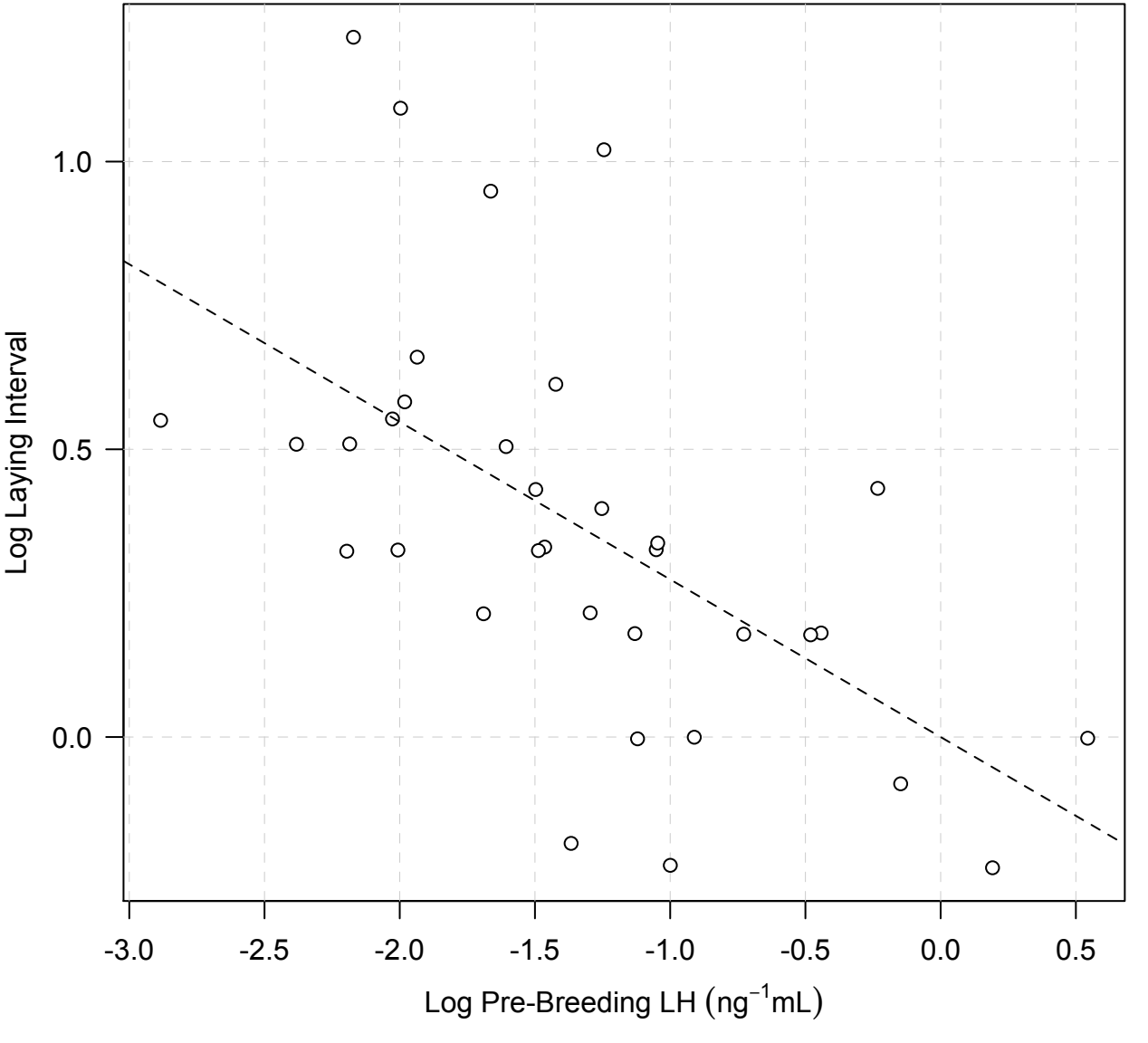
Figure 3. Prolactin (PRL) in the plasma breeding zebra finch females on the day the third egg was laid for control (A) and experimental breeding (B). Clutch sizes larger than those typically observed in the wild are noted as "atypical". The number of females laying a given clutch size are indicated in blue. A significant difference ($P = 0.002$) in PRL by clutch size was dependant on two high leverage, 10 egg clutches in the control breeding. There was no difference in PRL by clutch size for the normal range of clutches in the control breeding ($P = 0.634$), nor for bromocriptine breeding (all clutches: $P = 0.713$; 'normal' clutches: $P = 0.471$).

Figure 4. Change in breeding prolactin (PRL) and clutch size between control and experimental breeding attempts. The relationship between these two traits was significant including ($P = 0.035$) or excluding ($P = 0.043$) the two clutches for which there was only one observation (decreases in four and two eggs).

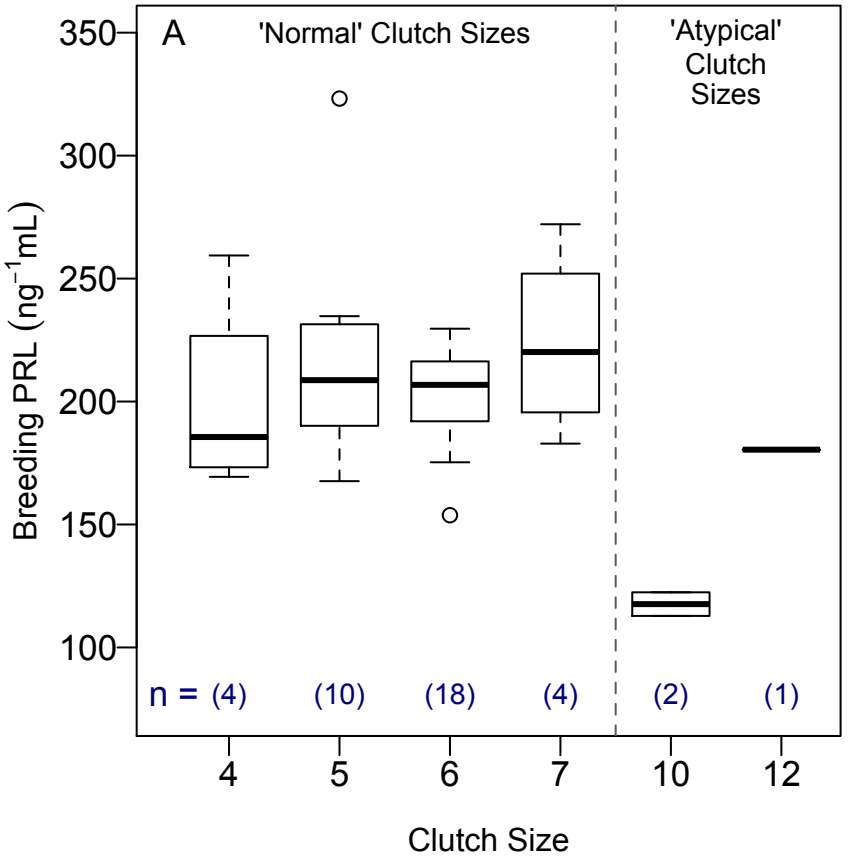
Figures

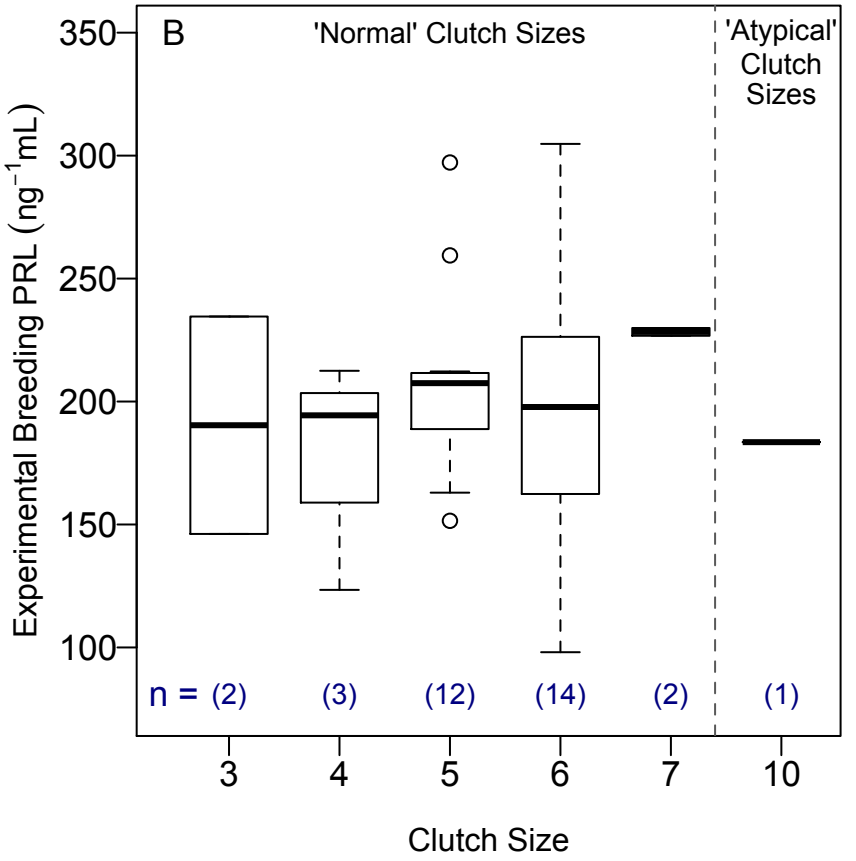

Log Pre-Breeding PRL (ng^{-1}mL)
4
3.5
3
2.5
-2.5
-2
-1.5
-1
-0.5
0
0.5
Log Pre-Breeding LH (ng^{-1}mL)

Figures

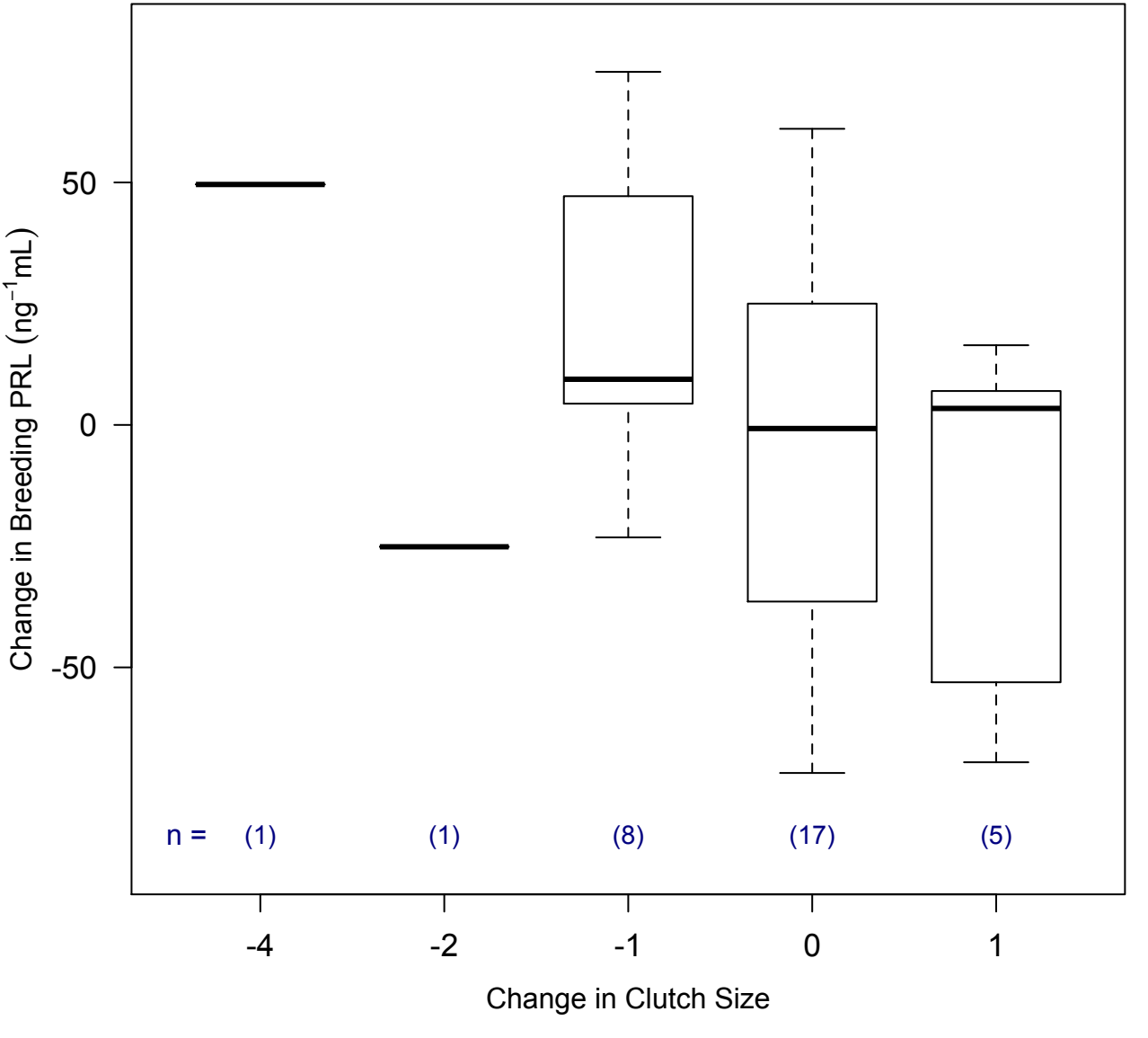

Log Laying Interval
1.0
0.5
0.0
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
Log Pre-Breeding LH (ng^{-1}mL)

Figures


A
'Normal' Clutch Sizes
'Atypical' Clutch Sizes
Breeding PRL (ng^{-1}mL)
350
300
250
200
150
100
n = (4) (10) (18) (4) (2) (1)
4 5 6 7 10 12
Clutch Size


B
'Normal' Clutch Sizes
'Atypical' Clutch Sizes
Experimental Breeding PRL (ng^{-1}mL)
350
300
250
200
150
100
n = (2) (3) (12) (14) (2) (1)
3 4 5 6 7 10
Clutch Size

Figures


50
0
-50
Change in Breeding PRL (ng^{-1}mL)
n = (1) (1) (8) (17) (5)
-4
-2
-1
0
1
Change in Clutch Size