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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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1 *Abstract*

2 Clutch size is a fundamental predictor of avian fitness, widely-studied from evolutionary
3 and ecological perspectives, but surprisingly little is known about the physiological
4 mechanisms regulating clutch size variation. The only formal mechanistic hypothesis for
5 avian clutch-size determination predicts an anti-gonadal effect of circulating prolactin
6 (PRL) via the inhibition of luteinizing hormone (LH), and has become widely-accepted
7 despite little experimental support. Here we investigated the relationship between pre-
8 breeding and breeding plasma PRL and LH and clutch-size in captive-breeding female
9 zebra finches (*Taeniopygia guttata*). Using a repeated-measures design, we followed
10 individual females from pre-breeding, through multiple breeding attempts, and
11 attempted to decrease PRL using the D₂-receptor agonist, bromocriptine. Clutch size
12 was independent of variation in pre-breeding PRL or LH, although pre-breeding LH was
13 negatively correlated with the time between pairing and the onset of laying. Clutch size
14 was independent of variation in plasma PRL on all days of egg-laying. Bromocriptine
15 treatment had no effect on plasma PRL, but in this breeding attempt clutch size was
16 also independent of plasma PRL. Finally, we found no evidence for an inverse
17 relationship between plasma PRL and LH levels, as predicted if PRL had inhibitory
18 effects via LH. Thus, our data fail to provide any support for the involvement of
19 circulating PRL in clutch size determination. These findings suggest that alternative
20 models for hormonal control of avian clutch size need to be considered, perhaps
21 involving downstream regulation of plasma PRL at the level of the ovary, or other
22 hormones that have not been considered to date.

23

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

1 **1. Introduction**

2 Clutch size is among the most important contributors to avian lifetime reproductive
3 success, and sets the upper limit on the number of young that can be successfully
4 fledged in any given reproductive event (Charmantier et al., 2006; McCleery et al.,
5 2004; Rockwell et al., 1987). Explaining the patterns and variability in clutch size has
6 been a major goal for both evolutionary biologists and ecologists (Godfray et al., 1991;
7 Klomp, 1970; Lack, 1947; Ricklefs, 2010; Williams, 1966). These studies have focused
8 largely on how evolutionary forces constrain and shape optimal clutch size (Charnov
9 and Krebs, 1974; e.g. Lack, 1947; Martin et al., 2006; Nager et al., 2000; Pettifor et al.,
10 1988; Ricklefs, 2010; Rowe et al., 1994; Williams, 1966), and the social and ecological
11 cues involved in individually fine-tuning that investment under varying conditions (Bolton
12 et al., 1993; Decker et al., 2012; e.g. Lack, 1947; Travers et al., 2010; Williams and
13 Miller, 2003; Zanette et al., 2011). However, understanding the physiological
14 mechanisms that coordinate life history traits like clutch size can elucidate ecological
15 and evolutionary drivers and constraints (Ricklefs and Wikelski, 2002; Williams, 2012a).
16 Nonetheless, the fundamental physiological and hormonal mechanisms that coordinate
17 clutch size and many other important life history traits remain poorly understood
18 (Haywood, 2013; Klomp, 1970; Sockman et al., 2006; Williams, 2012b).

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

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

26 The PRL-based model of clutch size determination generally focuses on variation
27 in circulating PRL levels 2-4 days after the first egg is laid (Haywood, 1993; Meijer et al.,
28 1990). However, several recent studies have suggested that pre-breeding hormone
29 levels might also influence, or potentially predict, subsequent reproductive performance
30 (Chastel et al., 2003; Crossin et al., 2012; Greives et al., 2012). For example, in a study

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

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

1 **2. Material and Methods**

2 *2.1. Animal care and breeding protocol*

3 Zebra finches were maintained in controlled environmental conditions (temperature 19–
4 23°C; humidity 35–55%; constant light schedule, 14 L: 10 D, lights on at 07.00). All birds
5 were provided with a mixed seed diet (*Panicum* and white millet, 1:3, 11.7% protein,
6 0.6% lipid and 84.3% carbohydrate by dry mass), water, grit and cuttlefish bone
7 (calcium) *ad libitum*, and received a multi-vitamin supplement in the drinking water once
8 per week. Breeding pairs were also provided with 6 g/pair/day of egg food supplement
9 (20.3% protein, 6.6% lipid) between pairing and clutch completion.

10 Before the experiment, all birds were housed in same-sex cages (61cm x 46cm x
11 41cm) but were not visually or acoustically isolated from the opposite sex. Individual
12 females used in experiments were 4-8 months of age (12-16 months of age for the
13 follow-up study), had been successfully bred at least once, and were always paired with
14 the same male to minimize variation in investment based on perceived mate quality.
15 Breeding pairs were housed individually in single cages (61cm x 46 cm x 41 cm), each
16 with an external nest-box (11.5cm x 11.5cm x 11.5cm). Females were weighed (\pm 0.1 g,
17 initial mass) at the time of pairing, just prior to blood sampling, and at clutch completion.
18 During breeding, nest-boxes were checked daily between 09.30 and 11.30 and all new
19 eggs were weighed (to 0.001 g) and numbered, to obtain data on egg size, clutch size
20 and laying interval (the time between pairing and laying of the first egg). A clutch was
21 considered complete when no additional eggs were produced over two consecutive
22 days. At clutch completion, eggs were removed and individuals were returned to same-
23 sex holding cages for a resting period of at least three weeks. Experiments and animal
24 husbandry were carried out under a Simon Fraser University Animal Care Committee
25 permit (no. 901B 94), in accordance with guidelines from the Canadian Committee on
26 Animal Care (CCAC).

27

28 *2.2. Blood sampling and hormone analysis*

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

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

14 Plasma immunoreactive prolactin (PRL) was determined using a radio-
15 immunoassay for recombinant-derived European Starling (*Sturnus vulgaris*) PRL
16 described by Bentley et al. (1997). Other than two blood samples for which there was
17 insufficient plasma, all samples were measured in duplicate. Day 3 samples were
18 measured in a single assay, diluted 1 in 3, and subsequently day 2 and 4 samples were
19 measured in a single assay, undiluted. The sensitivity of the assay, determined to be
20 the estimated concentration two standard deviations above the mean counts per minute
21 of the lowest standard, was $7.8 \text{ ng}\cdot\text{mL}^{-1}$. The intra-assay coefficient of variation of this
22 assay was 6.5%, and serial dilution of individual samples ran parallel along the standard
23 curve within the dilution range assayed. Luteinizing hormone (LH) was measured using
24 a micro-modified version of a previously described radioimmunoassay (Sharp et al.,
25 1987). Samples (day 3 only) were run in a single assay, in duplicate when sample
26 volume permitted (>90% of all samples), diluted 1 in 2.3 in radioimmunoassay (RIA)
27 buffer. Assay sensitivity was determined as described above, with a lower limit of
28 $0.087 \text{ ng}\cdot\text{mL}^{-1}$. Samples that fell below the detection limit of the assays were given the
29 median between the cut-off and the lowest measured value, and analyses using these
30 data yielded qualitatively similar results as when they were excluded. The intra-assay

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

4

5 *2.3. Bromocriptine treatment*

6 Manipulating PRL in birds for a sustained length of time through active or passive
7 immunization, or through exogenous PRL administration, has proven challenging,
8 (Sockman et al., 2000; A. Dawson and P. Sharp, unpublished data). Similarly, injection
9 of vasointestinal peptide (VIP) provides only short-term changes in circulating PRL
10 levels, and only in *non-breeding* birds (Christensen and Vleck, 2008). Therefore, we
11 used the dopamine (D₂ and D₃) receptor agonist, bromocriptine (2-bromo- α -ergocriptine
12 mesylate; Enzo, PA, USA) to manipulate plasma PRL levels. Bromocriptine binds to the
13 inhibitory D₂ receptor on secretory lactotroph cells in the pituitary, and has been widely
14 used to lower PRL in mammals, but less commonly in birds (see references below).
15 Females were randomly assigned to either one of two doses of bromocriptine (low, n =
16 13, 333 μ g/kg body weight or high, n = 14, 3333 μ g/kg body weight w/v in DMSO
17 (dimethylsulfoxide; Sigma-Aldrich, MO, USA), or vehicle only control (n = 11, 35-45 μ L
18 DMSO based on mass, as for bromocriptine). Doses were based on previous work in
19 mammals (Bales et al., 2002; Bridges and Ronsheim, 1990; Roberts et al., 2001) and
20 birds (Angelier et al., 2006; Jouventin and Mauget, 1996). Bromocriptine was
21 administered by intra-muscular injection into the pectoral muscle, daily between 1100
22 and 1300 hours beginning the day the first egg was laid and terminating at clutch
23 completion (see section 2.1). The timing of the first bromocriptine injection was chosen
24 to limit undue stress from injections and to prevent premature decreases in PRL, both of
25 which could have prevented gonadal development and the initiation of laying (Angelier
26 and Chastel, 2009; Maney et al. 1999; Small et al. 2007). On egg day three of the
27 bromocriptine experiment, injections were carried out immediately after blood sampling
28 (see section 2.2).

1

2 *2.4. Data analysis*

3 Data were first examined for normality, outliers, collinearity and interactions between
4 explanatory variables. Both hormones showed deviations from normality, which was
5 improved with log transformation. Log transformed data are described using median
6 and interquartile range; otherwise data are stated as mean \pm standard error.
7 Repeatability was calculated using previously described methods (Lessells and Boag,
8 1987). Since there were no statistical differences in the results found using mass alone
9 or the residuals of a regression of mass by tarsus, mass alone was used as the
10 measure of condition in all relevant analyses. For hormone analyses, only clutches
11 equal or greater to the day the blood sample was taken were included (≥ 3 eggs day 3
12 and experimental breeding, ≥ 2 eggs for day 2, ≥ 4 eggs for day 4). Several females laid
13 clutches larger than those normally observed in the wild (2-7 eggs; Zann, 1996). Since
14 clutch sizes larger than 7 are 'atypical' under normal breeding conditions, analyses were
15 run including and excluding these data. Results from both datasets are presented when
16 the model outcomes differed, otherwise results include larger than normal clutch sizes.
17 For the bromocriptine experiment we predicted individual increases in clutch size in
18 response to the treatment, specifically those greater than the range observed in free-
19 living birds.

20 Pre-breeding and simple breeding comparisons (excluding clutch size; see
21 below) were conducted using ANOVA or ordinary least squares regression. To examine
22 females through treatment and time (i.e. between pre-breeding and breeding; between
23 control breeding and bromocriptine breeding), we used linear mixed effects models for
24 repeated measures with individual female as a random factor, carried out in the
25 statistical package 'nlme' in R 2.12.2 (Pinheiro et al., 2011; R Core Development Team,
26 2011). This experimental and statistical design allowed us to make intra-individual
27 comparisons of the effects of treatment, so that treated females were compared to
28 themselves under the untreated breeding conditions (in addition to retaining a vehicle
29 only control group for bromocriptine, see section 2.3). For each stage, a small subset of
30 females did not provide sufficient plasma for both hormone assays, failed to breed, or

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

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

14

15 **3. Results**

16 *3.1. Relationship between pre-breeding LH, PRL and measures of body condition*

17 There were no significant relationships between pre-breeding mass or
18 hematocrit, i.e. measures of body condition, and pre-breeding LH ($F_{1,66} = 0.288$, $P =$
19 0.594 and $F_{1,66} = 0.128$, $P = 0.722$, respectively), or pre-breeding PRL ($F_{1,75} = 0.427$, P
20 $= 0.516$; $F_{1,75} = 3.729$, $P = 0.057$, respectively; Table 1). However, pre-breeding PRL
21 was weakly, but significantly and positively correlated with pre-breeding LH ($F_{1,65} =$
22 4.272 , $r^2 = 0.05$; $P = 0.043$), including after removing values at the detection limits of the
23 assay ($F_{1,55} = 3.46$, $r^2 = 0.091$; $P = 0.013$; Fig. 1).

24

25 *3.2. Relationships between pre-breeding LH and PRL, and breeding hormone levels* 26 *and reproductive traits*

27 Compared to pre-breeding levels, LH was significantly higher during the 3-egg
28 stage in breeding females (estimate for effect of breeding stage on LH \pm S.E.: $0.098 \pm$
29 0.051 ng/mL, $df = 31$, $t = 2.38$, $P = 0.024$; Intercept: 0.265 ± 0.039 , $df = 40$, $t = -9.77$, P
30 < 0.001 ; Table 1). Furthermore, individual variation in LH was repeatable between pre-

1 breeding and breeding stages ($R = 0.51$; 95% CI = 0.25, 0.77; $P < 0.002$). Pre-breeding
2 LH was negatively correlated with laying interval after controlling for the time elapsed
3 between pre-breeding blood sampling and subsequent pairing - females with higher pre-
4 breeding LH had shorter intervals between pairing and laying of the first egg ($F_{2,31} =$
5 15.52, $P < 0.001$; Fig. 2). However, pre-breeding LH was not significantly correlated with
6 either mean egg mass ($F_{2,30} = 1.66$, $P = 0.207$) or clutch size (Likelihood-ratio test: $\chi^2 =$
7 0.011, $df = 1$, $P = 0.915$) of the subsequent breeding attempt.

8 Breeding PRL levels at the 3-egg stage were markedly and significantly higher
9 than pre-breeding levels (Estimate for effect of breeding stage on PRL \pm S.E.: $180 \pm$
10 24ng/mL, $df = 38$, $t = 19.17$, $P < 0.001$; Intercept: 23.07 ± 2.03 , $df = 41$, $t = 37.21$, $P <$
11 0.001; Table 1). However, in contrast to LH, individual PRL levels were not repeatable
12 between pre-breeding and breeding stages ($P > 0.90$). Log laying interval, egg mass,
13 and clutch size were all independent of pre-breeding PRL levels ($P > 0.10$ in all cases).

14

15 *3.3. Relationships between breeding LH, PRL and reproductive traits*

16 Mean egg mass was significantly and positively correlated with body mass at
17 pairing ($F_{1,39} = 5.72$, $P = 0.022$), but not laying interval ($F_{1,39} = 1.29$, $P = 0.264$). In
18 contrast, clutch size was independent of mass at pairing (Likelihood-ratio test: $\chi^2 =$
19 0.873, $df = 1$, $P = 0.350$), but negatively correlated with laying interval (Likelihood-ratio
20 test: $\chi^2 = 9.234$, $df = 1$, $P = 0.002$). Neither egg mass or clutch size was significantly
21 correlated with individual variation in breeding plasma LH ($P > 0.15$ for both). Breeding
22 plasma PRL on egg day 3 was significantly correlated with variation in mean egg mass
23 ($F_{1,37} = 5.38$, $P = 0.026$) and clutch size ($\chi^2 = 9.17$, $df = 1$, $P = 0.002$; Fig. 3A), but these
24 effects were inconsistent and skewed by several influential data points (i.e. high
25 leverage points from clutch sizes outside the range normally observed in the wild [>7
26 eggs; Zann 1996]). Within the normal range of clutch sizes, both mean egg mass and
27 clutch size were independent of variation in breeding PRL at day 3 of egg-laying ($F_{1,34} =$
28 0.004, $P = 0.950$ and $\chi^2 = 0.227$, $df = 1$, $P = 0.634$, respectively; Fig. 3A). Similarly, in
29 the follow-up study, variation in clutch size was independent of variation in breeding

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

3

4 *3.4. LH, PRL, and reproductive traits for bromocriptine treatment breeding*

5 Luteinizing hormone levels decreased significantly between the control and
6 bromocriptine breeding attempts (estimate for effect of breeding attempt on LH \pm S.E.: -
7 0.133 ± 0.029 ng/mL, $df = 31$, $t = -3.20$, $P = 0.003$; Intercept: 0.32 ± 0.045 , $df = 34$, $t =$
8 7.46 , $P < 0.001$; Table 1), but this effect was not different for the control group or either
9 treatment (Breeding attempt *Treatment; Likelihood-ratio test: $\chi^2 = 1.56$, $df = 2$, $P =$
10 0.460). Similarly, hematocrit dropped significantly for the bromocriptine breeding
11 attempt (estimate for effect of Breeding attempt on hematocrit \pm S.E.: -0.03 ± 0.01 , $df =$
12 31 , $t = -5.30$, $P < 0.001$; Intercept: 0.481 ± 0.001 , $df = 34$, $t = 75.86$, $P < 0.001$; Table 1),
13 a change that also did not differ between control or treatment groups (Breeding attempt
14 *Treatment; Likelihood-ratio test: $\chi^2 = 0.51$, $df = 2$, $P = 0.776$).

15 Prolactin levels were not significantly different between the control and
16 bromocriptine breeding attempts (estimate for effect of breeding attempt on PRL \pm S.E.:
17 -1.64 ± 3.65 ng/mL, $df = 31$, $t = -0.22$, $P = 0.824$; Intercept: 197.26 ± 7.31 , $df = 34$, $t =$
18 139.82 , $P < 0.001$; Table 1). There were no differences in PRL by treatment group
19 (Treatment; Likelihood-ratio test: $\chi^2 = 2.93$, $df = 2$, $P = 0.230$), nor any interaction
20 between breeding attempt and treatment (Breeding attempt *Treatment; Likelihood-ratio
21 test: $\chi^2 = 1.12$, $df = 2$, $P = 0.571$). In fact, individual PRL levels between the control and
22 bromocriptine treatment breeding attempts were repeatable ($R = 0.54$; 95% CI = 0.28-
23 0.79 ; $P < 0.001$).

24 Clutch size also was not significantly different between the control and
25 bromocriptine breeding attempts (estimate for effect of breeding attempt on clutch size \pm
26 S.E.: -0.32 ± 0.90 eggs, $df = 32$, $t = -1.90$, $P = 0.07$; Intercept: 5.69 ± 0.20 , $df = 35$, $t =$
27 48.27 , $P < 0.001$; Table 1), and there were no interactions between breeding attempt
28 and treatment (Breeding attempt *Treatment; Likelihood-ratio test: $\chi^2 = 5.27$, $df = 2$, $P =$

1 0.072). Like PRL, clutch size showed individual repeatability between the control and
2 bromocriptine breeding attempts ($R = 0.66$; 95% CI = 0.46-0.86; $P < 0.001$).

3 4 3.5. Changes in PRL, LH, and clutch size between control and experimental breeding 5 attempts

6 Since there was no effect of treatment on PRL or clutch size between the control
7 and bromocriptine breeding attempts, we pooled treatment groups from the
8 experimental breeding for further analyses. As in the control breeding attempt, clutch
9 size was independent of plasma PRL for the experimental breeding ($\chi^2 = 0.519$, $df = 1$,
10 $P = 0.471$; Fig. 3B), including with clutch sizes larger than the range typically observed
11 in the wild ($\chi^2 = 0.135$, $df = 1$, $P = 0.713$). However, individual *changes* in PRL levels
12 between a female's control and experimental breeding attempts were significantly,
13 negatively correlated with individual *changes* in clutch size. This relationship remained
14 significant including ($\chi^2 = 4.116$, $df = 1$, $P = 0.043$) or excluding ($\chi^2 = 4.425$, $df = 1$, $P =$
15 0.035) two influential data points for which we had only a single observation for a given
16 change in clutch size. No such relationship was found for changes in PRL and changes
17 in egg mass ($F_{1,34} = 2.051$, $P = 0.163$), changes in PRL and changes in LH ($F_{1,30} =$
18 0.215 , $P = 0.647$), or changes in LH and clutch size ($\chi^2 < 0.001$, $df = 1$, $P = 0.979$) or
19 egg mass ($F_{1,30} = 0.345$, $P = 0.561$) between the control and experimental breeding
20 attempts.

21 22 4. Discussion

23 In this study we investigated individual variation in pre-breeding and breeding hormone
24 (PRL and LH) levels in relation to variation in reproductive traits (timing of laying, egg
25 mass, clutch size), specifically to test the hypothesis that variation in circulating PRL
26 levels mediates clutch size variation via the inhibition of LH (Haywood, 1993; Lea et al.,
27 1981; Meijer et al., 1990; Sockman et al., 2006). Clutch size was independent of
28 variation in pre-breeding PRL or LH, although pre-breeding LH was negatively
29 correlated with the time between pairing and the onset of egg-laying. We also found no
30 evidence for any inverse relationships between plasma PRL and plasma LH levels

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

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

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

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

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

1 hormones, and how they changed over time, by tracking individual hormonal profiles
2 through the transition between pre-breeding and breeding states. Breeding LH levels
3 were moderately though significantly higher than pre-breeding levels, and were
4 repeatable between pre-breeding and breeding states. In contrast, plasma PRL levels
5 increased dramatically (as high as 27 fold) between pre-breeding and egg day 3, and
6 PRL levels on egg day 3 were independent of pre-breeding PRL. Although LH levels on
7 day three were probably beginning to decline (based on rapid decreases in estradiol
8 around this time; Williams et al., 2005), our data still suggest an uncoupling of the
9 positive correlation between PRL and LH that we observed in pre-breeding females. An
10 uncoupling of these two hormones over time does not support the idea of a systemic
11 inhibitory effect of PRL on LH, since in our study both hormones increase with breeding,
12 yet vary independently between pre-breeding and breeding states. Accordingly, we also
13 found no significant relationship between breeding levels of PRL and LH. Furthermore,
14 while experimental bromocriptine treatment had no effect on circulating PRL (discussed
15 below), we again found no evidence for an inhibitory effect of PRL on LH in our
16 experimental breeding. Though correlational, the lack of empirical support for an
17 inhibitory effect of PRL on LH in this study, as well as in other passerines (Buntin et al.,
18 1999; Meier and Dusseau, 1968; Small et al., 2007), raises questions about the
19 universality of the PRL-dependent control of LH in the current mechanistic hypothesis,
20 and its applicability in this taxon.

21 In contrast to previous studies on mammals (Bridges and Ronsheim, 1990;
22 Palestine et al., 1987) and some avian species (Angelier et al., 2006; Jouventin and
23 Mauget, 1996; Reddy et al., 2007) we found that bromocriptine treatment had no effect
24 on circulating PRL levels in zebra finches for either the low or high dose groups, nor did
25 we observe a treatment effect on clutch size between the control and experimental
26 breeding. While a range of bromocriptine doses have been employed in birds, from as
27 low as $14 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (Reddy et al., 2007) to as high as $10,000 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (Badyaev
28 and Duckworth, 2005), our doses (low: $333 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$; high: $3,333 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) are
29 comparable to those successfully employed in other avian species (Angelier et al.,
30 2006: $1,500 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$; Jouventin and Mauget, 1996: $4,167 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) and

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

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

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

3 While any PRL-based mechanism for clutch size determination does not appear
4 to involve an absolute inhibitory threshold at the scale of the population, individual
5 differences in either the rate of increase or in the inhibitory threshold (relative PRL level
6 for inhibition for a given breeding attempt) remain plausible alternatives to, or
7 modifications of, the mechanistic model in its current form (Meijer et al., 1990; Williams,
8 2012b, p. 186). The only evidence we found to support a link between PRL and clutch
9 size was a negative relationship between individual *changes* in PRL between the control
10 and experimental breeding and individual *changes* in clutch size. If this finding is robust,
11 the fact that changes in PRL between breeding attempts were not associated with
12 changes in LH, nor were changes in LH associated changes in clutch size, may imply
13 downstream regulatory effects of PRL (e.g. at the level of the ovary). Although
14 speculative, this hypothesis is supported by work demonstrating the presence of PRL
15 receptors in ovarian follicles (Ohkubo et al., 1998), which can directly inhibit the effects
16 of follicle-stimulating hormone (FSH) and LH on, as well as estrogen and progesterone
17 secretion from, the avian ovary (Hrabia et al., 2004; Li and Yang, 1995).

18 Studying avian clutch size determination by looking at individual co-variation in
19 PRL and egg number may suggest more biologically-relevant alternatives to the
20 mechanistic hypothesis in its current form (Haftorn, 1981; Haywood, 1993; Meijer et al.,
21 1990), a hypothesis we found no support for in this study. Further experimental work
22 successfully uncoupling PRL from clutch size is necessary to reinforce this conclusion.
23 If the hormonal regulatory control of clutch size is superimposed upon individual
24 variation in downstream effectors (e.g. receptor expression in the ovary), repeated
25 measurements of individuals through time, as conducted in this study, have the benefit
26 of eliminating at least a portion of these potentially confounding effects, which might
27 bring questions about the endocrine control of this key life history trait into greater focus.
28 At present though, it seems most parsimonious to assume that the putative relationship
29 between circulating PRL early in egg-laying and clutch size simply reflects a temporal
30 coincidence, and that the increase in PRL at this time is functionally associated with

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

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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

^aMass, hematocrit, mean egg mass and clutch size values are mean ± standard error

^bLuteinizing hormone (LH) and prolactin (PRL) given as median and interquartile range

^cControl 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:

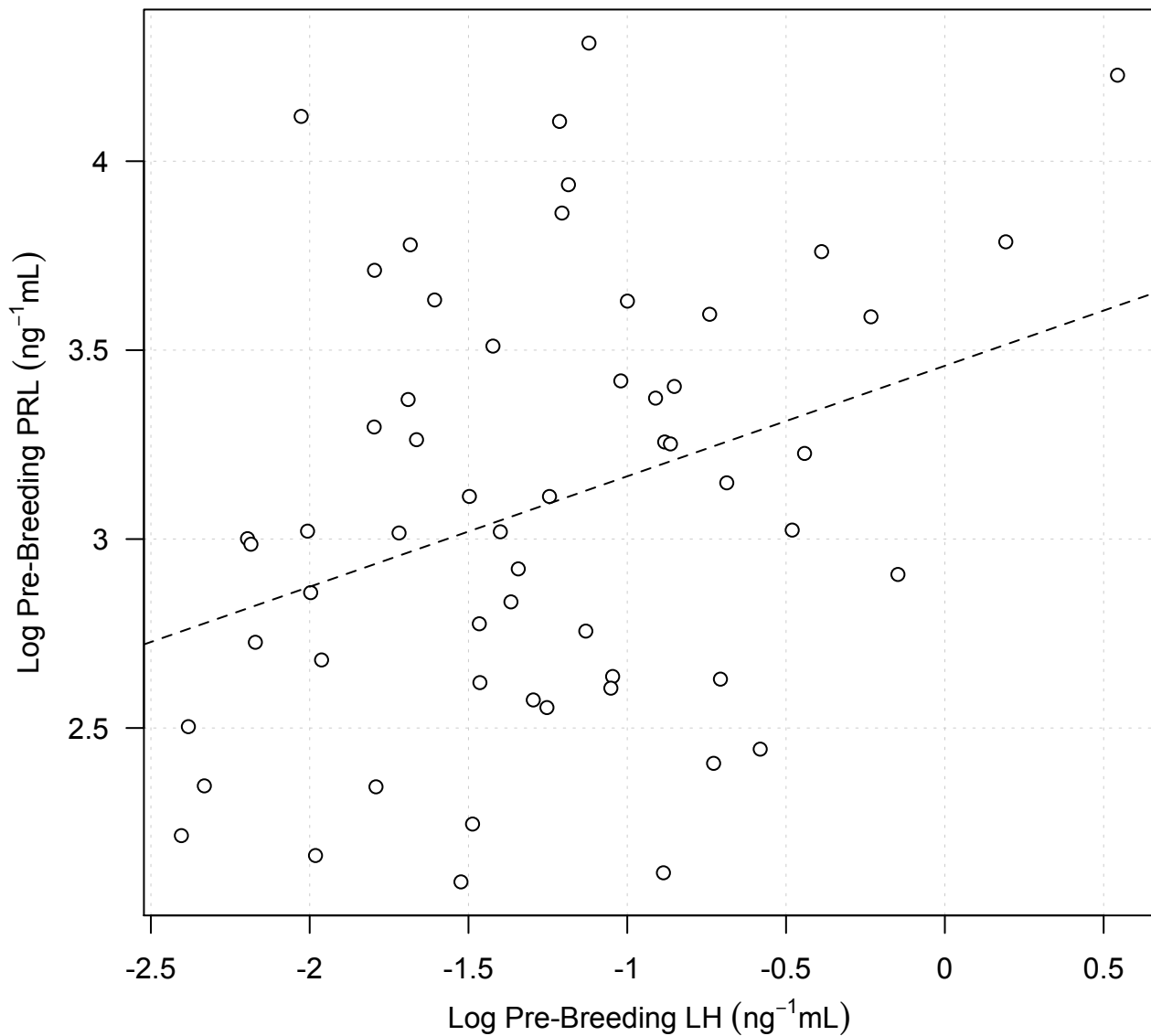
Figure 1. Relationship between pre-breeding log prolactin (PRL) and pre-breeding log luteinizing hormone. Correlation between these two traits was significant, including after removing values at the detection limits of the assay ($F_{1,55} = 3.46$, $r^2 = 0.091$; $P = 0.013$).

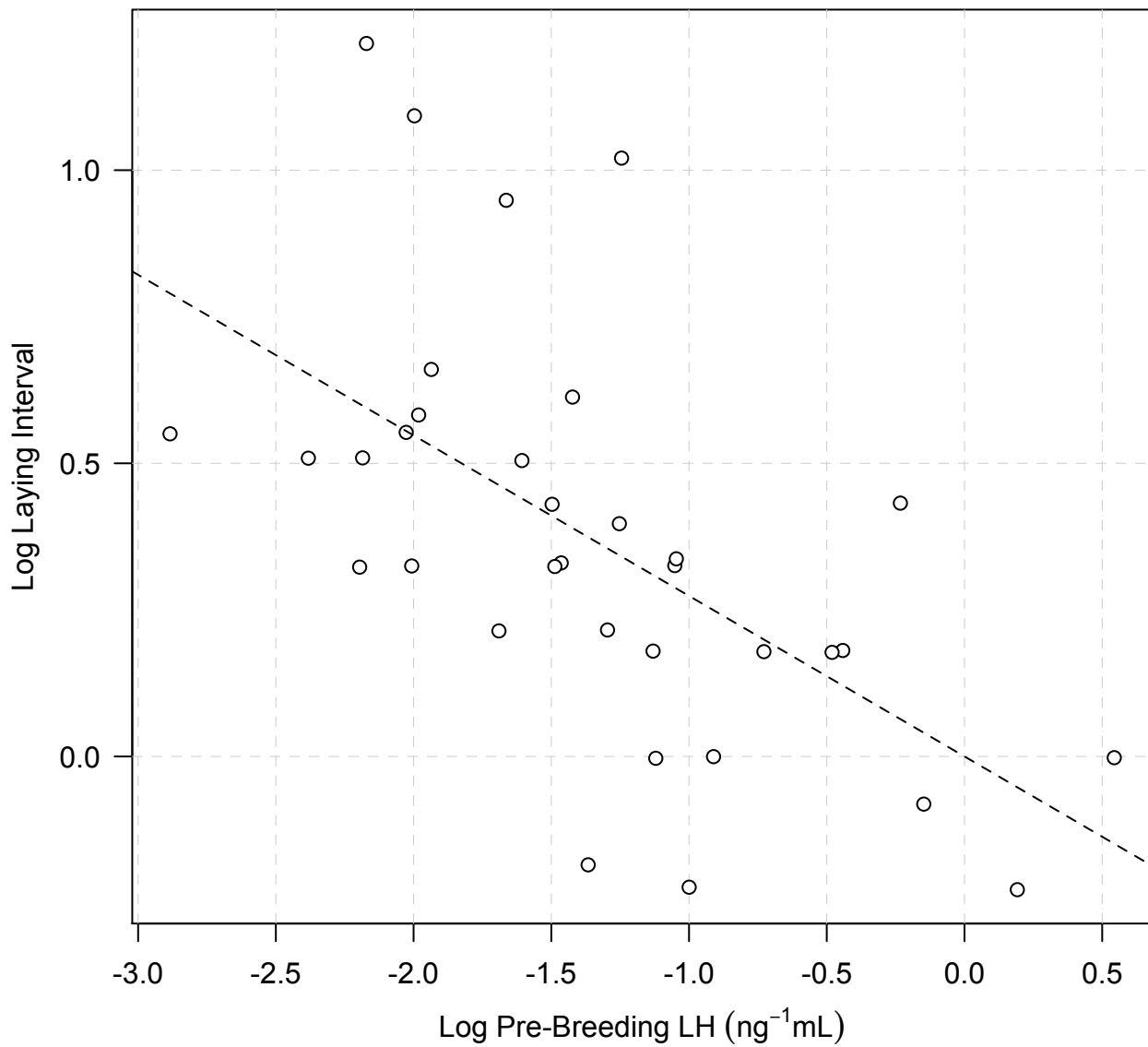
Figure 2. Relationship between pre-breeding luteinizing hormone (LH) and the interval between pairing and the first egg in subsequent pairing. Females were paired roughly two weeks following pre-breeding blood sampling, and the relationship between log LH and log laying interval was significant ($F_{2,31} = 15.52$, $P < 0.001$), 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: $P = 0.471$).

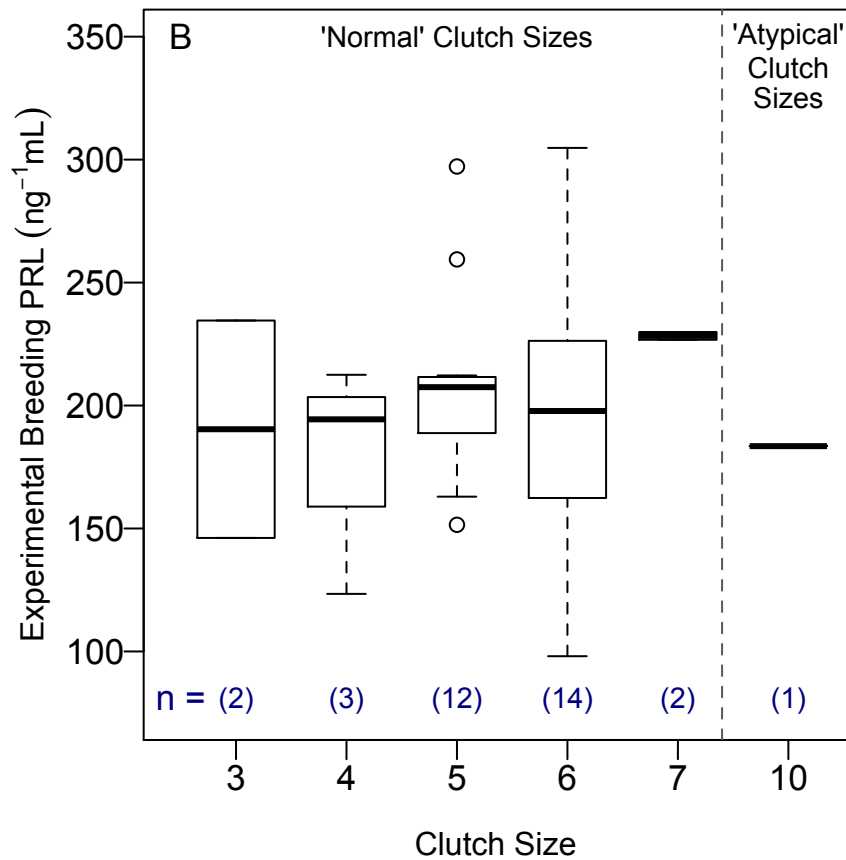
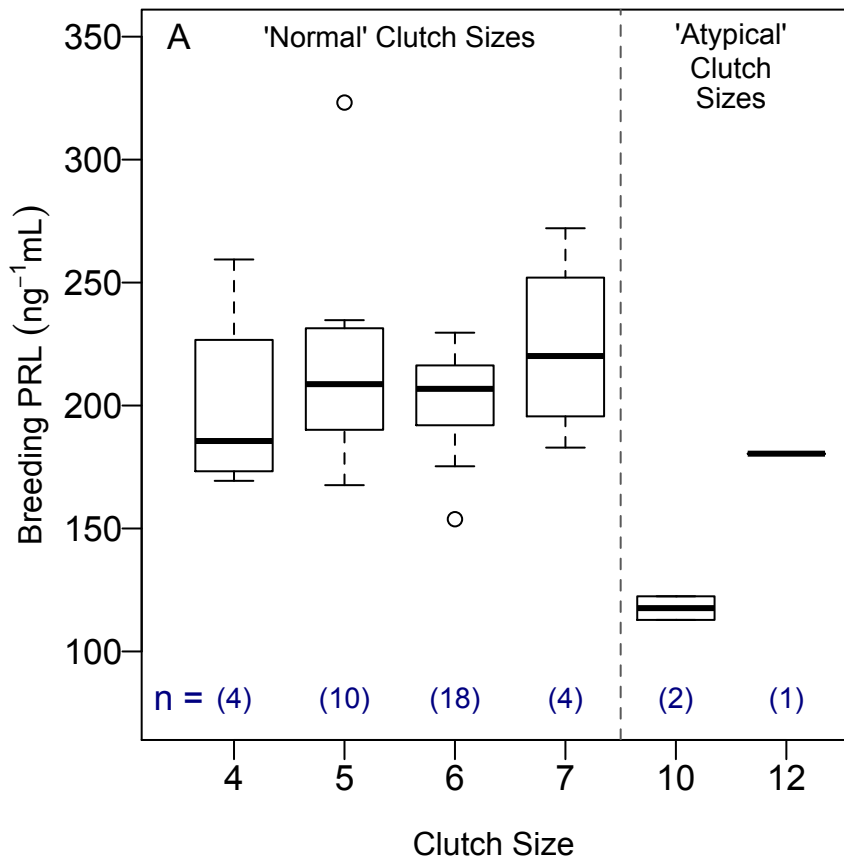
Figure 4. Change in breeding prolactin (PRL) and clutch size between control and experimental breeding attempts. The relationship between these two traits was significant including ($P = 0.035$) or excluding ($P = 0.043$) the two clutches for which there was only one observation (decreases in four and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

Figures





Figures



Figures

