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Uncoupling clutch size, prolactin, and luteinizing hormone using experimental egg removal

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Abstract

Clutch size is a key avian fitness and life history trait. A physiological model for clutch size determination (CSD), involving an anti-gonadal effect of prolactin (PRL) via suppression of luteinizing hormone (LH), was proposed over 20 years ago, but has received scant experimental attention since. The few studies looking at a PRL-based mechanistic hypothesis for CSD have been equivocal, but recent experiments utilizing a pharmacological agent to manipulate PRL in the zebra finch (*Taeniopygia guttata*) found no support for a role of this hormone in clutch size determination. Here, we take a complementary approach by manipulating clutch size through egg removal, examining co-variation in PRL and LH between two breeding attempts, as well as through experimentally-extended laying. Clutch size increased for egg removal females, but not controls, but this was not correlated with changes in PRL or LH. There were also no differences in PRL between egg removal females and controls, nor did PRL levels during early, mid- or late-laying of supra-normal clutches predict clutch size. By uncoupling PRL, LH and clutch size in our study, several key predictions of the PRL-based mechanistic model for CSD were not supported. However, a positive correlation between PRL levels late in laying and days relative to the last egg (clutch completion) provides an alternative explanation for the equivocal results surrounding the conventional PRL-based physiological model for CSD. We suggest that females coordinate PRL-mediated incubation onset with clutch completion to minimize hatching asynchrony and sibling hierarchy, a behavior that is amplified in females laying larger clutches.

Keywords: clutch size; prolactin; luteinizing hormone; avian reproduction; egg removal

1. INTRODUCTION

Clutch size is one of the most important and well-studied avian life history traits, setting the upper limit on the number of young fledged during a reproductive event (Charmantier et al., 2006; McCleery et al., 2004; Rockwell et al., 1987). Yet despite considerable interest across a range of disciplines, the physiological and endocrine mechanisms involved in the termination of laying and variation in clutch size remains poorly understood (Klomp, 1970; Ryan et al., 2014; Sockman et al., 2006; Williams, 2012). Current mechanistic hypotheses for avian clutch size determination suggest an anti-gonadal effect of prolactin (PRL) after reaching some threshold concentration early in laying (i.e., 2-4 days after the first egg is laid in several species), possibly through the inhibition of luteinizing hormone (LH) secretion from the anterior pituitary (Haywood, 1993a; Meijer et al., 1990; Williams, 2012). Under this scenario, endogenous increases in circulating PRL in response to photoperiod (Dawson and Goldsmith, 1985; Haftorn, 1981; Meijer et al., 1990) or tactile stimulation from the eggs during incubation (El Halawani et al., 1984; Hall and Goldsmith, 1983) would then influence how rapidly PRL levels reach the threshold for follicular inhibition (see Williams, 2012 Fig. 5.16b). If accurate, this mechanistic model could help explain broad patterns of variation in clutch size (e.g. the ubiquitous seasonal decline in clutch size with laying date in single-brooded species; Meijer et al., 1990; Rowe et al., 1994).

The involvement of PRL in incubation (El Halawani et al., 1984; Lea et al., 1981; March et al., 1994) and chick rearing (O'Dwyer et al., 2006; Angelier and Chastel, 2009; Miller et al., 2009) is relatively well-established (but see Adkins-Regan, 2005). In contrast, data supporting a mechanistic role for PRL in clutch size determination has been

derived from broad temporal associations between onset of incubation, clutch size, and plasma PRL, rather than from direct experimental evidence. In the few studies attempting to experimentally manipulate the *hormonal* component of the putative PRL-clutch size relationship, results were equivocal or contradictory. Sockman et al. (2000) found weak support for a negative association between plasma PRL levels early in laying and final clutch size in the American kestrel (*Falco sparverius)*, but experimental administration of ovine PRL had no effect on clutch size. Bromocriptine, a D2-receptor agonist often used for lowering PRL, failed to affect either clutch size or plasma PRL levels in zebra finches (*Taeniopygia guttata*; Ryan et al., 2014). However, this latter study found no support for a relationship between plasma PRL (measured at days 2-4 of egg-laying) and clutch size, nor was there evidence for an inhibitory, anti-gonadal, effect of PRL on LH (Ryan et al., 2014).

Here, we take a complementary approach to that reported in Ryan et al. (2014) by using egg removal to manipulate clutch size, i.e., the *phenotypic* component of the putative PRL-clutch size relationship, in captive-breeding zebra finches. We then analyze the correlated responses in plasma PRL and LH that would be predicted if these hormones are functionally linked to clutch size determination. Taking repeated individual measurements of PRL and LH between two breeding attempts allows us to study individual response in the form of the slope and intercept, referred to as 'physiological reaction norms' (sensu Williams, 2008). We also generate supra-normal clutch sizes through egg removal, which allows us to take multiple individual measurements of PRL and LH through an extended period of egg-laying, providing information about hormone dynamics unavailable from single point measurements alone. Based on current models

for clutch size determination (reviewed in Sockman et al., 2006; Williams, 2012, Chapt. 5) and previous work stating that clutch size determination in *T. guttata* occurs invariably on the day the 3rd egg is laid (Haywood, 1993a), we had a series of *a priori* predictions. We predicted: a) that egg removal females would have lower PRL and higher LH early in laying (i.e., the day the 3rd egg is laid), and; b) a negative relationship between PRL on the day the 3rd egg is laid and final clutch size, regardless of individual variation in response to egg removal (Williams and Miller, 2003). Alternatively, if elevated PRL remains the predominant mechanistic determinant of clutch size, but the timing of follicular inhibition itself varies with clutch size and is delayed by egg removal, we predicted: c) a negative relationship between clutch size and PRL at later time points (the days the 10th or 17th eggs were laid, for females who responded to egg removal by laying supra-normal clutches). Finally, by partitioning individual endocrine signatures into a slope and intercept, we predicted that females with the most rapid increases in PRL (e.g. between the 3rd and 10th eggs) would reach 'threshold' levels for follicular inhibition sooner, and so would lay fewer additional eggs compared to females exhibiting more gradual increases in PRL during this time (Williams, 2012). We predicted similar, though inverted, relationships between LH and clutch size, based on the proposed inhibitory effect of PRL on LH during egg laying.

2. MATERIAL AND METHODS

2.1. Animal care and general 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 spp*. red and white millet, 1:1, 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 per day of an egg food supplement (20.3 % protein, 6.6 % lipid) between pairing and clutch completion. Prior to the experiment, all birds were housed in same-sex cages (61 cm x 46 cm x 41 cm) but were not visually or acoustically isolated from birds of the opposite sex. For this study, birds were bred at least twice, so that intra-individual comparisons could be made between 'Breeding 1' and 'Breeding 2' breeding attempts. Prior to Breeding 1, all individuals were 4-10 months of age, had been successfully bred at least once, and were subsequently paired (~3 months later) with the same individual of the opposite sex to minimize variation in investment based on perceived mate quality. A large subset of the females used in Breeding 1 served as controls for another experiment (see Ryan et al., 2014 for a detailed analysis). Breeding pairs were housed individually in cages (61 cm x 46 cm x 41 cm), each with an external nest-box (11.5 cm x 11.5 cm x 11.5 cm). Females were weighed (± 0.1 g, initial mass) at the time of pairing, just prior to blood sampling, and at clutch completion. 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).

2.2. Experimental protocol

During Breeding 1, eggs were immediately returned to the nest after weighing, i.e., there was no egg removal or manipulation of clutch size. For Breeding 2, females

were assigned either to 'Egg Removal' (ER) or untreated 'Control' (CTL) groups (Fig. 1). For ER females, eggs 1 through 14 were removed from the nest on the day they were laid to induce continued laying and supra-normal clutch size. To look at the effect of egg contact after continued laying, eggs 15 and onwards for ER females were no longer removed, but were allowed to accumulate normally in the nest until clutch completion (Fig. 1). For untreated CTL females in Breeding 2, eggs were immediately returned to the nest in which they were laid after weighing (exactly as for Breeding 1). In all cases, a clutch was considered complete when no additional eggs were produced over two consecutive days. 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).

2.3. Hormone assays

For Breeding 1, females were blood sampled (max. 1 % body weight, from the brachial vein) on the day the 3rd egg was laid. Egg day 3 (~6 hours after lights on) was selected based on Haywood (1993a). The same timing was used for blood sampling of both the ER and CTL groups in Breeding 2. However, for Breeding 2, egg removal allowed us to take additional blood samples on the days the 10th and 17th eggs were laid (or at clutch completion if this occurred within one day of the 10th or 17th egg). These three blood samples were separated by roughly 7 day intervals, and allowed us to look at changes in PRL and LH levels in the absence of eggs in the nest within: a) normal (2-7 eggs), and; b) supra-normal clutch sizes (10+ eggs). By leaving the 15th, 16th, and 17th eggs, and blood sampling on egg day 17, we were also able to look at PRL and LH under

a third condition: well-beyond the normal clutch size, but still with only 3 eggs in the nest (Fig. 1). The third sampling condition allowed for a comparison of hormone levels between ER females laying ≥ 17 eggs, CTLs, and Breeding 1 where all females had only three eggs in the nest at the time of blood sampling and ultimately produced different numbers of eggs. Blood sampling was carried out between 11.30 and 13.30, based on the postulated temporal window previously described (Haywood, 1993a), and to control for any potential circadian fluctuations in hormone levels. Blood was thereafter centrifuged at 5000 *g* for 5 minutes, and plasma was stored at –20 °C until required for hormone assays.

Plasma immunoreactive prolactin (PRL) was determined using a radioimmunoassay for recombinant-derived starling (*Sturnus vulgaris)* PRL described by Bentley et al. (1997). Samples were measured in duplicate in a single assay, diluted 1 in 3 in assay buffer. 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 $ng \cdot mL^{-1}$, after correcting for dilution. 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 range assayed. Luteinizing hormone (LH) was measured using a micro-modified version of a previously described radioimmunoassay (Sharp et al., 1987). Samples were run in a single assay, in duplicate when sample volume permitted (>90 % of all samples), diluted 1 in 2.3 in RIA buffer. Assay sensitivity was determined as described above, with a lower limit of 0.087 $ng \cdot mL^{-1}$, after correcting for dilution. Since the inclusion of the small number of samples falling below the detection limits of the assays had no qualitative effect on the findings, all samples falling within the standard

curve were used and presented. The intra-assay coefficient of variation for this 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 range assayed.

2.4. Statistical analyses

Data were first examined for normality, outliers, collinearity and interactions between explanatory variables (Zuur et al., 2010). Only LH showed deviations from normality, which was corrected by log transformation. 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 a covariate in all relevant analyses. Mass was only included when significant or when affecting the significance of other covariates. Simple comparisons (excluding clutch size; see below) were conducted using ANOVA or ordinary least squares regression. For repeated measures analysis we used linear mixed effects models with individual female as a random factor using the statistical package 'nlme' in R 2.12.2 (Pinheiro et al., 2011; R Core Development Team, 2011). Breeding 1 starting sample size was 44 pairs, while Breeding 2 sample size was 39 pairs (27 ER pairs; 12 CTL pairs). In both breeding attempts, a subset of females were not available or failed to breed, laid fewer than 3 eggs (i.e., no hormone values for egg day three), or did not provide sufficient plasma for both hormone assays. Also, since individual response to egg removal treatment varied, only a subset of females who laid more than 3 eggs were still laying for the egg 10 blood sample, and only a subset who laid more than 10 egg

were still laying for the 17 egg blood sample. As a result, model degrees of freedom vary, particularly for between treatment comparisons.

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 variance structure to account for underdispersion (GLMMs carried out using R package "MASS"; Venables and Ripley 2002). Analyses of egg mass was conducted on mean egg mass per clutch. The relationship between PRL or LH and time in days relative to clutch completion was analyzed using linear mixed effect models with hormone levels as the response variable. All analyses were followed with standard model validation procedures to test the assumptions of the test employed. Statistical analyses are presented in the standard forms as follows: linear regression, F_{df}, P-value, R^2 (when significant); general linear regression, χ^2_{df}, N (number of observations), P-value; linear mixed effects and general linear mixed effects models, effect size (β) and 95 % confidence interval, t_{df} and P-value for significant effects or non-significant effects of interest, and χ^2_{df} and P-value for non-significant effects. Where multiple explanatory variables were found to affect a dependant variable, p-values are given for the full model including all significant variables (ANCOVA).

3. RESULTS

3.1. Clutch size, mass, PRL and LH for the first breeding attempt

During Breeding 1, females that were later assigned to either CTL or ER treatment groups for Breeding 2 did not differ in laying interval ($F_{1,35} < 0.01$, $P = 0.987$), mass at pairing ($F_{1,35} = 0.22$, $P = 0.644$), or clutch size ($\chi^2_1 = 0.77$, $N = 37$, $P = 0.378$).

There were also no treatment group differences in day 3 plasma PRL or LH at this time ($F_{1,32} < 0.01$, $P = 0.977$ and $F_{1,32} = 0.14$, $P = 0.713$, respectively). Clutch size during Breeding 1 was not associated with day 3 PRL ($\chi^2_1 = 0.23$, $N = 36$, $P = 0.634$) or LH ($\chi^2_1 = 0.49$, $N = 39$, $P = 0.484$), neither was it correlated with mass at the time of pairing nor mass lost during laying ($\chi^2_1 < 0.01$, $N = 39$, $P = 0.943$ and $\chi^2_1 = 0.85$, $N = 38$, $P = 0.357$, respectively; Table 1).

3.2. Changes in clutch size and mass between the first and second breeding attempts

Between Breeding 1 and Breeding 2, changes in clutch size for ER females and CTLs differed significantly in response to treatment (treatment × breeding attempt interaction: $\chi^2_1 = 30.62$, $P < 0.001$; including mass at pairing as a covariate). Clutch size increased significantly for ER females ($\beta = 7.38 \pm 2.24$ eggs, $t_{33} = 5.34$, $P < 0.001$), but did not change for CTLs ($\beta = -0.63 \pm 0.72$ eggs, $t_{33} = -0.78$, $P = 0.442$). However, individual ER females also exhibited marked variation in response to egg removal, laying from 3 *fewer* to 15 *additional* eggs in their Breeding 2 clutch compared with their Breeding 1 clutch; CTLs laid from 3 fewer to 1 additional egg (Fig. 2A). Part of the variation in Breeding 2 clutch size after controlling for treatment could be explained by a positive correlation with Breeding 1 clutch size ($\chi^2_1 = 18.04$, $N = 37$, $P < 0.001$) and egg mass ($\chi^2_1 = 18.37$, $N = 37$, $P < 0.001$). Between Breeding 1 and Breeding 2, body mass increased ($\beta = 0.52 \pm 0.17$ g, $t_{35} = 3.10$, $P = 0.004$) and laying interval decreased ($\beta = -1.9 \pm 0.30$ days, $t_{36} = -4.93$, $P < 0.001$) but there was no effect of treatment for either trait (treatment × breeding attempt interaction, $P > 0.1$ for both). Changes in mass were not

associated with changes in clutch size ($\chi^2_1 = 0.47, N = 28, P = 0.492$), but females with shorter laying intervals tended to lay larger clutches ($\chi^2_1 = 4.25, N = 29, P = 0.039$).

3.3. Changes in PRL and LH between the first and second breeding attempts

Plasma PRL decreased between Breeding 1 and Breeding 2 ($\beta = -34.83 \pm 6.74$ ng·mL^{-1}, $t_{28} = -5.17, P < 0.001$), and while this decrease was nearly twice as large for the ER females (-40.6 ng·mL^{-1} versus -21.5 ng·mL^{-1}) there were no differences by treatment group (treatment × breeding attempt interaction: $\chi^2_1 = 1.43, P = 0.231$; Fig. 2B). Furthermore, changes in PRL between Breeding 1 and Breeding 2 did not correspond to the observed changes in clutch size between the two breeding attempts ($\chi^2_1 = 0.96, N = 29, P = 0.328$). LH decreased for CTL females between the two breeding attempts, but not for ER females. There was a significant treatment × breeding attempt interaction ($\chi^2_1 = 4.84, P = 0.028$), with LH decreasing significantly for CTL ($\beta = -0.167 \pm 0.045$ ng·mL^{-1}, $t_{27} = -2.34, P = 0.027$), but not ER females ($\beta = -0.037 \pm 0.055$ ng·mL^{-1}, $t_{27} = 0.56, P = 0.577$; Table 1). Nevertheless, changes in LH between Breeding 1 and Breeding 2 did not correspond to the observed changes in clutch size between the two breeding attempts ($\chi^2_1 = 0.75, N = 29, P = 0.386$). Furthermore, the magnitude and direction of changes in PRL were not correlated with equivalent changes in LH ($F_{1,27} = 0.01$; $P = 0.755$).

3.4. Clutch size, mass, PRL and LH for the second breeding attempt

For Breeding 2, neither mass at pairing nor laying interval differed between ER and CTL females ($F_{1,35} = 0.07$, $P = 0.790$ and $F_{1,36} = 1.87$, $P = 0.179$, respectively). However, unlike Breeding 1, Breeding 2 clutch size was positively correlated with mass

at pairing ($\chi^2_1 = 5.36, N = 37, P = 0.021$), though this effect did not differ by treatment group (mass x treatment type interaction: $\chi^2_1 = 0.190, N = 37, P = 0.663$). Furthermore, mass lost during laying was unrelated to final clutch size ($F_{2,33} = 0.01$, P = 0.910, controlling for mass at pairing), and only marginally but non-significantly higher for ER females when compared to CTLs ($F_{2,33} = 4.04, P = 0.053$).

While plasma PRL on day 3 of Breeding 2 did not differ between ER and CTL females ($F_{1,32} = 0.960, P = 0.335$), LH during this time was higher for ER females compared to CTLs ($F_{1,32} = 7.07, P = 0.012, R^2 = 0.16$). However, neither PRL nor LH on egg day 3 of Breeding 2 was correlated with final clutch size, controlling for treatment ($\chi^2_1 = 0.72, N = 34, P = 0.396$ and $\chi^2_1 = 0.30, N = 34, P = 0.584$, respectively; Table 1). For ER females, clutch size was also independent of PRL and LH on day 10 ($\chi^2_1 = 1.99$, $N = 19, P = 0.158$ and $\chi^2_1 = 0.12, N = 19, P = 0.732$, respectively) and day 17 ($\chi^2_1 = 3.27$, $N = 9, P = 0.071$ and $\chi^2_1 = 0.80, N = 9, P = 0.371$, respectively), although statistical power in the latter cases was limited.

PRL increased in ER females between egg days 3, 10, and 17 ($\chi^2_2 = 20.35, P < 0.001$; Fig. 3). The increase in PRL between days 3 and 10 and 10 and 17 was confirmed with post hoc Tukey contrasts (adjusted $P < 0.05$ for all contrasts; Fig. 3). In contrast, plasma LH for ER females did not differ significantly between egg days 3, 10 and 17 ($\chi^2_2 = 4.91, P = 0.086$). There was no significant correlation between plasma PRL and plasma LH during breeding ($\chi^2_1 = 0.122, P = 0.726$) even when including sample day ($\chi^2_2 = 0.168, P = 0.919$). To investigate relationships between individual rate of change in plasma PRL and LH within Breeding 2 and its relationship to final clutch size, we calculated individual slopes from the difference in PRL between egg days 3 and 10 and

egg days 10 and 17 (i.e., sloped lines in Fig. 3). Final clutch size was independent of the rate of change in plasma PRL and LH between the two time points nearest to clutch completion (days 3 to 10 or 10 to 17: $\chi^2_1 = 0.447$, $P = 0.504$ for PRL; $\chi^2_1 = 1.07$, $P = 0.301$ for LH). There was also no correlation between the magnitude and direction of change in PRL and LH (using the value for the slope closest to clutch completion: $F_{1,16} = 0.06$, $P = 0.815$).

3.5. Plasma PRL and LH relative to timing of clutch completion

We analyzed plasma PRL and LH for the blood sample closest to the time of clutch completion in relation to days remaining of egg laying (i.e., day 3 sample for clutches of 3-9 eggs, day 10 for clutches of 10-16 eggs, day 17 for clutches >16 eggs). In contrast to clutch size, the number of days to the last egg takes into account that females may skip a lay day once or more prior to actual clutch completion. We found a positive relationship between plasma PRL and the time in days relative to the last laid egg ($\beta = -7.44 \pm 1.89$ ng·mL^{-1} for each day further from clutch completion, $t_{27} = -3.93$, $P < 0.001$; Fig. 4). The positive relationship between PRL and days relative to clutch completion included the significant effect of breeding attempt ($\beta = -14.83 \pm 5.77$ ng·mL^{-1}, $t_{27} = -2.57$, $P = 0.020$), but not treatment group, which was not significant ($\chi^2_1 = 0.09$, $P = 0.761$). Despite the lower plasma LH levels during Breeding 2 described above, LH was unrelated to the number of days remaining until the last laid egg ($\chi^2_1 = 0.08$, $P = 0.771$), controlling for the effect of breeding attempt ($\beta = -0.11 \pm 0.04$ ng·mL^{-1} for Breeding 2, $t_{27} = -2.22$, $P = 0.035$).

4. DISCUSSION

The objective of this study was to experimentally test the PRL-based mechanistic model for clutch size determination in captive-breeding zebra finches, using a complementary approach to that reported by Ryan et al. (2014). Consistent with our predictions, egg removal resulted in significant increases in clutch size, but with considerable individual variability in response to treatment. Changes in clutch size were not predicted by changes in plasma PRL, LH or mass at pairing. PRL decreased between Breeding 1 and 2 for both treatment groups, and LH decreased for CTL but not ER females, but the magnitude and direction of changes in PRL and LH were not correlated with changes in clutch size. Variation in clutch size was not associated with variation in circulating levels of either PRL or LH at the time when follicular inhibition is postulated to occur (the day the 3rd egg is laid; Haywood, 1993a). Although PRL concentrations increased between days 3 and 17 during extended laying of supra-normal clutches, the rate and direction of change in PRL, as well as static levels of PRL and LH on the days the 10^{th} and 17^{th} eggs were laid were all unrelated to final clutch size, again failing to confirm the predictions derived from the mechanistic model. However, plasma PRL levels for the sample taken closest to clutch completion were positively correlated with time in days relative to clutch completion for both breeding attempts and treatment groups. This last finding suggests an alternative explanation for the previously described, but equivocal, support for the PRL-based mechanism for clutch size determination as we discuss below.

Using data from a first breeding attempt (Breeding 1) as a 'baseline' allowed us to examine the co-variation in changes in PRL, LH and clutch size in response to egg removal for individual females during a second breeding attempt (Breeding 2). Tracking

changes in hormone-trait relationships may provide better insight into individually-variable strategies by generating 'physiological reaction norms' (Ryan et al., 2014; Vézina et al., 2006; Williams, 2008). While the majority (84 %) of females responded to egg removal by increasing clutch size, we observed marked individual variability in response. For both breeding attempts all females had access to *ad libitum* feed and a high-protein egg laying supplement, suggesting that our results do not reflect differences in resource availability (Gorman and Nager, 2003; Williams and Miller, 2003). The variability in final clutch size laid in response to egg removal was also not associated with changes in mass at the time of pairing between the two breeding attempts. For Breeding 2, but not Breeding 1, females who were heavier at pairing laid significantly larger clutches, but this does not appear to arise from larger females exhibiting greater response to egg removal, however – the effect of mass on clutch size did not differ by treatment type in Breeding 2, and was unrelated to any of the other parameters examined in this study. Perhaps more importantly, individual variation in response to egg removal (i.e., the number of additional eggs laid during Breeding 2) was predicted by individual variation in Breeding 1 clutch size and egg mass, consistent with individual differences in 'quality' or allocation strategies (Charnov and Krebs, 1974; Hamel et al., 2009; Lescroël et al., 2009) as shown previously for this species (Williams and Miller, 2003). Individual variability in response to egg removal therefore seems to be an extension of the natural variability in clutch size already present in un-manipulated laying zebra finches (Williams, 1996), which is integral to our experimental approach.

The overall declines in plasma PRL observed between Breeding 1 and 2 could reflect age-related declines in PRL-mediated reproductive investment (Angelier et al.,

2007). All birds used in this study were of uniform age and breeding experience, which would explain the consistent declines in PRL among all females. Though non-significant, the greater decrease in PRL for ER females that we observed would be compatible with the PRL-based mechanistic model. More intriguing is the decrease in LH for CTL, but not ER females, between Breeding 1 and 2. Declines in both LH and PRL may be part of an overall decrease in reproductive competence, while the absence of such declines in LH for ER females suggest plasticity in response to the perceived requirements of reproduction. This response to egg removal fits the predictions of the PRL-based mechanistic model for clutch size determination in that LH should remain high until PRL reaches some inhibitory threshold. However, an examination of the magnitude and direction of *individual* changes in PRL, LH, and clutch size revealed no relationship between these traits. Thus, egg removal appears to have exposed latent plasticity by extending the phenotypic range of clutch size, however this uncoupled rather than exaggerated any relationship between clutch size and the putative underlying hormones. The latter finding is inconsistent with a direct role for PRL in clutch size determination. We also found no evidence that individual variation in the magnitude and direction of changes in LH were associated with changes in PRL, as would be predicted by a systemic inhibitory effect of PRL on LH. Other studies have also not found evidence for inhibitory effect of PRL on LH (Buntin et al., 1999; Small et al., 2007), including in breeding female zebra finches (Ryan et al., 2014). If changes in LH precede changes in PRL, or if both hormones are regulated independently (Goldsmith et al., 1984; Sharp et al., 1988), the central role of PRL in clutch size determination via inhibition of LH would again not be supported, but opens the possibility for alternative mechanistic models. While it is

plausible that the individual relationships between PRL and LH described by the conventional model could vary inter-individually *and* intra-individually, for example through individually fluctuating slopes and thresholds through time, such a physiological model will provide limited predictive ability until the factors responsible for driving rate of hormonal change and thresholds are identified. In any case, it does seem likely that relationships between these traits, if associated, would exhibit more consistency within, rather than between individuals (Ryan et al., 2014). However, we did not ultimately observe correlations between PRL, LH and clutch size at the level of the individual that we would expect if these traits were mechanistically linked.

Restricting our analysis to the second breeding attempt (Breeding 2) where we manipulated egg laying, clutch sizes for ER females were significantly larger than those of CTL females, validating our experimental approach and consistent with previous studies in zebra finches (Haywood, 1993a; Williams and Miller, 2003). However, PRL on day 3 did not differ between the two treatment groups even though ER females, on average, went on to lay many more eggs. Despite considerable individual variability in hormone levels on day 3, PRL and LH were both unrelated to clutch size for both egg removal females and controls. Moreover, for supra-normal clutches, PRL and LH on the days the 10^{th} and 17^{th} eggs were laid were also not associated with final clutch size, though the power of our ability to detect effects was limited for the later time points. Millam et al. (1996) reported a negative relationship between PRL measured 17 days into incubation and clutch size in canaries, however this time point included females that had already finished laying. Since the only time when PRL could exert regulatory control over clutch size is between the recruitment and ovulation of the last follicle (Sockman et

al., 2006), and PRL and time spent incubating both increase rapidly near clutch completion (Sharp et al., 1998), findings including females well past laying are likely artifactual (Millam et al., 1996). Supporting this argument and in agreement with the findings of the current study, PRL during the 2nd, 7th, and 12th days of incubation in canaries, when all females were still laying, was unrelated to final clutch size (Millam et al., 1996).

In our study, PRL at several time points (days 3, 10 and 17) through laying of supra-normal clutches were not predictive of final clutch size. However, individuals vary in the timing and magnitude of their endocrine responses to specific breeding stimuli, e.g. white-crowned sparrows (*Zonotrichia leucophrys*) from different populations varied in their rate of plasma LH increase, and timing of brood patch and ovarian development, in response to the same long-day photoperiodic cue (Lewis, 1975; Wingfield et al., 1980). Marked individual differences in the *rate of change* in PRL and LH titres between pre-breeding and breeding females support similar individual variability in our captive-breeding zebra finches (Ryan et al., 2014). Thus, rate of change in hormone responses might be more informative in explaining individual variation in hormone-dependent traits than single, 'static' measurements of hormone titers. Specifically, if PRL or LH inhibit laying by reaching critical upper or lower threshold values respectively, the rate of change in hormone titers may be important signals in clutch size determination, particularly if thresholds are similar between females and/or if rates of change follow relatively predictable trajectories (Williams, 2008). We examined individual differences in the rate of change in PRL and LH between egg days 3 and 10 or days 10 and 17, predicting that the steepest slopes would be associated with the most rapid attainment of

any inhibitory threshold for egg laying (Sockman et al., 2006; Williams, 2012). However, there was no correlation between the changes in PRL and changes in LH through days 3, 10, and 17 of laying. Furthermore, we observed no significant associations between the rate of increase in PRL (or LH decrease) and total clutch size, or between the rate of increase in PRL (or decrease in LH) and the number of additional eggs subsequently laid. Thus, even when considering dynamic *changes* in hormone levels, our results fail to support a role for PRL in clutch size determination, consistent with the other findings of this study.

Carefully designing our experiments around the time postulated to be invariantly linked to the inhibitory signal for clutch size determination in zebra finches (Haywood, 2013; Haywood, 1993a), we found no support for any relationship between circulating levels of PRL and clutch size. This was true for ER females, which laid supra-normal clutch sizes, as well as control females. If, contrary to the conclusions of Haywood (1993a), the inhibitory signal disrupting follicle growth varies temporally in zebra finches as has been postulated in other species (i.e., Haywood, 1993b), a relationship between PRL and clutch size might have been revealed on days 10 or 17, or in the rate of change in PRL between these days. We found no evidence for such a relationship. However, the broad temporal associations between PRL, incubation, and the cessation of laying still warrants an explanation (Haftorn, 1981; Williams, 2012, chap. 5). In free-living zebra finches, incubation starts later for females laying larger clutches (Zann and Rossetto, 1991). Although hatching in captive zebra finches is typically asynchronous (Gilby et al., 2013; Mainwaring et al., 2010), females whose eggs are removed initiate incubation later (Gorman and Nager, 2003). Since incubation can be delayed, and the time between

follicle recruitment and laying is roughly four days (Haywood, 1993a), females, particularly those in the wild, appear to be coordinating incubation with clutch *completion*, rather than clutch *size*. Under this scenario, females nearest to clutch completion *irrespective of clutch size* should show elevated PRL relative to those further away, but because PRL is causally associated with onset of incubation, not clutch size determination *per se*.

Although not one of our original predictions, we examined variation in PRL at the last measured time point prior to clutch completion to test the hypothesis that PRL is coordinated with clutch completion, not clutch size. In individual females, variation in PRL at the last measured time point prior to clutch completion *did* predict the number of days to the cessation of laying, regardless of breeding attempt, treatment group, or final clutch size. A role for PRL in the coordination of onset of incubation with clutch completion in the absence of any effect on clutch size could explain the equivocal results for the PRL-based mechanistic model in previous studies (Millam et al., 1996; Ryan et al., 2014; Sockman et al., 2000). Since PRL measurements are generally taken early on or midway through laying, when clutch completion and development of full incubation is invariably closer for females laying smaller clutches, higher PRL would appear to correspond to fewer total eggs laid. This is not to say that the rate of incubation onset necessarily dictates clutch size, since the last follicle may have been ovulated at the time when both PRL levels and intensity of incubation coincidentally start to increase most rapidly. Rather, to the extent that variation in PRL levels reflect development of incubation behavior, females could be coordinating incubation and clutch completion, two independently regulated processes, perhaps to minimize hatching asynchrony and

sibling size hierarchies (Sockman et al., 2006). Such coordination would be most beneficial to females laying the largest clutches, either naturally or 'artificially' through egg removal, as we observed in our study. Thus it appears that the threads connecting incubation, PRL, egg laying, and clutch size may be intricately woven, but that PRL does not appear to be a simple causal factor in clutch size determination. Tactile stimulation from the eggs is important in the cessation of laying, and has a stimulatory effect on PRL, yet the variability in responses to our egg removal treatment demonstrates that it is not critical in the laying of a normal-sized clutch, nor in the rise in PRL during laying. For nearly all ER females, PRL slowly increased despite the absence of eggs, and even with egg removal some birds stopped laying; ER females who stopped laying were also not characterized by higher PRL levels than birds that continued laying. In conclusion, our data show that PRL and clutch size can be largely uncoupled, and higher PRL levels at clutch completion likely reflect simple temporal coordination with incubation onset rather than clutch size determination. Thus, although unlikely to be associated with the cessation of laying *per se*, the increase in PRL nearing clutch completion may reflect individually variable strategies in development of incubation behavior and hatching synchrony.

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3

Uncoupling clutch size, prolactin, and luteinizing hormone using experimental egg removal

Tables

Table 1. Clutch Size, prolactin (PRL), and luteinizing hormone (LH) [Mean ± SEM], and changes in these measures between two breeding attempts (Breeding 1 and Breeding 2) for female zebra finches. In Breeding 2, eggs were removed from the nest as they were laid (Egg removal) or left in the nest as with Breeding 1 (Controls).

	Breeding 1	Breeding 2		Breeding*Treatment Interaction
		Controls	Egg Removal[†]	
Clutch Size (eggs)	5.88 ± 0.25	5.67 ± 0.47	14.46 ± 0.75	$\chi^2_1 = 30.6.1$; $P < 0.001$[‡]
Mass at Pairing (g)	14.61 ± 0.20	15.06 ± 0.37	15.37 ± 0.18	$\chi^2_1 < 0.01$; $P = 0.989$
PRL (ng mL^{-1})	202.17 ± 5.92	178.23 ± 10.14	176.50 ± 6.26	$\chi^2_1 = 1.82$; $P = 0.177$
LH (ng mL^{-1})	0.47 ± 0.05	0.29 ± 0.10	0.42 ± 0.04	$\chi^2_1 = 4.84$; $P = 0.028$
Clutch Size ~ PRL	$\chi^2_1 = 0.23$; $P = 0.634$	$\chi^2_1 = 0.31$; $P = 0.577$	$\chi^2_1 = 0.67$; $P = 0.413$	
Clutch Size ~ LH	$\chi^2_1 = 0.49$; $P = 0.484$	$\chi^2_1 = 0.06$; $P = 0.815$	$\chi^2_1 = 0.25$; $P = 0.617$	

[†]Hormone levels for Egg Removal females during Breeding 2 presented here are from day 3 sample, as are Controls.
[‡]Mass at pairing included as a significant covariate in the model

Uncoupling clutch size, prolactin, and luteinizing hormone using experimental egg removal

Figure Captions:

Figure 1. Flowchart illustrating experimental design and analytical framework for testing the PRL-based mechanistic model for avian clutch size determination in captive zebra finches using egg removal. Intra-individual comparisons for both control (Blue) and egg removal (Red) females were made between Breeding 1 and Breeding 2 clutch sizes and PRL at the putative time of follicular inhibition (egg day 3). Comparisons between day 3 PRL and final clutch size were also made both within and between treatment groups for the Breeding 2. Finally, individual rate of change in PRL through the Treatment (days 3 to 10 and 10 to 17) and final clutch size were examined.

Figure 2. Individual changes in clutch size (**A**) and PRL (**B**) between Breeding 1 and Breeding 2 clutches for egg removal (ER; red) and control (CTL; blue) females. Clutch size increased significantly for ER females between Breeding 1 and 2, and was significantly larger for ER females during Breeding 2. PRL decreased between Breeding 1 and 2, but changes not differ between the two treatment groups for either breeding attempt.

Figure 3. Prolactin levels in laying female zebra finches. Prolactin for ER females laying 10 or more eggs are described by red lines, whereas those laying fewer than 10 are shown by solid red circles (ER) or blue triangles (CTL). Significant differences between sample days following Tukey adjustment for multiple comparisons are shown below.

Figure 4. Prolactin for the blood sample taken closest to clutch completion grouped by the number of days relative to last egg laid for all females in both breeding attempts. The model contained 73 observations from 44 females (pseudoreplication is controlled by including female as a random factor). Boxplots show median and quartile range, with individual points jittered and superimposed in blue (CTL) or red (ER). Prolactin was significantly associated with days remaining to clutch completion ($P < 0.001$), with or without breeding attempt and treatment group in the final model. One female was blood sampled the day following the last laid egg (+1).

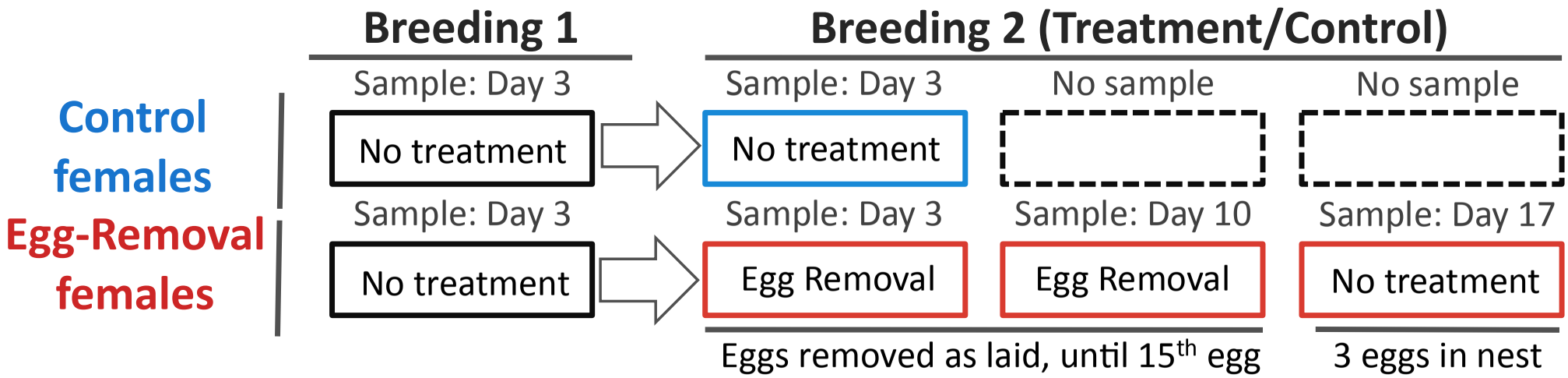

Breeding 1
Breeding 2 (Treatment/Control)
Control females
Egg-Removal females
Sample: Day 3
No treatment
Sample: Day 3
No treatment
Sample: Day 3
No sample
No sample
Sample: Day 3
No treatment
Sample: Day 3
Egg Removal
Sample: Day 10
Egg Removal
Sample: Day 17
No treatment
Eggs removed as laid, until 15th egg
3 eggs in nest

Figure 2AB

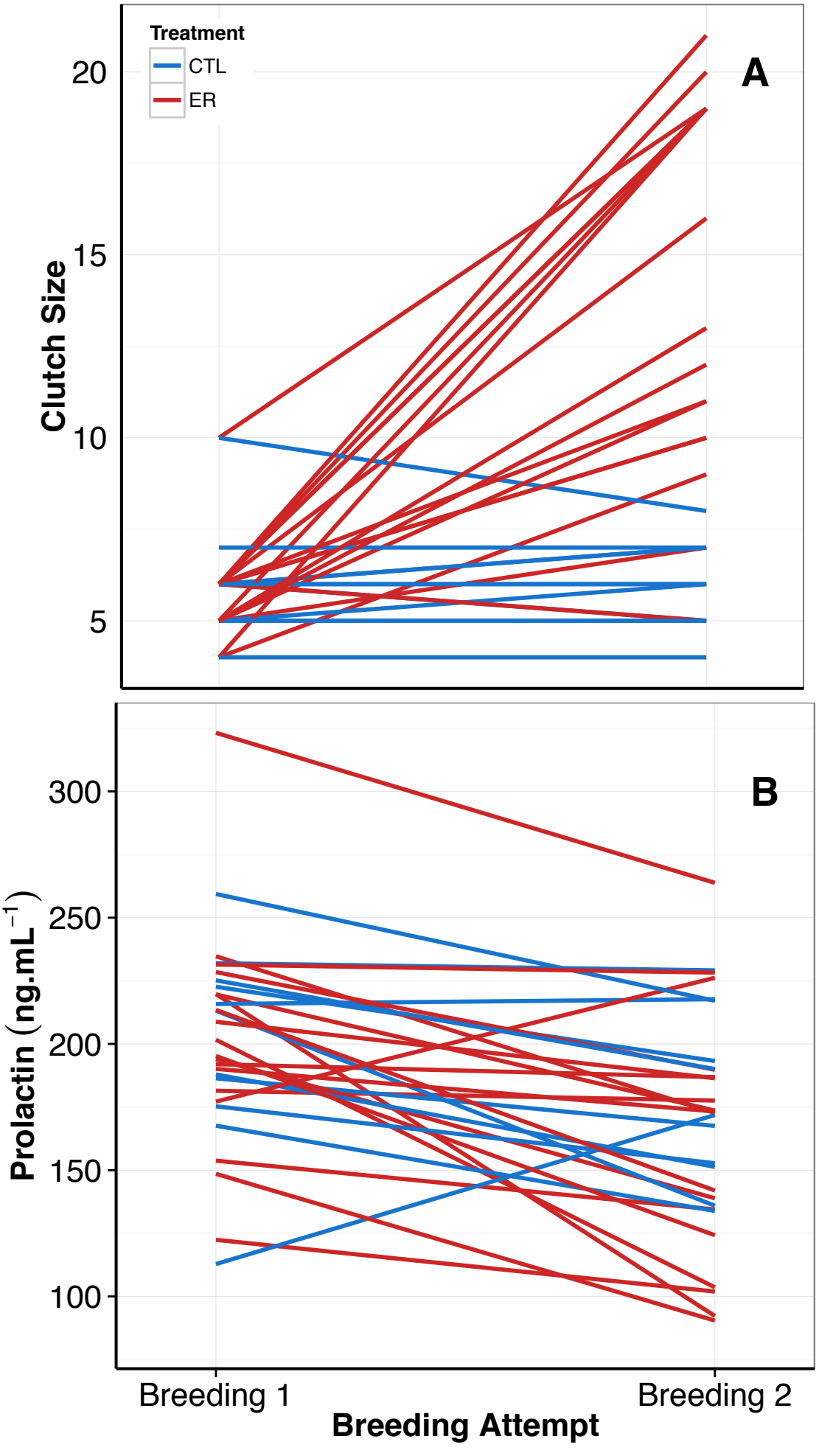

Treatment
CTL
ER
A
Clutch Size
20
15
10
5
B
Prolactin (ng.mL^{-1})
300
250
200
150
100
Breeding 1
Breeding 2
Breeding Attempt

Figure 3

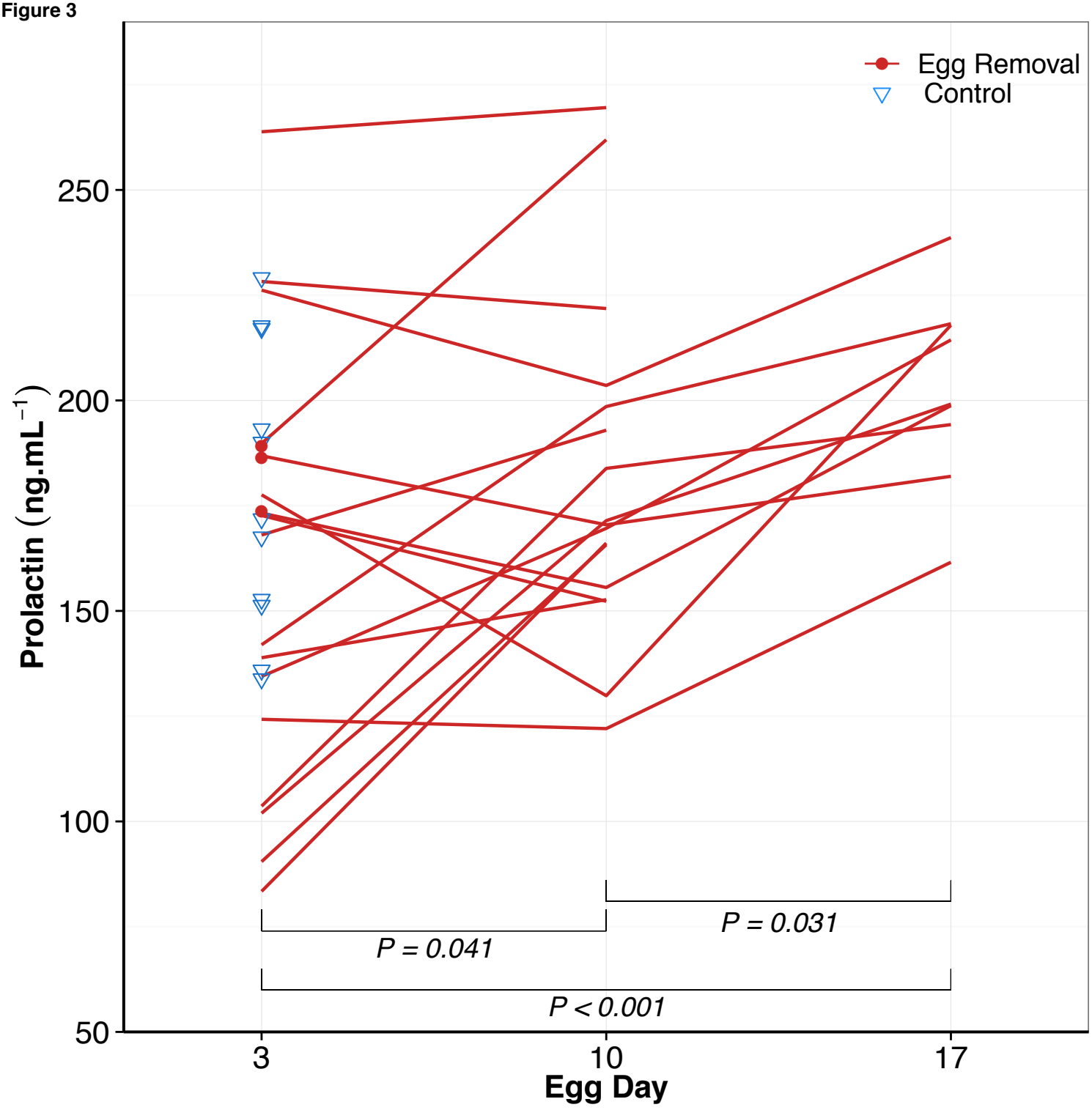
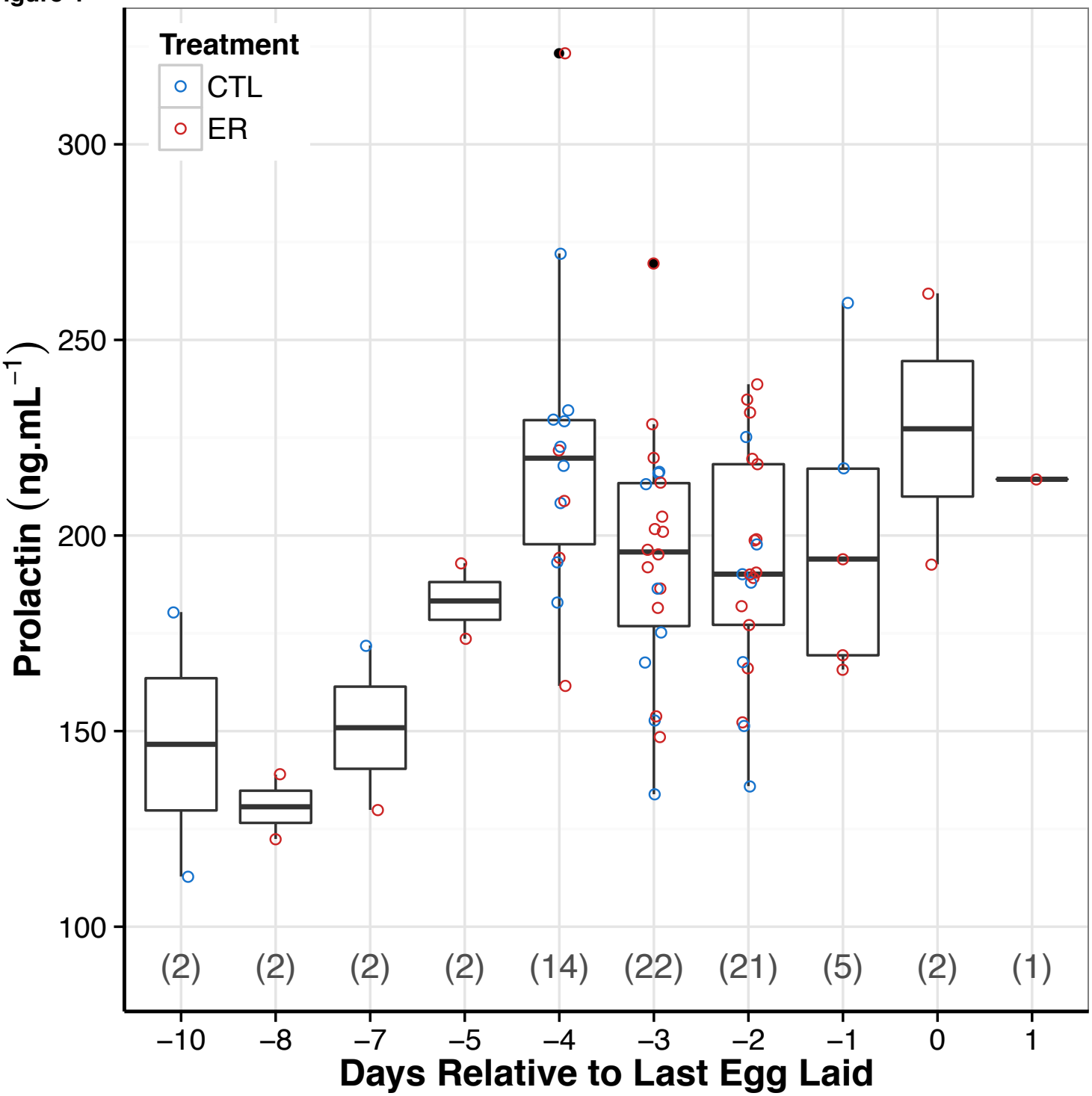

Egg Removal
Control
250
200
150
100
50
Prolactin (ng.mL^{-1})
3
10
17
Egg Day
P = 0.041
P = 0.031
P < 0.001

Figure 4


Treatment
CTL
ER
300
250
200
150
100
Prolactin (ng.mL^{-1})
(2)
(2)
(2)
(2)
(14)
(22)
(21)
(5)
(2)
(1)
−10
−8
−7
−5
−4
−3
−2
−1
0
1
Days Relative to Last Egg Laid