



## Divorce in the socially monogamous zebra finch: Hormonal mechanisms and reproductive consequences



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### ABSTRACT

Up to 80% of all bird species are socially monogamous. Divorce (switching partners) or pair disruption (due to the death of a partner) has been associated with decreased reproductive success, suggesting social monogamy is a strategy that may maximize fitness via coordination between partners. Previous studies have demonstrated the effects of divorce and pair disruption on immediate reproductive success. Here, we used a paired experimental design in the zebra finch (*Taeniopygia guttata*) to examine the hormonal mechanisms that modulate parental behavior and reproductive success in response to a partnership change (hereafter divorce). Specifically, we examined the effects of divorce on the avian stress hormone corticosterone (CORT) in both parents and nestlings, parental behaviors (incubation and nestling provisioning), prolactin (PRL), and reproductive success. We found that divorce resulted in delayed clutch initiation, reduced clutch mass, and an increase in nestling CORT response to a standardized stressor. These effects on reproductive investment and chick CORT response were not clearly determined by parental endocrine responses. Divorce had no effect on the level of parental CORT. PRL levels were highly correlated within a pair regardless of treatment, were negatively related to the investment that males made in incubation, and increased in experimental males as a result of pair disruption. This study demonstrates the fundamental impact which divorce has not only on reproduction, but also the physiological stress responses of offspring and suggests that in socially monogamous animals the maintenance of a stable partnership over time could be advantageous for long term fitness.

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### 1. Introduction

In contrast to all other taxonomic groups, birds display extraordinary rates of social monogamy with up to 80% of all avian species forming monogamous social pair bonds (Cockburn, 2006). Despite the widespread prevalence of social monogamy in birds, there are varying degrees of mate switching (divorce) between or within breeding seasons (Culina et al., 2015). However, even in species such as the zebra finch (*Taeniopygia guttata*) that rarely experience divorce (Zann, 1994), pair disruption still occurs due to the disappearance of a partner (Zann, 1994). Re-pairing after pair disruption (due to the loss of a partner) may incur fitness costs because of the limitation of available (unpaired) individuals in a local population (Zann, 1996), the delay in reproduction due to the time to form a pair relationship and coordinate parental behaviors (Adkins-Regan and Tomaszycski, 2007; Schweitzer et al., 2014), and negative effects on the quantity and quality of offspring produced. Examining the reproductive costs associated with pair

disruption (hereafter: divorce) can give insight into the underlying benefits of maintaining a long-term socially monogamous relationship and help to explain why divorce is rare or absent in some species.

Hormones are important modulators of social and reproductive behaviors (Adkins, 2005) and likely play a key role in mediating the costs of divorce via changes in behavior and reproductive physiology. The stress hormones, glucocorticoids (GCs) have well-known effects on reproduction and elevated levels have been reported in relation to a change in mate (Angelier et al., 2007) and experimental separation from a bonded partner (Remage-Healey et al., 2003). GCs are secreted via activation of the hypothalamic-pituitary-adrenal (HPA) axis and mobilize energy in order to meet the metabolic demands associated with acute stressors (stress induced GC secretion; reviewed in Wingfield and Sapolsky, 2003) and life-history transitions such as reproduction (baseline GC production; Sapolsky et al., 2000). Large increases in GCs levels, such as those associated with environmental stressors, trigger an “emergency life-history stage” where animals prioritize self-maintenance over reproduction (Wingfield et al., 1998). Elevated levels of corticosterone (CORT; the dominant avian GC) can inhibit reproduction via suppression of the hypothalamic-pituitary-

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gonadal (HPG) axis, disrupt parental behaviors (Silverin, 1986, 1990), and have been correlated with reduced parental care (Lendvai and Chastel, 2010; Miller et al., 2009) and reproductive success in free living birds (Crisuolo et al., 2005).

Elevated levels of CORT could also affect parental behavior and reproductive success indirectly via effects on prolactin (PRL) secretion (reviewed in Angelier and Chastel, 2009). PRL is a peptide hormone secreted by the anterior pituitary. In mammals, PRL has well established causal effects (Bridges et al., 1985; Bridges et al., 2001) and associations with maternal care (Freeman et al., 2000; Saito, 2015) and there is mixed support for its role in paternal care (Wynne-Edwards and Timonin, 2007; Ziegler, 2000). In birds, PRL has been implicated in the transition from sexual to parental activity (Sharp et al., 1988; Sockman et al., 2006) and is associated with parental behaviors such as incubation (Buntin et al., 1996; Sharp et al., 1988) and nestling provisioning (Duckworth et al., 2003; Smiley and Adkins-Regan, 2016b; Wang and Buntin, 1999), and reproductive success (i.e. number of nestlings produced and number of nestlings that survive to fledging; Smiley and Adkins-Regan, 2016a). Short-term elevations in CORT levels have been associated with decreased PRL levels and a decrease in parental behaviors (Angelier et al., 2009; Buysse et al., 1987; Spee et al., 2011). However, other studies have conversely found no effect of adrenocorticotrophic hormone (ACTH; Davison et al., 1980), CORT treatment (Nelson et al., 2015), or restraint stress (Krause et al., 2015) on circulating levels of PRL. Overall, there is mixed support for the role CORT in modulating PRL levels and, thus, having indirect effects on reproductive physiology and behavior. Studies that examine this pathway will add much to our understanding of the effects of CORT on parental behavior and reproductive success through indirect endocrine pathways.

Changes in parental hormone profiles and behavior following divorce could have negative effects on reproductive success by decreasing the quantity and quality of nestlings produced. In socially monogamous birds, pairs that have been together longer initiate clutches sooner and produce more fledglings compared to pairs that have made fewer reproductive attempts (Sanchez-Macouzet et al., 2014). In addition to affecting the quantity of nestlings produced, divorce could affect the quality of nestlings via changes in condition (via decreased provisioning) and stress exposure. Divorce could increase the level of stress nestlings experience by altering the quality or frequency of parental care resulting in elevated baseline (Kitaysky et al., 2006; Lynn and Kern, 2014; Pravosudov and Kitaysky, 2006; Rensel et al., 2010; Schmidt et al., 2014) and stress-induced CORT levels (Banerjee et al., 2012). Exposure to elevated levels of CORT during development can have system-wide and sustained effects on physiology, behavior, performance, and reproductive success (reviewed in Crino and Breuner, 2015; Henriksen et al., 2011; Monaghan and Haussmann, 2015; Schoech et al., 2011). In this way, the interactions between parents and the amount of care they provide nestlings could have sustained and important effects on nestling phenotype and fitness, via changes in HPA axis activity.

Here, we investigated endocrine mechanisms (CORT and PRL) that might underlie differences in reproductive success resulting from divorce in the domesticated zebra finch. Consistent with previous findings (Adkins-Regan and Tomaszycki, 2007), we predicted that divorced pairs would initiate nesting later than pairs that had previously bred together. Given the known effects of pair disruption on CORT (Remage-Healey et al., 2003), we predicted that divorce would increase parental CORT and that these hormonal changes would be associated with an increased latency to breed, reduced parental investment, and lower reproductive success. As previously discussed, evidence for the suppressive effects of CORT on PRL are equivocal. We tested this indirect pathway, by examining the effects of divorce on PRL and associations between PRL, CORT, parental behaviors, and reproductive success. Finally, we predicted that divorce would result in a decrease in both the quantity and quality (elevated stress responses and decreased condition) of offspring produced.

## 2. Material and methods

### 2.1. Experimental set-up and time course

Experimental work was conducted at Macquarie University. Domesticated zebra finches were sourced from a breeding colony at Macquarie University ( $n = 13$  males, 12 females) and from pet stores in Sydney, Australia ( $n = 6$  males, 7 females). All the birds used in this experiment were sexually mature and, at some point prior to our experiment, housed with birds of the opposite sex though not in housing conditions that allowed for breeding. Thus, they likely had sexual, but not reproductive experience. Birds were assigned a mate chosen at random from the pool of available birds and housed as single pairs in 24 cages ( $130 \times 70 \times 47$  cm) split across four temperature and humidity controlled rooms (set at 25 °C and 55% humidity). Birds were maintained on a 14:10 LD cycle and supplied with a nesting box and ad libitum nestling material (commercially available nesting string), dry finch seed mix, and water and were supplemented daily with hardboiled egg, cuttlebone, and green seeds. We initially established 24 zebra finch pairs. Three pairs did not breed and a male in the fourth pair died. We replaced these four pairs and of the total 28 pairs that were established, 19 successfully bred (i.e. fledged at least one chick). Following the first successful reproductive bout, males and females were separated and housed singly in same-sex rooms for a period of time between 11 and 14 days such that they were isolated from their partner both visually and acoustically (10 days is the median amount of time female zebra finches store sperm; Birkhead et al., 1989). Following this period of isolation half the birds were reunited with their original partner ( $n = 10$  control pairs) and half of the birds were paired with a different partner ( $n = 9$  divorce pairs). Birds in the divorce treatment were paired with individuals that had bred in the first round of breeding so that all birds in the experiment had the same level of breeding experience from the first round of breeding. After birds were united with a partner in either the control or divorce treatment they were allowed to breed again. In the second round of breeding, all pairs were returned to the male's original cage and successfully bred. To quantify the effects of divorce on parental physiology, behavior, and reproductive success we measured the following variables during both the first and the second reproductive bouts: egg incubation and nestling provisioning; HPA-axis activity and PRL titers; days to initiate a nest and a successful clutch; and nestling body size, condition, and HPA-axis activity (further described below) in nestlings. We defined a successful clutch as one that produced at least one fledgling. All nests that produced eggs that hatched ultimately fledged at least one nestling.

### 2.2. Incubation and nest attendance behavior

To quantify parental effort, we collected videos of pairs during incubation and nestling provisioning. We filmed the entrance of the nest box (from the outside the cage) 12 days after the first egg was laid to quantify the number of incubation bouts. Twelve days after the first nestling hatched we repeated the process to quantify nestling provisioning trips (when a bird completely entered the nest box). Incubation and nestling provisioning videos were an average of 6.25 h (st.dev. = 0.63) with the slight variation in length due to variable size of video clips produced by different cameras. We calculated incubation and nestling provisioning rate as the number of trips per hour and used these rates in statistical analyses. Videos were scored once by one of five different observers who were blind to treatment groups. To test for the effect of observer, we conducted a linear mixed model in which we included treatment, bout and the two-way interaction as fixed effects and bird identity and observer identity as random effects. We ran two models with this model structure, one for incubation rates and another for feeding rates. For each model, we compared the full model with a reduced model that deleted the observer identity effect using a log-likelihood ratio test (LRT) with a mixed  $\chi^2$  distribution of 0/1. For both models,

LRT < 0.01 and  $p > 0.99$ , strongly suggesting no observer bias and observer identity was, therefore, not included in behavioral analyses (see below).

### 2.3. Blood collection for quantification of CORT (adults and nestlings) and PRL (adults)

To measure CORT levels in adults, we collected blood samples the second day after birds were paired in the first and second (post-treatment) reproductive bouts. In a past study in zebra finches, experimental separation from a bonded partner resulted in elevated levels of CORT (Remage-Healey et al., 2003). Once birds were reunited with their original partner, CORT levels returned to normal, but, if birds were introduced to a novel partner CORT levels remained elevated 48 h after separation from the first partner (Remage-Healey et al., 2003). To measure HPA-axis activity we exposed birds to a standard restraint stress paradigm (Wingfield, 1994). Specifically, we obtained one 50  $\mu$ l blood sample within 3 min of entering the colony room (baseline CORT). After the first sample was collected, we placed birds in fabric bags for 15 min and then collected a second 50  $\mu$ l blood sample (stress-induced CORT). To collect blood, we punctured the alar vein with a 27-gauge needles and collected blood in heparinized microcapillary tubes. Immediately after collection, blood was kept on ice (<1 h) until it could be centrifuged to separate plasma from red blood cells (7000 rpm for 10 min). The isolated plasma was stored at  $-20^{\circ}\text{C}$  until assayed. Circulating PRL peaks during incubation and chick hatching in many avian species (e.g. Smiley and Adkins-Regan, 2016b; Sockman et al., 2000; Vleck et al., 2000). To compare PRL levels to measures of parental care (incubation and nestling provisioning), we collected blood from adults on the day after the first egg hatched using identical methods except that samples were collected within 5 min of entering the colony room. We collected blood to quantify CORT and PRL once per reproductive bout to minimize disturbance and the sustained effects acute stressors can have on HPA axis activity and corticosteroid-binding globulin (Lynn et al., 2010; Malisch et al., 2010).

We measured HPA-axis activity in nestlings at 28 days post-hatching using the same methods used in adults. We measured the HPA-axis responses of nestling once to minimize disturbances that could influence parental behaviors that were evaluated in this study.

### 2.4. Nestling morphological and corticosterone measurements

We monitored nest boxes daily for signs of hatching. On hatch day, we marked nestlings with a unique combination of leg markings using a black Sharpie marker. We banded nestlings with a numbered plastic leg band between seven and eight days post-hatching. At 12 and 28 days post-hatching we weighed nestlings to the nearest 0.1 g and measured tarsus length (posterior to anterior tarsus) and wing chord (carpus to longest primary feather) to the nearest 0.1 mm. We used tarsus length and mass to calculate condition using the scaled mass index (Peig and Green, 2009). The scaled mass index uses a scaling relationship derived from the population of interest to calculate expected mass of each individual at a given body size (Peig and Green, 2009). Scaled mass values are in units of grams and are hereafter referred to as 'condition.' Nestlings were considered to have successfully fledged if they survived to 28 days post-hatching.

### 2.5. Corticosterone assays

The following assays were conducted at Deakin University by OLC. We quantified corticosterone titers using Enzyme Immunoassay (EIA) kits (Cat No. ADI 900-097, Enzo Life Sciences). Samples were spiked with 1 pg of tritiated CORT (78.1 Ci/mmol; Cat. No. NET39901MC, Perkin Elmer) prior to steroid extraction to determine recovery percentage. We extracted CORT from raw plasma using a double wash of dichloromethane (Cat. No. 400142, Thermo Fisher Scientific). Samples were then

dried under nitrogen gas and reconstituted in buffer solution (1:25 ratio). We adjusted hormone values for the average sample recovery (87.49%). We ran our reconstituted samples at half volume against a six point standard curve ranging from 20,000 to 15.53 pg/ml. An external standard of 500 pg/ml was run on every plate and used to calculate inter-plate variation. All samples and standards were run in triplicate. Plates were read on a FLUOstar Omega microplate reader at 405 nm corrected at 595 nm. Intra- and inter-plate variation was 6.5 and 6.8% respectively.

### 2.6. PRL assays

Plasma PRL samples were analyzed at Cornell University by KOS using previously described methods (Smiley and Adkins-Regan, 2016b). Samples were analyzed across five plates. All samples were run in duplicate with the exception of three samples for which there was not enough plasma for a duplicate and thus were run singly. One sample was excluded from analyses because it could not be detected by the assay. Samples were randomly assigned to a plate and each plate had a similar distribution of samples from both pre- and post-treatment and from treatment groups.

Briefly, 96 well ELISA plates (Nunc MaxiSorp) were coated with 0.1 ml of goat anti-rabbit IgG (Jackson ImmunoResearch) diluted 1:2000 in 0.05 M phosphate buffer (pH = 7.4) and incubated overnight at  $4^{\circ}\text{C}$ . Twenty-four hours later, wells were blocked by adding 0.1 ml of blocking solution containing 0.15 M phosphate buffer solution (PBS; pH = 7.2), 0.4% casein (Cat. No 37528, Thermo Scientific), and 0.25 M ethylenediaminetetraacetic acid (EDTA) and incubated for 2 h at room temperature. After blocking, plates were washed (ELX 405 AutoPlate Washer, Biotek Instruments, Inc.) three times using wash buffer containing  $10\times$  PBS diluted 1:50 and 0.05% Tween-20. Fifty microliter samples, either 10  $\mu$ l of plasma diluted in 40  $\mu$ l of assay buffer containing 0.15 M PBS (pH 7.2), 0.1% casein, and 0.25 M EDTA, or serially diluted chicken PRL standard (Dr. AF Parlow, National Hormone and Peptide Program) in assay buffer were added to wells. We then added 25  $\mu$ l of biotinylated PRL tracer (kindly provided by I. Rozenboim and R. Heiblum) diluted 1:50,000 in assay buffer, followed by 25  $\mu$ l of rabbit anti-chicken PRL (Dr. AF Parlow, National Hormone and Peptide Program) diluted 1:45,000 in assay buffer to wells. Plates were incubated overnight at  $4^{\circ}\text{C}$ . Following incubation, plates were washed and 0.1 ml of streptavidin horseradish peroxidase diluted 1:50,000 in assay buffer was added to each well and incubated for 2 h at room temperature. Plates were washed again and 0.1 ml of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) reagent was dispensed across all wells. The color reaction was read 45 min later (450  $\mu\text{m}$ ; Synergy HT plate reader, Biotek).

The intra- and inter-assay coefficients of variation (CV) were calculated using high and low pools of zebra finch plasma. The intra-assay CV was 9.11% (high pool) and 20.30% (low pool). The inter-assay CV was 9.42% (high pool) and 17.21% (low pool).

### 2.7. Statistical analyses

All statistics were performed using R (v3.2).

#### 2.7.1. Parental hormones

Central to this study's hypothesis is that the effects of divorce on reproductive costs are related to changes in hormone levels. We asked if divorce altered parental CORT and PRL profiles by testing for changes in baseline CORT ( $\Delta\text{CORT}_{\text{base}}$ ), stress induced CORT ( $\Delta\text{CORT}_{\text{peak}}$ ), the magnitude of the stress response ( $\Delta\text{CORT}_{\text{fold}}$ : ratio of  $\text{CORT}_{\text{peak}}$  to  $\text{CORT}_{\text{base}}$ ), and PRL ( $\Delta\text{PRL}$ ) across reproductive bouts. We performed separate linear mixed models in which we used the difference in hormone concentration between first and second bout as the response variable, except for  $\Delta\text{CORT}_{\text{fold}}$  in which we used the difference in log-transformed values (i.e. proportional change). We included sex and

divorce treatment (new or same partner) as main effects and sex \* treatment as an interaction effect. We also included pair identity (PAIRID) as a random effect, which allowed us to test for correlations in a pair's hormonal responses to treatment. Using log likelihood ratio test (LRT), we compared models with and without the interaction, and removed the interaction if  $p > 0.10$ . Model assumptions were assessed using graphical analyses. Presented effect sizes are least square means with  $\pm 1$  standard error. Ninety-five percent confidence intervals for PAIRID correlation coefficient were calculated using parametric bootstrap.

### 2.7.2. Breeding parameters – parental behaviors, clutch size, egg mass

For nestling provisioning and incubation rates (trips per hour), we ran the similar linear models structure as above for the hormone analyses (as we have separate response measures for each bird in the pair), but included two additional covariates: average  $\Delta\text{CORT}_{\text{base}}$  per pair and average  $\Delta\text{PRL}$  per pair. We included two-way interactions and removed nonsignificant interactions at  $p > 0.10$  using LRT. For the other breeding parameters (nest, egg, and nestling variables), the response measure is at the level of the pair. Therefore, we ran a linear model with treatment, average  $\Delta\text{CORT}_{\text{base}}$ , average  $\Delta\text{PRL}$ , and two-way interactions. Again, we performed model selection on the two-way interactions. For the control groups, we only included one datum per pair. For the treatment pairs, we included only the male (paternal) data for the time to nest, as males are mainly responsible for the initiating nest construction (Zann, 1996). For egg variables (time to clutch initiation, egg mass, egg number), we only included the female (maternal) data as females are mainly responsible for these variables. For nestling morphological measures including condition, we ran the model with just female and then with just male values. Patterns were the same for models run with male or female values, so we only report the models with female values. For all models, graphical analyses of residuals were performed to assess model assumptions.

### 2.7.3. Nestling CORT profiles

CORT levels for nestlings were modelled using a mixed linear model. Unlike the previous analyses, there are no baseline values. We had nestling  $\text{CORT}_{\text{base}}$ ,  $\text{CORT}_{\text{peak}}$ , and  $\text{CORT}_{\text{fold}}$  (ratio of  $\text{CORT}_{\text{peak}}$  to  $\text{CORT}_{\text{base}}$ ) as response variables in separate models.  $\text{CORT}_{\text{fold}}$  provides a measure of the magnitude of the stress response. Hormone measures were log-transformed. Male and female PRL and  $\text{CORT}_{\text{base}}$  levels were added as covariates, as well as clutch size, nestling sex, and clutch order. We also included time at which the sample was measured (e.g. around 1 min for baseline and 15 min for stressed). As before, two way interactions were included between the covariates and treatment, but removed if not significant through backward model selection. For the covariance structure, we included PAIRID as a random effect. Nestling identity (CHICKID) was nested in PAIRID. Graphical analysis of residuals was conducted to assess model fit.

## 3. Results

### 3.1. Effect of divorce on parental hormonal profiles

#### 3.1.1. CORT profiles

Contrary to our prediction, divorce had no significant effect on parental CORT levels. For all CORT measures, we found no support for an interaction between sex and treatment ( $p > 0.10$ ) and hence removed it from the final model. We predicted that divorced birds in the second reproductive bout would have a greater change in CORT levels from the first reproductive bout ( $\Delta\text{CORT}$ ) compared to control birds. However, we found no significant difference between  $\Delta\text{CORT}$  between divorce and control birds (Fig. 1;  $0.38 \pm 0.72$  vs.  $-0.6 \pm 0.70$ , respectively;  $t_{13} = 1.03$ ,  $p = 0.32$ ). Similarly, divorce did not have a significant effect on the change in stress-induced CORT ( $\Delta\text{CORT}_{\text{peak}}$ ; Fig. 1;  $t_{13} = 0.16$ ,  $p = 0.88$ ), with divorced birds ( $-3.04 \pm 1.88$ ) having similar estimates to control birds ( $-3.5 \pm 1.75$ ), nor did divorce affect change in fold

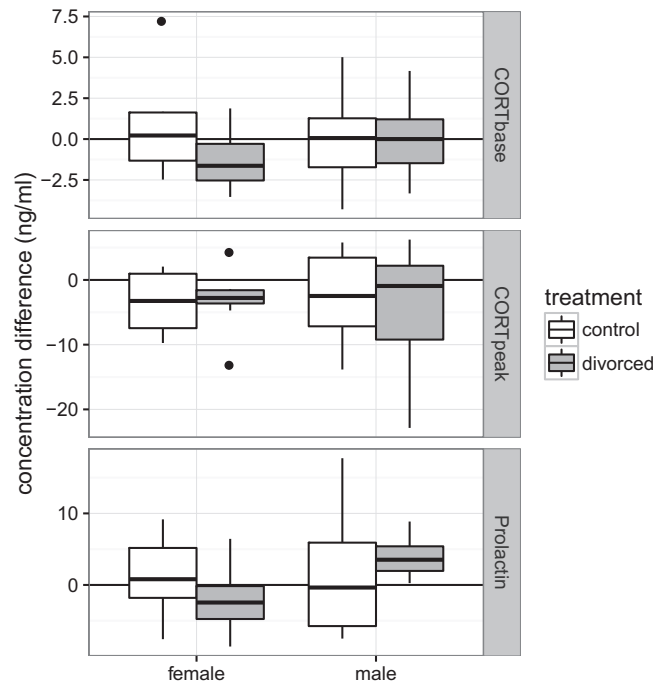


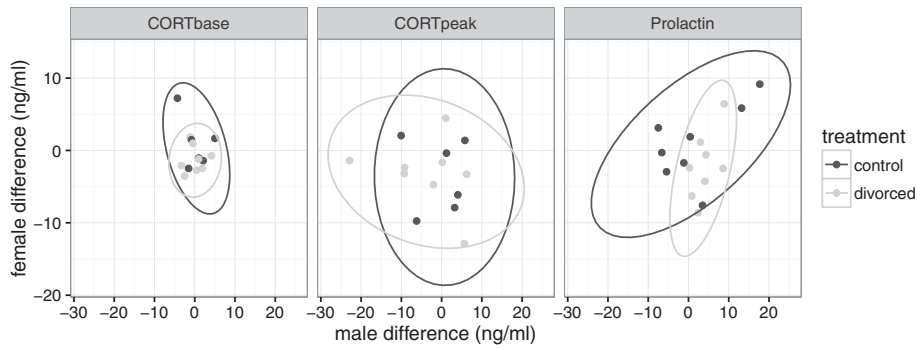
Fig. 1. Boxplot of change in hormone levels between the second and first reproductive bout for baseline CORT ( $\Delta\text{CORT}_{\text{base}}$ ), stress-induced CORT ( $\Delta\text{CORT}_{\text{peak}}$ ), and prolactin ( $\Delta\text{PRL}$ ). Open boxplots indicate values from the control group and grey bars indicate values from the divorce group. Outliers (points) were not excluded from analysis.

increase in CORT ( $\Delta\text{CORT}_{\text{fold}}$ ;  $t_{13} = 0.3$ ,  $p = 0.77$ ). Sex did not significantly affect CORT:  $\Delta\text{CORT}_{\text{base}}$  ( $t_{13} = 0.26$ ,  $p = 0.80$ ),  $\Delta\text{CORT}_{\text{peak}}$  ( $t_{13} = 0.012$ ,  $p = 0.99$ ), and  $\Delta\text{CORT}_{\text{fold}}$  ( $t_{13} = -0.6$ ,  $p = 0.58$ ). Finally, the correlation in responses between pairs were low for  $\Delta\text{CORT}_{\text{base}}$  (mean = 0.00; (95%CI 0.00, 0.58),  $\Delta\text{CORT}_{\text{peak}}$  (mean = 0.00, (95%CI 0.00, 0.61)), and  $\Delta\text{CORT}_{\text{fold}}$  (mean = 0.00, (95%CI 0.00, 0.60).

#### 3.1.2. PRL profiles

Divorce affected the change in PRL levels from the first to the second reproductive bout ( $\Delta\text{PRL}$ ), though the effect depended on the bird's sex (Fig. 2: sex\*treatment: LRT = 3.6,  $p = 0.065$ ).  $\Delta\text{PRL}$  were very similar between males ( $1.9 \pm 2.0$ ) and females ( $1.2 \pm 1.9$ ) in the control treatment. In contrast, divorced males significantly increased PRL concentrations ( $4.0 \pm 2.1$ ) in comparison to females ( $-2.1 \pm 2.1$ ) ( $t_{14} = -2.9$ ,  $p = 0.05$ , Tukey-corrected). The directionality of these effects sizes suggests that divorce could be leading to increased PRL for males and a decrease in females, but a comparison of treatment vs. control means for each sex did not find statistical support for this assertion (female:  $\Delta\beta = -3.3 \pm 2.8$ ,  $t_{14} = -1.2$ ,  $p = 0.26$ ; males:  $\Delta\beta = 2.2 \pm 2.9$ ,  $t_{14} = 0.74$ ,  $p = 0.47$ ) with our current sample size.

We did find support that individuals in a pair adjust their PRL concentrations similarly (Fig. 2c), with birds in a pair having a tendency to either both increase or decrease their PRL concentrations from their baseline values from the first reproductive bout. We estimated the within pair correlation to be 0.51 (95% CI: 0.12, 0.82). Since the control birds have previous breeding experience with their mates, it might be expected that the correlation may differ between individuals in control and divorced pairs. Therefore, we ran separate models for control and treatment, keeping sex as a fixed factor and PAIRID as a random factor. The PAIRID correlations were 0.49 (95% CI: 0.00, 0.88) and 0.55 (95% CI: 0.00, 0.90) for control and divorced, respectively. As the above analysis only tests that birds in a pair adjust their PRL similarly, we next asked if raw PRL concentrations are similar between birds in a pair due to this adjustment. Therefore, we ran two linear mixed models as above using raw PRL instead of  $\Delta\text{PRL}$ ; one for the first bout and the second for the second bout. The PAIRID correlations were low and



**Fig. 2.** Scatterplot of the association between male and female hormone differences (between the second and the first reproductive bout) in a pair. Each dot represents a bird pair. Divorced pairs are represented by black dots and control pairs are represented by grey dots. Ellipsis shows 95% confidence interval.

nonsignificant:  $0.06$  (95%CI:  $0.00, 0.54$ ) and  $0.15$  (95%CI:  $0.00, 0.66$ ) for baseline and treatment bouts respectively.

### 3.2. Effect of divorce on reproductive success

#### 3.2.1. Nest/clutch initiation

The change in time to initiate a nest within a reproductive bout did not differ between control ( $-13.6 \pm 4.7$  days) and divorced pairs ( $-18.0 \pm 5.7$  days) (Fig. 3:  $\Delta\beta = -4.3 \pm 7.4, t_{10} = -0.6, p = 0.57$ ). Neither average  $CORT_{base}$  ( $-0.3 \pm 3.9, t_{10} = -0.1, p = 0.94$ ) nor average PRL ( $-1.5 \pm 3.3, t_{10} = -0.4, p = 0.67$ ) were associated with time to initiate a nest. Conversely, and as predicted, control females significantly decreased the time to lay a clutch in their second reproductive bout compared to their first reproductive bout ( $-55.5 \pm 17.2$  days,  $t_{15} = -3.2, p = 0.009$ ). Divorced females did not significantly adjust the time to lay a clutch in the second reproductive bout compared to the first reproductive bout ( $-2.8 \pm 14.4$  days,  $t_{15} = -0.2, p = 0.85$ ). There was no association between the time for females to lay a clutch and average  $CORT_{base}$  ( $12.4 \pm 12.0, t_{10} = 1.0, p = 0.33$ ) or PRL ( $-9.2 \pm 10.2, t_{10} = -0.9, p = 0.39$ ).

#### 3.2.2. Incubation/feeding rate

Neither sex ( $\Delta\beta = 0.04 \pm 0.10, t_{11} = 0.4, p = 0.7$ ), or treatment ( $\Delta\beta = -0.014 \pm 0.10, t_{14} = -0.13, p = 0.90$ ) affected the change in incubation rates between the first and second reproductive bouts (Fig. S1). There was no association between of  $CORT_{base}$  and incubation rates ( $-0.03 \pm 0.06, t_{11} = -0.6, p = 0.57$ ) or incubation rates and PRL ( $0.08 \pm 0.1, t_{11} = 0.8, p = 0.42$ ). However, there was a significant association between nestling feeding rates and the average parental  $CORT_{base}$ , but only in the control group (Fig. 4;  $F_{1,9} = 12.1, p = 0.007$ ). Nestling feeding rates increased with parental  $CORT_{base}$  in the control group ( $1.5 \pm 0.5, t_9 = 3.2, p = 0.01$ ), but did not change

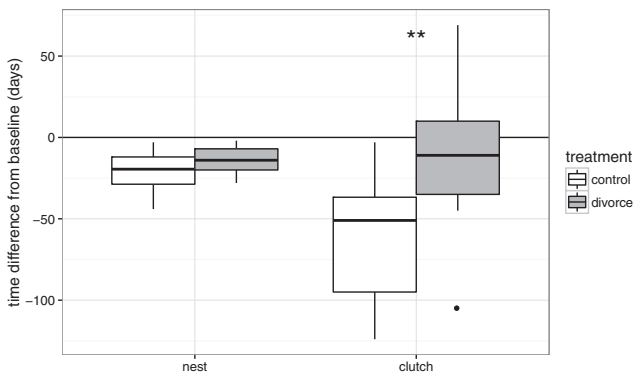
significantly with  $CORT_{base}$  in the divorced group ( $-0.4 \pm 0.3, t_9 = -1.2, p = 0.25$ ). There was no association between nestling feeding rates and parental PRL ( $-0.04 \pm 0.5, t_9 = -0.07, p = 0.94$ ).

#### 3.2.3. Egg mass and clutch size characteristics

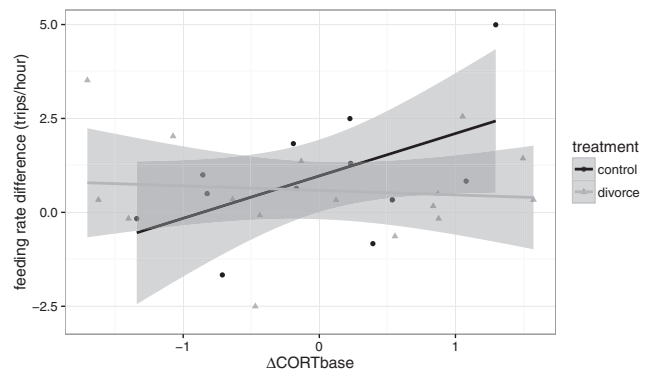
Divorced pairs produced eggs of significantly lower mass compared to the control pairs (Fig. S2;  $-0.06 \pm 0.024, t_9 = -2.6, p = 0.03$ ). However, this was driven by an increase in the mass of eggs by females that bred with the same partner for a second time. Females that lost their initial partner had no significant change in egg mass from the first reproductive bout ( $0.01 \pm 0.018, t_9 = 0.4, p = 0.68$ ), while control females had significantly heavier eggs in the second reproductive bout compared to the first bout ( $0.07 \pm 0.02, t_9 = 4.0, p = 0.003$ ). There were no significant associations between PRL ( $0.06 \pm 0.05, t_9 = 1.2, p = 0.26$ ) or  $CORT_{base}$  ( $-0.02 \pm 0.02, t_9 = -0.85, p = 0.42$ ) on egg mass. Clutch size did not differ between treatment groups, but divorce had no effect on clutch size (Fig. S2;  $t_{16} = -0.17, p = 0.52$ ) both control and divorced females had a non-significant increase of clutch size from the first reproductive bout ( $0.9 \pm 0.41, t_{17} = 2.2, p = 0.06$ ;  $0.7 \pm 0.39, t_{16} = 1.9, p = 0.09$ , respectively). There were no significant associations between PRL ( $-0.28 \pm 0.63, t_9 = -0.44, p = 0.67$ ) or  $CORT_{base}$  ( $-0.79 \pm 0.52, t_9 = -1.5, p = 0.16$ ) on clutch size. Similar to clutch size, the number of fledglings did not differ between treatment groups ( $0.6 \pm 0.88, t_{10} = 0.7, p = 0.52$ ). There were also no significant associations between PRL ( $0.19 \pm 1.1, t_9 = 0.2, p = 0.86$ ) or  $CORT_{base}$  ( $-1.4 \pm 0.9, t_9 = -1.63, p = 0.13$ ) and the number of fledglings produced.

#### 3.2.4. Nestling condition

Divorce did not affect nestling condition either on day 12 post-hatching (Fig. S2;  $0.3 \pm 1.1, t_{10} = -0.3, p = 0.79$ ) or on day 28 post-hatching ( $-0.18 \pm 1.1, t_{16} = -0.2, p = 0.88$ ). Furthermore, there



**Fig. 3.** The change in time between the second and first reproductive bout for pairs to start constructing a nest and to initiate a successful clutch. Outliers (points) were not excluded from analysis. \*\* indicates  $p < 0.01$ .



**Fig. 4.** The relationship between average baseline CORT ( $\Delta CORT_{base}$ ) of a pair and nestling provisioning rate ( $\Delta CORT_{base}$  is Z-transformed). Grey shading represents 95% confidence interval.

were no significant changes in condition either on day 12 or day 28 post-hatching from the first reproductive bout for either control or divorce birds (Fig. S2), nor any significant effect of PRL or  $CORT_{base}$  on nestling condition.

### 3.2.5. Nestling hormones

There was no difference in baseline or stress induced levels of CORT between nestlings raised by divorced or control parents. However, the proportional increase from baseline and stress induced CORT levels (i.e. fold increase) was higher in nestlings reared by divorced parents (Fig. 5; difference =  $0.03 \pm 0.01$ ,  $t_{58} = 2.15$ ,  $p = 0.04$ ). Male (paternal) PRL levels were negatively associated with both nestling baseline and stress induced CORT (estimate =  $-0.15 \pm 0.07$ ,  $t_{12} = -2.1$ ,  $p = 0.05$ ). (see Table 1)

## 4. Discussion

Here, we investigated the effects of divorce on CORT and PRL and examined the associations between these hormones and parental behavior and reproductive success. Similar to past findings (Adkins-Regan and Tomaszycski, 2007), divorce affected reproductive success; divorced females took longer to initiate a clutch and produced clutches with overall lower mass compared to control females. We found no evidence that CORT modulates parental behaviors or reproductive success in response to divorce either directly or through indirect effects on PRL levels. Divorce had no effect on nestling body size or condition, but, interestingly, nestlings reared by divorced parents had higher stress responses compared to nestlings reared by control parents.

Control females took fewer days to lay a clutch in the second reproductive bout compared to the first reproductive bout; conversely, there was no change in the time it took divorced females to initiate a clutch between the second and first reproductive bouts. In a study similar to ours, Adkins-Regan and Tomaszycski (2007) suggested that the delayed clutch initiation in divorced birds may be due to elevated levels of CORT. Indeed, the short-term experimental separation of paired zebra finches has been shown to increase CORT levels (Remage-Healey et al., 2003)

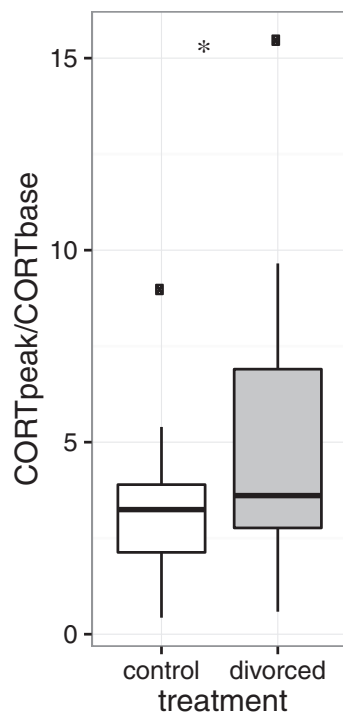


Fig. 5. The fold increase in nestling CORT response (stress-induced/baseline) in the second reproductive bout from nestlings reared by divorced or control parents. \* indicates  $p < 0.05$ .

and CORT has suppressive effects on egg production in zebra finches (Salvante and Williams, 2003). In our study, we found no support for this proposed mechanism; divorce did not affect baseline nor stress-induced levels of CORT two days after males and females were paired. Our results mirror those of Schweitzer et al. (2014) who did not detect elevated levels of CORT in female zebra finches 1–4 days following six days of isolation from their original partner. Cumulatively, this evidence suggests that CORT is not modulating changes in reproductive behavior or physiology following divorce in zebra finches.

In addition to shortening clutch initiation, control females also produced heavier clutches compared to divorced females (8.04% heavier clutch mass). Life history theory predicts that individuals should invest heavily in a reproductive effort when the fitness returns are expected to be high (Roff, 1992; Stearns, 1992). The production of eggs is costly for females (reviewed in Williams, 2005) and limiting investment in egg mass with a novel partner may allow females to minimize the cost of a reproductive event when she has limited information on the quality of her mate. In other studies, egg mass has been shown to be positively correlated with mass and growth rates in nestlings (Newbrey and Reed, 2009) and the allocation of carotenoids, antibodies, and lipids to egg yolk can have both immediate and sustained effects on nestling body size at hatching, health and growth (e.g. Berthouly et al., 2007; Buechler et al., 2002; De Neve et al., 2008; Saino et al., 2003; Saino et al., 2010). Overall, the contribution to egg mass and composition by females can have large and sustained effects on nestling health and survival and, thus, can affect the fitness of both parents.

Juveniles (sampled 28 days post-hatching) reared by divorced parents had higher levels of CORT responses (fold increase) compared to juveniles reared by control parents. Elevated levels of CORT output in juveniles from divorced parents could be due to increased exposure to pre-natal CORT and/or differences in the post-natal environment. In birds, developing embryos can be exposed to prenatal CORT through the deposition of maternal CORT into the egg yolk (e.g. Hayward and Wingfield, 2004) and elevated prenatal exposure to CORT can increase HPA axis activity (Hausmann et al., 2012). However, in zebra finches, Schweitzer et al. (2014) found no effect of pair disruption on the amount of yolk CORT concentrations. Additionally, we found no effect of divorce on maternal CORT levels suggesting that exposure to prenatal CORT does not explain elevated juvenile CORT output in our experiment. HPA axis activity in nestlings can also be affected by postnatal stressors such as deprivation of maternal care (Banerjee et al., 2012; Rensel et al., 2010) and periodic cooling that would occur during breaks in incubation (Lynn and Kern, 2014). We found no difference between treatment groups in either incubation behavior or the amount of parental care young nestlings received. Despite the fact that there were not absolute differences, it is possible that divorced pairs were less coordinated with their parental care and/or that they fed nestlings less once they had fledged but were still nutritionally dependent. In birds, exposure to stress during development can have system-wide effects that can be sustained across life-history stages (reviewed in Crino and Breuner, 2015; Henriksen et al., 2011; Schoech et al., 2011). Although there were no differences between treatment groups in the size or condition of nestlings produced, elevated HPA axis activity such as we described could affect nestling immune responses (Chin et al., 2013; Kriengwatana et al., 2013), learning ability (Buchanan et al., 2003; Crino et al., 2014a; Spencer et al., 2003), survival (Monaghan et al., 2012), and reproduction (Crino et al., 2014b). These sustained effects on nestling phenotype mediated by increased exposure to CORT during development or by long-term changes in HPA axis activity could affect nestling and, hence, parental fitness and reveal hidden effects that divorce can have on fitness in socially monogamous birds.

Although divorce did not affect CORT levels in our study, CORT did predict parental behaviors across treatment groups. Both incubation time and nestling provisioning bouts were positively correlated with baseline CORT levels in females, but not in males. Although elevated levels of stress-induced CORT can disrupt reproduction (reviewed in

Table 1

Variable	Control-first	Control-second	Control-both	Control-female	Control-male	Divorce-first	Divorce-second	Divorce-female	Divorce-male
Parental CORTbase (ng/ml)	4.5 ± 2.83 (17)	3.8 ± 2.81 (17)		0.9 ± 3.51 (6)	0.0 ± 2.89 (8)	3.0 ± 1.90 (19)	2.5 ± 1.59 (16)	−1.2 ± 1.88 (8)	0.0 ± 2.42 (8)
Parental CORTpeak (ng/ml)	12.0 ± 6.16 (17)	7.0 ± 7.04 (17)		−3.4 ± 5.10 (6)	−2.7 ± 7.27 (8)	7.9 ± 5.81 (19)	4.1 ± 3.34 (16)	−3.1 ± 4.80 (8)	−3.8 ± 9.67 (8)
Parental CORTfold	3.1 ± 1.46 (17)	2.7 ± 3.27 (17)		0.6 ± 4.26 (6)	−1.2 ± 2.09 (8)	3.7 ± 4.01 (19)	1.8 ± 1.01 (16)	−2.5 ± 5.84 (8)	−1.4 ± 2.50 (8)
Prolactin (ng/ml)	25.5 ± 22.54 (20)	20.7 ± 5.94 (20)		1.2 ± 5.00 (10)	−10.8 ± 31.00 (10)	21.5 ± 12.76 (19)	22.1 ± 12.89 (18)	−3.3 ± 5.51 (9)	3.6 ± 3.23 (9)
Nest initiation (days)	23.7 ± 13.21 (20)	2.0 ± 1.45 (20)	−22.1 ± 14.75 (9)			19.9 ± 10.79 (19)	4.2 ± 3.66 (18)	−15.8 ± 10.80 (9)	−14.3 ± 8.65 (9)
Clutch initiation (days)	68.1 ± 37.27 (20)	5.9 ± 1.55 (20)	−62.2 ± 38.39 (10)			36.6 ± 28.01 (19)	26.0 ± 26.37 (18)	−14.0 ± 48.32 (9)	−5.4 ± 37.95 (9)
% incubation	0.5 ± 0.20 (20)	0.5 ± 0.17 (20)	0.0 ± 0.21 (20)			0.5 ± 0.29 (19)	0.5 ± 0.19 (18)	0.0 ± 0.27 (9)	0.1 ± 0.33 (9)
Feeding (trips/h)	2.1 ± 1.33 (18)	2.6 ± 1.32 (20)	0.5 ± 1.68 (18)			2.1 ± 0.85 (19)	2.7 ± 1.21 (18)	0.2 ± 1.34 (9)	0.9 ± 1.26 (9)
Avg. egg mass (g)	1.10 ± 0.136 (20)	1.14 ± 0.121 (20)	0.04 ± 0.079 (10)			1.02 ± 0.039 (19)	1.03 ± 0.042 (18)	0.02 ± 0.042 (9)	0.01 ± 0.056 (9)
Clutch size (eggs)	3.9 ± 0.72 (20)	4.8 ± 0.62 (20)	0.5 ± 1.29 (4)			3.7 ± 0.65 (19)	4.6 ± 0.86 (18)	0.5 ± 1.29 (4)	1.0 ± 1.00 (3)
Fledglings produced	2.7 ± 0.92 (20)	3.7 ± 1.22 (20)	1.0 ± 1.58 (5)			2.3 ± 1.05 (19)	3.9 ± 1.02 (18)	1.7 ± 2.16 (6)	2.2 ± 1.92 (5)
Nestling condition (g) day 12	10.5 ± 1.09 (18)	9.5 ± 1.78 (18)	−1.0 ± 2.34 (9)			10.8 ± 1.20 (19)	10.2 ± 0.98 (18)	−0.7 ± 1.65 (9)	−0.5 ± 1.78 (9)
Nestling condition (g) day 28	13.3 ± 1.37 (18)	13.5 ± 1.02 (18)	0.2 ± 1.66 (9)			13.2 ± 1.19 (19)	13.1 ± 1.15 (18)	−0.1 ± 1.81 (9)	−0.1 ± 1.47 (9)

Landys et al., 2006; Wingfield and Sapolsky, 2003), elevated baseline levels of CORT have been associated with increased parental behavior and reproductive success in birds (Bonier et al., 2011; Love et al., 2004; Ouyang et al., 2011). For example, in female European starlings (*Sturnus vulgaris*) levels of free CORT (not bound to binding globulins) peak with chick rearing, potentially to enable females to meet the increased metabolic demands associated with nestling provisioning (Love et al., 2004). Interestingly, in our study, female, but not male provisioning rates were positively associated with baseline CORT levels. Additionally, we found that females from divorced pairs produced clutches with smaller masses (see above), but they did not feed nestlings less than control females. This suggests that, in response to divorce females invest less at early stages of reproduction, but increase investment at later stages.

We found that, overall, divorce increased PRL levels in male, but not in female zebra finches. PRL has been associated with increased parental behavior in birds (reviewed in Angelier and Chastel, 2009; Angelier et al., 2016). Regardless of treatment group, we found that males had a negative association between PRL levels and incubation behavior and a positive association between baseline CORT and PRL levels. During reproduction, elevated levels of PRL are associated with parental behaviors such as incubation and nestling provisioning (Angelier et al., 2009; Buntin et al., 1996; Duckworth et al., 2003; Sharp et al., 1988) while elevated levels of baseline CORT may enable animals to meet the metabolic demands associated with these parental behaviors (Bonier et al., 2009; Bonier et al., 2011; Crossin et al., 2012; Romero, 2002). Contrary to the positive association between PRL and chick incubation that has previously been described (Smiley and Adkins-Regan, 2016a), we found that PRL levels were negatively associated with incubation behavior in males. This negative relationship could have resulted from the stress of the handling prior to the blood sample. In general, prolactin decreases in birds from acute stressors (Angelier et al., 2009). However, (Chastel et al., 2005) found that prolactin levels slightly increase initially following an acute capture/restraint stressor in black-legged kittiwakes (*Rissa tridactyla*), before showing a significant decrease in PRL. Therefore, it is possible that males have a more reactive initial prolactin-stress response than females, but this hypothesis remains to be tested. We also found that pairs had a high degree of similarity in PRL levels in the second reproductive bout. In our experiment, males and females were force paired so the similarity between PRL

levels is a result of plasticity in PRL production and not in assortative mating. The similarity in PRL levels could be indicative of behavioral and physiological coordination between mates as has been described in other species (Ouyang et al., 2014).

## 5. Conclusions

The immediate reproductive consequences of divorce and pair disruption have been quantified in a range of socially monogamous bird species (e.g. Adkins-Regan and Tomaszycki, 2007; Cockburn et al., 2003; Dreiss and Roulin, 2014; Heg et al., 2003; Naves et al., 2007; Ramsay et al., 2000; Saino et al., 2002; Sanchez-Macouzet et al., 2014). In contrast, only a few studies have examined the underlying hormonal mechanisms associated with mate switching in relation to their effects on parental behavior and reproductive success (Angelier et al., 2007). Our study is the first to examine interactions between CORT and PRL in relation to divorce in a socially monogamous species and also to show that divorce can affect nestling phenotype via increasing stress responses.

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