

# Female Zebra Finches (*Taeniopygia guttata*) Are Chronically but Not Cumulatively “Anemic” during Repeated Egg Laying in Response to Experimental Nest Predation

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## ABSTRACT

Recently it has been recognized that reproduction itself, or the regulatory processes controlling reproduction, might contribute to physiological costs of reproduction. Reproductive anemia, a decrease in hematocrit and hemoglobin concentration, might provide one such mechanism underlying the costs of egg production in birds. In this study, we investigated the effect of repeated cycles of egg production in response to experimental nest predation (egg removal) on hematological traits in female zebra finches (*Taeniopygia guttata*). We predicted that if the negative effect of egg production on hematocrit and hemoglobin concentration was cumulative, with anemia being proportional to reproductive effort, then females laying more clutches, or laying successive clutches without recovery during incubation, would show greater reproductive anemia. In contrast, if females maintain hematocrit and hemoglobin concentration at some minimum functional level independent of reproductive effort, then there should be no difference in hematological traits among females laying two or more successive clutches. Our results supported the second of these hypotheses: egg-laying females had reduced hematocrit (−7.5%) and hemoglobin concentrations (−10%), but the extent of reproductive anemia did not differ among females laying either two or three successive clutches, with or without recovery during incubation, or in females laying 7–21 eggs. Females maintained low hematocrit and hemoglobin for 20–35 d, and we suggest that prolonged periods of anemia might be common and functionally important in free-living birds, for example, where females produce multiple successive clutches in response to high levels of nest

predation or where they initiate a second clutch while still rearing first brood chicks.

## Introduction

Cost of reproduction is a key life-history trade-off that predicts an inverse relationship between investment in current reproduction and future fecundity and survival, but the physiological mechanisms underlying such costs remain poorly understood (Williams 2005; Harshman and Zera 2006). Numerous field studies have confirmed that costs of reproduction are associated specifically with egg production in birds: females that are experimentally manipulated to lay additional eggs show decreases in egg quality, chick viability, survival to fledging (Monaghan et al. 1995; Nager et al. 2000), maternal chick-provisioning ability (Monaghan et al. 1998), and maternal survival (Nager et al. 2001; Visser and Lessells 2001). Traditionally, these costs have been explained in terms of the resource reallocation hypothesis, which suggests that resources necessary for egg formation are reallocated from other physiological functions or female self-maintenance (e.g., immune function) to reproductive effort (Harshman and Zera 2006). More recently, it has been proposed that reproduction itself, or the regulatory (physiological) processes controlling reproduction, might also generate physiological costs to the female (Partridge et al. 2005; Harshman and Zera 2006) that might be independent of allocation of resources per se (i.e., non-resource-based costs [Williams 2005]). Reproductive anemia—a decrease in hematocrit, red blood cell count, and hemoglobin concentration in females associated with egg production—might be one such mechanism underlying costs of egg production (Williams et al. 2004a; Wagner et al. 2008a, 2008b). Anemia has been widely documented in egg-laying females (deGraw et al. 1979; Jones 1983; Morton 1994; Gayathri and Hegde 2006). Initial changes in hematocrit are probably due to hemodilution: osmotic movement of water from extracellular spaces into the blood, which increases plasma volume in order to maintain plasma osmolarity in the face of marked increases in plasma lipids and proteins associated with yolk precursor production (de Graw et al. 1979; Reynolds and Waldron 1999; Challenger et al. 2001). However, anemia also involves transient estrogen-dependent suppression of erythropoiesis—or red blood cell production—and, subsequently, increased reticulocytosis (Wagner et al. 2008a, 2008b). Reproductive anemia is therefore associated with negative pleiotropic effects of the essential reproductive hormone estrogen (sensu

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Ketterson and Nolan 1999), which produce longer-term effects—for example, a slower recovery—that persist beyond the period of egg production itself (Wagner et al. 2008b).

It remains unknown whether reproductive anemia is proportional to reproductive effort such that females that lay more or larger eggs or that lay eggs more frequently (e.g., in replacement clutches) become more anemic and, therefore, potentially pay higher costs of egg production. Kalmbach et al. (2004) showed that experimentally increasing egg production in female great skuas (*Stercorarius skua*) increased anemia in terms of a greater reduction in hematocrit and red blood cell number compared with control females, and they suggested that anemia was proportional to egg-laying effort rather than there being a “fixed” decline. In contrast, Wagner et al. (2008a) suggested that female zebra finches breeding on a low-quality diet traded off reproduction and hematological status, maintaining hematocrit, hemoglobin, and red blood cell number at some minimum functional level at the cost of reduced reproductive investment (see also Garcia et al. 1986). If anemia is positively correlated with reproductive effort, with an “additive” effect of increased or repeated egg production, this could have widespread significance in many free-living birds, for example, where birds initiate a second clutch while still rearing offspring from a first brood (Verhulst and Hut 1996; Gruebler and Naef-Daenzer 2008; Wagner et al. 2008b) or where females produce many successive clutches in response to high levels of nest predation (Grzybowski and Pease 2005; Zanette et al. 2006).

In this article, we investigated the effect of repeated cycles of egg production on hematological traits in the female zebra finch (*Taeniopygia guttata*). Specifically, we caused birds to lay either two or three successive clutches by egg removal (experimental nest predation) with or without the opportunity for physiological recovery during incubation. We predicted that if the negative effect of egg production on hematocrit and hemoglobin concentration was additive or cumulative, then (1) females laying three successive clutches with no incubation (recovery) would show the greatest reproductive anemia and (2) females laying two successive clutches with an intervening period of incubation would show lower reproductive anemia at the end of their replacement clutch than females laying two clutches with no incubation. All birds were provided with a high-quality diet to reduce the effect of resource availability, since we were primarily interested in anemia related to repeated cycles of exposure to reproductive hormones (estrogens) during egg production. After females laid two to three clutches, we let birds rear chicks to investigate the effect of re-laying on hematocrit and hemoglobin concentrations after a phase of chick rearing just before or at fledging of the first-brood chicks.

## Material and Methods

### General Husbandry and Breeding

Zebra finches (*Taeniopygia guttata*) were housed under controlled environmental conditions (temperature 19°–23°C, humidity 35%–55%, constant light schedule of 14L : 10D, lights on at 0700 hours). Before the experiment, all birds were housed

in same-sex cages but were not visually or acoustically isolated from the opposite sex. Females were randomly paired with an experienced male, and breeding pairs were housed individually in cages (61 cm × 46 cm × 41 cm) equipped with an external nest box (15 cm × 14.5 cm × 20 cm). During breeding, nest boxes were checked daily between 0900 and 1100 hours to obtain data on laying interval (the number of days elapsed between pairing and laying of the first egg), egg sequence, egg mass ( $\pm 0.001$  g), and clutch size. Clutches were considered complete when no additional eggs were produced over two consecutive days. All birds received a mixed seed diet (panicum and white millet 1 : 1; approximately 11.7% protein, 0.6% lipid, and 84.3% carbohydrate), water, grit, and cuttlefish bone (calcium) ad lib. and a multivitamin supplement in the drinking water once per week. All experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (657B-96) following guidelines of the Canadian Committee on Animal Care.

### Experimental Protocol

Females were randomly paired with an experienced male, and pairs were randomly assigned to one of three treatment groups. (1) High reneesting/no recovery (HRNR) females were allowed to lay one clutch, and then their eggs were removed 2 d after clutch completion. When females laid a replacement clutch, egg removal was repeated such that females laid a total of three successive clutches with no incubation, that is, no opportunity for physiological recovery between breeding attempts. (2) Low reneesting/recovery (LRR) females were allowed to lay one clutch and incubate these eggs for 10 d before egg removal, allowing for physiological recovery before their laying a second replacement clutch. (3) Low reneesting/no recovery (LRNR) females had their eggs removed 2 d after their first clutch completion, laying a total of two clutches with no incubation and with no opportunity for physiological recovery between breeding attempts (Fig. 1). We paired a total of  $n = 51$  females, but only 28 females completed laying of either a second or third successive clutch in their respective treatments. The final sample sizes were therefore  $n = 10$  pairs for HRNR,  $n = 8$  pairs for LRR, and  $n = 10$  pairs for LRNR. Laying interval was the time (d) between pairing and laying egg 1 of the first clutch. Relaying interval was the time (d) between egg removal and laying egg 1 of the replacement clutch. After laying their second or third successive clutch, females in all treatments were allowed to incubate and rear chicks to fledging from their final clutch, and body mass ( $\pm 0.001$  g), tarsus length, and wing length were recorded for all surviving chicks. Body mass was recorded for females at initial pairing and at the one-egg stage and at clutch completion for each clutch. Breeding pairs were provided with 6 g/pair/d of a high-quality egg food supplement (one whole boiled hen's egg, 13 g cornmeal, 13 g breadcrumbs; 20.3% protein, 6.6% lipid) from 7 d before initial pairing until fledging, and a small portion of romaine lettuce (4 cm × 4 cm) was added daily to each cage.

Blood samples were taken from all females four different

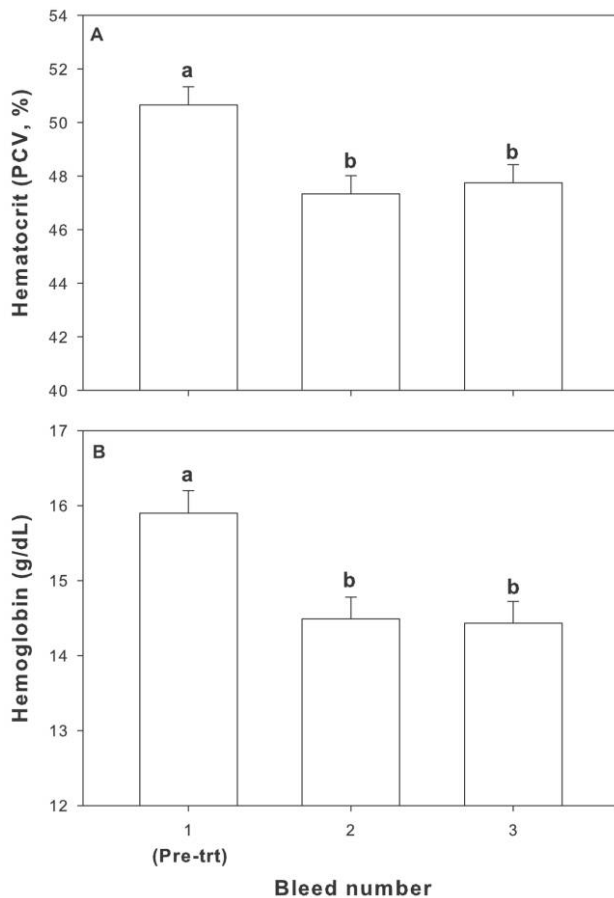


Figure 1. Variation in hematocrit (A) and hemoglobin (B) in female zebra finches before breeding (pretreatment) and at clutch completion after laying two or three successive clutches. Values are least squares means  $\pm$  SE.

times. Seven days before initial pairing, a pretreatment blood sample was taken from all birds. Blood samples during egg production were taken at two intervals depending on the experimental group. HRNR females were sampled 3 d after the final egg was laid (i.e., at clutch completion) after their second and third clutches. LRR females were sampled 1 d after their 10 d incubation/recovery period, which began the day after the last egg of their first clutch was laid, and again at clutch completion after their second clutch. LRNR females were sampled at clutch completion after their first and second clutches. These two blood samples were therefore obtained at similar dates, but birds should have been in different reproductive or physiological states based on the number of clutches laid and the time spent incubating eggs. Finally, we sampled all females at chick fledging of their final clutch, 21 d after mean hatching date. All blood samples ( $\sim 50 \mu\text{L}$ ) were collected from the brachial vein within 3 min of capture (to avoid potential capture-related stress effects) between 0930 and 1130 hours PST.

#### Physiological Measurements

Hematological variables were measured with standard techniques developed for human blood that have been commonly

used on birds (Campbell and Ellis 2007). Hematocrit (% packed cell volume) was measured with digital calipers ( $\pm 0.01 \text{ mm}$ ) following centrifugation of whole blood for 3 min at 13,000 g. Hemoglobin (g/dL whole blood) was measured using the cyanomethemoglobin method (Drabkin and Austin 1932) modified for use with a microplate spectrophotometer (BioTek Powerwave 340, BioTek Instruments, Winooski, VT) by using  $5 \mu\text{L}$  whole blood diluted in 1.25 mL Drabkin's reagent (D5941 Sigma-Aldrich, Oakville, Ontario), with absorbance measured at 540 nm. Intra- and interassay coefficients were 1.1% ( $n = 12$ ) and 2.2% ( $n = 6$ ), respectively, for hemoglobin assays.

We were able to confirm directly whether individual females were egg-producing on the basis of records of egg laying: egg formation takes ca. 4 d in this species, so blood from any bird sampled  $\geq 5$  d before or after they laid an egg was unlikely to have elevated plasma estradiol or yolk precursor levels (Salvante and Williams 2002). For females at fledging, we also measured plasma triglyceride levels—an index of yolk-targeted very low-density lipoprotein (yolk precursor) production or egg development (Mitchell and Carlisle 1991; Williams and Martyniuk 2000; this assay does not measure plasma phospholipids)—using an analytical assay for free glycerol and total glycerol (Sigma-Aldrich). Plasma triglyceride was calculated as the difference between total glycerol and free glycerol. Intra-assay coefficient of variation was 4.0% ( $n = 10$ ), and interassay coefficient of variation was 9.7% ( $n = 6$ ) for plasma triglyceride calculated using a chicken hen plasma pool.

#### Statistical Analysis

All statistical analyses were carried out using SAS software (ver. 9.1; SAS Institute 2003). We analyzed treatment (HRNR, LRNR, LRR) and time (bleed number or clutch number) effects using mixed models (proc MIXED) with treatment and time as main effects, individual as a random effect, and other variables included as covariates where relevant (based on Williams 1996 or our preliminary analysis; e.g., body mass as a covariate for egg mass analyses, laying interval as a covariate in clutch size analyses). Post hoc tests for differences between means were corrected for multiple comparisons using Tukey-Kramer adjustment. All values are presented as least squares means  $\pm$  SE unless otherwise stated.

#### Results

There was no difference in pretreatment female body mass, male (partner) body mass, and hematocrit or hemoglobin for females assigned to the three treatment groups that subsequently laid multiple successive clutches; that is, females were assigned randomly for these traits ( $P > 0.20$  in all cases). In addition, there was no difference in initial body mass ( $P > 0.09$ ) and hematocrit ( $P > 0.70$ ) or hemoglobin concentration ( $P > 0.80$ ) for females that did or did not go on to lay multiple successive clutches. Body mass was independent of treatment, time (bleed), and the treatment  $\times$  time interaction for successive breeding attempts at the one-egg stage ( $P > 0.80$  in all

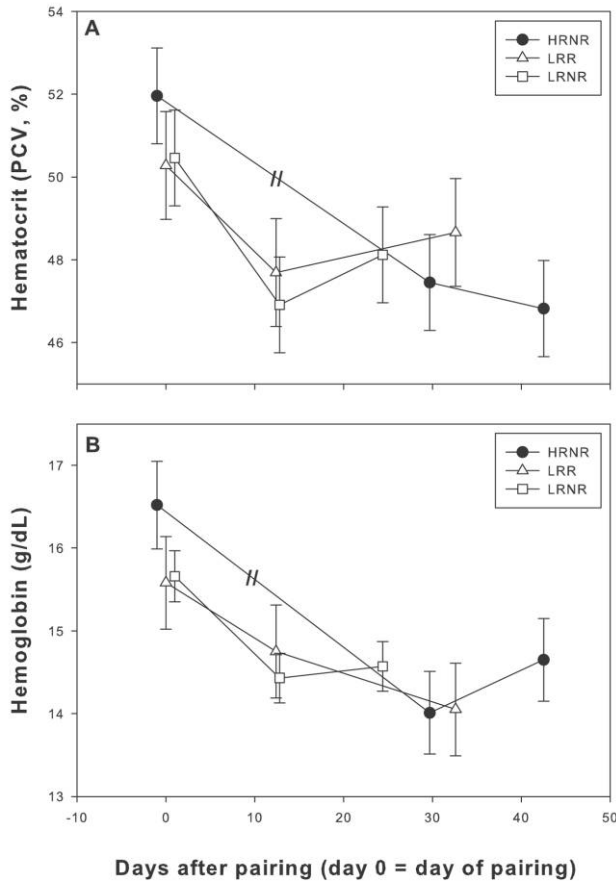


Figure 2. Variation in hematocrit (A) and hemoglobin (B) concentration over time in females laying two or three successive clutches: high reneating/no recovery (HRNR) females laid three successive clutches with no recovery; low reneating/recovery (LRR) females laid two successive clutches with 10 d for physiological recovery during incubation; low reneating/no recovery (LRNR) females laid two successive clutches with no recovery. Values are least squares means  $\pm$  SE.

cases) and at clutch completion ( $P > 0.50$  in all cases), so we do not control for body mass in subsequent analyses.

*Effect of Re-Laying, with and without Recovery, on Hematocrit and Hemoglobin*

There was no effect of treatment ( $F_{2,25} = 0.04, P > 0.95$ ) or a treatment  $\times$  time interaction ( $F_{4,50} = 1.07, P > 0.30$ ) on hematocrit (Fig. 2). However, there was a highly significant time effect ( $F_{2,50} = 12.92, P < 0.001$ ). Hematocrit was significantly lower at clutch completion for both bleed 2 (Tukey-Kramer adjusted,  $P < 0.001$ ) and bleed 3 ( $P < 0.001$ ) compared with pretreatment values, but mean hematocrit for bleeds 2 and 3 were not significantly different ( $P > 0.40$ ). Overall, hematocrit decreased by 7.5% from  $50.9\% \pm 0.7\%$  in pretreatment females to  $47.4\% \pm 0.7\%$  at bleed 2 (Fig. 1A). There was no effect of treatment ( $F_{2,24.3} = 0.15, P > 0.85$ ) or a treatment  $\times$  time interaction ( $F_{4,48.8} = 0.95, P > 0.40$ ) on hemoglobin concentration. However, there was a highly significant time effect

( $F_{2,48.8} = 9.40, P < 0.001$ ). Hemoglobin was significantly lower at both clutch completion bleeds 2 ( $P < 0.001$ ) and 3 ( $P < 0.001$ ) compared with pretreatment values (Fig. 1B), but mean hemoglobin for bleeds 2 and 3 were not significantly different ( $P > 0.90$ ). Overall, hemoglobin concentration decreased by 10.6% from prebreeding ( $15.92 \pm 0.31$  g/dL) to bleed 2 ( $14.39 \pm 0.30$  g/dL; Fig. 1B). Females maintained low hematocrit and hemoglobin for between 20–40 d depending on the number of clutches they laid (Fig. 2).

Total number of eggs laid had no significant effect on the change in hematocrit ( $F_{1,27} = 0.01, P > 0.90$ ; treatment and interaction term,  $P > 0.40$ ) or hemoglobin concentration ( $F_{1,26} = 0.00, P > 0.90$ ; treatment and interaction term,  $P > 0.90$ ) between pretreatment and clutch completion for the final clutch (Fig. 3). Similarly, change in hematocrit and hemoglobin was independent of the total mass of eggs laid ( $P > 0.60$  in both cases).

*Effect of Re-Laying, with and without Recovery, on Reproductive Traits*

There was no difference in mean egg mass, clutch size, or clutch mass for the first breeding attempts among treatment groups

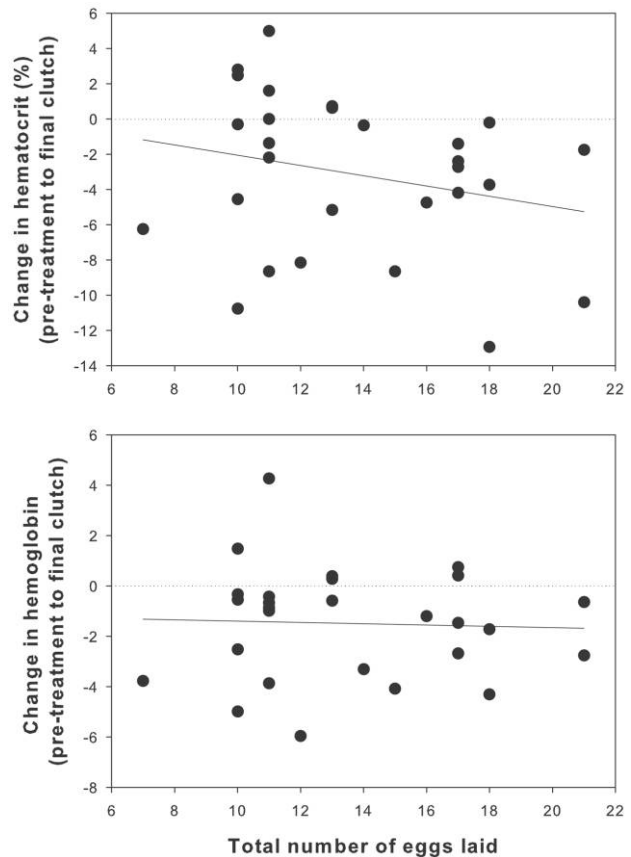


Figure 3. Relationship between total number of eggs laid and change in hematocrit (top) and change in hemoglobin (bottom) between pre-treatment and clutch completion for the final clutch laid.

( $P > 0.10$  in all cases). Time to laying for the first clutch was almost twice as long in HRNR females ( $8.1 \pm 1.2$  d) compared with LRR females ( $4.9 \pm 1.3$  d) and LRNR females ( $4.5 \pm 1.2$  d), but this difference was not significant ( $F_{2,26} = 2.61$ ,  $P = 0.094$ ). However, laying interval was independent of pre-breeding hematocrit, treatment, or the hematocrit  $\times$  treatment interaction ( $P > 0.30$  in all cases), so we did not consider this difference further. Re-laying interval for the first to second clutch did not differ by treatment ( $F_{2,27} = 0.81$ ,  $P > 0.40$ ; HRNR,  $5.9 \pm 0.4$  d; LRR,  $5.3 \pm 0.4$  d; LRNR,  $5.1 \pm 0.5$  d). Similarly, re-laying interval for HRNR females between their second and third clutch ( $5.3 \pm 0.3$  d) was not different from the other treatment intervals for the first to second clutches ( $F_{2,27} = 0.09$ ,  $P > 0.90$ ).

Mean egg mass did not vary with treatment ( $F_{2,22.6} = 0.91$ ,  $P > 0.40$ ), clutch number ( $F_{2,30.4} = 2.32$ ,  $P > 0.10$ ), or the treatment  $\times$  clutch number interaction ( $F_{2,30.3} = 0.19$ ,  $P > 0.80$ ; including female body mass as a covariate; Table 1). There was a marginally significant treatment–clutch number interaction for clutch size ( $F_{2,31.1} = 3.21$ ,  $P = 0.054$ , including laying interval as a covariate) but no effect of treatment ( $F_{2,27.9} = 0.68$ ,  $P > 0.50$ ) or clutch number ( $F_{2,31.5} = 0.73$ ,  $P > 0.40$ ) in the overall model. However, clutch size did not vary with clutch number for any treatment analyzed separately ( $P \geq 0.09$  in all cases; Table 1). There was a significant treatment  $\times$  clutch number interaction for total clutch mass ( $F_{2,32.9} = 5.62$ ,  $P < 0.01$ ; including laying interval as a covariate; Table 1), but again, clutch mass did not vary with clutch number for any treatment when analyzed separately ( $P > 0.10$  in all cases). For these analyses of clutch size and clutch mass, we excluded data for one LRNR female bird that laid an abnormally large clutch size of 14 eggs (Williams 1996); including these data increased the nonsignificance of all results.

There was no significant effect of treatment on chick mass or tarsus or wing length at fledging ( $P > 0.40$  in all cases). LRNR females had smaller mean brood size at fledging ( $1.9 \pm 0.6$  chicks) compared with HRNR ( $3.5 \pm 0.6$  chicks) and LRR females ( $4.1 \pm 0.6$  chicks,  $F_{1,26} = 3.20$ ,  $P = 0.06$ ; controlling for

clutch size). However, this was due to the fact that more LRNR females that laid a final clutch failed to rear any chicks (brood size at fledging = 0). When these females were excluded, there was no difference in brood size between HRNR ( $4.40 \pm 0.4$  chicks), LRNR ( $3.3 \pm 0.5$  chicks), and LRR broods ( $4.0 \pm 0.4$  chicks;  $F_{1,20} = 1.45$ ,  $P > 0.25$ ).

#### Hematocrit and Hemoglobin in Chick-Rearing Females at Fledging

For chick-rearing females sampled at fledging ( $n = 22$ ), we classified birds that laid eggs  $\leq 5$  d after blood sampling as egg producing and those that laid  $> 6$  d after sampling as non-egg producing. Egg-producing females ( $n = 12$ ) had significantly lower hematocrit ( $44.8\% \pm 1.4\%$  vs.  $49.9\% \pm 1.5\%$ ;  $F_{1,21} = 6.13$ ,  $P < 0.025$ ) and higher plasma triglyceride levels ( $F_{1,21} = 7.29$ ,  $P < 0.025$ ) compared with non-egg-producing females ( $n = 10$ ). Sample sizes were too small to look for laying  $\times$  treatment interaction effects on hematology at fledging, and there were no significant treatment effects on hematocrit or hemoglobin among either egg-producing or non-egg-producing females ( $P > 0.30$  in all cases). Overall, egg-producing females had lower hematocrit compared with their pretreatment values ( $-6.0\% \pm 1.7\%$ ,  $t_{12} = 3.47$ ,  $P < 0.01$ ), but hematocrit was not different compared with clutch completion values ( $P > 0.20$ ), although among egg-producing females, HRNR females had the lowest hematocrit ( $41.7\% \pm 3.4\%$ ) compared with LRR ( $45.5\% \pm 2.9\%$ ) and LRNR females ( $46.2 \pm 2.6\%$ ).

#### Discussion

In this study, we investigated the effect of repeated cycles of egg production in response to experimental “nest predation” (egg removal) on hematological traits in the female zebra finch (*Taeniopygia guttata*). We predicted that if the negative effect of egg production on hematocrit and hemoglobin concentration was additive or cumulative, with anemia being proportional to reproductive effort (Kalmbach et al. 2004), then fe-

Table 1: Reproductive output in relation to treatment and clutch number

Treatment and Clutch	<i>n</i>	Egg Mass (g)	Clutch Size	Clutch Mass (g)
HRNR	10			
1		$1.132 \pm .029$	$6.1 \pm .3$	$6.72 \pm .49$
2		$1.169 \pm .029$	$5.7 \pm .3$	$6.70 \pm .49$
3		$1.168 \pm .029$	$5.7 \pm .3$	$6.78 \pm .49$
LRR	8			
1		$1.129 \pm .032$	$5.3 \pm .3$	$6.11 \pm .55$
2		$1.149 \pm .032$	$5.8 \pm .3$	$6.79 \pm .55$
LRNR	10			
1		$1.091 \pm .029$	$6.0 \pm .3$	$7.29 \pm .49$
2		$1.112 \pm .028$	$5.1 \pm .3$	$5.55 \pm .49$

Note. HRNR, high reneating/no recovery females; LRR, low reneating/recovery females; LRNR, low reneating/no recovery females. Values are estimates (least squares means  $\pm$  SE) from proc MIXED model.

males laying more clutches or laying successive clutches without recovery during incubation would show greater reproductive anemia (lower hematocrit and hemoglobin concentration). In contrast, if females maintain hematocrit and hemoglobin concentration at some minimum functional level independent of reproductive effort (Wagner et al. 2008a), then we predicted that there would be no difference in hematological traits among females laying two or more successive clutches.

Our results are clearly more consistent with the second of these hypotheses: the extent of reproductive anemia did not differ among female zebra finches laying either two or three successive clutches with or without recovery during incubation. Although our sample sizes were relatively small, the repeated-measures design we used adds power to these analyses, and we did detect highly significant decreases in hematocrit during laying of the first clutch typical of reproductive anemia in this and other species. In previous experiments using the same repeated-measures design, we have been able to detect differences in hematocrit of as little as 4% with sample sizes of 8–10 per treatment (T. D. Williams, unpublished data), and in this experiment, we detected a highly significant effect of egg production on hematocrit at fledging with only slightly larger sample sizes (10–12 per group). In addition, the decrease in hematocrit and hemoglobin in laying birds was independent of the total number of eggs laid (range 7–21 eggs) and the total mass of eggs laid. We confirmed that egg laying itself was associated with marked reproductive anemia; that is, at clutch completion, hematocrit and hemoglobin concentrations were 7.5% and 10% lower, respectively, than at prebreeding levels, as previously demonstrated for this species (Wagner et al. 2008a, 2008b) and typical of other species (e.g., deGraw et al. 1979; Jones 1983; Morton 1994; Gayathri and Hegde 2006). There was no evidence for an additive or cumulative effect of multiple successive breeding attempts or recovery interval on either hematocrit or hemoglobin levels. However, females did not show any sign of recovery of hematocrit or hemoglobin concentration after their initial clutch while laying subsequent clutches, even in females that had the potential for 10-d recovery during incubation. These results are consistent with a slow, prolonged nature of recovery from reproductive anemia (Wagner et al. 2008a). Females maintained low hematocrit and hemoglobin for 20–30 d (up to 36 d in HRNR females, assuming a significant decrease in hematocrit by clutch completion of their first clutch 12 d after pairing; see Fig. 2).

We provided all females with a high-quality diet because we wanted to exclude any potential resource-dependent effects on changes in hematology (see Wagner et al. 2008b). We conclude that our high-quality diet was effective in this regard because there was no difference in body mass, egg mass, or clutch size in females laying successive clutches, and egg and clutch size were relatively large and typical for zebra finches on a high-quality diet (e.g., see Selman and Houston 1996; Williams 1996). Thus, we tested the idea that any additive effect of laying successive clutches of eggs was due to “non-resource-dependent” mechanisms (*sensu* Williams 2005), specifically negative pleiotropic effects of repeated or continuous exposure to en-

dogenous reproductive estrogens (Wagner et al. 2008a). Females laid the first egg of replacement clutches on average 5 d after egg removal, and rapid yolk development takes 4 d/yolk in zebra finches (Haywood 1993). Assuming that plasma estradiol and yolk precursor levels decreased to baseline by clutch completion (Salvante and Williams 2002; Williams et al. 2004b), then females must have reinitiated estrogen-dependent vitellogenesis within 1 d of egg removal. In other words, for females laying three successive clutches, plasma estradiol levels would have been elevated more or less continuously for up to 30 d, with lower levels at most for 1–2 d on average immediately after the last egg of each clutch was laid but before egg removal. Despite this prolonged, almost continuous exposure to elevated plasma estradiol, which is known to suppress erythropoiesis (Blobel and Orkin 1996; Wagner et al. 2008a), there were no cumulative effects on reproductive anemia. Consistent with this result, Wagner et al. (2008a) showed that experimental elevation of plasma estradiol levels did not cause a further decrease in hematocrit or hemoglobin concentration in egg-laying female zebra finches (although the antiestrogen tamoxifen inhibited development of anemia). We interpret these data as supporting the idea that although females show rapid onset of anemia during their initial egg-laying bout, they maintain a constant minimum level of hematocrit and hemoglobin during subsequent bouts of egg production (Wagner et al. 2008a).

Although we found no evidence for any cumulative effects of reproductive effort on the hematological traits that we measured, we believe that prolonged periods with low hematocrit and hemoglobin (5%–10% lower than nonbreeding levels) might have important consequences for reproductive success in free-living birds, assuming that lower hematocrit negatively influences aerobic capacity or flight performance (Carpenter 1975; Saino et al. 1997; Hammond et al. 2000). In a wide range of species, females will produce multiple successive clutches in response to high levels of nest predation, with egg development and re-laying being initiated immediately after nest failure (Martin 1995; Grzybowski and Pease 2005; Zanette et al. 2006). Furthermore, in this study we confirmed that females that initiate laying of a natural second clutch around the time of fledging of their first brood undergo a second bout of reproductive anemia (as suggested by Wagner et al. 2008a). Many birds have a prolonged period of postfledging parental care that is thought to be important for fledgling survival (Verhulst and Hut 1996; Gruebler and Naef-Daenzer 2008). Thus, a reduction in hematocrit in egg-laying females during this period might have important consequences for sex-specific patterns of postfledging parental care, for example, the female’s provisioning effort.

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