Hematological changes associated with egg production: direct evidence for changes in erythropoiesis but a lack of resource dependence?

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SUMMARY

Reductions in hematological parameters among laying birds are well reported, but the cause of this anemia is not known. We tested specific predictions generated from several, non-mutually exclusive hypotheses for mechanisms underlying reproductive anemia associated with egg production (hemodilution, transient suppression of erythropoiesis, resource dependence) in relation to (1) the time-course of development and recovery from anemia, (2) changes in specific hematological traits, and (3) the effect of diet quality, in female zebra finches (*Taeniopygia guttata*). Female zebra finches showed marked decreases in hematocrit (~6%), red blood cell counts (~8%), and plasma hemoglobin concentration (~9%) during egg production, even on a high-quality *ad libitum* diet, consistent with an effect of hemodilution associated with yolk precursor production. However, our results provide strong support for the hypothesis that erythropoiesis is transiently suppressed during egg-laying and that the recovery from anemia is relatively long-lasting, extending through incubation and hatching periods. Decreased hematocrit, red blood cell counts, and hemoglobin concentration did not recover at clutch completion, but showed evidence of recovery to baseline pre-breeding levels at hatching. More importantly, there was significant time-dependent variation in the proportion of reticulocytes, which increased at clutch completion but peaked at hatching 10–12 days after clutch completion, and in mean red blood cell volume, which showed a significant increase at clutch completion; consistent with enhanced production and release of larger immature cells into the circulation following suppression of erythropoiesis. Finally, we found no evidence for resource dependence of anemia associated with egg production in relation to diet quality, i.e. exogenous lipid and protein resources available to the laying female. This study demonstrates that transient suppression of erythropoiesis and, subsequently, increased reticulocytosis, are key components of reproductive anemia in egg-laying females.

Key words: anemia, cost of reproduction, egg production, erythropoiesis, estrogen, zebra finch.

INTRODUCTION

Although physiological mechanisms underlying 'costs of reproduction' remain poorly understood (Zera and Harshman, 2001; Harshman and Zera, 2007), this trade-off between current reproduction and future fecundity and/or survival has traditionally been assumed to involve reallocation of resources among different, competing physiological systems (Stearns, 1992). More recently it has been suggested that the reproductive process itself or the regulatory (physiological) networks controlling reproduction (e.g. Partridge et al., 2005; Harshman and Zera, 2007) might induce costs of reproduction. Hormones are particularly strong candidates for generating as well as regulating such trade-offs because hormones can have pleiotropic effects, both positive and negative, on multiple physiological systems (e.g. Finch and Rose, 1995; Ketterson and Nolan, 1999; Williams et al., 2005; Zera et al., 2007). Here we focus on one potential physiological mechanism that might mediate costs of reproduction: the development of anemia during egg production in birds (Williams et al., 2004a; Williams, 2005). Anemia – a reduction in hematocrit, hemoglobin and red blood cell number (Campbell and Ellis, 2007) – during egg production has been documented in numerous studies [(e.g. deGraw et al., 1979; Jones, 1983; Morton, 1994; Gayathri and Hegde, 2006; Wagner et al., 2008) and references therein], and may persist into later stages of incubation and chick rearing in some cases (Williams et al., 2004a). In addition, Kalmbach et al. (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. Since anemia should reduce total oxygen carrying capacity of the blood and compromise aerobic performance, this might explain negative effects of increased egg production effort on subsequent reproductive stages (i.e. incubation and chick rearing) that have been widely reported in birds (e.g. Monaghan et al., 1998; Nager et al., 2001).

Several potential mechanisms have been proposed to explain the development of anemia during egg production. Decreased hematocrit might be an indirect effect of estrogen-dependent hepatic production of yolk precursors and mobilization of calcium ions (Morton, 1994; Salvante and Williams, 2002), osmotically active compounds that are transported in the blood at high concentrations during egg production. This in turn may trigger an increase in plasma volume due to osmotic movement of water from extra-cellular spaces into the blood (e.g. hemodilution) to maintain plasma osmolarity or viscosity at a constant level (Reynolds and Waldron, 1999), which would decrease hematocrit (red blood cells per unit plasma volume) but not total cell number. Some authors have proposed that the reduction in hematocrit reflects a transient suppression of erythropoiesis (e.g. red blood cell production) during egg production in order to redirect energy to meet the increased metabolic demands of the reproductive organs (Ronald et al., 1968), or because essential

factors required for erythropoiesis are preferentially allocated to the production of egg components (Jones, 1983; Gayathri and Hegde, 2006; Kasprzak et al., 2006). Alternatively, development of anemia might represent a direct, negative pleiotropic effect of estrogen, which is present at high levels during egg production and has essential reproductive functions (Kalmbach et al., 2004; Williams et al., 2004b; Williams et al., 2005). Estrogens inhibit the differentiation, proliferation and survival of white and red blood cell precursors in the bone marrow (Blobel and Orkin, 1996; Medina et al., 2000; Perry et al., 2000). Blocking estrogen receptors using the anti-estrogen tamoxifen inhibits the development of anemia in egg-laying birds supporting a role for estrogen-dependent suppression of erythropoiesis in anemia (Wagner et al., 2008). The candidate mechanisms that have been proposed involve very different predictions about (1) the time-course of development and recovery from anemia, (2) whether specific changes would occur among a subset or all hematological variables (hematocrit, hemoglobin concentration, red blood cell size and number, proportion of immature red blood cells or reticulocytes; see Table 1), and (3) the extent to which development of anemia should be influenced by resource availability or diet quality.

In the present study, we tested predictions generated from these different, non-mutually exclusive hypotheses for the causal mechanisms underlying anemia associated with egg production using female zebra finches (*Taeniopygia guttata*; see Table 1). Specifically, if hemodilution is solely responsible for the observed decrease in hematocrit, we predicted that, (1) red blood cell number and hemoglobin per unit plasma volume would also decrease during egg production, but that these changes would be reversed at clutch completion when plasma yolk precursor concentration returned to non-breeding levels (Salvante and Williams, 2002), and (2) there would be no changes in mean red blood cell size in relation to cell age [e.g. newly produced red blood cells are larger than mature cells (Campbell and Ellis, 2007)] or in the proportion of reticulocytes, since dilution *per se* would not change rates of red blood cell turnover (production and degradation of cells). By contrast, if anemia involved transient, estrogen-dependent suppression of erythropoiesis, we predicted that (1) decreased hematocrit, cell number and hemoglobin concentration would not recover at clutch completion since it takes 7–14 days for regenerative erythropoiesis to restore red blood cell numbers following experimentally induced anemia (Domm and Taber, 1946; Clark et al., 1988), and (2) there would be a marked increase in immature red blood cells (reticulocytes) post-laying and a corresponding increase in mean cell volume [i.e. reticulocytes are larger than mature cells (Campbell and Ellis, 2007)]. Finally, if anemia during egg production is resource dependent, we predicted that females on a low-quality diet (e.g. protein and lipid deficient) would show larger decreases in hematocrit, red blood cell counts and hemoglobin concentration compared with females on a high-quality diet.

MATERIALS AND METHODS
Study species and husbandry
Zebra finches (*Taeniopygia guttata* Vieillot) were housed under controlled environmental conditions (temperature 19–23°C, humidity 35–55%, constant light schedule of 14 h:10 h L:D, lights on at 07:00 h). 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 libitum*, 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. Prior to the experiment, all birds were housed in same-sex cages, but were not visually or acoustically isolated from the opposite sex. The females selected for this experiment were approximately 8 months of age and had been bred twice previously at 4 and 6 months of age, i.e. they were all experienced breeders. Females were randomly paired with an experienced male, and breeding pairs were housed individually in cages (61×46×41 cm) equipped with an external nest box (15×14.5×20 cm). Body mass (±0.001 g) and tarsus length (±0.01 mm) of both birds was recorded at the time of pairing. Nest boxes were checked daily between 09:00 and 11:00 to obtain data on laying interval (the number of days elapsed between pairing and laying of the first egg), egg sequence, egg mass (±0.001 g), and clutch size. Clutches were considered complete when no additional eggs were produced over two consecutive days.

Experimental protocol
Twenty-nine breeding pairs were randomly assigned to either a low-quality (*N*=14) or high-quality diet treatment (*N*=15). Between pairing and clutch completion, females on the low-quality diet continued to receive the standard seed diet *ad libitum* (see above), while those on the high-quality diet received the standard seed diet plus a daily egg food supplement (6 g day^{-1}, 20.3% protein:6.6% lipid). Initial observations showed that hatching success was extremely low on the low-quality diet, confirming that this diet *was* poor quality, so all pairs receiving this diet were separated and returned to same-sex holding cages at clutch completion. As such, we present data from the hatching and chick-rearing periods for females receiving the high-quality diet only: breeding pairs receiving the high-quality diet were permitted to incubate eggs and rear chicks to fledging, with egg-food provided again during the chick-rearing period. Pairs were left undisturbed from clutch completion until the hatching period, at which point nest boxes were checked daily to determine hatching success per clutch. Immediately after hatching,

Table 1. Summary of predictions generated from 'hemodilution' *versus* 'suppression of erythropoiesis' hypotheses for changes in hematological parameters during the female breeding cycle

Trait	Hemodilution	Suppression of erythropoiesis
Hematocrit	Decrease at 1-egg stage	Decrease at 1-egg stage
	Recovery *at* clutch completion	Recovery *after* clutch completion
Red blood cell number	Decrease at 1-egg stage	Decrease at 1-egg stage
	Recovery *at* clutch completion	Recovery *after* clutch completion
Hemoglobin concentration	Decrease at 1-egg stage	Decrease at 1-egg stage
	Recovery *at* clutch completion	Recovery *after* clutch completion
Red blood cell volume	No change	Increase at clutch completion or hatching (increased proportion of larger, immature reticulocytes)
Proportion of reticulocytes	No change	Increase at clutch completion or hatching (increased reticulocytosis)

chicks were weighed and marked with non-toxic dye to indicate hatch order, and then individually banded at 8 days of age. The mass of each chick was recorded at 7, 10, 14 and 21 days post-hatch to monitor growth rates. At 30 days of age, final brood size for each nest was recorded, and weight, tarsus length, and bill length of each chick was measured. After a 3 month rest period, the same matched pairs were bred again under the opposite diet regime than previously assigned, such that repeated measures data was obtained for 23 pairs that initiated egg-laying on both diet regimes (of the original 29 females two died in the intervening rest period (both from the high-quality diet trial) and four females either did not lay eggs or laid only one egg and were eliminated from the second low-quality diet trial).

Blood sampling and hematological analysis

To monitor hematological parameters across the breeding cycle, female birds were blood sampled at five intervals. (1) pre-breeding – at pairing (N=29); (2) egg-laying – day of laying of first egg (N=29); (3) clutch completion – after two consecutive days without laying an egg (N=25); (4) hatching – the day the first chick hatched (N=18); and (5) fledging – on average 21 days post-hatching (N=12). For the diet-quality analysis we used blood samples obtained for 23 females at prebreeding, the 1-egg stage, and clutch completion only. All blood samples (~50 µl) were collected within 3 min of capture from the brachial vein (to avoid potential capture-related stress effects) between the hours of 09.30 and 11.30.

Hematological variables were measured with standard techniques developed for human blood and commonly used on birds (Campbell and Ellis, 2007). Hematocrit (Hct; %) was measured with digital calipers (±0.01 mm) following centrifugation of whole blood for 3 min at 13,000 g. Hemoglobin (Hb; g dL^{-1} 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, USA), using 5 µl whole blood diluted in 1.25 ml Drabkin's reagent (D5941 Sigma-Aldrich Canada, Oakville, Ontario, Canada) with absorbance measured at 540 nm. Intra- and inter-assay coefficients were 1.7% and 3.9%, respectively. Erythrocyte counts (RBC; number of cells×10^6 µl^{-1}) were determined from duplicate samples (1 µl blood diluted 1/200 with modified Natt and Herrick's solution (Natt and Herrick, 1952; Robertson and Maxwell, 1990) with an improved Neubauer hemocytometer (Fisher Scientific, Ottawa, Ontario, Canada). The average variation among duplicate RBC samples from the same bird was 6.9%, and measurement error (determined from repeated sampling) was 8.9%, which is to be

expected with this technique (Campbell and Ellis, 2007). From these measurements we calculated mean red cell volume (MCV; femtolitres or fl) using the formula Hct/RBC×10=MCV (Campbell and Ellis, 2007). The proportion of reticulocytes (%Ret=number of immature red blood cells/total red blood cells×100) was estimated from whole blood smears after supravital staining with new Methylene Blue (R4132, Sigma Aldrich Canada, Oakville, Ontario, Canada). A total of 1000 red blood cells were counted per slide, and reticulocytes were distinguished from mature erythrocytes by their relatively larger size and less condensed chromatin (Campbell and Ellis, 2007). Red blood cells were classified as reticulocytes if at least five reticulum (RNA) aggregations were visible in the cytoplasm or if a distinct ring of reticulum was surrounding the nucleus (Fernandez and Grindem, 2006). The same individual counted all blood smears (E.C.W.), and slides were randomly coded prior to analysis so that the examiner was blind to the identity of the female and reproductive stage being scored. If blood samples collected at various stages were of insufficient volume to permit all analyses listed above, priority was given to measuring hematocrit (final sample sizes are listed in Table 2).

Statistical analysis

All statistical analyses were carried out using SAS software version 9.1 (SAS 2003). Separate repeated-measures analyses were performed to (1) examine variation in body mass and hematological parameters across the entire breeding cycle in females on the high quality diet (pre-breeding, 1-egg, clutch completion, hatching and fledging stages), and (2) examine the effect of diet quality on changes in body mass and hematological parameters during egg-production (pre-breeding, 1-egg and clutch completion). To examine temporal variation in body mass and hematological parameters, we used repeated-measures mixed linear models (MIXED procedure) with reproductive stage included in the model as a fixed effect and individual as a random effect. For the second analysis (effect of diet quality), diet type and diet × stage were also included as fixed effects in the model. For all repeated measures analyses, the denominator degrees of freedom for tests of fixed effects and *post-hoc* tests were computed using the Kenward-Roger method as recommended by Littell et al. (Littell et al., 2006). The between- and individual-variance components (e.g. random effects) were calculated and evaluated *via* comparison with a Gaussian distribution expected if the variance/covariance was equal to zero. *Post-hoc* tests for differences between means were corrected for multiple comparisons using the Tukey–Kramer adjustment formula. Clutch size was the only variable that was not approximately normal in distribution

Table 2. Sample sizes for hematological analyses in each experiment

	Pre-breeding	1-egg stage	Clutch completion	Hatching	Fledging
A. Experiment 1: temporal variation in hematological parameters					
Body mass	29	29	25	18	12
Hematocrit	29	29	25	18	12
Hemoglobin	29	29	25	16	12
Red blood cell number	29	29	23	18	12
Mean cell volume	29	29	23	18	12
Reticulocyte counts	20	20	20	16	11
B. Experiment 2: effect of diet quality on temporal variation in hematological parameters					
Body mass	23	23	23		
Hematocrit	23	23	23		
Hemoglobin	22	22	19		
Red blood cell number	22	23	19		
Mean cell volume	22	23	19		
Reticulocyte counts	18	14	9		

(Kruskal–Wallis test; UNIVARIATE procedure) and was therefore log-transformed prior to analyses. All values presented are least square means ± s.e.m. unless otherwise stated.

RESULTS

Treatment order (i.e. the order in which females experienced the different diet regimes) was initially included as a fixed effect in all repeated measures analyses but was not found to be a significant term in any of the models ($P>0.1$ for all) and was therefore omitted.

Body mass variation and mass independence of hematological variables

Body mass varied across the complete reproductive cycle for all females ($N=29$) on the high-quality diet ($F_{4,23}=35.91$, $P<0.0001$; Fig. 1A) as we have previously described (e.g. Salvante and Williams, 2002; Wagner et al., 2008). The between-individual variance component estimate associated with the model was 0.61 ± 0.22 ($Z=2.77$, $P<0.003$) and the within-individual variance component was 0.49 ± 0.08 ($Z=6.28$, $P<0.0001$). In relation to diet quality, there was a highly significant diet × reproductive stage interaction for change in body mass between pre-breeding and clutch completion, i.e. the pattern of change in body mass varied by diet ($F_{2,53}=7.96$, $P<0.001$; Fig. 1C). The between-individual variance component estimate associated with the model was 0.58 ± 0.23 ($Z=2.51$, $P<0.015$) and the within-individual variance component was 0.75 ± 0.06 ($Z=12.85$, $P<0.0001$). On the high quality diet, mean body mass increased from pre-breeding to the 1-egg stage, then decreased to levels significantly lower than preceding stages at clutch completion (pre-breeding vs clutch completion, $t_{45}=4.36$, $P<0.008$; 1-egg vs clutch completion $t_{45}=8.92$, $P<0.0001$ (Fig. 1C). By contrast, there was no significant change in mean body mass across reproductive stages on the low quality diet ($P>0.05$ for all comparisons; Fig. 1C).

As previous studies have found a relationship between body condition and hematocrit (reviewed by Fair et al., 2007), body mass was initially included as a covariate in all repeated-measures analyses of hematological variables. However, body mass did not significantly influence the variables of interest ($P>0.1$ for all) except in the analysis of hematocrit across the complete reproductive cycle ($P=0.002$). Therefore, body mass was retained as a covariate for analysis of hematocrit and omitted from all other analyses of hematological traits (hemoglobin concentration, red blood cell number, mean cell volume, and reticulocyte count).

Variation in hematocrit

Hematocrit varied significantly with breeding stage ($F_{4,84}=13.28$, $P<0.0001$; Fig. 1B), including mass as a covariate in the model ($F_{1,104}=5.44$, $P=0.02$). The between-individual variance component estimate was 13.92 ± 5.5 ($Z=2.53$, $P=0.0026$) and the within-individual variance component was 10.51 ± 1.28 ($Z=8.24$, $P<0.0001$). Hematocrit decreased significantly from pre-breeding to the 1-egg stage ($t_{78}=6.99$, $P<0.0001$), and remained significantly lower than pre-breeding levels at clutch completion ($t_{80}=4.4$, $P<0.0003$; Fig. 1B). At hatching, hematocrit was not significantly different from clutch completion or pre-breeding values ($P>0.15$ for both) i.e. hematocrit appeared to recover to 'baseline' levels (Fig. 1B). However, at fledging, hematocrit decreased to levels significantly lower than the preceding stage (hatching vs fledging: $t_{80}=2.86$, $P<0.05$) as well as pre-breeding baseline levels ($t_{81}=4.42$, $P<0.0003$; Fig. 1B).

For the diet-quality experiment ($N=23$), there was a significant diet × reproductive stage interaction for hematocrit ($F_{2,60}=4.18$, $P<0.02$; Fig. 1D). However, post-hoc pairwise analyses did not detect any significant differences in hematocrit at any stage between the different diets ($P\geq0.2$ for all comparisons), and change in hematocrit between breeding stages was independent of diet quality for all pair-
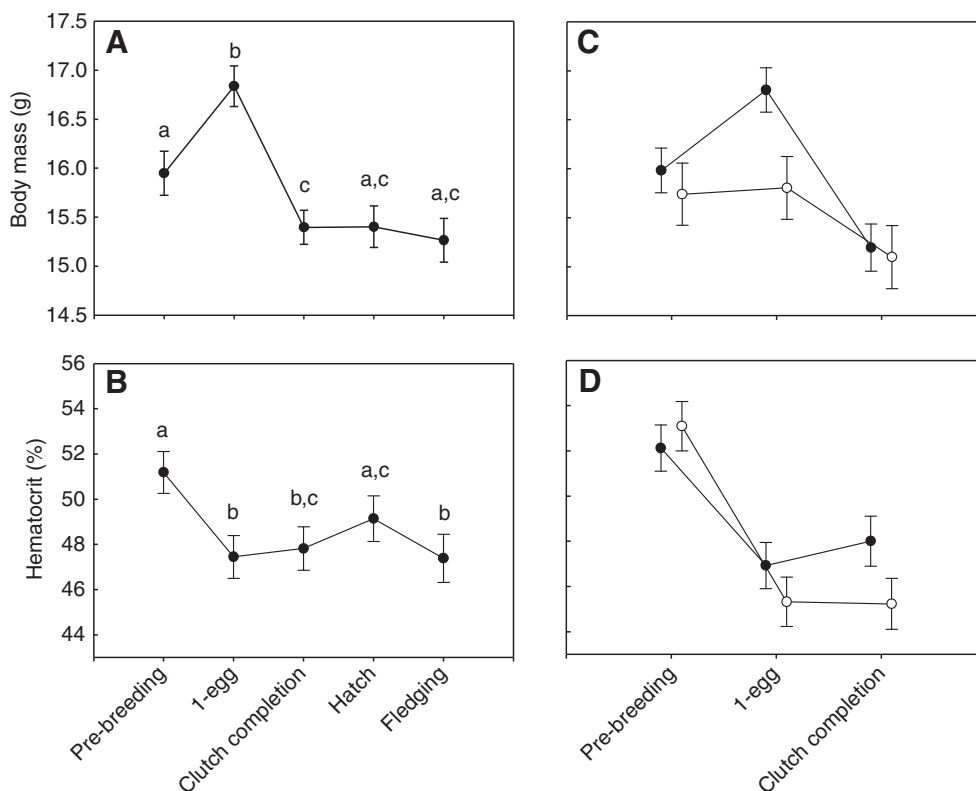

Fig. 1. Variation in (A) body mass and (B) hematocrit across the reproductive cycle on a high-quality diet (different lowercase letters indicate significant differences in means at $P<0.05$ using Tukey–Kramer adjustment); and variation in (C) body mass and (D) hematocrit across the egg-laying cycle on a high-quality (filled circles) versus low-quality (open circles) diet. Values are least-square means ± s.e.m.

wise comparisons ($P \geq 0.10$). After removing the interaction term from the model, the main effects of diet quality ($F_{1,22}=1.24$, $P=0.28$) and body mass ($F_{1,100}=2.30$, $P=0.13$) were not significant, but reproductive stage had a highly significant effect on hematocrit ($F_{2,38}=35.14$, $P<0.0001$). The between-individual variance component estimate associated with the model was 8.55 ± 3.56 ($Z=2.40$, $P<0.02$) and the within-individual variance component was 0.52 ± 0.09 ($Z=5.55$, $P<0.0001$). Among females on the high-quality diet, mean hematocrit decreased significantly from pre-breeding birds to the 1-egg stage ($t_{43}=5.12$, $P<0.0001$), and remained significantly lower than pre-breeding values at clutch completion ($t_{52}=3.76$, $P<0.005$; Fig. 1D, Table 3). On the low-quality diet, hematocrit decreased significantly from pre-breeding to the 1-egg stage ($t_{42}=7.05$, $P<0.0001$), and again remained significantly lower than pre-breeding values at clutch completion ($t_{46}=7.13$, $P<0.001$; Fig. 1D, Table 3).

Variation in plasma hemoglobin concentration
Plasma hemoglobin concentration varied significantly with breeding stage ($F_{4,51}=4.85$, $P<0.02$; Fig. 2A); the between-individual and within-individual variance component estimates associated with the model were 1.86 ± 0.78 ($Z=2.38$, $P<0.01$) and 2.27 ± 0.27 ($Z=8.25$, $P<0.01$), respectively. There were no significant differences in hemoglobin concentration between the pre-breeding, 1-egg, clutch completion or hatching stages ($P>0.1$ for all comparisons), but hemoglobin concentration at the fledging stage was significantly lower than all other stages ($P<0.05$) except clutch completion ($P=0.5$; Fig. 2A). By contrast, for the diet-quality analysis, the diet $\times$ reproductive stage interaction was not significant ($F_{2,51}=0.45$, $P=0.64$; Fig. 2C). After removing the interaction term from the model, the main effect of diet quality was not significant ($F_{1,32}=0.50$, $P=0.48$), but there was a significant main effect of reproductive stage on variation in hemoglobin concentration ($F_{2,50}=13.44$, $P<0.0001$). The between-individual and within-individual variance component estimates associated with the model were -0.33 ± 0.74 ($Z=-0.44$, $P=0.66$) and 0.44 ± 0.10 ($Z=4.44$, $P<0.0001$) respectively. For the pooled diet groups, hemoglobin concentration decreased from 16.19 ± 0.35 mg dl^{-1} in pre-breeding birds to 14.51 ± 0.34 mg dl^{-1} at the 1-egg stage ($t_{47}=4.49$, $P<0.0001$), and hemoglobin concentration remained significantly lower than pre-breeding levels at clutch completion (14.33 ± 0.36 mg dl^{-1}; $t_{51}=4.69$, $P<0.0001$).

Variation in red blood cell number and mean cell volume
Red blood cell (RBC) number varied significantly with breeding stage ($F_{4,78}=11.85$, $P<0.001$; Fig. 2B). The between-individual and within-individual variance component estimates were 0.31 ± 0.11

($Z=2.61$, $P<0.005$) and 0.28 ± 0.05 ($Z=6.17$, $P<0.0001$) respectively. Mean RBC decreased significantly from pre-breeding to the 1-egg stage ($t_{76}=5.01$, $P<0.0001$), and remained significantly lower than pre-breeding levels at clutch completion ($t_{78}=6.09$, $P<0.0001$; Fig. 2B). This was followed by a significant increase in RBC from clutch completion to hatching and fledging stages ($P<0.006$ for both comparisons); mean RBC at hatching and fledging were not significantly different from pre-breeding values ($P>0.3$ for both comparisons; Fig. 2B). For the diet-quality analysis the diet $\times$ reproductive stage interaction was not significant ($F_{2,52}=0.85$, $P=0.44$; Fig. 2D). After the interaction term was removed from the model, the main effect of diet quality was not significant ($F_{1,30}=0.01$, $P=0.9$) but reproductive stage did have a significant effect on variation in RBC number ($F_{2,48}=18.03$, $P<0.001$). The between-individual and within-individual variance component estimates were 0.06 ± 0.11 ($Z=0.54$, $P=0.6$) and 0.51 ± 0.09 ($Z=5.65$, $P<0.0001$), respectively. For pooled data, pre-breeding RBC number ($4.97\times10^6\pm0.14\times10^6$ cells μl^{-1}) was significantly higher than RBC at the 1-egg ($4.21\times10^6\pm0.14\times10^6$ cells μl^{-1}; $t_{46}=5.49$, $P<0.0001$) and clutch completion stages ($4.22\times10^6\pm0.14\times10^6$ cells μl^{-1}; $t_{50}=5.19$, $P<0.0001$).

Mean red blood cell volume varied significantly with breeding stage ($F_{4,78}=3.13$, $P<0.02$; Fig. 3A). The between-individual and within-individual variance component estimates were 133.76 ± 58.97 ($Z=2.27$, $P<0.025$) and 255.89 ± 42.06 ($Z=7.93$, $P<0.0001$), respectively. There were no significant differences found in mean cell volume among pre-breeding, 1-egg and hatching stages ($P>0.05$ for all comparisons; Fig. 3A). However, mean cell volume at clutch completion was significantly greater than pre-breeding mean cell volume ($t_{77}=-2.74$, $P<0.05$), whereas mean cell volume at the fledging stage was significantly lower than mean cell volume at clutch completion ($t_{82}=3.13$, $P<0.025$; Fig. 3A). For the comparison between diets, the interaction diet $\times$ reproductive stage was not significant ($F_{2,48}=0.14$, $P=0.871$; Fig. 3C). After the interaction term was removed from the model, neither diet quality ($F_{1,36}=0.38$, $P=0.54$) nor reproductive stage ($F_{2,56}=1.20$, $P=0.31$) had a significant effect on variation in red blood cell volume. The between-individual and within-individual variance component estimates were 25.57 ± 52.99 ($Z=0.48$, $P=0.63$) and 0.18 ± 0.11 ($Z=1.69$, $P=0.09$), respectively.

Variation in reticulocyte counts
Reticulocyte counts showed significant variation across different stages of the reproductive cycle ($F_{4,40}=3.47$, $P<0.02$; Fig. 3B); the between-individual and within-individual variance component estimates were 1.32 ± 1.94 ($Z=0.68$, $P=0.25$) and 27.14 ± 6.67 ($Z=4.07$, $P<0.005$), respectively. There was no significant difference found

Table 3. Reproductive output and variation in hematocrit of female zebra finches in relation to diet quality

Trait	High-quality diet	Low-quality diet
Pre-breeding body mass (g)	16.0±0.2	15.7±0.3
1-egg body mass (g)	16.8±0.2	15.8±0.3
Mean egg mass (g)***	1.080±0.033	0.893±0.034
Clutch size (number of eggs)***	5.9±0.4	3.6±0.5
Clutch mass (g)***	6.44±0.52	3.16±0.53
Laying interval (days)	7.1±0.5	6.2±0.6
Change in hematocrit (%)		
Pre-breeding to 1-egg	−5.9±1.3 (−8.8 to −3.1)	−8.5±1.3 (−11.2 to −5.8)
One-egg stage to clutch completion	+1.8±1.2 (−0.9 to +4.4)	+0.7±1.1 (−1.8 to +3.2)
Pre-breeding to clutch completion	−4.2±1.5 (−7.4 to −1.0)	−7.8±1.5 (−11.0 to −4.6)

Values are least-squared means ± s.e.m. with 95% confidence intervals in parentheses; ***$P<0.001$ ($N=23$).
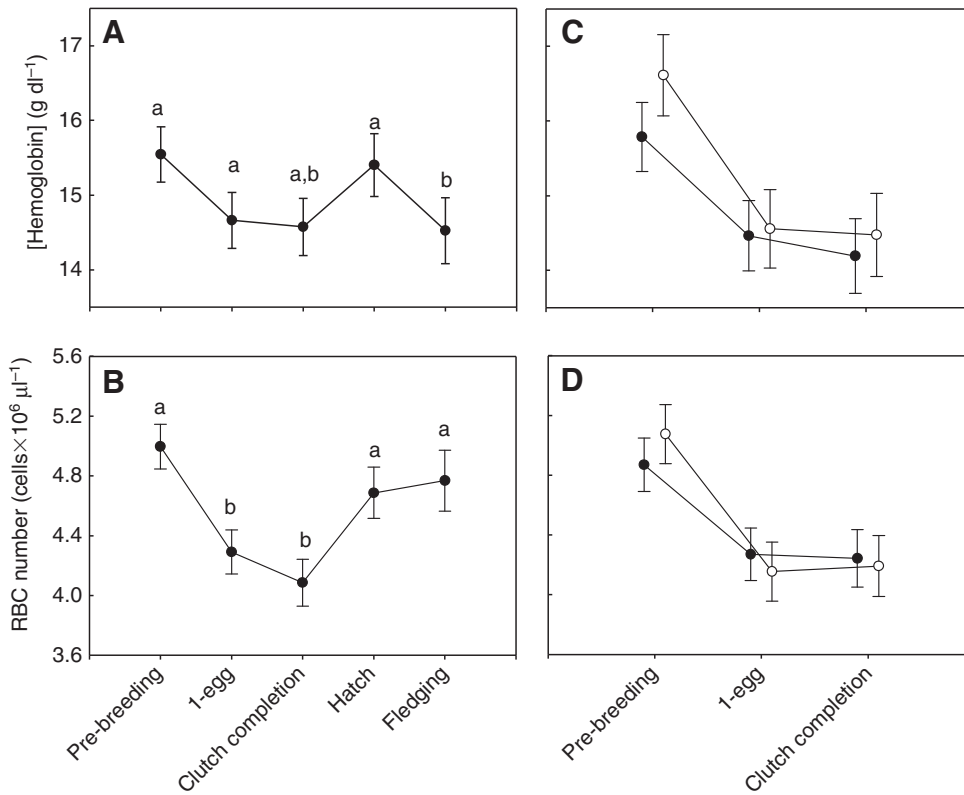
Fig. 2. Variation in (A) hemoglobin concentration and (B) red blood cell (RBC) number across the reproductive cycle on a high-quality diet (different lowercase letters indicate significant differences in means at $P<0.05$ using Tukey–Kramer adjustment); and variation in (C) hemoglobin and (D) red blood cell number across the egg-laying cycle on a high-quality (filled circles) *versus* low-quality (open circles) diet. Values are least-square means ± s.e.m.

in reticulocyte counts between pre-breeding, 1-egg, clutch completion or fledging stages ($P>0.05$ for all comparisons; Fig. 3B). However, the reticulocyte count at hatching was significantly greater than at the pre-breeding ($t_{40}=-2.85$, $P<0.05$) and 1-egg stage ($t_{40}=-3.31$, $P<0.02$; Fig. 3B). For the diet quality experiment, the interaction between diet×reproductive stage was not significant ($F_{2,27}=0.49$, $P=0.62$; Fig. 3D). After removing the interaction term from the model, the main effect of diet quality was not significant ($F_{1,18}=2.26$, $P=0.16$), but reproductive stage had a significant effect on variation in reticulocyte counts ($F_{2,27}=5.56$, $P<0.0085$). The between-individual and within-individual variance component estimates were 34.52 ± 10.83 ($Z=3.19$, $P<0.001$) and -0.23 ± 0.14 ($Z=-1.64$, $P=0.10$), respectively. On both diets, the reticulocyte count at clutch completion ($15.5\pm1.3\%$) was significantly higher than at the pre-breeding ($10.3\pm1.3\%$; $t_{37}=-2.67$, $P<0.03$) and 1-egg stages ($9.4\pm1.2\%$; $t_{39}=-3.2$, $P<0.008$).

Effects of diet quality on reproductive output

On the high-quality diet (all females, $N=29$), mean egg size was 1.106 ± 0.021 g, mean clutch size was 5.9 ± 0.5 eggs and mean clutch mass was 6.444 ± 0.497 g. For those females that hatched chicks ($N=18$; 62% hatching success), mean brood size at hatch was 3.1 ± 0.3 chicks, and number of chicks fledged was 2.0 ± 0.4. Correlational analyses showed no relationship between hematological parameters measured at pre-breeding, 1-egg or clutch completion stages and mean egg size, clutch size or brood size at hatch and fledging stages ($P>0.05$ in all cases), and no relationship between the change in hematological parameters from pre-breeding to the 1-egg stage or from 1-egg to clutch completion stage and reproductive traits ($P>0.05$ in all cases). Diet quality significantly influenced reproductive output: females on the high-quality diet produced larger clutches with larger mean egg mass (Table 3). There was no correlation between reproductive output and hematological

parameters at any stage or the change in these parameters across stages on either the high- or low-quality diet ($P>0.05$ in all cases).

DISCUSSION

We tested specific predictions generated from several, non-mutually exclusive hypotheses for mechanisms underlying reproductive anemia associated with egg production (hemodilution, transient suppression of erythropoiesis, resource dependence) in relation to (1) the time-course of development and recovery from anemia, (2) changes in specific hematological traits, and (3) the effect of diet quality. Female zebra finches showed marked decreases in hematocrit (~6%), red blood cell counts (~8%), and plasma hemoglobin concentration (~9%) during egg production, even with a high-quality *ad libitum* diet. It is likely that the initial decrease in these traits involves hemodilution given the marked changes in plasma yolk precursor, calcium and water balance at onset of laying (Morton, 1994; Reynolds and Waldron, 1999; Salvante and Williams, 2002). However, our results provide strong support for the hypothesis that erythropoiesis is transiently suppressed during egg-laying and that the recovery from anemia is relatively long-lasting, extending through incubation and hatching periods. Decreased hematocrit, red blood cell counts and hemoglobin concentration did not recover at clutch completion, but showed evidence of recovery to baseline pre-breeding levels at hatching, consistent with the estimated timescale for regenerative erythropoiesis following experimentally induced anemia [7–14 days (Domm and Taber, 1946; Clark et al., 1988)]. More importantly, there was significant time-dependent variation in (1) the proportion of reticulocytes, which increased at clutch completion but peaked at hatching 10–12 days after clutch completion, and (2) in mean red blood cell volume, which showed a significant increase at clutch completion. This is consistent with enhanced production and release of larger immature cells into the circulation following suppression
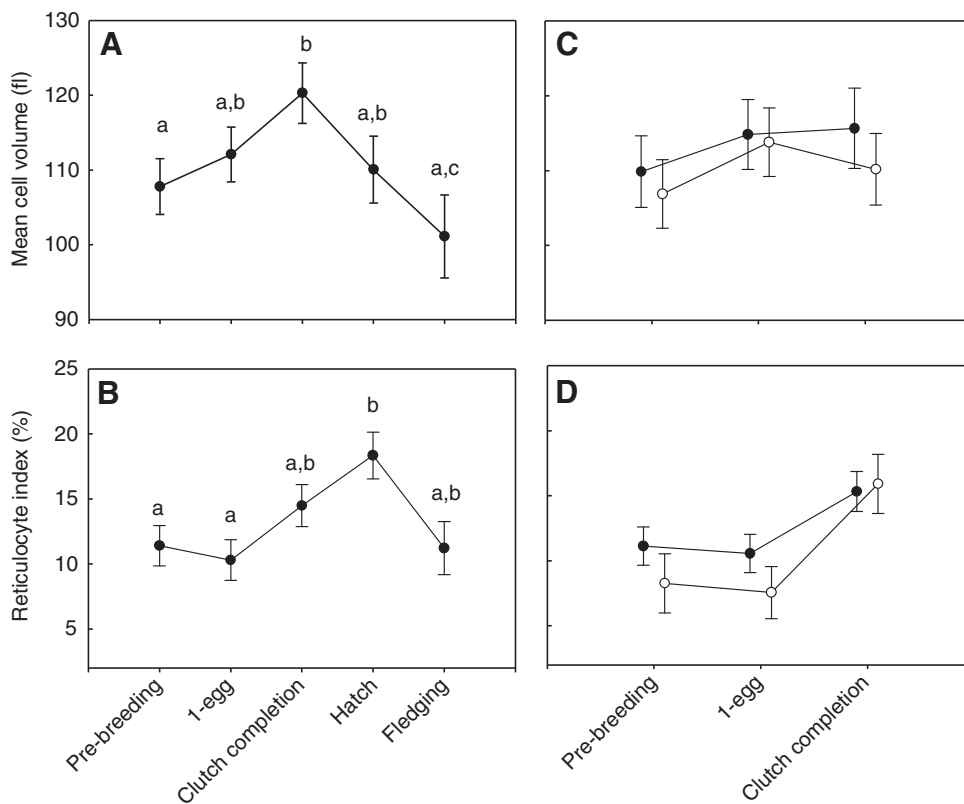
Fig. 3. Variation in (A) mean cellular volume and (B) reticulocyte index (%) across the reproductive cycle on a high-quality diet (different lowercase letters indicate differences in means at *P*<0.05 using Tukey–Kramer adjustment); and variation in (C) mean cellular volume and (D) reticulocyte index across the egg-laying cycle on a high-quality (filled circles) *versus* low-quality (open circles) diet. Values are least-square means ± s.e.m.

of erythropoiesis. Finally, we found no evidence that development of anemia associated with egg production was affected by diet quality, i.e. exogenous lipid and protein resources available to the laying female.

Of note, there were two key differences between experiments regarding temporal variation in hematological traits. In the analysis of temporal variation across the entire reproductive cycle, we found that there was no significant difference in mean hemoglobin concentration and reticulocyte counts between pre-breeding, 1-egg and clutch completion stages, whereas in the diet quality experiment, we found that these traits varied significantly across these stages. Inconsistencies between the two analyses can be attributed to differences in sample size (i.e. not all individuals were included in the diet quality experiment) as well as the significant degree of intra-individual variation in hematological traits typical of this population (see Wagner et al., 2008).

A reduction in hematocrit during egg production has been reported in numerous avian species (reviewed by Williams et al., 2004a; Wagner et al., 2008), ranging from –1.5% in the great tit (Horak et al., 1998a) to –10% in the red-billed quelea (Jones, 1983) relative to pre-breeding levels. Furthermore, Kalmbach et al. (Kalmbach et al., 2004) concluded that the extent of anemia was proportional to egg laying effort. The magnitude of this change in hematocrit is comparable to putative 'adaptive' adjustments of hematocrit proposed to facilitate oxygen uptake and transfer during periods of intense metabolic activity (reviewed by Saino et al., 1997a; Saino et al., 1997b). For example, increased hematocrit (range 1–20%) is associated with acclimatization to cold temperatures (Kubena et al., 1972; Carey and Morton, 1976), low oxygen partial pressures (Jaeger and McGrath, 1974; Keys et al., 1986; Prats et al., 1996), endurance exercise or migration (Palomeque and Planas, 1978; Soler et al., 1999; Landys-Ciannelli et al., 2002) and experimentally elevated flight costs (Saino et al.,

1997a; Cuervo and De Ayala, 2005). This supports that changes in hematocrit associated with egg production (~6% decrease) are biologically relevant and could potentially have a negative impact on future reproduction *via* effects on oxygen carrying capacity and aerobic capacity (Williams et al., 2004a; Williams, 2005).

Reticulocytosis, the enhanced release of reticulocytes from the bone marrow due to stimulatory effects of the hormone erythropoietin, is considered the best single indicator of intensified erythropoiesis in response to the tissue hypoxia associated with anemia (Fernandez and Grindem, 2006). Although the proportions of reticulocytes present at the prebreeding and 1-egg stages in our study were within the reference range reported for small birds (~10%) (Campbell and Ellis, 2007), we noted an approximate 5–7% increase in the proportion of reticulocytes during later breeding stages (clutch completion and hatching) that is consistent with the time-course of the reticulocyte response to anemia. Reticulocytosis is initially observed 2–4 days after induction of anemia (i.e. suppression of red blood cell production or blood loss), typically peaks at 4–7 days, and gradually declines within 2–3 weeks of the original insult (Fernandez and Grindem, 2006). To our knowledge, our study is the first to examine changes in proportion of reticulocytes across the reproductive cycle, although previous studies have investigated changes in the hematopoietic organs in this context (reviewed by Kendall, 1995; Kalmbach et al., 2004). For example, Jones (Jones, 1983) documented that the thymus became enlarged and actively produced red blood cells in the red-billed quelea (*Quelea quelea*) during incubation, and suggested that this functioned to augment erythropoiesis in the bone marrow and compensate for anemia during egg-laying. The changes observed in mean red blood cell volume in the present study were also consistent with the hypothesis that erythropoiesis is suppressed during egg production. During initial stages of egg-laying, estrogen would inhibit the production of new red blood cells, but existing

red blood cells would mature and splenic degradation of senescent erythrocytes would presumably continue (John, 1994). At clutch completion the decrease in plasma estrogen to non-breeding levels (Williams et al., 2004b; Williams et al., 2005) would release erythropoiesis from inhibition. This would result in a higher proportion of reticulocytes in the plasma, which are larger due to less condensed chromatin (Campbell and Ellis, 2007). In other words, fewer red blood cells would be present at clutch completion, but a greater proportion of these cells would be of larger and mean cellular volume would increase; again, consistent with a regenerative response to anemia (Fernandez and Grindem, 2006). It is possible that recovery from anemia, with enhanced and sustained levels of erythropoiesis during incubation, is regulated by high plasma prolactin levels that are present during incubation (Sockman et al., 2006), as prolactin promotes erythropoiesis in mice (Jepson and Lowenstein, 1964), and has been characterized as a hematopoietic growth factor (Constantinescu et al., 1999; Welniak et al., 2001).

In the present study, we found evidence for a further reduction in hematocrit and hemoglobin concentration at the end of the chick-rearing period (fledging), though at a lower level than that seen during laying. In general, reductions in hematocrit during chick rearing have been attributed to a decline in body condition of provisioning parents and/or reallocation of resources to meet increased energetic demands of chick rearing (Morton, 1994; Pap, 2002), although these correlative results contrast with the fact that hematocrit is positively correlated with experimentally enlarged brood sizes in the great tit (Horak et al., 1998b). Although the energetic demands of chick rearing are presumably moderated in captive species (e.g. absence of predators, reduced foraging costs, *ad libitum* food), metabolic rate is still significantly higher in captive-breeding zebra finches during chick rearing compared with pre-breeding or incubating birds (Vezina et al., 2006). However, an alternate explanation in our study is that the decrease in hematocrit at fledging may have been due to a second phase of estrogen-dependent anemia and suppression of eythropoiesis in females preparing to lay a second clutch (i.e. re-laying). Data from a follow-up study (E.C.W. and T.D.W., unpublished data) support this: hematocrit did not decrease linearly throughout the chick-rearing period as would be expected if the energetic demands of chick provisioning were negatively impacting maternal body condition. Rather, hematocrit remains at a constant level during chick rearing, and does not show a decrease until the end of the fledging period, which is when females would be initiating egg production for a second clutch (E.C.W. and T.D.W., unpublished data).

We predicted that if reproductive anemia functioned to allow reallocation of nutrients or energy to egg production, then hematological changes should be more marked in females bred on a protein- and lipid-deficient diet, where the demand for endogenous reserves would presumably be greater. Although there was a significant interaction between diet quality and reproductive stage in the overall model for hematocrit, we could not detect any statistically significant differences in mean hematocrit in relation to diet quality within any breeding stage, or in the *change* in hematocrit between breeding across stages. In addition, diet quality had no effect on hemoglobin concentration or red blood cell number although these traits also decreased significantly during egg-laying. Therefore, we found little evidence to support that diet quality affected the development or extent of anemia during egg production. To our knowledge, no other studies have examined whether diet quality (e.g. protein and lipid content) has an effect on the degree of change in hematological parameters during egg production, although effects of diet restriction on hematology have been investigated in commercial species (Kubena et al., 1972; Garcia et al., 1986; Maxwell et al., 1990). Food restriction is a common practice for improving biological and economical performance in the domestic fowl, and only severe food restriction programs cause hematological parameters to deviate from the 'normal' range (Maxwell et al., 1990). However, there is another possible explanation for the apparent lack of effect of resource availability on hematological traits in our study: diet quality did have a significant effect on primary reproductive effort. Females on the high-quality diet produced bigger clutches of larger eggs, and these eggs were likely of better quality (as indicated by the low hatching success rate for eggs laid on the low-quality diet). This suggests that when diet quality was poor, egg-laying females might have traded-off reproduction for hematological status, maintaining hematocrit, hemoglobin and red blood cell number at some minimum functional level at a potential cost of reduced reproductive investment. Our body mass data support this idea: although on the high-quality diet females were significantly heavier at the 1-egg stage, there was no difference in mean body mass at clutch completion between trials, suggesting that females were not in poorer condition at cessation of laying on the low-quality diet. Maintenance of erythropoiesis at the cost of reduced reproductive output and egg/offspring viability has been demonstrated experimentally in egg-laying Japanese quail fed an iron-deficient diet (Garcia et al., 1986). In comparison to control females, when iron-deficient quail were provided with a radio-labeled iron supplement, a significantly higher amount of labeled iron was detected in the hematopoietic organs (i.e. bone marrow, liver, spleen) and erythrocytes, but no difference was found in amount of labeled iron found in the oviduct or eggs (Garcia et al., 1986). In other words, although iron deposited in the yolk is essential for embryo development and postnatal survival, once iron was provided to iron-deficient females it was preferentially diverted to restoring red blood cell production and hematocrit levels in the laying female herself and not to reproductive output (Garcia et al., 1986).

In conclusion, our study demonstrates that transient suppression of erythropoiesis and, subsequently, increased reticulocytosis are key components of reproductive anemia in egg-laying females. Moreover, reticulocytosis, which is a classic physiological response associated with 'remission' or recovery from anemia is prolonged in egg-laying females (see also Kalmbach et al., 2004) extending into incubation and chick rearing. Thus, reproductive anemia could provide a physiological basis for cost of egg production underlying the negative 'carry-over' effects of increased egg production effort on subsequent reproductive stages (i.e. incubation and chick rearing) that have been widely reported in birds (e.g. Monaghan et al., 1998; Nager et al., 2001). Future work should focus on linking these physiological changes in hematology to fitness, and confirming that this connectivity operates *via* effects on oxygen-carrying capacity and aerobic capacity, preferably using experimental manipulation of hematological parameters in egg-laying females.

LIST OF ABBREVIATIONS

Hb	hemoglobin
Hct	hematocrit
MCV	mean cell volume
RBC	red blood cell count
r	Pearson correlation coefficient
% Ret	percent reticulocytes

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