

## Hematological changes associated with egg production: estrogen dependence and repeatability

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### SUMMARY

The 'cost of reproduction' (i.e. the trade-off between current reproduction and future fecundity and/or survival) is a central concept in life history theory, yet we still know very little about the physiological mechanisms underlying such costs. Recently it has been recognized that reproduction itself or the regulatory (hormonal) mechanisms underlying reproduction might result in 'costs' (cf. resource-allocation based mechanisms). As one example, it has been suggested that the decrease in hematocrit observed during egg production in birds might be due to antagonistic pleiotropic effects of estrogens. This could generate costs of reproduction by reducing oxygen-carrying capacity during subsequent aerobically demanding stages such as chick provisioning. Here we show that the reduction in hematocrit during egg-laying is dependent on receptor-mediated actions of endogenous estrogens: blocking estrogen receptors using the anti-estrogen tamoxifen reduces the decrease in hematocrit during egg production in female zebra finches (*Taeniopygia guttata*) such that hematocrit at the 1-egg stage is not significantly different than pre-breeding, baseline values. We also show that both pre-breeding hematocrit and the decrease in hematocrit associated with egg production are repeatable, and that females with the highest pre-breeding hematocrit values tend to show the largest decreases in hematocrit during egg production. We suggest that hematological changes during egg production are a good candidate mechanism for a regulatory-network based trade-off involving antagonistic pleiotropic effects of estrogens, which otherwise have essential reproductive functions.

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

### INTRODUCTION

The 'cost of reproduction', a central and long-standing component of life history theory, contends that the benefits of increased investment into a current reproductive bout are offset by costs in terms of current condition, future fecundity and survival (Stearns, 1992). Studies where investment in egg production has been experimentally manipulated have provided considerable empirical evidence in support of this concept (Monaghan et al., 1995; Daan et al., 1996; Monaghan and Nager, 1997; Monaghan et al., 1998; Nager et al., 2001). However, we still know very little about the physiological mechanism(s) underlying such costs (Williams, 2005; Harshman and Zera, 2007; Zera et al., 2007). Most studies to date have focused on relatively simple models of resource-based allocation trade-offs (e.g. Gustafsson et al., 1994; Houston et al., 1995; Oppliger et al., 1997; Veasey et al., 2001; Kullberg et al., 2002; Martin et al., 2003), in which energy and/or nutrients are reallocated to egg production and away from other physiological functions with negative consequences. More recently, it has been suggested that costs of reproduction might also be caused by the reproductive process itself or the regulatory (hormonal) mechanisms underlying reproduction (e.g. Partridge et al., 2005; Harshman and Zera, 2007). Hormones are particularly strong candidates for regulating such trade-offs due to their many pleiotropic effects, both positive and negative (Ketterson and Nolan, 1992; Finch and

Rose, 1995; Rose and Bradley, 1998; Ketterson and Nolan, 1999; Reed et al., 2006).

Here we test the hypothesis that decreased hematocrit during egg production reflects a pleiotropic effect of estrogen, the principle female reproductive hormone, which otherwise has essential functions during egg production. Numerous studies in birds have documented a reduction in hematocrit, hemoglobin concentration, and red blood cell counts during egg production (Jones, 1983; Keys et al., 1986; Morton, 1994; Merino and Barbosa, 1997; Horak et al., 1998; Davey et al., 2000; Sheridan et al., 2004; Gayathri and Hegde, 2006). In some cases, the reduction in hematocrit may persist through incubation and chick rearing stages (Williams et al., 2004a). Williams et al. (Williams et al., 2004a) proposed that these associated changes in hematological parameters may play a role in shaping the costs of egg production *via* reducing the total oxygen carrying capacity of the blood, which could negatively impact aerobic performance during subsequent energetically demanding reproductive stages such as chick provisioning (Monaghan et al., 1998; Nager et al., 2001).

Kern et al. (Kern et al., 1972) suggested that the decrease in hematocrit during initial stages of egg production is most likely due to osmoregulatory processes (hemodilution) associated with estrogen-dependent changes in lipid metabolism and the rapid increase of yolk precursors in the blood (Challenger et al., 2001), which induces a compensatory increase in total plasma volume

(Kern et al., 1972; Reynolds and Waldron, 1999). However, plasma concentrations of yolk precursors decrease rapidly upon ovulation of the last follicle, reaching non-breeding levels at clutch completion (Challenger et al., 2001; Salvante and Williams, 2002). If the observed reduction in hematocrit was due to hemodilution alone, then hematocrit should be restored to normal (non-breeding) levels at clutch completion, which is not the case (Williams et al., 2004a; Williams, 2005). One explanation for the persistence of the decrease in hematocrit is that the high levels of estrogens required to drive egg production also have a transient inhibitory effect on erythropoietic (red blood cell) stem cells (Clermont and Schraer, 1979). Estrogen treatment has been shown to induce anemia in several mammalian and avian species (reviewed by Blobel and Orkin, 1996) and molecular studies have demonstrated that estrogen inhibits erythroid gene expression, delays progenitor cell maturation, and induces apoptosis in erythroid cell lineages in vitro (Blobel et al., 1995; Blobel and Orkin, 1996; Perry et al., 2000). Since the estimated lifespan of avian red blood cells is 30–42 days (Rodnan et al., 1957), transient suppression of erythropoiesis during egg production could have relatively long-lasting effects on the proportion of red blood cells in circulation due to continued cell turnover.

In this study, we investigated whether the reduction in hematocrit during egg production is estrogen dependent in the female zebra finch (*Taeniopygia guttata*) using experimental manipulations with 17 $\beta$ -estradiol and the anti-estrogen tamoxifen citrate, and a robust repeated-measures breeding design (i.e. each individual female acted as her own control). We predicted that treatment with exogenous 17 $\beta$ -estradiol would result in a larger reduction in hematocrit during egg production, whereas treatment with tamoxifen citrate would result in a smaller reduction in hematocrit at the onset of egg-laying with reference to unmanipulated and sham-treatment breeding trials for each female. Utilizing the repeated-measures experimental design, we also examined individual variability and repeatability of hematological parameters (hematocrit, hemoglobin, red blood cell size and number) and of the change in hematocrit associated with egg production.

## MATERIALS AND METHODS

### Study species and breeding conditions

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). The 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 (692B-94) following guidelines of the Canadian Committee on Animal Care. Prior to the experiment, all birds were housed in same-sex cages (61×46×41 cm), but were not visually or acoustically isolated from the opposite sex. The females selected for this experiment were between 6 and 9 months in age. Randomly assigned breeding pairs were housed individually in cages (61×46×41 cm) equipped with an external nest box (15×14.5×20 cm). Body mass ( $\pm 0.001$  g), bill length ( $\pm 0.01$  mm) and tarsus length ( $\pm 0.01$  mm) of both birds was recorded at the time of pairing. Breeding pairs were provided with an egg-food supplement (20.3% protein:6.6% lipid) daily from pairing to clutch completion. Nest boxes were checked daily between 09:00 h and

11:00 h to obtain data on laying interval (the number of days elapsed between pairing and deposition of the first egg), egg sequence, egg mass ( $\pm 0.001$  g), and clutch size. A clutch was considered complete when no additional eggs were produced over two consecutive days. At clutch completion, eggs were removed and breeding pairs were returned to same-sex holding cages for a 3-week recovery period prior to subsequent breeding attempts, i.e. no experimental manipulations were performed during this time and pairs did not rear chicks.

### Experimental protocol

A repeated-measures design was used to control for variation among individual females in the traits of interest (i.e. hematological and reproductive variables). Twenty-nine inexperienced females were randomly paired with 29 inexperienced males, and the same matched breeding pairs were used in four successive breeding attempts (experimental trials), alternating with 3-week rest periods. (1) Unmanipulated ( $N=23$ ): females did not receive injections; (2) estradiol treatment ( $N=24$ ): females were injected intramuscularly (i.m.) every other day from pairing until clutch completion with 25.5  $\mu\text{g}$  17 $\beta$ -estradiol (E8875 Sigma-Aldrich Canada Ltd., Oakville ON, Canada) in 30  $\mu\text{l}$  canola oil (1.5  $\mu\text{g}$  g<sup>-1</sup> body mass); (3) sham treatment ( $N=26$ ): females were injected i.m. every other day from pairing until clutch completion with 30  $\mu\text{l}$  canola oil; and (4) tamoxifen treatment ( $N=21$ ): females were injected i.m. every other day from pairing until clutch completion with 170  $\mu\text{g}$  of tamoxifen citrate (T9262 Sigma-Aldrich Canada Ltd.) in 30  $\mu\text{l}$  1,2-propanediol (10  $\mu\text{g}$  g<sup>-1</sup> body mass). Treatment doses were based on our previous work, which found that injections of 25.5  $\mu\text{g}$  17 $\beta$ -estradiol every other day increased estrogen concentration in laying females twofold in comparison to control females but remained within the physiological range of this species (Williams et al., 2005), and that injections of 170  $\mu\text{g}$  of tamoxifen citrate every other day reduced egg size by ~10% but did not alter clutch size or negatively impact maternal condition, whereas more frequent injections negatively affected egg quality, hatchability and offspring growth (Wagner and Williams, 2007) (T.D.W., unpublished data).

In each experimental trial, pre-breeding blood samples were collected from females on the day of pairing to measure hematological parameters (hematocrit, hemoglobin concentration, red blood cell number, mean red cell volume). A second blood sample was collected at the 1-egg stage (i.e. on the day that the first egg was laid) to measure the same hematological parameters as well as plasma estradiol levels. If blood samples collected at the 1-egg stage were of insufficient volume to permit all analyses listed above, priority was given to measuring hematocrit and aliquoting necessary plasma for estradiol determination (final sample sizes are listed in Table 1). All blood samples were collected from the brachial vein within 3 min of capture (to avoid potential capture-related stress effects) on hematological parameters between 09:30 h and 11:30 h.

### Hematological analyses

Hematological variables were measured with standard techniques developed for human blood and commonly used on birds (Campbell, 1995). Hematocrit (Hct; %) was measured following centrifugation of whole blood for 3 min at 13 000 g. Hemoglobin (Hb; g 100 ml<sup>-1</sup> whole blood) was measured using the cyanomethemoglobin method (Drabkin and Austin, 1932) modified for use with a microplate spectrophotometer (BioTek Powerwave 340, BioTek Instruments, Ltd., Winooski, VT, USA), using 5  $\mu\text{l}$

whole blood diluted in 1.25 ml Drabkin's reagent (D5941 Sigma-Aldrich Canada Ltd.) with absorbance measured at 540 nm. Intra- and inter-assay coefficients were 1.71% and 3.90%, respectively. Erythrocyte counts (RBC; number of cells  $\times 10^6 \mu\text{l}^{-1}$ ) were determined from duplicate samples [1  $\mu\text{l}$  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, ON, 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 expected with this technique (Campbell, 1995). As the same examiner (E.C.W.) scored all red blood cell counts, we expect the measurement error to be consistent across different breeding stages and experimental trials. From these measurements we calculated mean red cell volume (MCV; in fl) with the formula  $\text{Hct}/\text{RBC}=\text{MCV}$  (Archer, 1965). For breeding birds, additional blood collected was centrifuged at 2200 g for 10 min to separate the plasma layer, which was decanted and frozen at  $-20^\circ\text{C}$  until assayed for estradiol.

#### Estradiol determination

Plasma samples and controls were passed through C18 columns (octadecylsilane; CUC18156, United Chemical Technologies SPE columns, Chromatographic Specialties Inc., Brockville, ON, Canada) following the procedure described (Williams et al., 2005). Prior to the solid phase extraction procedure, 1 ml of doubly distilled (dd) water was added to each plasma sample (50  $\mu\text{l}$  unless sample volume could only provide 25  $\mu\text{l}$  or 10  $\mu\text{l}$ ). Using vacuum filtration, each column was primed with 3 ml of HPLC-grade methanol, followed by 10 ml of dd water, followed by the entire diluted plasma sample, and then washed with 10 ml of dd water. Estradiol was eluted with 5 ml of 80% methanol into 7 ml borosilicate vials (03-337-26, Fisher Scientific, Ottawa, ON, Canada). Each sample was then evaporated to dryness under vacuum with gentle shaking and reconstituted in 300  $\mu\text{l}$  of 10% methanol.

A pool of zebra finch plasma was used to quantify the recovery of estradiol. Following vortexing, the plasma pool was divided into three aliquots, each containing 500  $\mu\text{l}$  of plasma. One aliquot was diluted with 400  $\mu\text{l}$  of the assay buffer, one was spiked with 400  $\mu\text{l}$  of the 1 ng  $\text{ml}^{-1}$  standard (400 pg spike) and the third remained as raw plasma. Each solid phase extraction run ( $N=6$ ) contained one 50  $\mu\text{l}$  raw sample, two 50  $\mu\text{l}$  diluted samples and one 50  $\mu\text{l}$  spiked sample. Recovery, calculated as the proportion of the 3.7 pg spike in the assay well that was recovered (duplicate determinations for each of six quantifications of the spiked sample – average of 12 diluted sample duplicate determinations across three plates) was 89%.

The concentration of estradiol in each extracted sample was then determined using a  $17\beta$ -estradiol enzyme immunoassay kit (Ecologena/Japan EnviroChemicals Ltd., Abraxis LLC, Warminster, PA, USA) exactly as indicated in the instructions. In duplicate, 100  $\mu\text{l}$  of reconstituted sample (equivalent to 16.7  $\mu\text{l}$ , 8.3  $\mu\text{l}$  or 3.3  $\mu\text{l}$  of the original plasma) was mixed with 100  $\mu\text{l}$  of antigen-enzyme conjugate solution to yield a 5% methanol

Table 1. Sample sizes for hematological analyses at the pre-breeding and 1-egg stages in each experimental trial

Trial/stage	Unmanipulated ( $N=23$ )	Estradiol ( $N=24$ )	Sham ( $N=26$ )	Tamoxifen ( $N=21$ )
Pre-breeding				
Hematocrit	23	23	26	21
Hemoglobin	N/A	23	26	21
Red blood cell number	23	22	26	21
Mean cell volume	23	22	26	21
1-egg stage				
Hematocrit	22	23	26	18
Hemoglobin	N/A	19	23	20
Red blood cell number	13	17	13	20
Mean cell volume	13	17	13	18
Plasma estradiol	22	23	26	19

Hemoglobin concentration was not measured in the unmanipulated trial.

Estradiol, females injected with 25.5  $\mu\text{g}$   $17\beta$ -estradiol; Sham, females injected with 30  $\mu\text{l}$  canola oil; Tamoxifen, females injected with 170  $\mu\text{g}$  of tamoxifen citrate; N/A, not applicable.

solution. Of that 200  $\mu\text{l}$ , 100  $\mu\text{l}$  (equivalent to 8.3  $\mu\text{l}$ , 4.2  $\mu\text{l}$  or 1.7  $\mu\text{l}$  of the original plasma sample of 50  $\mu\text{l}$ , 25  $\mu\text{l}$ , or 10  $\mu\text{l}$ , respectively) was then transferred into an antibody-coated plate for quantification. A total of three assay plates were run, each of which included all samples, in duplicate, from two solid phase extraction runs. One sample fell outside the range of assay sensitivity and was further diluted prior to re-analysis on a subsequent plate. Assay variability was calculated from controls at 9 pg/well and 21.5 pg/well, yielding intra-assay coefficients of variability of 2.3% and 5.3% and inter-assay coefficients of variability of 5.3% and 6.7%, respectively.

#### Statistical analysis

All statistical analyses were carried out using SAS software version 9.1 (SAS Institute, 2003). A repeated-measures mixed linear model (MIXED procedure) was used to compare temporal variation in body mass and hematological parameters across trials, with reproductive stage, treatment and stage  $\times$  treatment interaction included in the model as fixed effects and individual as a random effect. Treatment effects on plasma estradiol concentration and reproductive traits were analyzed using repeated-measures mixed linear models, with treatment included as a fixed effect and individual as a random effect in the model. The effect of reproductive stage on hematocrit was analyzed separately for each trial using generalized linear models (GLM procedure). *Post-hoc* tests for differences between means were adjusted for multiple comparisons following the Tukey–Kramer method. Repeatability of pre-breeding hematological parameters and the change in hematocrit from pre-breeding to the 1-egg stage were determined using nested ANOVA (NESTED procedure) following Lessells and Boag (Lessells and Boag, 1987). Clutch size was the only variable that was not approximately normal in distribution (Kruskal–Wallis test; UNIVARIATE procedure) and was therefore log-transformed prior to analyses. All values presented are least-squares means  $\pm$  s.e.m. unless otherwise stated.

## RESULTS

### Body mass variation and interrelationships with other variables

There was a highly significant treatment  $\times$  reproductive stage interaction for body mass ( $F_{6,72}=8.25$ ,  $P<0.0001$ ; Table 2). In the first, unmanipulated trial, females were initially at a higher body

Table 2. Treatment effects on body mass dynamics and reproductive traits

Trait	Unmanipulated	Estradiol	Sham	Tamoxifen
Sample size	22	23	26	19
Pre-breeding body mass (g)	16.2±0.2**	14.8±0.2	15.0±0.2	15.0±0.3
1-egg body mass (g)	15.8±0.2	15.7±0.2	15.9±0.2	15.3±0.2
Clutch completion body mass (g)	15.3±0.3	14.8±0.3	14.7±0.2	14.6±0.3
Plasma [estradiol] (ng ml <sup>-1</sup> )	1.14±0.48	3.84±0.47**	1.11±0.44	1.16±0.52
Mean egg mass (g)	1.026±0.021	1.072±0.020	1.044±0.019	0.897±0.020**
Clutch size	5.1±0.5	4.9±0.5	5.4±0.5	4.5±0.5
Clutch mass (g)	5.435±0.526	5.192±0.519	5.620±0.497	4.033±0.539
Laying interval (days)	8.30±0.50*	7.15±0.51	6.61±0.48	6.42±0.53

Clutch size was log-transformed prior to analysis but is presented here unaltered for clarity.

Values are least-squares means ± s.e.m. Asterisks indicate significant differences in sample means \* $P < 0.05$ , \*\* $P < 0.001$  (corrected for multiple comparisons). Estradiol, females injected with 25.5 µg 17β-estradiol; Sham, females injected with 30 µl canola oil; Tamoxifen, females injected with 170 µg of tamoxifen citrate.

mass than all other trials ( $P < 0.0002$  for all comparisons), and there was a linear decline in body mass such that mass at clutch completion was marginally lower than pre-breeding values ( $P > 0.07$ ; Table 2). However, in the estradiol- and sham-treatment trials, body mass increased significantly from pre-breeding to the 1-egg stage ( $P < 0.001$  in both cases), then decreased ( $P < 0.001$  in both cases) such that body mass at clutch completion was not significantly different than at pre-breeding ( $P > 0.25$  for both; Table 2). A similar temporal pattern was observed among tamoxifen-treated females, although the increase in body mass from pre-breeding to the 1-egg stage was not significant ( $P > 0.5$ ), but the decrease in mass from the 1-egg stage to clutch completion was significant ( $P < 0.015$ ; Table 2).

Excluding pre-breeding data from the first, unmanipulated trial (but not data for clutch completion for this trial, see Discussion for rationale), there was no difference in mean body mass at pre-breeding ( $F_{2,67}=0.34$ ,  $P > 0.7$ ) and clutch completion stages ( $F_{3,85}=1.34$ ,  $P > 0.25$ ) between treatments, indicating that female condition was not adversely affected by hormonal manipulations or successive breeding attempts. In addition, all hematological parameters (hematocrit, hemoglobin concentration, red blood cell count and mean cell volume) were independent of pre-breeding body mass ( $P > 0.1$  in all cases), and plasma estradiol concentration was independent of 1-egg stage body mass ( $P > 0.1$  for all trials).

#### Variation in plasma estradiol concentration and reproductive output

Experimental treatment had a highly significant effect on mean plasma estradiol concentration at the 1-egg stage ( $F_{3,59}=8.48$ ,  $P < 0.0001$ ; Table 2). There was no difference in plasma estradiol levels at the 1-egg stage among unmanipulated, sham-treated and tamoxifen-treated females ( $P > 0.7$  for all comparisons, 95% CI=1.02–1.22 ng ml<sup>-1</sup>). However, plasma estradiol levels were significantly elevated in the estrogen-treated females ( $P < 0.001$  for all pairwise comparisons; 95% CI=2.22–3.77 ng ml<sup>-1</sup>).

Controlling for differences in female body mass at laying, experimental treatment had a significant effect on mean egg mass ( $F_{3,66}=10.2$ ,  $P < 0.0001$ ; Table 2); mean egg mass was reduced by approximately 10% in tamoxifen-treated females compared with all other breeding trials. However, treatment had no effect on clutch size (controlling for differences in laying interval:  $F_{3,48}=0.94$ ,  $P > 0.45$ ; Table 2) or clutch mass ( $F_{3,46}=2.66$ ,  $P > 0.05$ ; Table 2). Controlling for differences in clutch size, laying interval was significantly longer in the first, unmanipulated trial compared with all other trials ( $F_{3,42}=3.16$ ,  $P < 0.035$ ; Table 2).

#### Treatment effects on hematological parameters

The change in hematological parameters from pre-breeding to the 1-egg stage was independent of the number of days elapsed between blood samples for all four experimental trials ( $P > 0.15$  for all). Among individual females, pre-breeding measurements of hematocrit ( $F_{3,53}=2.14$ ,  $P > 0.1$ ), hemoglobin ( $F_{2,43}=2.06$ ,  $P > 0.1$ ), red blood cell number ( $F_{3,68}=0.46$ ,  $P > 0.7$ ), and mean cell volume ( $F_{3,71}=0.52$ ,  $P > 0.65$ ) did not differ between trials indicating that hematological variables recovered to baseline, pre-breeding levels during the 3-week recovery period between the four successive breeding trials, and that treatment order, breeding experience and age did not alter these parameters (Table 3).

There was a highly significant treatment–reproductive stage interaction for hematocrit ( $F_{3,35}=7.32$ ,  $P < 0.0006$ ) and this was due to maintenance of a higher hematocrit at the 1-egg stage in tamoxifen-treated females (Table 3). Hematocrit decreased significantly from pre-breeding to the 1-egg stage in the unmanipulated ( $-4.3 \pm 1.1\%$ ;  $F_{1,21}=14.54$ ,  $P < 0.001$ ; Fig. 1A), estradiol-treated ( $-5.5 \pm 0.7\%$ ;  $F_{1,22}=60.83$ ,  $P < 0.0001$ ; Fig. 1B) and sham-treated females ( $-5.3 \pm 0.7\%$ ;  $F_{1,25}=66.41$ ,  $P < 0.0001$ ; Fig. 1C), but there was no significant difference in hematocrit from pre-breeding to the 1-egg stage in the tamoxifen-treated females ( $F_{1,17}=3.77$ ,  $P > 0.07$ ; Fig. 1D).

The interaction between treatment–reproductive stage was not significant for red blood cell number ( $F_{3,37}=0.84$ ,  $P = 0.5$ ; Table 3), hemoglobin ( $F_{2,31}=0.96$ ,  $P = 0.4$ ; Table 3), or mean cell volume ( $F_{3,37}=0.07$ ,  $P = 0.98$ ; Table 3), but there was a significant main effect of reproductive stage for red blood cell number ( $F_{1,38}=28.6$ ,  $P < 0.0001$ ; Table 3) and hemoglobin concentration ( $F_{1,37}=13.67$ ,  $P < 0.0007$ ; Table 3). From pre-breeding to the 1-egg stage, red blood cell number decreased by  $-6.64 \times 10^5$  cells µl<sup>-1</sup>, and hemoglobin concentration decreased by  $-1.23$  g 100 ml<sup>-1</sup> for all experimental trials pooled. Mean cell volume did not change significantly from pre-breeding to the 1-egg stage for all trials pooled ( $F_{1,38}=2.36$ ,  $P = 0.13$ ; Table 3).

#### Individual variation and repeatability of hematological parameters

Pre-breeding hematocrit was highly variable among individuals (45–63%; Fig. 1) but was also highly repeatable with individual female, explaining 63.4% of the total variation ( $F_{13,42}=7.93$ ,  $P < 0.0001$ ). Pre-breeding hemoglobin concentration across the estradiol-treatment, sham-treatment and tamoxifen-treatment trials was also repeatable with individual female explaining 35.9% of the total variation ( $F_{13,28}=2.68$ ,  $P < 0.015$ ; hemoglobin data were not

available for the first, unmanipulated trial). However, pre-breeding measurements of red blood cell number ( $F_{13,42}=0.93$ ,  $P=0.5$ ) and mean cell volume ( $F_{13,42}=1.26$ ,  $P=0.3$ ) were not repeatable across trials.

With the exception of tamoxifen-treated females, the majority of individual females showed a robust and consistent decrease in hematocrit between pre-breeding and the 1-egg stage (Fig. 1). Pre-breeding and 1-egg hematocrit were positively correlated in the estradiol- ( $r_{23}=0.60$ ,  $P<0.0025$ ; Fig. 2B) and sham-treated females ( $r_{26}=0.73$ ,  $P<0.0001$ ; Fig. 2C), and this relationship was positive but not significant in unmanipulated females ( $r_{22}=0.31$ ,  $P=0.16$ ; Fig. 2A) and tamoxifen-treated females ( $r_{18}=0.44$ ,  $P<0.07$ ; Fig. 2D). There was some variation among individual females in the magnitude of the decrease in hematocrit (calculated as 1-egg to pre-breeding values) within experimental trials: the change in hematocrit ranged from  $-17.3$  to  $+3.8\%$  in the unmanipulated trial,  $-13.0$  to  $+1.1\%$  in the estradiol-treatment trial,  $-11.3$  to  $+2.1\%$  in the sham-treatment trial and  $-13.3$  to  $+6.0\%$  in the tamoxifen-treatment trial (Fig. 3). Furthermore, the change in hematocrit was negatively correlated with pre-breeding hematocrit for all trials (unmanipulated:  $r_{22}=-0.74$ ,  $P<0.0001$ ; estradiol-treatment  $r_{23}=-0.61$ ,  $P<0.002$ ; sham-treatment:  $r_{26}=-0.51$ ,  $P<0.007$ ; tamoxifen-treatment:  $r_{18}=-0.57$ ,  $P<0.015$ ), i.e. females with the highest pre-breeding hematocrit values tended to show the largest decrease in hematocrit

during egg production (Fig. 3). Nevertheless, for all experimental trials the change in hematocrit from pre-breeding to the 1-egg stage was repeatable, with individual female explaining 31.6% of the total variation ( $F_{13,42}=2.85$ ,  $P=0.005$ ). Excluding the tamoxifen-treatment and unmanipulated trials (see Discussion), repeatability of the reduction in hematocrit for the sham- and estradiol-treatment trials was 66.7% ( $F_{13,14}=5.0$ ,  $P<0.003$ ).

Table 3. Temporal variation in hematological variables

Trait	Pre-breeding	1-egg stage	F value	P value
<b>Hematocrit (%)</b>				
Unmanipulated	51.6±1.0	47.9±0.7	14.54	<0.001
Estradiol	53.4±0.9	47.9±0.8	66.41	<0.0001
Sham	53.2±1.0	47.6±0.8	60.83	<0.0001
Tamoxifen	51.6±1.0	50.1±0.9	NS	0.08
<b>Hemoglobin (g 100 ml<sup>-1</sup>)</b>				
Estradiol	15.57±0.43	14.33±0.56	–	–
Sham	16.47±0.37	15.58±0.28	–	–
Tamoxifen	16.35±0.44	14.66±0.31	–	–
All trials (pooled)	15.57±0.43	14.33±0.56	13.67	0.0007
<b>Red blood cell number (cells×10<sup>6</sup> μl<sup>-1</sup>)</b>				
Unmanipulated	5.10±0.25	4.49±0.27	–	–
Estradiol	5.41±0.30	4.81±0.29	–	–
Sham	5.30±0.14	4.40±0.22	–	–
Tamoxifen	5.22±0.10	4.76±0.09	–	–
All trials (pooled)	5.25±0.10	4.59±0.13	20.22	<0.0001
<b>Mean cell volume (fl)</b>				
Unmanipulated	106.53±5.37	109.55±7.15	–	–
Estradiol	103.22±4.28	106.24±6.27	–	–
Sham	102.74±4.18	108.96±4.30	–	–
Tamoxifen	100.08±1.64	105.91±2.39	–	–
All trials (pooled)	102.97±2.08	108.17±2.86	NS	0.1

Separate repeated-measures ANOVAs were performed for each experimental trial to test for main effect of reproductive stage on hematocrit. Repeated-measures analyses to test for main effect of reproductive stage on hemoglobin, red blood cell number, and mean cell volume were performed on pooled data from all trials.

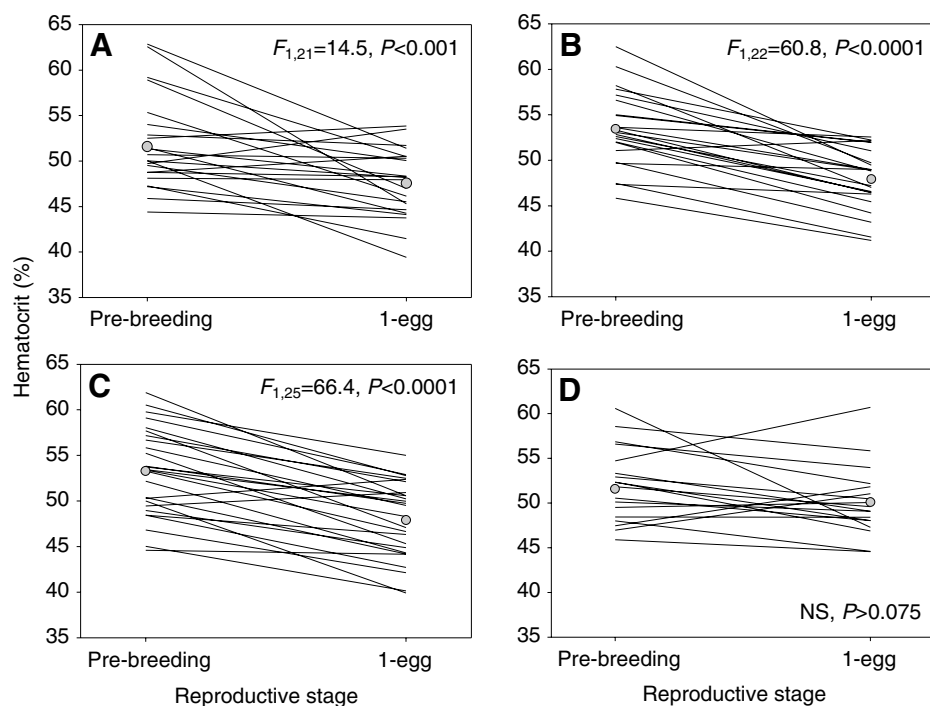


Fig. 1. Reaction norms for change in hematocrit from pre-breeding to the 1-egg stage in the (A) unmanipulated ( $N=22$ ), (B) estradiol-treatment ( $N=23$ ), (C) sham-treatment ( $N=26$ ) and (D) tamoxifen-treatment ( $N=18$ ) trials. Plotted lines represent data from individual females and grey filled circles represent the mean hematocrit calculated at each stage.

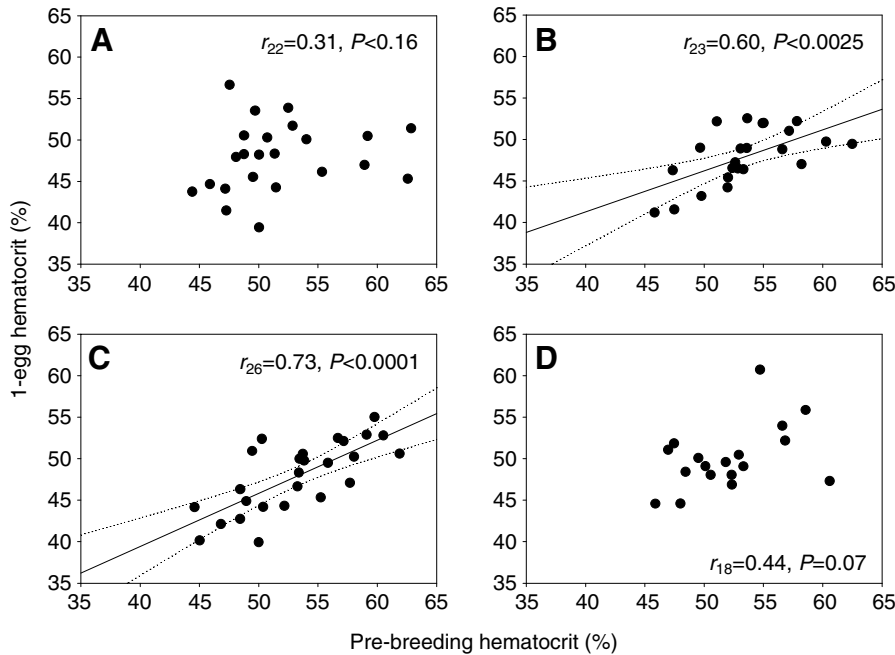


Fig. 2. The relationship between pre-breeding and 1-egg hematocrit among individual females in the (A) unmanipulated trial ( $N=22$ ), (B) estradiol-treatment ( $N=23$ ), (C) sham-treatment ( $N=26$ ) and (D) tamoxifen-treatment ( $N=18$ ) experimental trials. Significant regression lines are presented with 95% confidence intervals (dotted lines).

### DISCUSSION

In this study we have shown that the association between egg production and decreased hematocrit is robust and consistent in female zebra finches even under relatively benign conditions (i.e. in captivity with *ad libitum* food). Although exogenous estradiol treatment did not enhance the reduction in hematocrit (contrary to one of our initial predictions) blocking estrogen receptors with the anti-estrogen tamoxifen resulted in a smaller reduction in hematocrit, to the extent that tamoxifen-treated females did not show a significant change in hematocrit during egg production. Thus, our experimental results support the hypothesis that the decrease in hematocrit during egg production is dependent on the receptor-mediated action of endogenous estrogens. With the exception of

tamoxifen-treated females, most females showed a consistent decrease in hematocrit of approximately 5% in the unmanipulated, sham and estradiol treatments. Both pre-breeding hematocrit and the change in hematocrit associated with egg production were repeatable among individual females, although females with the highest pre-breeding hematocrit values tended to show the largest decrease in hematocrit during egg production. Finally, we found no evidence of compensation for the decrease in hematocrit among other hematological parameters measured: decreased hematocrit during egg production was also associated with a significant decrease in red blood cell number and hemoglobin levels.

Body mass variation, estradiol and tamoxifen treatment effects on reproductive traits in the present study were similar to those

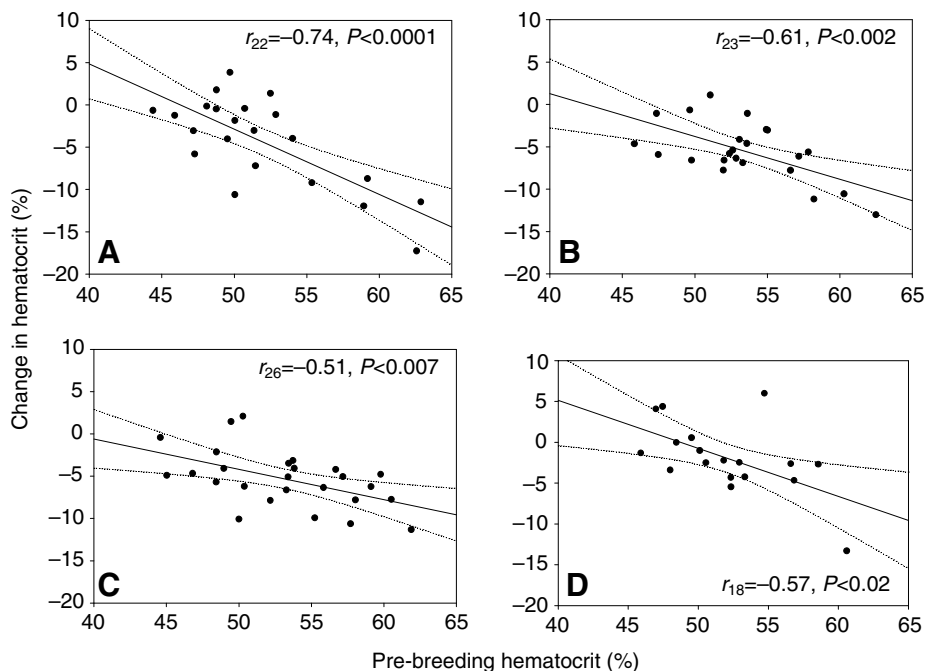


Fig. 3. The change in hematocrit from pre-breeding to the 1-egg stage as a function of pre-breeding hematocrit for (A) unmanipulated ( $N=22$ ), (B) estradiol ( $N=23$ ), (C) sham ( $N=26$ ) and (D) tamoxifen ( $N=18$ ) experimental trials. Regression lines are presented with 95% confidence intervals (dotted lines).

reported in our previous studies (Williams, 1996a; Christians and Williams, 1999; Williams, 1999; Williams, 2000; Wagner and Williams, 2007). In our experiment, all females were exposed to the four treatments in the same sequential order, and it is possible that treatment order, age, or breeding experience might have influenced our results. However, we do not believe that this was the case for the following reasons. First, effects of estradiol and tamoxifen injections at the physiological doses we used are short-term and transient (Johnson and van Tienhoven, 1981; Tsang and Grunder, 1984; Williams, 2000). We also demonstrated directly that hematological parameters returned to 'baseline' pre-breeding values during each of the 3-week recovery periods, and that these pre-breeding hematological variables were repeatable across trials. Second, Williams (Williams, 1996b) has shown that successive breeding attempts with no recovery period between laying bouts does not affect egg and clutch size. In addition, Williams and Christians (Williams and Christians, 2003) demonstrated that age and breeding experience do not significantly influence primary reproductive effort (egg size, clutch size) in this population of zebra finches, the only exception being a longer laying interval in the first breeding attempt, which is consistent with the results of this experiment. During the initial reproductive bout (unmanipulated trial) of the current experiment, females also showed changes in body mass typical of inexperienced breeders (Williams, 1996a) (E.C.W., unpublished data): pre-breeding body mass was significantly higher than all other trials, and in contrast to the pattern observed in estradiol- and sham-treatment trials, females showed a linear decline in body mass from pre-breeding to clutch completion. There was some evidence that hematocrit was similarly affected by breeding inexperience, but for the first trial only. In comparison to the estradiol- and sham-treatment trial, the mean change in hematocrit from pre-breeding to the 1-egg stage was slightly less (~1%) and there was a greater range in the magnitude of this change among individuals, suggesting that breeding experience may alter body condition somewhat through unknown mechanisms. However, the effects of estradiol and tamoxifen were consistent in comparison with both the unmanipulated and sham-treatment groups, and the latter group was included specifically to control for breeding experience. Third, in our study only females in the last (tamoxifen) treatment showed any treatment effect and this was a reduction of the decrease in hematocrit: the opposite to what would be predicted if successive breeding attempts were causing a decline in hematological variables (e.g. maternal condition). Reproductive traits did not vary significantly across the first three trials as might be expected if birds were experiencing 'reproductive exhaustion', and the specific tamoxifen-induced decrease in egg size (with no change in clutch size) in the last trial is entirely consistent with the direct effects of tamoxifen shown in previous studies (Williams, 2000; Williams, 2001; Wagner and Williams, 2007). Finally, in a preliminary experiment (E.C.W., unpublished data) utilizing a different treatment order we obtained results that are consistent with those reported here.

In the present study, tamoxifen inhibited the reduction in hematocrit but estradiol did not enhance the reduction in hematocrit during egg production, and initially these results appear to be contradictory. However, similar differential effects on estrogen-dependent reproductive traits have been documented here and in previous studies: tamoxifen treatment causes a robust decrease in egg size (Williams, 2000; Williams, 2001; Wagner and Williams, 2007), whereas exogenous estradiol does not increase egg size (Christians and Williams, 1999; Williams, 1999). Although previous studies have reported that estradiol treatment induces

anemia (range -2 to -15%) in domestic fowl (Domm and Taber, 1946; Sturkie and Eiel, 1966), pilgrim geese (Hunsaker, 1968), Japanese quail (Nirmalan and Robinson, 1972; Nirmalan and Robinson, 1973; Garcia et al., 1984), rain quail (Deshmukh and Suryawanshi, 1982) and white-crowned sparrows (Kern et al., 1972), these studies all used non-breeding birds, which would have low baseline levels of endogenous estrogens. To our knowledge, our study is the first to assess effects of physiological levels of estradiol on hematological parameters in laying females specifically within the context of egg production. We suggest two reasons for the differential effects of anti-estrogen treatment (tamoxifen) *versus* estrogen treatment on hematocrit: (1) estrogen-mediated physiological effects might be effectively 'maximized' at normal endogenous plasma estradiol concentrations, or (2) despite the experimentally induced increase in circulating estrogens, homeostatic mechanisms may act to maintain hematocrit above some minimum 'threshold' level to maintain a minimum oxygen-carrying capacity required to meet enhanced metabolic demands and/or to facilitate transport of egg constituents to the oviduct. It is possible that in egg-laying females estrogen receptors in bone marrow (the site of erythropoiesis) might be saturated at endogenous estrogen concentrations and thus unresponsive to estrogen supplementation, and/or that estrogen receptor number or receptor sensitivity might be downregulated to modulate pleiotropic effects of the high levels of endogenous estrogens present during egg production (Williams et al., 2004b; Williams et al., 2005). This is consistent with previous work demonstrating that exogenous estradiol treatment stimulates estrogenic processes in non-breeding birds, but does not enhance the same processes in breeding females, e.g. hepatic synthesis and release of yolk precursors (Williams, 1999). Alternatively, homeostatic mechanisms (hemoconcentration or hemodilution) may act to maintain hematocrit within an optimum range that best meets the increased metabolic demands during egg production (Vezina et al., 2003; Vezina et al., 2006); therefore, any inhibitory effects of exogenous estradiol on erythropoiesis may be masked as a result. Given that hematocrit is a critical determinant of blood viscosity (Gaudard et al., 2003), it may not be advantageous for females to deviate from a set range because this would compromise blood flow dynamics and influence efficiency of oxygen and/or nutrient delivery to tissues (Nikinmaa, 1990; Hebert et al., 1997) during key reproductive stages. In addition, hematocrit might be maintained within a set range to maximize transfer efficiency of proteins, lipids, water and trace elements from the plasma reservoir to the oviduct (Reynolds and Waldron, 1999). This idea of some minimum threshold at which egg-producing females maintain hematocrit is supported by our observation that females with relatively low pre-breeding hematocrit showed the smallest change in hematocrit during egg production. However, the fact that decrease in hematocrit itself was repeatable suggests that any threshold hematocrit level might vary among individuals and that the decrease in hematocrit during egg-laying may be 'programmed' both within- and among-individual females, possibly reflecting individual variation in a lower physiological limit below which aerobic capacity would be compromised.

Our study supports the hypothesis that the reduction in hematological parameters during egg production is dependent on the receptor-mediated action of endogenous estrogens, and thus we suggest that this mechanism is a good candidate for a regulatory-network-based trade-off involving antagonistic pleiotropic effects of estrogens, which otherwise have essential reproductive functions during egg production. However, the present study does not

distinguish between the specific proximate mechanisms underlying these changes in hematological parameters that were potentially disrupted by tamoxifen treatment. Our main objective was to manipulate hematocrit levels at the onset of egg-laying, and because of our experimental design, we did not investigate potential long-term effects of changes in hematocrit at later stages of egg production and incubation. Therefore, we could not separate estrogen-dependent hemodilution effects from direct inhibition of erythropoiesis, and we did not capture any delayed effects of estradiol on red blood cell production, e.g. estradiol suppression of erythrocyte production at multiple points in the maturation pathway, such as gene transcription, cell differentiation and hemoglobin production (Blobel and Orkin, 1996). Future studies could clarify this issue by collecting blood samples from estradiol- and tamoxifen-treated females at later stages in the reproductive cycle. In addition, whereas tamoxifen prevented a decrease in hematocrit during egg production, we did not detect any treatment effects on other hematological parameters (i.e. there was no difference in the decrease in hemoglobin and red blood cell number in tamoxifen-treated females). Whether this is the result of too small sample numbers of these more variable, less repeatable traits or whether this reflects differential actions of estrogens, e.g. estrogen-independent components of the hematological response, remains to be determined.

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