1997; Veasey et al., 2001; Kullberg et al., 2002; Martin et al.,
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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.

(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*

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

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

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 decreaselerns)ot aell numbtrerit sedoglobaselhesls.t intreatmeffectst in

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

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

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