ANEMIA: A PHYSIOLOGICAL MECHANISM UNDERLYING THE COST OF EGG PRODUCTION.

by

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ABSTRACT

The 'cost of reproduction' (i.e. trade-offs between current reproduction and future fecundity and/or survival) is a central concept in life history theory, yet the physiological mechanisms underlying such costs remain unknown. Recently it has been recognised that physiological mechanisms regulating reproduction might result in costs, for example, hormones may have antagonistic pleiotropic effects in non-reproductive organs and tissues. Here we investigate the development of anemia during egg production, which may be due to estrogenic inhibition of erythropoiesis. Anemia could function as a cost via reducing oxygen carrying-capacity of the blood, compromising aerobic performance during subsequent reproductive stages. In this thesis, we provide evidence that the development of anemia is not strictly dependent on resource level (e.g. diet quality), but is directly dependent on endogenous estrogens. Therefore, 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.

Keywords: anemia; cost of reproduction; egg production; erythropoiesis; estrogen; zebra finch

Subject Terms: Birds -- Physiology; Birds -- Reproduction; Zebra finch

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: GENERAL INTRODUCTION

Introduction

Life history theory (e.g. the age-specific schedule of fecundity and mortality, Stearns 1992), relates the phenotype of an individual to its fitness, and is essential to understand the role of natural selection in adaptive evolution (Barnes & Partridge 2003). Phenotype is expressed as demographic traits: birth, age and size at maturity, growth and reproductive investment, number and size of offspring, lifespan, senescence and death (Stearns 1992) and these traits may complement each other or interact to form constrained relationships, the balance of which determines individual fitness (Roff 1992; Stearns 1992). Trade-offs, or negative associations between traits, are thought to have a pivotal role in the evolution of life history patterns (Zera & Harshman 2001), as life histories must involve compromises between adaptation and constraint (Barnes & Partridge 2003). Of fundamental importance to the study of phenotypic evolution is the concept of a 'cost of reproduction'. By definition, the cost of reproduction can take one of three forms: increased investment in the current reproductive bout can negatively influence a) the probability of surviving to the next age class (e.g. 'survival' cost), b) the reproductive value of the next age class (e.g. 'reproductive' cost), or c) both survival and future reproduction (Stearns 1992). The concept of a cost of reproduction has been a powerful tool in addressing ultimate questions (i.e. why trade-offs between reproduction versus survival, growth, and condition would exist), but less frequently addressed are the proximate questions, (i.e. the mechanistic basis of these trade-offs themselves). Therefore, the objective of this thesis was to characterize a physiological mechanism, the development of anemia during egg production, which could act as one proximate effector (*sensu* Harshman & Zera 2007) of the cost of reproduction in birds.

Overview: Cost of Reproduction

There is considerable empirical evidence supporting the existence of a cost of reproduction in birds. Traditionally, studies have focused primarily on constraints operating during the chick-rearing stage, in accordance with Lack's (1947) influential model of clutch size evolution. Lack proposed that clutch size is "far below the potential limit of egg production", and that clutch size was not constrained by the number of eggs a female could produce and incubate successfully. Instead he argued that clutch size was constrained by the high demands of chick-rearing, so that the number of eggs laid matched the number of offspring that could be raised successfully to independence (Lack 1947). Accordingly, experimentally induced deviations from this optimum brood size should have negative effects on other life-history traits, compromise fitness and reveal reproductive costs (Sinervo 1999). In support, studies have shown that experimentally increased brood sizes are associated with reduced return rates (e.g. survival; Nur 1984; Dijkstra et al. 1990; Daan et al. 1996) and/or fecundity in subsequent reproductive attempts (Slagsvold 1984; Roskaft 1985; Lessells 1986; Gustafsson & Part 1990; Pettifor 1993; Tinbergen & Both 1999).

More recent work has challenged the assumption that egg production costs are negligible by clearly demonstrating that females do incur costs associated specifically with egg-laying (e.g. Monaghan & Nager 1997). Reproduction can be manipulated by giving parents additional chicks (increased rearing costs; see previous paragraph) or by removing eggs to induce the female to lay replacement eggs (increased egg production and rearing costs). It has been demonstrated that female birds with experimentally

increased egg production and rearing costs produce chicks of lesser quality (i.e. lower hatch mass and growth rates) with lower survival rates compared to chicks produced by females with increased rearing costs only (Monaghan et al. 1995). These effects are due to reductions in the chick-rearing capacity of experimental females, i.e. a trade-off between egg production and maternal body condition (Monaghan et al. 1998). In addition, several studies have reported that increased egg production is associated with decreased return rates and relatively long-lasting reductions in (maternal) body condition and/or fecundity (Nager et al. 2001; Visser & Lessells 2001; Kalmbach et al. 2004). Thus, costs of egg production appear to be as important as those operating during chick-rearing when making reproductive decisions that maximize fitness and minimize the overall cost of reproduction. However, as the functional bases of these costs remain unclear, we can not directly compare costs by stage, or determine if a female is able to minimize or buffer some costs of reproduction via physiological or hormonal adjustment.

Functional Explanations for the Cost of Reproduction

From fruit flies (Sgro & Partridge 1999) to humans (Penn & Smith 2007), the pervasiveness of the cost of reproduction implies that underlying mechanisms may be evolutionarily conserved (Partridge et al. 2005). The possibility of a universal causal mechanism has stimulated considerable research attention, but two conceptually different perspectives have dominated: 1) that the cost of reproduction is driven by conflicting requirements for resources (Williams 1966; van Noordwijk & de Jong 1986), or 2) that reproduction itself, or the regulatory processes controlling it, directly induce costs (Rose

& Bradley 1998; Ketterson & Nolan 1999; Barnes & Partridge 2003; Williams 2005). Each perspective will be discussed in more detail in the following sections.

Resource Allocation Trade-offs

Presumably, costly processes such as reproduction, somatic maintenance and repair, growth, and movement compete for resources, and trade-offs emerge because it is impossible to maximize allocation to all of these processes simultaneously (Partridge et al. 2005). Resources preferentially diverted to reproduction are unavailable for somatic repair and maintenance; therefore, the outcome is an inverse relationship between reproductive rate and lifespan (i.e. reproductive bouts shorten lifespan in an additive manner; Partridge et al. 2005). However, which physiological functions are potentially compromised in this manner and how this resource reallocation trade-off operates on a mechanistic level remains unclear. I will discuss the two most commonly cited resource allocation trade-offs: 1) protein depletion from pectoral (flight) muscle (Houston et al. 1995a), and 2) compromised immune function (Gustafsson et al. 1994).

In many species, pectoral muscle mass decreases during egg production, and it has been suggested that this reflects utilization of muscle sarcoplasm reserves to meet enhanced protein requirements (Houston et al. 1995b). This could function as a cost if flight muscle depletion impaired flight performance, which in turn reduced chick-rearing ability and/or increased likelihood of predation (Veasey et al. 2000; Veasey et al. 2001). However, while flight performance is compromised during egg production in some species (Lee et al. 1996; Kullberg et al. 2002), there is no evidence that this effect is directly attributable to flight muscle depletion. It seems equally likely that impaired flight

ability reflects increased wing-loading in gravid females (Lee et al. 1996). In addition, not all birds show a decrease in muscle mass during egg production (Williams 1996b; Woodburn & Perrins 1997; Kullberg et al. 2002), or do not show an enhanced loss of muscle mass in response to increased egg production following egg removal (e.g. increased protein requirements; Christians 2000). Furthermore, Kullberg et al. (2005) demonstrated that a) there was no difference in flight performance between non-breeding versus egg-laying female zebra finches (tested following deposition of first egg, or at the "1-egg stage") and b) that breeding zebra finches reduced wing load by 9% from the 1-egg stage to clutch completion, *improving* flight speed by 12%. This suggests that muscle depletion is either unrelated to decreased flight performance, or that initial costs may be balanced out if the decrease in muscle mass facilitates flight performance during later stages. There may not be a simple trade-off between flight muscle depletion and flight performance as initially proposed, or at the very least, the relationship between flight muscle depletion and reproductive costs is more complex than originally thought (Cottam et al. 2002; Kullberg et al. 2005; Williams 2005).

Egg production has also been associated with increased susceptibility to parasite infection (Gustafsson et al. 1994; Oppliger et al. 1997), and reduced antibody and cell-mediated components of the immune response (Deerenberg et al. 1997; Moreno et al. 1999; Christe et al. 2002; Pap & Markus 2003; Morales et al. 2004), which is often attributed to a resource-based trade-off between reproductive effort and immunity (Gustafsson et al. 1994; Lochmiller & Deerenberg 2000). Essentially, this trade-off could operate through a) a shift in general energy and/or nutrient allocation away from the

immune system to reproduction (Gustafsson et al. 1994; Sheldon & Verhulst 1996), or b) transfer of a specific limiting factor (carotenoids, immunoglobulins, or lysozymes; Saino et al. 2002a; Saino et al. 2002b; Morales et al. 2006) away from the maternal immune system and into eggs (i.e. maternal effects) to promote embryo/chick immunocompetence (i.e. the ability to mount an effective immune response). However, several other studies have found either no relationship (Williams et al. 1999) or a positive relationship (Apanius & Nisbet 2006) between reproductive effort and immune response, and even a positive relationship between immune response during reproduction and future survival costs (Hanssen et al. 2004). How can these discrepancies between studies be explained? Compromised immune defenses may have a role in mediating immediate costs of reproduction, however, detecting and understanding such trade-offs is confounded by the complexity and redundancy of the immune system (Lee 2006; Martin et al. 2006). Discrepancies between studies may be due to measuring a single component rather than an integrated measure of immune response (Norris & Evans 2000; Salvante 2006), or the possibility that an optimal immune response is not necessarily a strong response (Martin et al. 2006). Again, as with studies of flight muscle depletion, the relationship between immune function and reproductive effort appears to be far more complex than originally thought, and there is little evidence that either resource allocation trade-off described here can have long-term consequences for future fecundity or survival.

Non-resource based Trade-offs

As described above, most studies have focused on resource based allocation trade-offs as the basis of physiological costs of reproduction, and non-resource based

mechanisms have received comparatively little research attention. As suggested by Barnes and Partridge (2003), a non-resource based mechanism would involve reproduction itself or the processes that enable it directly inflicting somatic damage, which would result in a causal, inverse relationship between reproductive rate and lifespan (Partridge et al. 2005). In particular, hormones may be key mediators of costs, as they are essential to regulating reproductive processes but can also have widespread pleiotropic effects in non-target tissues, both positive and negative (Ketterson & Nolan 1992; Ketterson & Nolan 1999; Williams 2005). Much of the work in this field has focused on the pleiotropic effects of androgens in breeding male birds (Wingfield et al. 2001; Reed et al. 2006). Given the many hormones involved in expression of reproductive behaviour and physiology in females (see Williams 1998 for review), it is surprising that costs associated specifically with female hormones remain largely unexplored. The focus of this thesis is one such mechanism, the development of anemia during egg production, which may represent a regulatory-network based trade-off mediated by antagonistic pleiotropic effects of estrogen that otherwise has essential reproductive functions during egg production.

Anemia during Egg Production

Anemia during egg production has been documented in females of several diverse avian species (Table 1.1) and may persist through incubation and chick rearing in some cases (Williams et al. 2004a). The decrease in hematocrit during initial stages of egg production is often attributed to osmoregulatory processes (Kern et al. 1972; Morton 1994; Reynolds & Waldron 1999). The onset of egg production is characterized by

estrogen-dependent changes in lipid metabolism and a rapid increase in the levels of associated yolk precursors in the blood (Challenger et al. 2001; Salvante & Williams 2002). To maintain fluid balance and cell integrity, the homeostatic response to counteract increased plasma concentration of solutes may be hemodilution - an increase in total plasma volume (Reynolds & Waldron 1999). Hemodilution would effectively reduce the proportion of blood cells per unit volume of blood, resulting in decreased hematocrit. However, plasma concentration of yolk precursors decreases rapidly upon ovulation of last follicle, reaching non-breeding levels at clutch completion (Challenger et al. 2001; Salvante & Williams 2002). If anemia is due to hemodilution alone, then hematocrit should be restored to normal (non-breeding) levels at clutch completion. This does not occur; therefore, additional factors must be involved.

One explanation for the persistence of anemia through to later reproductive stages 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 & Schraer 1979). Estrogen treatment has been shown to induce anemia in several mammalian and avian species (Blobel & Orkin 1996). Furthermore, molecular studies have demonstrated that estrogen inhibits gene expression in erythrocyte precursor cells (erythroid cells), delays progenitor cell maturation, and induces apoptosis in avian erythroid cell lineages *in vitro* (Blobel et al. 1995; Blobel & Orkin 1996). Elevated levels of estrogen during the breeding period (Williams et al. 2004b; Williams et al. 2005) may have similar inhibitory effects *in vivo.* As 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 due to continued cell turnover (John 1994). Williams (2005) suggested that pleiotropic effects of estrogen drive the development and duration of anemia in breeding birds. Given that hematocrit is positively correlated with flight ability and aerobic capacity (Carpenter 1975; Viscor et al. 1985; Hammond et al. 2000), anemia may represent an obligatory cost of reproduction attributable to pleiotropic effects of an essential reproductive hormone.

The Zebra Finch (*Taeniopygia guttata*)

The biology of the zebra finch has been reviewed extensively by Zann (1996). In brief, zebra finches are ubiquitous throughout continental Australia, with a pattern of distribution encompassing all climatic zones that prevail on the continent (tropical, subtropical, transitional, and temperate). Their preferred habitat is open grassland with a scattering of trees and bushes to provide nesting sites and shade. The diet of zebra finches consists almost exclusively of grass seeds. Half-ripened seeds are essential for reproduction, so the breeding season coincides with wet summer months when warm, moist conditions facilitate the seeding of most species of grasses. Zebra finches are highly social, forming colonies in the wild that roost, feed, and flock together (ranging from 7-47 nesting pairs). Mating partners in particular establish a strong pair bond, although extra-pair paternity (2.4% of young) has been observed occasionally. Clutch size ranges from 2-7 eggs, egg mass from 0.75 – 1.25 g, and females produce 1-2 broods per breeding season depending on colony location. Both parents participate in parental care, and rearing success varies from 87-100% of nestlings fledged. Juveniles reach

sexual maturity within 3 months of hatching, average lifespan from hatching is 51 days, and maximum lifespan ranges from 3-5.6 years.

The zebra finch is a popular, and in some cases preferred, model species for laboratory research in a wide range of biological disciplines (reviewed in Zann 1996). For our purposes, zebra finches are an ideal model for the study of reproductive decisions and costs in passerine species. Not only do zebra finches breed well in captivity, repeated handling and manipulations (e.g. injections, blood sampling) appear to have little to no effect on individual condition (e.g. body mass, hematocrit; E. Wagner unpublished data) or reproductive output (e.g. egg mass, clutch size; Chapters 2 and 3). The breeding biology of both captive and wild zebra finches has been well-studied (Williams 1996a; Williams 1996b; Zann 1996), and in our colony, females exhibit marked inter-individual variation in traits of interest: egg mass (0.670 – 1.261 g), clutch size (2 – 12 eggs), and baseline (e.g. non-breeding) hematocrit (39.0% - 62.9%), red blood cell count (3.19 – 8.51 $*10^6$ cells/μl), and hemoglobin concentration (12.42 – 20.54 g/dL; E. Wagner unpublished data).

As with any study on a captive species, there are some limitations in extrapolating our results to free-living birds. Selective pressures on captive species can differ dramatically from those acting on free-living species (e.g. Kohane & Parsons 1988). For example, foraging costs are minimized and predation/parasitism risk is eliminated, and as a result, the probability that mating pairs will survive to reproduce again is much higher than free-living counterparts (Zann 1996). These aspects could influence the trade-off between self-maintenance and current reproduction such that a female could allocate

resources preferentially to self-maintenance because she is likely to reproduce again. However, captive conditions could also have the opposite effect. Because laboratory conditions are held constant, females would not have to maintain elevated body reserves to cope with an unpredictable environment (i.e. fluctuations in food/water availability, ambient temperature), and could potentially allocate more resources to reproduction. It is important to consider these issues when interpreting results; however, our rationale for using a captive model species was to control potentially confounding environmental effects and to moderate inter-individual variation in body condition to elucidate reproductive costs. Mechanisms underlying the cost of reproduction are likely to be conserved or similar across avian species; however, future studies of free-living species would illuminate how proximate mechanisms interact with each other and/or the environment at multiple levels of organization.

Summary of Thesis Chapters

This thesis consists of two research chapters, each presented in manuscript form with its own summary, discussion and literature cited sections. I use the term "we" rather than "I" in these chapters to reflect the important contributions of my collaborators to these studies. The names of the collaborators for each chapter are listed on the first page.

In Chapter 2, we sought to distinguish between the mechanistic explanations for the development of anemia during egg production (i.e. estrogen-dependent effects of hemodilution and suppression of erythropoiesis versus a resource-driven allocation trade-off between reproduction and red blood cell production). The key differences between these proposed mechanisms and the predictions for this chapter were: 1) hemodilution

effects would act on a shorter timescale compared to suppression of erythropoiesis, and 2) adaptive reallocation decisions would be strongly influenced by resource availability, whereas estrogenic effects would be relatively resource-independent. To this end, we assessed a) changes in multiple hematological parameters (hematocrit, red blood cell size and number, plasma and cellular hemoglobin concentration) across the entire breeding cycle (i.e. prebreeding, egg-laying, incubation, hatching and fledging of chicks), in the zebra finch, and b) the degree to which hematological changes during egg production were dependent on resource level (diet quality) available to egg-laying females.

In Chapter 3, we directly tested the hypothesis that anemia is an estrogen-dependent effect in egg-laying birds, i.e. that it represents a pleiotropic hormonal effect, *via* hormonal manipulations during egg production. Using a repeated-measures design, we administered 17β-estradiol and the anti-estrogen tamoxifen citrate to breeding female zebra finches and assessed treatment effects on hematological parameters (hematocrit, red blood cell size and number, plasma hemoglobin concentration). We predicted that treatment with exogenous 17β-estradiol would increase the level of anemia during egg production, while treatment with tamoxifen citrate would decrease the level of anemia at the onset of egg-laying with reference to unmanipulated and sham-treatment breeding trials for each female.

In Chapter 4, I briefly review implications of the main results from preceding chapters, and discuss possibilities for future research.

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Table 1.1. Summary of studies that have documented a reduction in hematocrit during egg production in various avian species

Species	Pre-breeding hematocrit (%)	Egg-laying hematocrit (%)	Change in hematocrit	Reference
Great tit	50.5	49	-1.5%	Horak et al. 1998
American kestrel	39	35	-4.0%	Rehder et al. 1982
Shearwater	47	43	-4.0%	Davey et al. 2000
Montane sparrow	55	51	-4.0%	Morton 1994
Rock pigeon	44	39	-5.0%	Gayathri et al. 2004
Brown-headed cowbird	56	51	-5.0%	Keys et al. 1986
Pied flycatcher	52	42	-10.0%	Silverin 1981
Red-billed quelea	55	45	-10.0%	Jones 1983
White-crowned sparrow	50	40	-10.0%	deGraw et al. 1979

CHAPTER 2: CHANGES IN HEMATOLOGICAL PARAMETERS DURING THE REPRODUCTIVE CYCLE OF THE FEMALE ZEBRA FINCH: IS THE DEVELOPMENT OF ANEMIA RESOURCE-DEPENDENT?

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Summary

Egg production is commonly associated with development of anemia in a wide range of avian species; however, the mechanism underlying this decrease in hematocrit remains unclear. Two alternate hypotheses have been proposed: 1) that erythropoiesis is suppressed to differentially allocate energy and/or resources to meet the high demands of egg production, and 2) that the high levels of estrogen regulating egg production have negative pleiotropic effects, inhibiting the production of red blood cells in the bone marrow. To determine whether anemia during egg production is a resource-driven allocation trade-off or a resource-independent pleiotropic effect in the zebra finch (*Taeniopygia guttata*), we investigated a) changes in multiple hematological parameters across the entire breeding cycle to capture additional energetically-demanding stages (i.e. chick-rearing), and b) the degree to which hematological changes during egg production were dependent on resource level (diet quality) available to egg-laying females. Our results support that erythropoiesis is suppressed during egg production: hematocrit (Hct), hemoglobin concentration (Hb), and red blood cell counts (RBC) decreased significantly at the onset of egg-laying, remained low at clutch completion but showed evidence of recovery to pre-breeding levels at the hatching stage. Interestingly, we observed a secondary decrease in Hct and Hb during chick-rearing, which may be due to resource reallocation during the energetically-demanding chick-rearing phase, or a pleiotropic effect of estrogens that may be elevated in preparation for relaying a second clutch. In the diet quality experiment, we found limited evidence that anemia is a resource-driven trade-off. Females appeared to show a larger decrease in hematocrit at the 1-egg and

clutch completion stages on the low-quality versus high-quality diet; however, there was no difference between diets in temporal variation of any other hematological parameters examined (i.e. diet*stage interaction was not significant). The effect of diet quality on reproductive output was much more apparent: females initiated laying earlier, and produced larger, heavier clutches on the high-quality diet. As such, we can not state conclusively that the development of anemia is resource-independent. Instead, we suggest that when diet quality was poor, egg-laying females most likely maintained red blood cell production at some minimum 'threshold' level and hence their own condition at a potential cost of reduced egg size, number, and viability.

Introduction

Life history theory predicts a trade-off between current reproductive effort and maternal condition, future fecundity and/or survival (Williams 1966; Stearns 1992), and studies of birds have provided considerable evidence supporting this 'cost of reproduction' (Monaghan & Nager 1997; Zera & Harshman 2001; Williams 2005). In passerines, reproduction imposes significant energetic demands across the breeding cycle (Williams & Vezina 2001; Vezina et al. 2006), and the traditional explanation for how a female meets these enhanced energetic demands has been the differential allocation of resources (Stearns 1992). This theory predicts that since a female has access to a fixed amount of resources, she must reallocate resources among different physiological systems in response to environmental, social and/or internal cues in order to optimize the trade-off between self-maintenance and reproductive effort (Stearns 1992). When resources are limited, reproduction will be more costly because to maintain reproductive output, proportionally fewer resources will be available to divert towards self-maintenance, potentially compromising survival and future fecundity (Rutstein et al. 2005). In support, studies have consistently demonstrated that a poor quality diet and/or increased investment in egg production, incubation, and chick-rearing results in reduced egg and chick viability, maternal survival and future reproductive effort (Roskaft 1985; Daan et al. 1996; Monaghan & Nager 1997; Monaghan et al. 1998; Rutstein et al. 2005)

Recently, an alternative view of the cost of reproduction has come to light. Rather than a purely resource-driven allocation trade-off, the reproductive process itself or the regulatory (physiological) networks controlling reproduction may directly induce costs of

reproduction (e.g. Partridge et al. 2005; Harshman & Zera 2007). Hormones are particularly strong candidates for regulating such trade-offs due to their many pleiotropic effects, both positive and negative (e.g. Ketterson & Nolan 1992; Finch & Rose 1995; Ketterson & Nolan 1999; Williams et al. 2005; Reed et al. 2006). To critically evaluate these two competing hypotheses we need a better understanding of the proximate mechanisms underlying costs of reproduction, which despite considerable research attention, remain unclear (Harshman & Zera 2007). In this paper, we focus on one specific physiological mechanism, the development of anemia during egg production. Anemia may function as a physiological mechanism underlying the cost of reproduction by 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 (Williams et al. 2004a; Williams 2005). In addition, speculation regarding the cause of anemia has been divided between the resource allocation and hormonal pleiotropy explanations (see below), so this particular mechanism presents an excellent opportunity to differentiate between these competing hypotheses.

A significant decrease in hematocrit levels from pre-breeding to egg-laying stages has been observed in a wide range of avian species (Ronald et al. 1968; deGraw et al. 1979; Keys et al. 1986; Ronald & George 1988; Morton 1994; Strakova et al. 1994; Merino & Barbosa 1997; Davey et al. 2000; Strakova et al. 2001; Gayathri et al. 2004; Sheridan et al. 2004; Williams et al. 2004a; Gayathri & Hegde 2006). Several authors have proposed that erythropoiesis is transiently suppressed during egg production in

order to redirect energy to meet the increased metabolic demands of the reproductive organs (Ronald et al. 1968), or that anemia is the result of essential factors required for erythropoiesis (i.e. proteins, lipids, trace elements) being preferentially allocated to the production of egg components (Jones 1983; Gayathri & Hegde 2006; Kasprzak et al. 2006). Alternatively, Williams (2005) suggested that the development of anemia is a pleiotropic effect of estrogen, which is present at high levels during initial stages of egg production (Williams et al. 2004b; Williams et al. 2005). It has been demonstrated that estrogen inhibits the differentiation, proliferation, and survival of white and red blood cell precursors in the bone marrow (Medina et al. 2000; Perry et al. 2000), resulting in anemia (see Blobel & Orkin 1996 for review). Estrogen may also act indirectly, by stimulating hepatic production of yolk precursors and mobilization of calcium ions (Salvante & Williams 2002; Morton 1994), osmotically active compounds transported in the blood at high concentrations during initial stages of egg production. This in turn may trigger hemodilution, the movement of water from body stores into the blood via osmosis to maintain plasma osmolarity at a constant level (Reynolds & Waldron 1999). The key differences between these proposed mechanisms and the basis for the current experiment are that hemodilution effects would act on a shorter timescale relative to suppression of erythropoiesis, and adaptive reallocation decisions would be strongly influenced by resource availability, whereas estrogenic effects would be relatively resource-independent.

In this study, we had two main objectives: 1) to distinguish between short-term effects of hemodilution versus potentially long-term effects of suppressed erythropoiesis,

we measured hematocrit, hemoglobin concentration, red blood cell count, and red blood cell indices (mean cell volume and mean cellular hemoglobin) at pre-breeding, egg-production, incubation, hatching and fledging of chicks in female zebra finches (*Taeniopygia guttata*), and 2) to determine if the development of anemia is dependent on resource availability, we investigated the degree to which hematological changes during egg production were dependent on the quality of diet provided to egg-laying female zebra finches.

Predictions for the first experiment are summarized in Table 2.1, and are as follows. If hemodilution effects are solely responsible for the observed decrease in hematocrit, we predicted that red blood cell number and hemoglobin would also decrease during egg production, but that these hematological parameters would recover at clutch completion (i.e. once plasma yolk precursor concentration had returned to non-breeding levels; Salvante & Williams 2002). In addition, we would not expect to see any age-related changes in red blood cell size or cellular hemoglobin concentration across the breeding cycle. In contrast, if erythropoiesis was also transiently suppressed during egg production, we predicted that hematological parameters would not recover until hatching, as it would take 7-14 days post-suppression for regenerative erythropoiesis to restore red blood cell numbers to pre-breeding levels (Nikinmaa 1990). In addition, as immature red blood cells are larger in size but contain less hemoglobin than mature cells (Campbell 1995), we predicted that mean cell volume would decrease and mean cellular hemoglobin would increase during egg production, but that both would show the opposite trend as immature cells were released into circulation post-suppression (i.e. at clutch completion).

For the second experiment, if the development of anemia during egg-laying is a resource-based reallocation trade-off, we predicted that a) females would also develop anemia during the energetically demanding chick-rearing stage, b) the degree of anemia would be positively correlated with reproductive effort (i.e. egg size, clutch size, hatching success, and fledging success) such that females that invested more into reproduction would have fewer resources available to maintain erythropoiesis, and c) females on a low-quality diet would show larger decreases in hematocrit, red blood cell counts, hemoglobin concentration, and red blood cell indices during egg-laying than females on a high-quality diet. In contrast, if the development of anemia is not a resource-based reallocation trade-off attributable to pleiotropic effects of estrogen, then a) the development of anemia would be restricted to the egg-production phase, b) the degree of anemia should be independent of reproductive effort, and c) there should be no significant difference in the change in hematological parameters during egg production between females on a high- versus low-quality diet.

Materials and Methods

Study Species and Breeding Conditions

Zebra finches (*Taeniopygia guttata*) were housed under controlled environmental conditions (temperature 19–23 °C, humidity 35–55%, constant light schedule of 14L:10D, lights on at 07:00). 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 in age and had been bred twice previously at 4 and 6 months of age. 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 x 14.5 x 20 cm). Body mass (± 0.001 g), bill length (± 0.01 mm), 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 deposition of the first egg), egg sequence, egg mass (± 0.001 g), and clutch size. A clutch was 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 regime (n = 15). Between pairing and clutch completion, the low-quality diet group continued to receive the standard seed diet *ad libitum* (described above), while the high-quality diet group received the standard diet plus a daily egg food supplement (6g/day, 20.3% protein:6.6% lipid). Originally we intended for breeding pairs on the low-quality diet to rear chicks; however, initial observations indicated that hatching success was extremely poor on the low-quality diet, so all pairs were separated and returned to same-sex holding cages after clutch completion. Breeding pairs in the

high-quality diet group were permitted to incubate eggs and rear chicks to fledging, with egg-food provided again during the chick-rearing period. For this group, breeding 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, chicks were weighed and marked with non-toxic dye to indicate hatch order, and then individually banded at eight 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, 2 females died in the intervening rest period (1 from each diet-quality trial), and 4 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 schedule, female birds on the high-quality diet were blood sampled at five intervals: 1) pre-breeding: at pairing (n = 29), 2) egg-laying: day of laying of 1st egg (n = 29), 3) clutch completion: after two consecutive days without laying an egg (n = 25), 4) hatching: the day the first chick had hatched (n = 18) and 5) fledging of chicks, on average 21 days post-hatching (n = 12). For the low-quality diet experiment, blood samples were obtained for 23 females at

prebreeding, 1-egg stage, and clutch completion. All blood samples (~50 μl) were collected within 3 minutes of capture from the brachial vein.

Hematological variables were measured with standard techniques developed for human blood and commonly used on birds (Campbell 1995). Hematocrit (Hct, %) was measured with digital calipers (± 0.01 mm) following centrifugation of whole blood for 3 minutes at 13000 g. Hemoglobin (Hb, mg/dL whole blood) was measured using the cyanomethemoglobin method modified for use with a microplate spectrophotometer, using 5 uL whole blood diluted in 1.25 ml Drabkin's solution (Drabkin & Austin 1932) and absorbance measured at 540 nm. Intra- and inter-assay coefficients were 1.71% and 3.90% respectively. Erythrocyte counts (RBC, number of cells x 10^6/μL) were determined from duplicate samples (1 uL blood diluted 1/200 with modified Natt & Herrick's solution (Natt & Herrick 1952; Robertson & Maxwell 1990) with an improved Neubauer hemocytometer. 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). From these measurements we calculated mean red cell volume (MCV, femtolitres or fL) with the formula Hct/RBC x 10 = MCV, and mean cellular hemoglobin (MCH, picograms or pg) with the formula Hb/RBC x 10 (Archer 1965). Mean cellular hemoglobin represents the amount of hemoglobin contained within one cell.

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 hematological

parameters across the entire breeding cycle of 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 hematological parameters across stages (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. *Post-hoc* tests for differences between means were corrected for multiple comparisons using Tukey-Kramer (unplanned comparisons) or Dunnett (planned contrasts) adjustment formulas. 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 ± s.e.m. unless otherwise stated.

Results

Variation in body mass

Body mass varied across the complete reproductive cycle for all females (n = 29) on the high-quality diet ($F_{4,23} = 35.91, p < 0.0001$; Figure 2.1a). Mean body mass increased significantly (+0.90 ± 0.14 g) from a pre-breeding value of 15.95 ± 0.22 g to a value of 16.84 ± 0.21 g at the 1-egg stage ($t_{28} = -6.58, p < 0.0001$). Body mass then decreased significantly (-1.44 ± 0.15g) from the 1-egg stage to a mean body mass of 15.40 ± 0.17 g at clutch completion ($t_{26} = 9.84, p < 0.0001$). There were no significant

changes in body mass at later stages ($p > 0.95$ for all comparisons); body mass remained stable at 15.40 ± 0.21 g at hatching and 15.26 ± 0.22 g at fledging.

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 ($F_{2,53} = 7.96$, $p < 0.001$; Figure 2.1b). On the high quality diet, mean body mass increased significantly by 0.82 ± 0.16 g from pre-breeding (15.98 ± 0.23 g) to the 1-egg stage (16.80 ± 0.23 g), and then decreased by -1.61 ± 0.18 g to 15.19 ± 0.24 g at clutch completion, a value significantly lower than pre-breeding and 1-egg values (pre-breeding vs. clutch completion: $t_{45} = 4.36$, $p < 0.008$; 1-egg vs. clutch completion $t_{45} = 8.92$, $p < 0.0001$). In contrast, there was no significant change in mean body mass across reproductive stages on the low quality diet (pre-breeding – 15.71 ± 0.32 g, 1-egg – 15.80 ± 0.32 g, clutch completion 15.10 ± 0.32 g; $p > 0.05$ for all comparisons).

Mass-independence of and inter-relationships between hematological variables

Pre-breeding hematocrit, hemoglobin concentration, red blood cell count, and mean cell volume were independent of pre-breeding body mass ($p > 0.1$ in all cases); therefore body mass was not included as a covariate in subsequent analyses of hematological parameters.

To determine if any of the multiple hematological measurements were redundant, we examined correlations between hematological variables at each stage of the reproductive cycle. Hematocrit was positively correlated with red blood cell count at pre-breeding, clutch completion, and hatching stages ($p < 0.015$ for all correlational analyses,

Table 2.2) and positively correlated with hemoglobin concentration at the fledging stage ($p < 0.05$, Table 2.2) but was not correlated with any other hematological variable at any other reproductive stage (Table 2.2). Plasma hemoglobin concentration was positively correlated with mean cellular hemoglobin concentration at pre-breeding, 1-egg, clutch completion, and hatching stages ($p < 0.001$ for all correlational analyses, Table 2.2), and was positively correlated with mean cell volume at the 1-egg and clutch completion stages ($p < 0.05$ for both, Table 2.2), but was not correlated with any other hematological variable at any reproductive stage (Table 2.2). There was a strong negative correlation between red blood cell number and both mean cell volume and mean cellular hemoglobin at each reproductive stage ($p < 0.0125$ for all correlational analyses, Table 2.2); although this might be expected as both MCV and MCH are calculated from RBC, and are strongly correlated with each other at each reproductive stage as a result ($p < 0.001$ for all, Table 2.2). No single hematological measurement was correlated with all other respective measurements at any reproductive stage; therefore all were retained in subsequent analyses.

Hematocrit

Hematocrit varied significantly across the complete reproductive cycle ($F_{4,80} = 14.77, p < 0.0001$, Figure 2.2a). Hematocrit decreased from 52.5 ± 0.9% at pre-breeding to 46.4 ± 0.9% at the 1-egg stage ($t_{78} = 6.99, p < 0.0001$), and hematocrit remained significantly lower (48.3 ± 1.0%) than pre-breeding levels at clutch completion ($t_{80} = 4.4$, $p < 0.0003$). At hatching, hematocrit (50.9 ± 1.1%) was not significantly different than clutch completion or pre-breeding values ($p > 0.15$ for both) i.e. hematocrit appeared to

recover to 'baseline' levels. However, at fledging, hematocrit decreased once more to 47.19 ± 1.25%, a value significantly lower than the preceding stage (hatching vs. fledging: $t_{80} = 2.86$, $p < 0.05$) as well as pre-breeding baseline levels ($t_{81} = 4.42$, $p < 0.0003$).

For the diet-quality experiment (n = 23), there was a significant diet*reproductive stage interaction for hematocrit ($F_{2,60} = 4.18$, $p < 0.02$, Figure 2.2b). However, *post-hoc* pairwise analyses did not detect any significant differences between mean hematocrit values at any stage comparing the different diets ($p > 0.2$ for all comparisons), and separate analyses of the change in hematocrit from pre-breeding to the 1-egg stage (controlling for pre-breeding hematocrit; $F_{1,18} = 3.01$, $p = 0.1$), from the 1-egg stage to clutch completion (controlling for 1-egg stage hematocrit; $F_{1,18} = 2.33$, $p = 0.14$), and from pre-breeding to clutch completion (controlling for pre-breeding hematocrit; $F_{1,18} = 2.77$, $p = 0.12$) also showed no significant differences between diet types. There was no main effect of diet quality ($F_{1,22} = 1.24$, $p = 0.28$), but reproductive stage had a highly significant effect on hematocrit in both trials ($F_{2,38} = 35.14$, $p < 0.0001$). Mean hematocrit decreased significantly by -5.9% from pre-breeding (52.4 ± 1.0%) to the 1-egg stage (46.4 ± 1.1%) in the high-quality diet trial ($t_{43} = 5.12$, $p < 0.0001$), and remained significantly lower than pre-breeding values at clutch completion (48.2 ± 1.1%; $t_{52} = 3.76$, $p < 0.005$; Figure 2.2b, Table 2.3). In comparison, hematocrit showed a significant decrease of -8.5% from pre-breeding (53.8 ± 1.1%) to the 1-egg stage (45.3 ± 1.1%) in the low-quality diet trial ($t_{42} = 7.05$, $p < 0.0001$), and remained significantly lower than

pre-breeding values at clutch completion (44.6 ± 1.2%; $t_{46} = 7.13$, $p < 0.001$; Figure 2.2b, Table 2.3).

Plasma Hemoglobin Concentration

Plasma hemoglobin concentration varied significantly across the complete reproductive cycle ($F_{4,51} = 4.85$, $p < 0.02$, Figure 2.3a). While there were no significant differences found in hemoglobin concentration between pre-breeding (15.97 ± 0.51 g/dL), 1-egg (14.71 ± 0.35 g/dL), clutch completion (14.61 ± 0.38 g/dL), or hatching stages (15.82 ± 0.59 g/dL; $p > 0.1$ for all comparisons), hemoglobin concentration at the fledging stage (13.85 ± 0.42 g/dL) was significantly lower than all other stages (prebreeding $t_{23} = 3.1$, $p < 0.05$; 1-egg $t_{19} = 3.17$, $p < 0.05$; hatching $t_{18} = 3.6$, $p < 0.02$) except clutch completion ($p = 0.5$).

For the diet-quality experiment, the diet*reproductive stage interaction was not significant ($F_{2,51} = 0.45$, $p = 0.64$, Figure 2.3b), but there was a significant main effect of reproductive stage on hemoglobin concentration ($F_{2,49} = 14.13$, $p < 0.0001$). For the pooled diet groups, pre-breeding mean hemoglobin concentration (16.19 ± 0.35 mg/dL) decreased to 14.51 ± 0.34 mg/dL at the 1-egg stage ($t_{47} = 4.49$, $p < 0.0001$), and remained significantly lower than pre-breeding levels at clutch completion (14.33 ± 0.36 mg/dL; $t_{51} = 4.69$, $p < 0.0001$).

Red Blood Cell Number

Red blood cell counts varied significantly across the complete reproductive cycle ($F_{4,78} = 11.85$, $p < 0.001$, Figure 2.4a). Mean red blood cell number at the pre-breeding stage was 5.00 ± 0.15 cells $*10^6/\mu L$. Red blood cell number decreased to 4.29 ± 0.15 cells

*10^6/μL at the 1-egg stage ($t_{76} = 5.01$, $p < 0.0001$), and remained significantly lower than pre-breeding levels at clutch completion (4.09 ± 0.16 cells *10^6/μL; $t_{78} = 6.09$, $p < 0.0001$). There was a significant increase from clutch completion levels to 4.69 ± 0.17 cells *10^6/μL at hatching ($t_{78} = -3.54$, $p < 0.006$) followed by a slight increase to 4.77 ± 0.20 cells *10^6/μL at fledging (NS, $p = 0.99$). Mean red blood cell number at hatching and fledging were not significantly different compared with pre-breeding values ($p > 0.3$ for both comparisons).

For the diet-quality experiment, the diet*reproductive stage interaction was not significant ($F_{2,52} = 0.85$, $p = 0.44$, Figure 2.4b), but there was a significant main effect of reproductive stage on red blood cell counts ($F_{2,49} = 14.13$, $p < 0.0001$). On both diets, pre-breeding red blood cell count (4.97 ± 0.14 cells *10^6/μL) was significantly higher than RBC at the 1-egg (4.21 ± 0.14 cells *10^6/μL; $t_{46} = 5.49$, $p < 0.0001$) and clutch completion stages (4.22 ± 0.14 cells *10^6/μL; $t_{50} = 5.19$, $p < 0.0001$).

Red Blood Cell Indices

Mean cell volume showed significant variation across different stages of the reproductive cycle ($F_{4,78} = 3.13$, $p < 0.02$, Figure 2.5a). There were no significant differences found in mean cell volume between pre-breeding (107.79 ± 3.72 fL), 1-egg (112.08 ± 3.67 fL), or hatching stages (110.06 ± 4.47 fL; $p > 0.05$ for all comparisons). However, mean cell volume at clutch completion (120.29 ± 4.03 fL) was significantly greater than pre-breeding mean cell volume ($t_{77} = -2.74$, $p < 0.05$), while mean cell volume at the fledging stage (101.12 ± 5.54 fL) was significantly lower than mean cell volume at clutch completion ($t_{82} = 3.13$, $p < 0.025$). There was no significant temporal

variation across the reproductive cycle in mean cellular hemoglobin ($F_{4,77} = 2.26$, $p = 0.07$, Figure 2.6a).

There was no significant diet*reproductive stage interaction for mean cell volume ($F_{2,48} = 0.14$, $p = 0.87$, Figure 2.5b) or mean cellular hemoglobin ($F_{2,49} = 0$, $p = 1.0$, Figure 2.6b). The main effect of reproductive stage was also not significant for either red blood cell indices (MCV: $F_{2,56} = 1.24$, $p = 0.30$; MCH: $F_{2,56} = 1.52$, $p = 0.23$).

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), 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 ($p > 0.05$ for all), 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 ($p > 0.05$ for all).

Diet composition strongly influenced reproductive output (Table 2.2). On the high-quality diet, mean egg mass was significantly greater ($F_{1,22} = 16.88$, $p < 0.0005$), and females laid a significantly larger number of eggs ($F_{1,20} = 15.85$, $p < 0.0007$). Consequently, mean clutch mass was significantly larger in the high-quality diet trial ($F_{1,22} = 18.69$, $p < 0.0003$). However, there was no difference in laying interval between the two diets ($F_{1,25} = 1.2$, $p = 0.3$). 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 ($p > 0.05$ for all).

Discussion

In this study, we confirmed that hematocrit decreases significantly (-6.2%) from pre-breeding to the 1-egg stage in female zebra finches, and we demonstrated that decreased hematocrit at the onset of egg-laying is accompanied by reduced red blood cell counts (-7.1 $*10^5$ cells/μL) and plasma hemoglobin concentration (-1.25 mg/dL) relative to the pre-breeding stage. Our results provide strong support for the hypothesis that erythropoiesis is transiently suppressed during egg-laying: decreases in hematocrit, red blood cell counts, and hemoglobin concentration were sustained through to clutch completion, but showed evidence of recovery to baseline pre-breeding levels at hatching, which is consistent with the estimated timescale for regenerative erythropoiesis following experimentally induced anemia (7-14 days; Domm & Taber 1946, Clark et al. 1988). Further evidence that erythropoiesis is transiently suppressed is that mean cell volume showed a significant increase at clutch completion, possibly indicating enhanced production and release of larger immature cells into the circulation at this stage (i.e. post-suppression). Interestingly, at fledging, we observed a significant secondary decrease in hematocrit and hemoglobin concentration to levels not significantly different than egg-laying values, suggesting females also became anemic during the chick-rearing period.

A reduction in hematocrit during egg production has been reported in several avian species (e.g. Rehder & Bird 1983; Morton 1994; Merino & Barbosa 1997; Gayathri et al. 2004; Sheridan et al. 2004; Williams et al. 2004a; Gayathri & Hegde 2006), 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. The magnitude of this change in hematocrit is comparable to 'adaptive' adjustments of hematocrit believed to facilitate oxygen uptake and transfer during periods of intense metabolic activity (reviewed in 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 & Morton 1976), low oxygen-partial pressures (Jaeger & McGrath 1974; Keys et al. 1986; Clemens 1990; Ruiz et al. 1995; Prats et al. 1996), endurance exercise or migration (Palomeque & Planas 1978; Piersma & Everaarts 1996; Soler et al. 1999; Landys-Ciannelli et al. 2002), and experimentally elevated flight costs (Saino et al. 1997a; Cuervo & De Ayala 2005). In light of these results, we suggest that the decrease in hematocrit observed in this study is biologically relevant and could potentially have a negative impact on aerobic capacity during egg production.

We also observed a significant decrease in hematocrit (-3.7%) and hemoglobin concentration (-2.0 mg/dL) from hatching to fledging, an effect that may be independent of endogenous estrogen levels. In general, reductions in hematocrit during chick-rearing stages have been attributed to a decline in body condition and/or reallocation of resources to meet increased energetic demands of chick-rearing (Morton 1994; Moreno et al. 1998; Pap 2002), although these explanations are challenged by evidence that hematocrit is positively correlated with experimentally enlarged brood sizes in the great tit (Horak et al. 1998b). Nonetheless, while 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 increased in comparison to pre-breeding and incubation stages in zebra finches (Vezina et al. 2006), and the possibility that anemia during chick-rearing reflects reallocation of resources away from erythropoiesis to meet the energetic demands of chick-rearing remains to be explored. Alternatively, the decrease in hematocrit at fledging may have been due to a secondary increase in estrogen levels as females prepared to lay a second clutch, possibly resulting in hemodilution effects and/or estrogenic suppression of erythropoiesis once more.

Mean cell volume did not change significantly from pre-breeding to the 1-egg stage, but was significantly elevated at clutch completion in comparison to prebreeding levels (+12.5 fL), which is consistent with our 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 unabated (John 1994). At clutch completion, plasma estrogen decreases to non-breeding levels (Williams et al. 2004b; Williams et al. 2005) releasing erythropoiesis from inhibition. A higher proportion of reticulocytes, immature erythrocytes significantly larger than mature erythrocytes due to less condensed chromatin (Campbell 1995), would be present in circulation compared to preceding stages. Fewer red blood cells would be present at clutch completion, but a greater proportion of these cells would be of larger size and mean cellular volume would increase, indicating a regenerative response to anemia (Dein 1986).

We found that mean cell volume was significantly lower at fledging than at clutch completion (-19.2 fL), possibly reflecting enhanced erythropoietic activity during incubation. Jones (1983) documented that the thymus became enlarged and actively produced red blood cells in the red-billed quelea (*Quelea quelea*) during incubation, which he suggested functioned to augment erythropoiesis in the bone marrow and compensate for anemia during egg-laying. Interestingly, erythropoiesis during incubation could also be enhanced by prolactin, a hormone present at elevated levels during incubation (Sockman et al. 2006) that promotes erythropoiesis in mice (Jepson & Lowenstein 1964) and has been characterized as a hematopoietic growth factor (Bellone et al. 1997; Constantinescu et al. 1999; Welniak et al. 2000; Welniak et al. 2001). A burst of erythropoietic activity during incubation would quickly restore hematocrit and red blood cell number (as indicated by recovery of these variables at hatching), and would also result in a larger proportion of cells of the same age in circulation. As these cells matured over time, there would be a larger proportion of similarly-sized cells decreasing in size, and thus a trend towards a smaller mean cell volume in later stages such as hatching and fledging. If erythropoiesis was secondarily suppressed during chick-rearing, then this age effect may have been more pronounced, as fewer reticulocytes would be present to balance the high proportion of smaller mature cells. In addition, while hematocrit and hemoglobin concentration decreased significantly from hatching to fledging, red blood cell number did not change. This suggests that although the proportion of cells present per unit volume decreased, the number of cells per unit volume did not because the cells were smaller in size.

Contrary to predictions, mean cellular hemoglobin showed no change across the entire reproductive cycle. If erythropoiesis was suppressed via estrogen-dependent or independent means, we would expect to see an increase in mean cellular hemoglobin during egg production, as mature erythrocytes tend to contain higher levels of hemoglobin than immature cells (Campbell 1995). Reactivation of erythropoiesis during incubation would result in decreased mean cellular hemoglobin levels at later stages as these immature cells entered circulation (Dein 1986). However, since data from all other hematological parameters support our hypothesis that erythropoiesis is suppressed during egg production; it may be an issue of statistical power, particularly since there appears to be a trend towards predicted results.

The interaction between diet composition and reproductive stage was significant for hematocrit measurements, suggesting that anemia during egg production was influenced by resource availability. However, we did not detect any significant differences in either mean hematocrit at each stage or in the change in hematocrit across stages between diet trials. In addition, diet quality had no effect on hemoglobin concentration or red blood cell number although these traits also decreased significantly during egg-laying. We also found no relationship between the degree of anemia and reproductive effort in either experiment. Therefore, we found limited evidence that anemia during egg production is resource-dependent, and the resource reallocation hypothesis was not strongly supported. To our knowledge, no other studies have examined whether resource level (e.g. diet quality) 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 restriction programs cause hematological parameters to deviate from the 'normal' range (Maxwell et al. 1990).

Diet quality did have a far greater impact on primary reproductive effort (i.e. egg and clutch size) and current breeding success. On the high-quality diet, females produced larger, heavier clutches of better quality eggs, as indicated by a nearly 100% hatch failure rate in the low-quality diet trials. Depending on the quality of diet available, females may have used different strategies to balance potentially conflicting demands of reproduction and self-maintenance. We suggest that when diet quality was poor, egg-laying females most likely maintained red blood cell production and hence their own condition at a potential cost of reduced egg size, number, and viability. Body mass data also supports this conclusion; 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. As such, we can not conclude that the development of anemia during egg production is strictly resource-independent because it is possible that if females were induced to invest an equivalent amount of energy and/or resources in reproductive output (e.g. via egg removal) during the low-quality diet trial, a larger decrease in hematocrit would be observed during egg-laying in comparison to the high-quality diet trial.

Preferential 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-labelled iron supplement, a significantly higher amount of labelled iron was detected in the hematopoietic organs (i.e. bone marrow, liver, spleen) and erythrocytes, but no difference was found in amount of labelled iron found in the oviduct or eggs produced (Garcia et al. 1986). Iron deposited in the yolk is essential for embryo development and postnatal survival, yet once available, it was preferentially diverted to restoring red blood cell production and hematocrit levels, not reproduction (Garcia et al. 1986). Mineral deposition in the yolk has been studied in poultry, and trace elements such as iron, copper, zinc, manganese and selenium all appear to play a role in normal avian development (Richards 1989; Richards 1997; Ramesh et al. 2000). Notably, the same trace elements are essential for red blood cell production (Dessypris & Sawyer 2004). Although lipid and protein requirements for egg production have been well characterized in the zebra finch (e.g. Williams 1996), requirements for trace minerals during egg production and the relative amounts of these minerals deposited in eggs is not known. If mineral requirements for egg production versus erythropoiesis in the zebra finch were determined, future studies could employ more targeted manipulations of dietary components in the diet rather than an overall reduction in protein, lipids, and trace elements as was used here. A diet deficient in select minerals and/or vitamins but similar to the high quality diet in all other aspects might allow a female to produce viable eggs, a limitation of the current experiment.

In conclusion, we confirm that the development of anemia during egg production is robust even under relatively benign conditions of captive breeding (with an ad lib, high-quality diet). In addition, the rapid onset of anemia during egg-laying is associated with marked decreases in hemoglobin concentration and number of circulating red blood cells. Our data challenges the hypothesis that anemia is a purely resource-driven differential allocation decision, but since females did not invest the same amount of energy and/or resources into reproduction on the low- versus high-quality diet, we can not unequivocally conclude that anemia is independent of resource level. Instead we suggest that when resources were limited, egg-laying females most likely maintained red blood cell production at some minimum 'threshold' level and hence their own condition at a potential cost of reduced reproductive output. This observation may be more consistent with the hypothesis that anemia is a consequence of hormonal pleiotropy, as resource-independent mechanisms are related to reproduction but appear to be independent of variation in fecundity (i.e. 'all-or-nothing costs', Williams 2005). Future research is necessary to determine if the reproductive costs of preferential maintenance of red blood cell production and/or maternal condition during resource limitation are balanced by increased survival and future fecundity.

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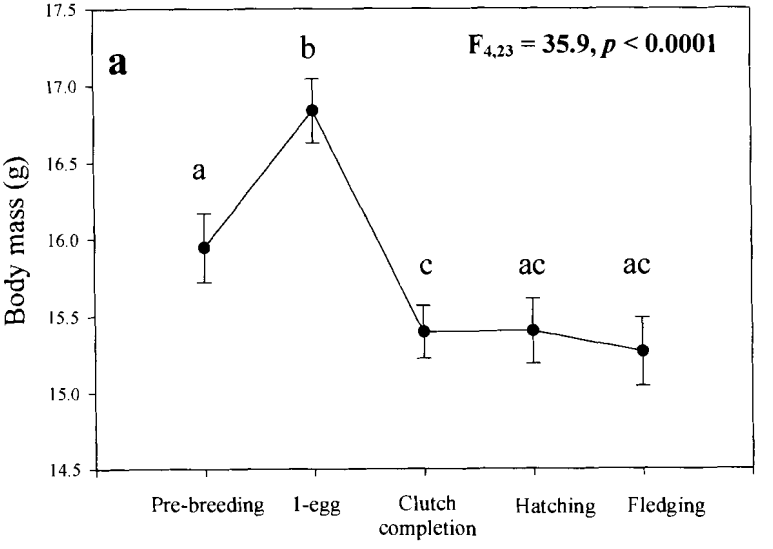
Figure 2.1. a) Change in body mass across reproductive cycle of female zebra finches on a high-quality diet. Different lowercase letters indicate significant difference in mean hematocrit values at a level of $p < 0.05$ (corrected for multiple comparisons using Tukey-Kramer adjustment).
b) Comparison of change in body mass across egg-laying cycle for female zebra finches (n = 23) on a high-quality (filled circles) versus low-quality (open circles) diet. Data presented are least-squares means ± standard error of mean.



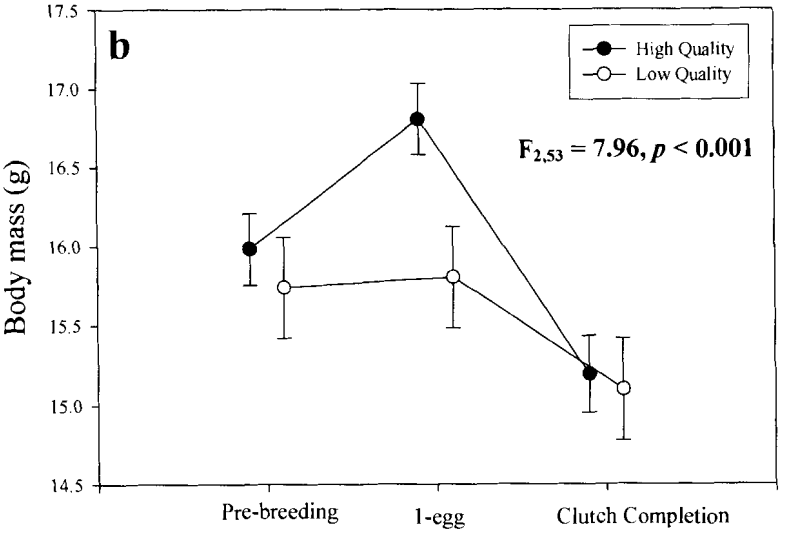
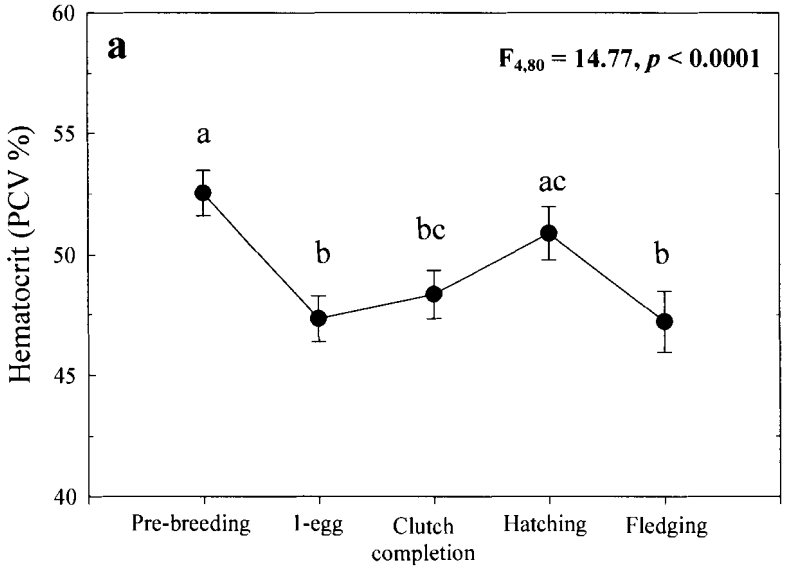


Figure 2.2. a) Change in hematocrit across reproductive cycle of female zebra finches on a high-quality diet. Different lowercase letters indicate significant difference in mean hematocrit values at a level of $p < 0.0005$ (corrected for multiple comparisons using Tukey-Kramer adjustment).
b) Comparison of change in hematocrit across egg-laying cycle for female zebra finches on a high-quality (filled circles) versus low-quality (open circles) diet. Data presented are least-squares means ± standard error of mean.



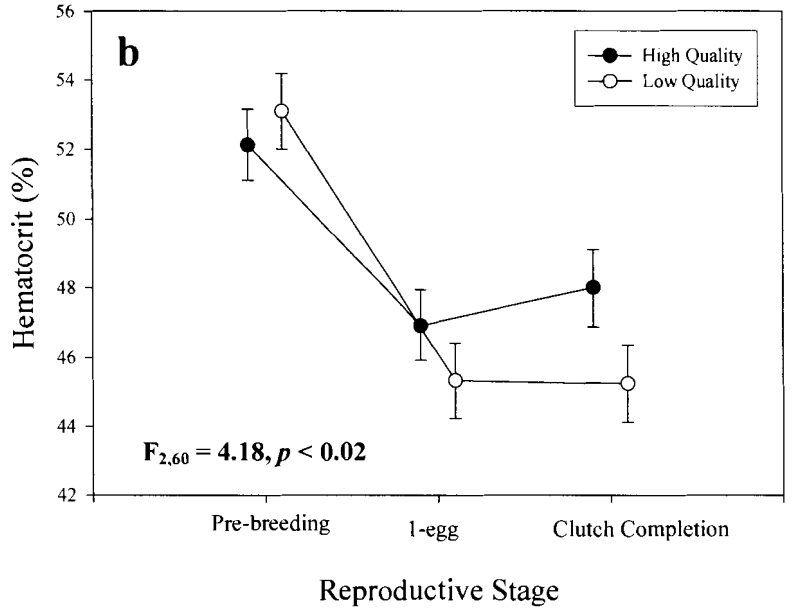
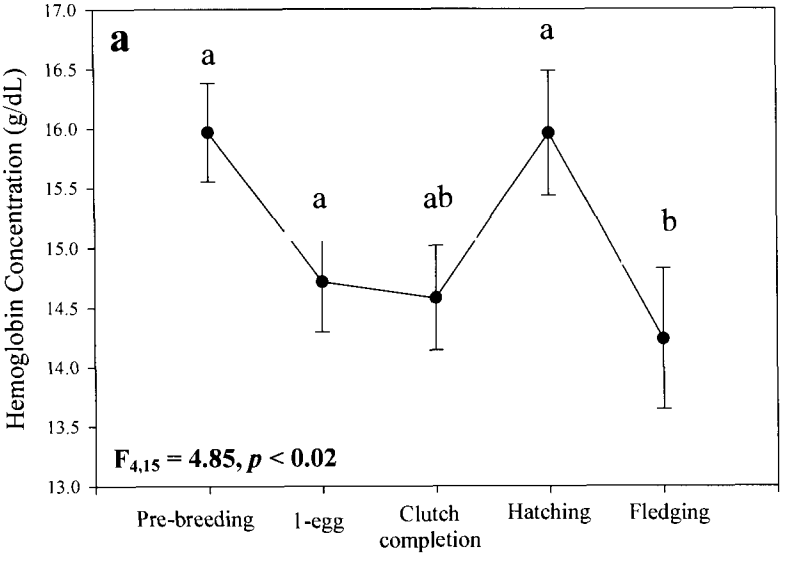


Figure 2.3. a) Change in hemoglobin concentration across reproductive cycle of female zebra finches on a high-quality diet. Different lowercase letters indicate significant difference in mean hemoglobin levels at a level of $p < 0.05$ (corrected for multiple comparisons with Tukey-Kramer adjustment).
b) Comparison of change in hemoglobin concentration across egg-laying cycle for female zebra finches (n = 23) on a high-quality (filled circles) versus low-quality (open circles) diet. Data presented are least-squares means ± standard error of mean.



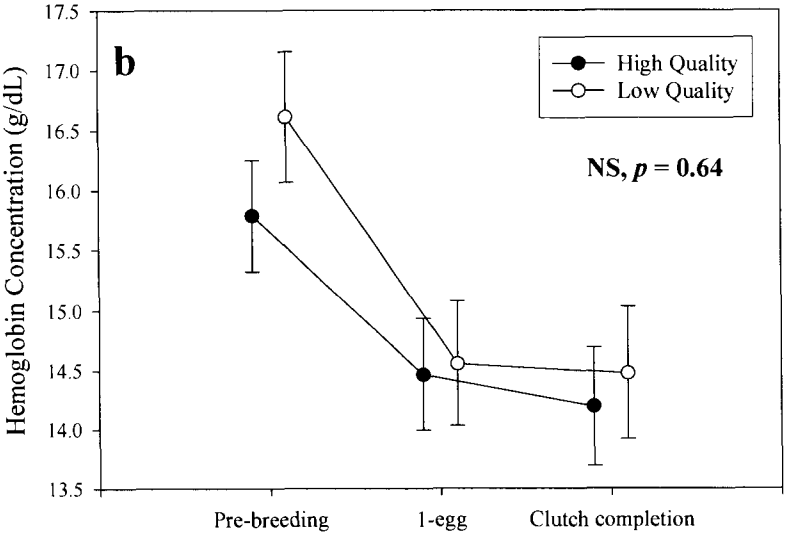
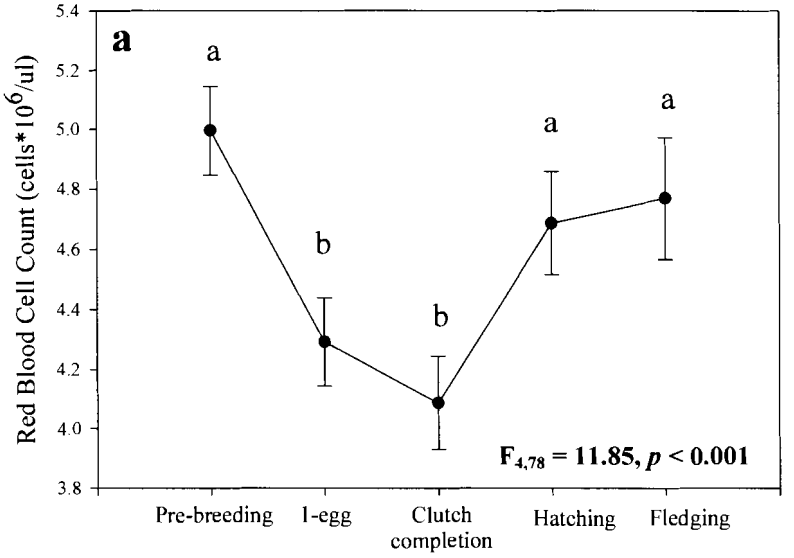


Figure 2.4. a) Change in red blood cell number across reproductive cycle of female zebra finches on a high-quality diet. Different lowercase letters indicate significant difference in mean cell number at a level of $p < 0.01$ (corrected for multiple comparisons using Tukey-Kramer adjustment).
b) Comparison of change in red blood cell number (RBC) across egg-laying cycle for female zebra finches (n = 23) on a high-quality (filled circles) versus low-quality (open circles) diet. Data presented are least-squares means ± standard error of mean.



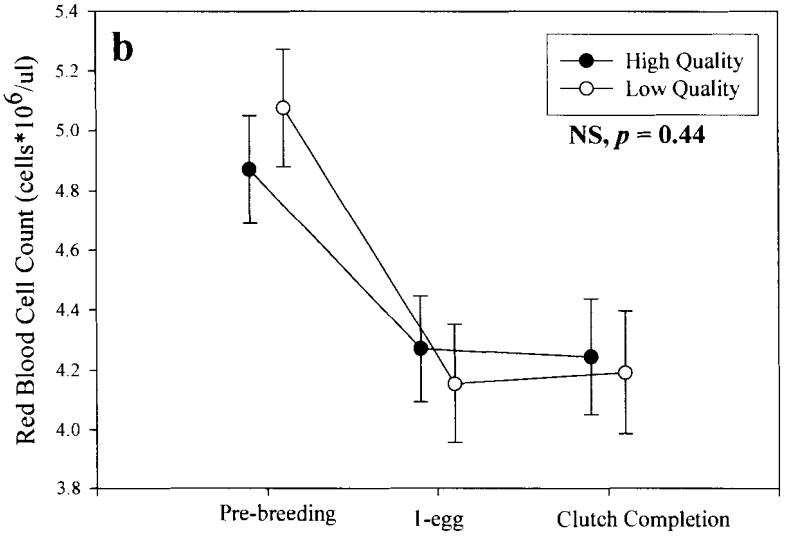
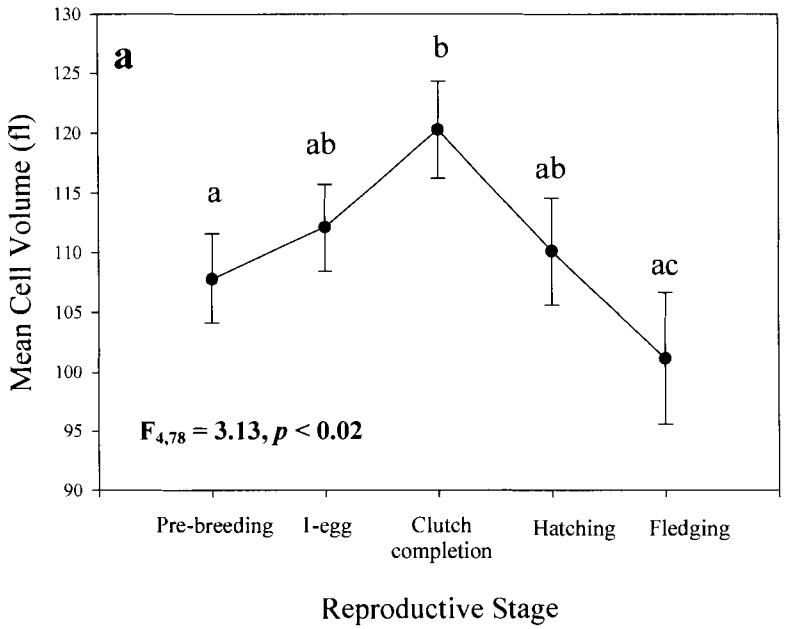


Figure 2.5. a) Change in mean cell volume across reproductive cycle of female zebra finches on a high-quality diet. Different lowercase letters indicate significant difference in mean cell number at a level of $p < 0.05$ (adjusted for multiple comparisons using Tukey-Kramer method).
b) Comparison of change in mean cell volume across egg-laying cycle for female zebra finches (n = 23) on a high-quality (filled circles) versus low-quality (open circles) diet. Data presented are least-squares means ± standard error of mean.



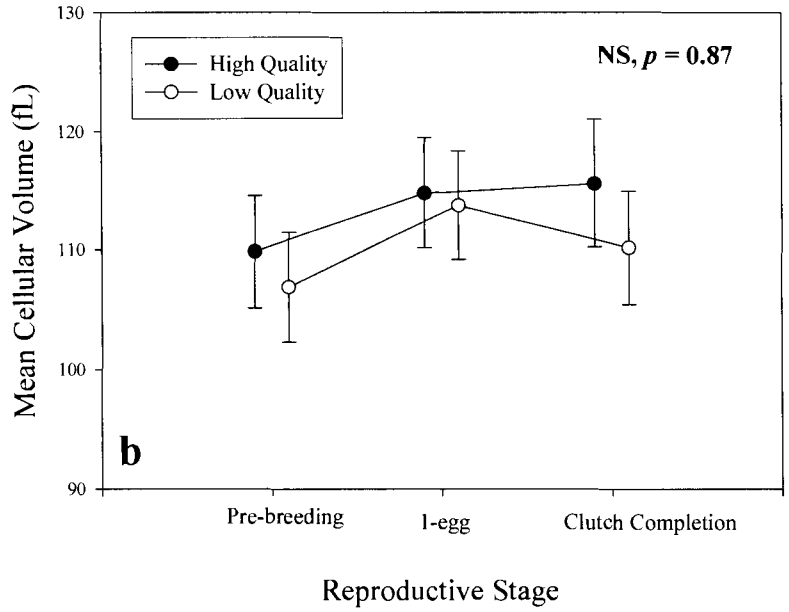
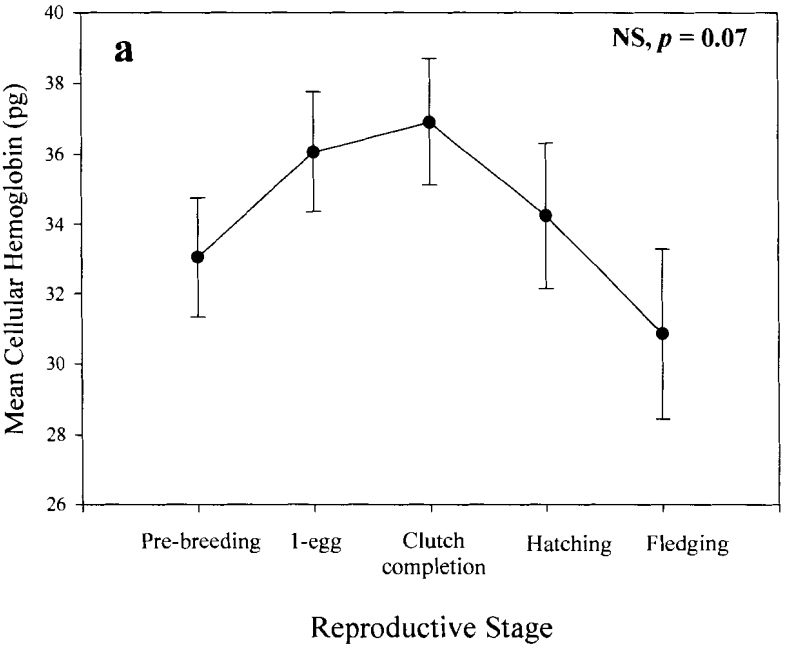


Figure 2.6. a) Mean cellular hemoglobin across the reproductive cycle of the female zebra finches on a high-quality diet.
b) Comparison of change in mean cellular hemoglobin across the egg-laying cycle for female zebra finches (n = 23) on a high-quality (filled circles) versus low-quality (open circles) diet. Data presented are least-squares means ± standard error of mean.



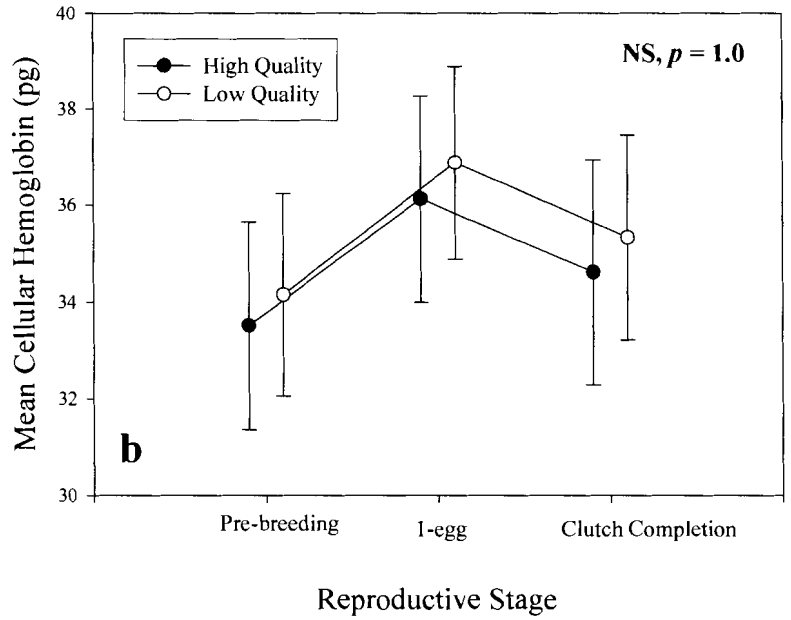


Table 2.1. Summary of predictions generated from 'Hemodilution' versus 'Suppression of Erythropoiesis' hypotheses in relation to temporal changes in hematological parameters during the female reproductive cycle.

Trait	**Hemodilution**	**Suppression of Erythropoiesis**
Hematocrit	↓ at 1-egg stage Recovery at clutch completion	↓ at 1-egg stage Recovery at hatching
Red blood cell number	↓ at 1-egg stage Recovery at clutch completion	↓ at 1-egg stage Recovery at hatching
Red blood cell volume	No change	↑ at clutch completion Recovery at hatching
Hemoglobin concentration	↓ at 1-egg stage Recovery at clutch completion	↓ at 1-egg stage Recovery at hatching
Mean cellular hemoglobin	No change	↑ at 1-egg stage Recovery at hatching

Table 2.2 Inter-correlation matrices for hematological parameters at each reproductive stage: a) pre-breeding, b) 1-egg, c) clutch completion, d) hatching, and e) fledging. From top to bottom, values in each cell are Pearson correlation coefficients, *p*-values (* indicates significance at $\alpha = 0.05$), and sample size.

a) Pre-breeding	Hematocrit	Hemoglobin	Red blood cell number	Mean cell volume
Hemoglobin	0.18470 0.3375 29			
Red blood cell number	0.49436 **0.0075*** 28	0.00763 0.2459 28		
Mean cell volume	-0.02052 0.9175 28	0.08309 0.6742 28	-0.85844 **< 0.0001*** 28	
Mean cellular hemoglobin	-0.28762 0.1378 28	0.61900 **0.0004*** 28	-0.74702 **< 0.0001*** 28	0.71092 **< 0.0001*** 28

b) 1-egg stage	Hematocrit	Hemoglobin	Red blood cell number	Mean cell volume
Hemoglobin	0.28488 0.1417 28			
Red blood cell number	0.35167 0.0614 29	-0.22675 0.2459 28		
Mean cell volume	0.16421 0.3947 29	0.37503 **0.0492*** 28	-0.83680 **< 0.0001*** 29	
Mean cellular hemoglobin	-0.06133 0.7565 28	0.65431 **0.0002*** 28	-0.84073 **< 0.0001*** 28	0.87444 **< 0.0001*** 28

c) Clutch completion	Hematocrit	Hemoglobin	Red blood cell number	Mean cell volume
Hemoglobin	0.33682 0.1160 23			
Red blood cell number	0.59357 **0.0028*** 23	-0.21309 0.2459 24		
Mean cell volume	-0.02376 0.9143 23	0.47403 **0.0223*** 25	-0.80139 **< 0.0001*** 23	
Mean cellular hemoglobin	-0.23676 0.2767 23	0.68515 **0.0002*** 24	-0.83889 **< 0.0001*** 24	0.87708 **< 0.0001*** 23

d) Hatching	Hematocrit	Hemoglobin	Red blood cell number	Mean cell volume
Hemoglobin	0.47170 0.0651 16			
Red blood cell number	0.56908 **0.0137*** 18	0.05211 0.848 16		
Mean cell volume	0.02231 0.9300 18	0.3052 0.2504 16	-0.80162 **< 0.0001*** 18	
Mean cellular hemoglobin	0.06831 0.8015 16	0.7523 **0.0008*** 16	-0.60806 **0.0125*** 16	0.74364 **< 0.001*** 16

e) Fledging	Hematocrit	Hemoglobin	Red blood cell number	Mean cell volume
Hemoglobin	0.59278 **0.0422*** 12			
Red blood cell number	0.30722 0.3581 11	0.02671 0.9379 11		
Mean cell volume	0.4351 0.1811 11	0.30096 0.3685 11	-0.71983 **0.0125*** 11	
Mean cellular hemoglobin	-0.04368 0.8985 11	0.49337 0.1230 11	-0.85379 **0.0008*** 11	0.77429 **0.005*** 11

Table 2.3. The effect of diet quality on reproductive output and variation in hematocrit across reproductive stages in zebra finch females (n = 23) bred on a high-quality (egg food supplement + seed) versus low-quality (seed only) diet. Clutch size was log-transformed prior to analysis but is presented here unaltered for clarity. Values are least-squared means ± standard error of mean with 95% confidence intervals in parentheses.
* indicates significance level of $p < 0.001$.

Trait	**High-Quality Diet**	**Low-Quality Diet**
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) *	6.441 ± 0.521	3.158 ± 0.533
Laying interval (days)	7.1 ± 0.5	6.2 ± 0.6
Change in hematocrit (%) from pre-breeding to 1-egg stage	-5.9 ± 1.3 (-8.8 – -3.1)	-8.5 ± 1.3 (-11.2 – -5.8)
Change in hematocrit (%) from 1-egg stage to clutch completion	+1.8 ± 1.2 (-0.9 – +4.4)	+0.7 ± 1.1 (-1.8 – +3.2)
Change in hematocrit (%) from pre-breeding to clutch completion	-4.2 ± 1.5 (-7.4 – -1.0)	-7.8 ± 1.5 (-11.0 – -4.6)

CHAPTER 3: ANEMIA ASSOCIATED WITH EGG PRODUCTION IS ESTROGEN-DEPENDENT: A BASIS FOR THE COST OF REPRODUCTION?

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Summary

The 'cost of reproduction' (i.e. trade-offs 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 recognised that reproduction itself or the regulatory (physiological) mechanisms underlying reproduction might result in 'costs' (cf. the more traditional resource-allocation based mechanisms). The antagonistic pleiotropic effects of hormones potentially represent such a mechanism, where the endocrine networks regulating reproduction have multiple effects in other body systems with negative non-reproductive effects considered resource-independent costs. Previously we have proposed a specific mechanism, the development of anemia (decreased hematocrit) during egg production in birds, which may play a role in associated costs of reproduction by reducing oxygen-carrying capacity during subsequent aerobically-demanding stages such as chick-provisioning. Here we demonstrate that anemia during egg-laying is dependent on endogenous estrogens: blocking estrogen receptors using the anti-estrogen tamoxifen partly inhibits the development of anemia in female zebra finches (*Taeniopygia guttata*) such that hematocrit of tamoxifen-treated laying females is not significantly different from pre-breeding or non-breeding values, and is significantly higher than control laying birds. Thus, 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.

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. In birds, 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 & Nager 1997; Monaghan et al. 1998; Nager et al. 2001). However, we still know very little about the physiological mechanism(s) underlying these costs (Vezina & Williams 2003; Williams 2005). Most studies to date have focused on relatively simple models of resource-based allocation trade-offs (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. Alternatively, it has been suggested that costs of reproduction might instead be caused by the reproductive process itself or the regulatory (physiological) mechanisms underlying reproduction (e.g. Partridge et al. 2005; Williams 2005). Hormones are particularly strong candidates for regulating such trade-offs due to their many pleiotropic effects, both positive and negative (Ketterson & Nolan 1992; Finch & Rose 1995; Rose & Bradley 1998; Ketterson & Nolan 1999; Reed et al. 2006).

Here we test the hypothesis that anemia (i.e. a decrease in hematocrit) during egg production is a pleiotropic effect of estrogen, the principle female reproductive hormone, which is present at high levels during initial stages of egg-laying. Thus, this mechanism is

a good candidate for a regulatory-network based mechanism underlying the cost of reproduction. Numerous studies in birds have documented the occurrence of anemia during egg production (Jones 1983; Keys et al. 1986; Morton 1994; Merino & Barbosa 1997; Horak et al. 1998; Davey et al. 2000; Sheridan et al. 2004; Gayathri & Hegde 2006) and anemia may persist through incubation and chick rearing in some cases (e.g. Williams et al. 2004a). Williams et al. (2004a) proposed that anemia 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.

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 & Waldron 1999). However, plasma concentration of yolk precursors decreases rapidly upon ovulation of the last follicle, reaching non-breeding levels at clutch completion (Challenger et al. 2001; Salvante & Williams 2002). If the observed anemia 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 anemia 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 & Schraer 1979). Estrogen treatment has been shown to induce anemia in several

mammalian and avian species (see Blobel & Orkin 1996 for review) 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 & 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 if anemia during egg production is estrogen-dependent in the female zebra finch (*Taeniopygia guttata*) using experimental manipulations with 17β-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 17β-estradiol would increase the level of anemia during egg production, while treatment with tamoxifen citrate would decrease the level of anemia at the onset of egg-laying with reference to unmanipulated and sham-treatment breeding trials for each female.

Materials and Methods

Study Species and Breeding Conditions

Zebra finches (*Taeniopygia guttata*) were housed under controlled environmental conditions (temperature 19–23 °C, humidity 35–55%, constant light schedule of 14L:10D, lights on at 07:00). 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 – 9 months in age. Randomly-assigned breeding pairs were housed individually in cages (61× 46 × 41 cm) equipped with an external nest box (15 x 14.5 x 20 cm). Body mass (± 0.001 g), bill length (± 0.01 mm), and tarsus length (± 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, and again during the chick-rearing period. 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 deposition of the first egg), egg sequence, egg mass (± 0.001 g), and clutch size. A clutch was considered complete when no additional eggs were produced over two consecutive days.

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 three-week rest periods: 1) Unmanipulated (n = 23): females were not manipulated; 2) Estradiol treatment (n = 24): females were injected

intramuscularly (i.m.) every other day from pairing until clutch completion with 25.5 μg 17β-estradiol (Sigma) in 30 μL canola oil (1.5 μg/g BW); 3) Sham-treatment (n = 26): females were injected i.m. every other day from pairing until clutch completion with 30 μL canola oil; and 4) Tamoxifen-treatment (n = 21): females were injected every other day i.m. from pairing until clutch completion with 170 μg of tamoxifen citrate (Sigma) in 30 μL 1,2-propanediol (10 μg/g BW).

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 3.1). All blood samples were collected from the brachial vein within 3 minutes of capture between the hours of 09:30 and 11:30.

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 minutes at 13000 g. Hemoglobin (Hb, g/dL whole blood) was measured using the cyanomethemoglobin method (Drabkin & Austin 1932) modified for use with a microplate spectrophotometer, using 5 uL whole

blood diluted in 1.25 ml Drabkin's solution with absorbance measured at 540 nm. Intra- and inter-assay coefficients were 1.71% and 3.90% respectively. Erythrocyte counts (RBC, number of cells x 106/μL) were determined from duplicate samples (1 uL blood diluted 1/200 with modified Natt & Herrick's solution; Natt & Herrick 1952; Robertson & Maxwell 1990) with an improved Neubauer hemocytometer. 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 (ECW) scored all red blood cell counts, we expect the measurement error to be consistent across different breeding stages and experimental trails. From these measurements we calculated mean red cell volume (MCV, femtolitres or fL) with the formula Hct/RBC = MCV (Archer 1965). For breeding birds, additional blood collected was centrifuged at 5000 rpm for 10 minutes to separate the plasma layer, which was decanted and frozen at -20C until assayed for estradiol.

Estradiol Determination

Plasma samples and controls were passed through C18 columns (CUC18156 United Chemical Technologies SPE columns, Chromatographic Specialties, Inc., Brockville, Ontario) following the procedure described in Williams et al. (2005). Prior to the solid phase extraction procedure, 1mL of doubly distilled (dd) water was added to each plasma sample (50 μl unless sample volume could only provide 25 μl or 10 μl). 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

7mL borosilicate vials (03-337-26, Fisher Scientific, Ottawa, Ontario). Each sample was then evaporated to dryness under vacuum with gentle shaking and reconstituted in 300 µl 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 3 aliquots, each containing 500µl of plasma. One vial was diluted with 400 µl of the assay buffer, one vial was spiked with 400 µl of the 1ng/mL standard (400 pg spike), and the third vial remained as raw plasma. Each solid phase extraction run (N = 6) contained one 50 µl raw sample, two 50 µl diluted samples and one 50 µl 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 17β-estradiol enzyme immunoassay kit (Ecologiena/ Japan EnviroChemicals Ltd., Abraxis LLC, Warminster, PA) exactly as indicated in the instructions. In duplicate, 100 µl of reconstituted sample (equivalent to 16.7 µl, 8.3 µl or 3.3 µl of the original plasma) was mixed with 100 µl of antigen-enzyme conjugate solution to yield a 5% methanol solution. Of that 200 µl, 100 µl (equivalent to 8.3 µl, 4.2 µl or 1.7 µl of the original plasma sample of 50 µl, 25 µl, or 10 µl respectively) was then transferred into an antibody-coated plate for quantification. A total of 3 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 reanalysis 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*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 analysed 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 corrected using Tukey-Kramer (unplanned comparisons) adjustment formulas. 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 (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 ± s.e.m. unless otherwise stated.

Results

Body mass variation and interrelationships with other variables

There was a highly significant treatment*reproductive stage interaction for body mass ($F_{6,72} = 8.25$, $p < 0.0001$; Figure 3.1). 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). A similar temporal pattern was observed among tamoxifen-treated females (Figure 3.1); although the increase in body mass from pre-breeding to the 1-egg stage was not significant ($p > 0.5$), the decrease in mass from the 1-egg stage to clutch completion was significant ($p < 0.015$). In the unmanipulated trial, females were initially at a higher body 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 significantly lower than prebreeding values ($p > 0.07$).

Excluding prebreeding (but not clutch completion, see Discussion for rationale) data from the unmanipulated trial, there was no difference in mean body mass at prebreeding ($F_{2,67} = 0.34$, $p > 0.7$) 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 ($F_{3,59} = 8.48$, $p < 0.0001$, Figure 3.2, Table 3.2). 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). 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).

Controlling for differences in female body mass, treatment had a significant effect on mean egg mass ($F_{3,66} = 10.2$, $p < 0.0001$, Table 3.2); 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 ($F_{3,48} = 0.94$, $p > 0.45$; controlling for laying interval; Table 3.2) or clutch mass ($F_{3,46} = 2.66$, $p > 0.05$; Table 3.2). Controlling for differences in clutch size, laying interval was significantly longer in the unmanipulated trial versus all other trials ($F_{3,42} = 3.16$, $p < 0.035$, Table 3.2).

Treatment effects on hematological parameters

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 three-week recovery period between breeding attempts (Table 3.3).

The interaction between treatment*reproductive stage was significant for hematocrit ($F_{3,35} = 7.32, p < 0.0006$), as was the main effect of reproductive stage on hematocrit ($F_{1,33} = 29.35, p < 0.0001$; Table 3.3). Hematocrit decreased significantly from pre-breeding to the 1-egg stage in the unmanipulated (-4.3 ± 1.1%; $F_{1,21} = 14.54, p < 0.001$; Figure 3.3a), estradiol-treatment (-5.5 ± 0.7%; $F_{1,22} = 60.83, p < 0.0001$; Figure 3.3b), and sham-treatment trials (-5.3 ± 0.7%; $F_{1,25} = 66.41, p < 0.0001$; Figure 3.3c), but there was no significant difference in hematocrit from pre-breeding to the 1-egg stage in the tamoxifen trial ($F_{1,17} = 3.77, p > 0.07$; Figure 3.3d). Most females showed a decrease in hematocrit of approximately 5% in the estradiol- and sham-treatment trials (Figure 3.4), but there was some variation between-individuals in the magnitude of the anemia response within experimental trials (unmanipulated = -17.3 – +3.8%, estradiol-treatment -13.0 – +1.1%; sham-treatment = -11.3 - +2.1%; tamoxifen-treatment = -13.3 - +6.0%; Figures 3.3 and 3.4).

The interaction between treatment*reproductive stage was not significant for red blood cell number ($F_{3,37} = 0.84, p = 0.5$; Table 3.3), hemoglobin ($F_{2,31} = 0.96, p = 0.4$; Table 3.3), or mean cell volume ($F_{3,37} = 0.07, p = 0.98$; Table 3.3), but there was a significant main effect of reproductive stage for red blood cell number ($F_{1,38} = 28.6, p < 0.0001$; Table 3.3) and hemoglobin concentration ($F_{1,37} = 13.67, p < 0.0007$; Table 3.3). From pre-breeding to the 1-egg stage, red blood cell number decreased by -6.64 cells*10^5/μl, and hemoglobin concentration decreased by -1.23 g/dL 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 3.3).

Repeatability and temporal variation of hematological parameters

Fourteen females bred in all four experimental trials, allowing for the investigation of repeatability in pre-breeding hematological variables. Comparing pre-breeding hematocrit over all experimental trials, 63.4% of the total variation was explained by among-individual variability ($F_{13,42} = 7.93$, $p < 0.0001$). Pre-breeding hemoglobin concentration across the estradiol-treatment, sham-treatment, and tamoxifen-treatment trials (not measured in unmanipulated trial) was also repeatable (35.9%, $F_{13,28} = 2.68$, $p < 0.015$). 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.

There was a significant positive correlation between prebreeding and 1-egg hematocrit in the estradiol ($r_{23} = 0.60$, $p < 0.0025$; Figure 3.5b) and sham trials ($r_{26} = 0.73$, $p < 0.0001$; Figure 3.5c), and this relationship was positive but not significant in unmanipulated ($r_{22} = 0.31$, $p = 0.16$; Figure 3.5a) and tamoxifen-treated ($r_{18} = 0.44$, $p < 0.07$; Figure 3.5d) females. There was also a significant negative correlation between the change in hematocrit (pre-breeding – 1-egg stage) and 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: $r_{18} = -0.57$, $p < 0.015$; Figure 3.6), i.e. females with the highest pre-breeding hematocrit values tended to show the largest decreases in hematocrit during egg production. For all experimental trials, repeatability of the change in hematocrit from pre-breeding to the 1-egg stage was 31.6% ($F_{13,42} = 2.85$, $p = 0.005$), and excluding the tamoxifen-treatment and unmanipulated

trials (see Discussion for rationale), repeatability between the sham- and estradiol-treatment trials was 66.7% ($F_{13,14} = 5.0, p < 0.003$).

In contrast, there was no correlation between hemoglobin concentration, red blood cell number, or mean cell volume measured at pre-breeding versus the 1-egg stage in any of the experimental trials ($p > 0.05$ in all cases). Limited data was available for these parameters (see Table 3.1); therefore, repeatability analyses were not performed.

Discussion

In this study, we have shown that the association between egg production and anemia is robust and consistent in zebra finches, even under relatively benign conditions (e.g. in captivity with *ad libitum* food). With the exception of tamoxifen-treated females, most females showed a consistent decrease in hematocrit of approximately -5% in the unmanipulated, sham-, and estradiol-treatment trials. Furthermore, we found evidence that pre-breeding hematocrit and the anemia response associated with egg production was repeatable among individual females. We found no evidence of compensation for the decrease in hematocrit among other hematological parameters measured: anemia during egg production was also associated with a significant decrease in red blood cell number and hemoglobin levels. Although exogenous estradiol treatment did not enhance anemia (contrary to one of our initial predictions) blocking estrogen receptors with the anti-estrogen tamoxifen reduced the development of anemia in female zebra finches. Thus, our experimental results confirm that the decrease in hematocrit during egg production is most likely dependent on endogenous estrogens.

Body mass variation and treatment effects on reproductive traits (i.e. egg mass, clutch size) were similar to those reported in previous studies (Williams 1996; Christians & Williams 1999; Williams 1999; Williams 2000; Wagner & Williams 2007). During the initial reproductive bout (unmanipulated trial), females showed changes in body mass typical of inexperienced breeders (Williams 1996; E.C. Wagner 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. In comparison to the estradiol- and sham-treatment a trial, the mean change in hematocrit was slightly less (~1%) and there was a greater range in the magnitude of the anemia response among individuals, suggesting that breeding experience may alter body condition somewhat through unknown mechanisms. Breeding inexperience also influenced the time it took to lay the first egg (laying interval was +1 day longer than all other trials; Williams 1996), but in agreement with previous studies, had no effect on primary reproductive effort (i.e. egg mass and clutch size; Williams 1996). In this study, tamoxifen inhibited the development of anemia but estradiol did not enhance development of anemia, and initially these results appear to be contradictory. However, similar seemingly paradoxical effects on reproductive traits have been documented here and in previous studies: tamoxifen treatment caused a robust decrease in egg size (Williams 2000; Williams 2001; Wagner & Williams 2007), whereas exogenous estradiol had no effect on egg size (Christians & Williams 1999; Williams 1999).

Although previous studies have reported that estradiol treatment induces anemia (range -2 to -15%) in domestic fowl (Domm & Taber 1946; Sturkie & Eiel 1966), pilgrim geese (Hunsaker 1968), Japanese quail (Nirmalan & Robinson 1972; Nirmalan & Robinson 1973; Garcia et al. 1984), rain quail (Deshmukh & Suryawanshi 1982) and white-crowned sparrows (Kern et al. 1972), these studies all used non-breeding birds. To our knowledge, our study is the first to assess effects of physiological levels of estradiol on hematological parameters within the context of egg production. We suggest two reasons for the different effects of anti-estrogen (tamoxifen) versus estrogen treatment: 1) that breeding females may be effectively 'maximized' on estradiol in the endogenous form, 2) that homeostatic mechanisms may act to maintain hematocrit above a minimum threshold level to meet enhanced metabolic demands and/or facilitate egg production.

It is possible that in an egg-laying female, estrogen receptors in bone marrow may be saturated with endogenous estrogens and thus unresponsive to estrogen supplementation, and/or estrogen receptor number and sensitivity may 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, for example, hepatic synthesis and release of yolk precursors (vitellogenin or VLDL-y; Williams 1999). Alternatively, homeostatic mechanisms (e.g. 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. Alternatively, hematocrit may 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 & Waldron 1999).

Interestingly, despite the high degree of individual variability in hematocrit (45-60%), we found evidence that the mean decrease in hematocrit during egg production was similar (approximately -5%) across individuals in the sham- and estradiol-treatment trials, and that there was a negative relationship between the change in hematocrit and pre-breeding hematocrit, i.e. females with higher pre-breeding hematocrit levels tended to show larger decreases in hematocrit. In addition, the change in hematocrit from pre-breeding to the 1-egg stage was repeatable within individuals (66.7%), which is comparable to estimates of repeatability for other physiological and reproductive traits in the zebra finch, including resting metabolic rate (53%; Vezina & Williams 2005) and plasma vitellogenin levels during egg production (85 - 93%; Salvante & Williams 2002), egg mass and clutch size (82% and 58% respectively; T.D. Williams unpublished data). Taken together, these results suggest that while the "optimal" hematocrit may vary among individuals, the decrease in hematocrit during egg-laying may be "programmed"

both within- and among-individual females, possibly reflecting a lower physiological limit below which aerobic capacity would be compromised.

While this study supports the hypothesis that anemia during egg-production is estrogen dependent, it does not distinguish between the proximate mechanisms underlying the development of anemia that have potentially been disrupted. Our main objective was to manipulate hematocrit levels at the onset of egg-laying, and due to our experimental design, we did not investigate potential long-term effects at later stages of egg production and incubation. Therefore, we could not separate estrogen-dependent hemodilution effects from direct inhibition of erythropoiesis, and did not capture any delayed effects of estradiol on red blood cell production, i.e. estradiol can suppress erythrocyte production at multiple points in the maturation pathway (e.g. gene transcription, cell differentiation, hemoglobin production; see Blobel & Orkin 1996 for review). 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, while 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 between trials in the decrease in hemoglobin and red blood cell number). As demonstrated in the preceding chapter, temporal changes should be correlated among these variables, and it is difficult to see how tamoxifen could prevent the estrogen-dependent decrease in hematocrit without simultaneously preventing a decrease in hemoglobin and red cell number. Further research is needed to increase statistical power and determine if changes in hemoglobin and red blood cell number during egg production

are affected by tamoxifen treatment, or if they represent estrogen-independent components of the anemia response.

In conclusion, the results from the tamoxifen trial confirm that this mechanism is a good candidate for a regulatory-network based trade-off involving pleiotropic effects of estrogens, which otherwise have essential reproductive functions during egg production. Differences between treatment effects of the anti-estrogen tamoxifen and exogenous estradiol highlight the complexity of using hormonal manipulations in studies such as this. The selection of an appropriate hormonal tool can be challenging as hormones tend to regulate a suite of interacting processes, and hormonally-induced effects can be highly specific to physiological state and environmental context. The results of this study emphasize the value of using multiple complementary approaches to experimentally manipulate phenotype.

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Figure 3.1. Effects of the interaction between treatment and reproductive stage on body mass dynamics in breeding female zebra finches. Open circles represent the unmanipulated experience trial (n = 23), filled squares represent the exogenous estradiol treatment trial (n = 24), inverted open triangles represent the sham-treatment trial (n = 26), and filled triangles represent the tamoxifen treatment trial (n = 21). All values are least-squares means ± standard error of mean.

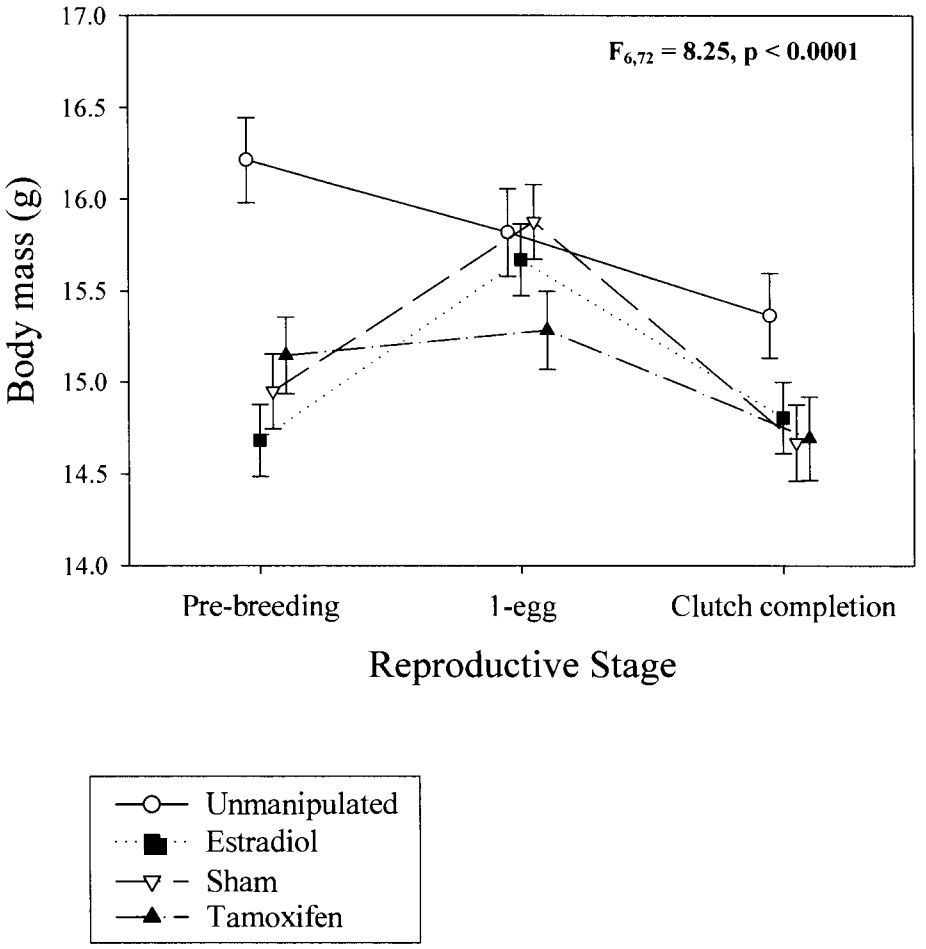

Figure 3.2. Treatment effects on plasma estradiol concentration. Sample sizes are as follows: unmanipulated (n = 23), estradiol treatment (n = 24), sham treatment (n = 26), and tamoxifen treatment (n = 21). All values are least-squares means ● standard error of mean. * indicates a significant difference between sample means at a significance level of $p < 0.001$ (corrected for multiple comparisons using Tukey-Kramer adjustment).

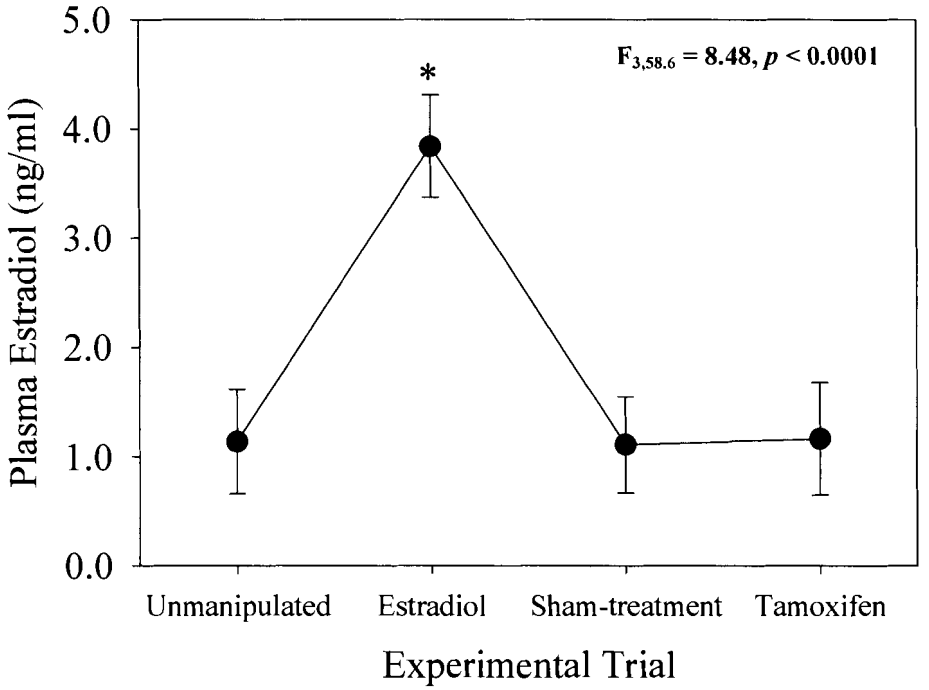

Figure 3.3. The change in hematocrit from pre-breeding to the 1-egg stage in the a) unmanipulated (n = 22), b) estradiol-treatment (n = 23), c) sham-treatment (n = 26), and d) tamoxifen-treatment (n = 18) trials. Plotted lines represent data from individual females and grey filled circles represent the mean hematocrit calculated at each stage.

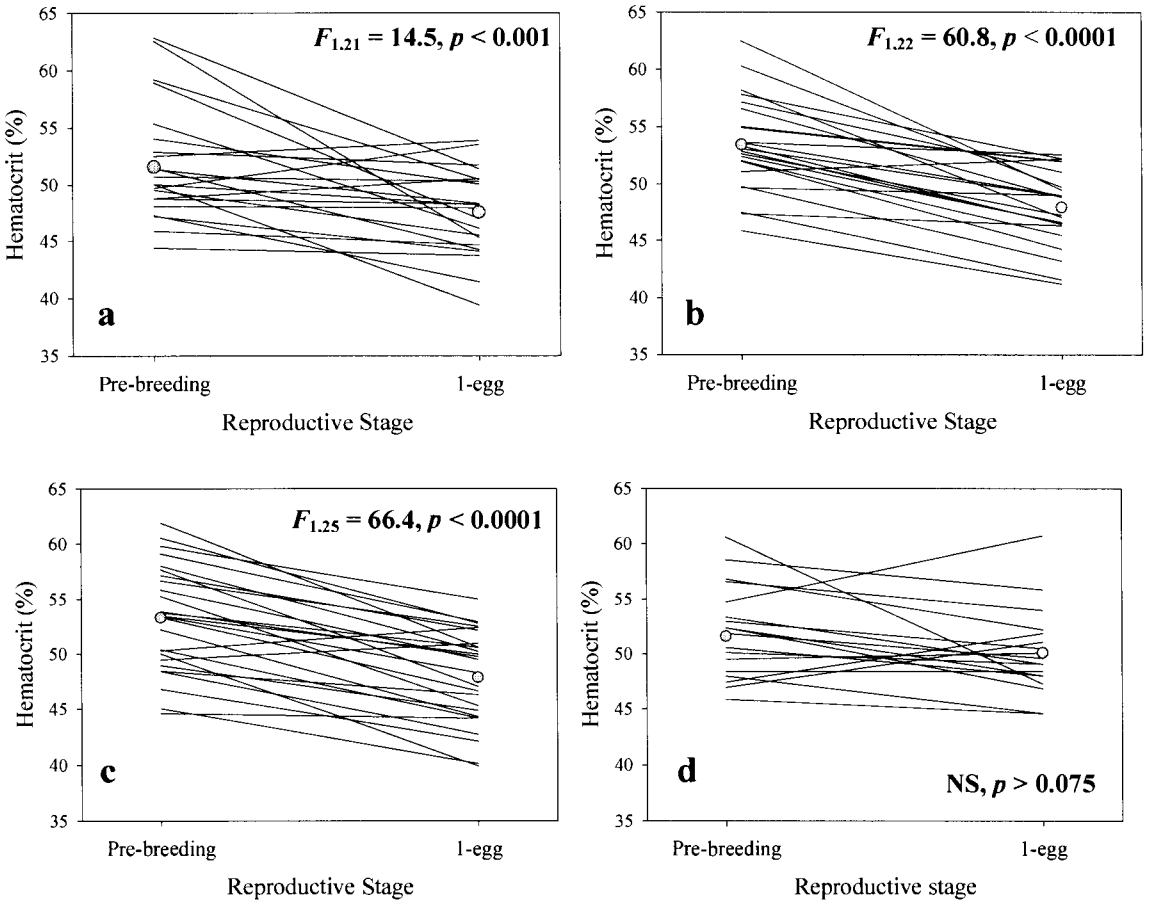

Figure 3.4. Histograms showing the pattern of distribution in magnitude of the anemia response (i.e. the decrease in hematocrit from pre-breeding to the 1-egg stage) between individuals for the a) unmanipulated trial (n = 22), b) estradiol-treatment (n = 23), c) sham-treatment (n = 26), and d) tamoxifen-treatment (n = 18) experimental trials.

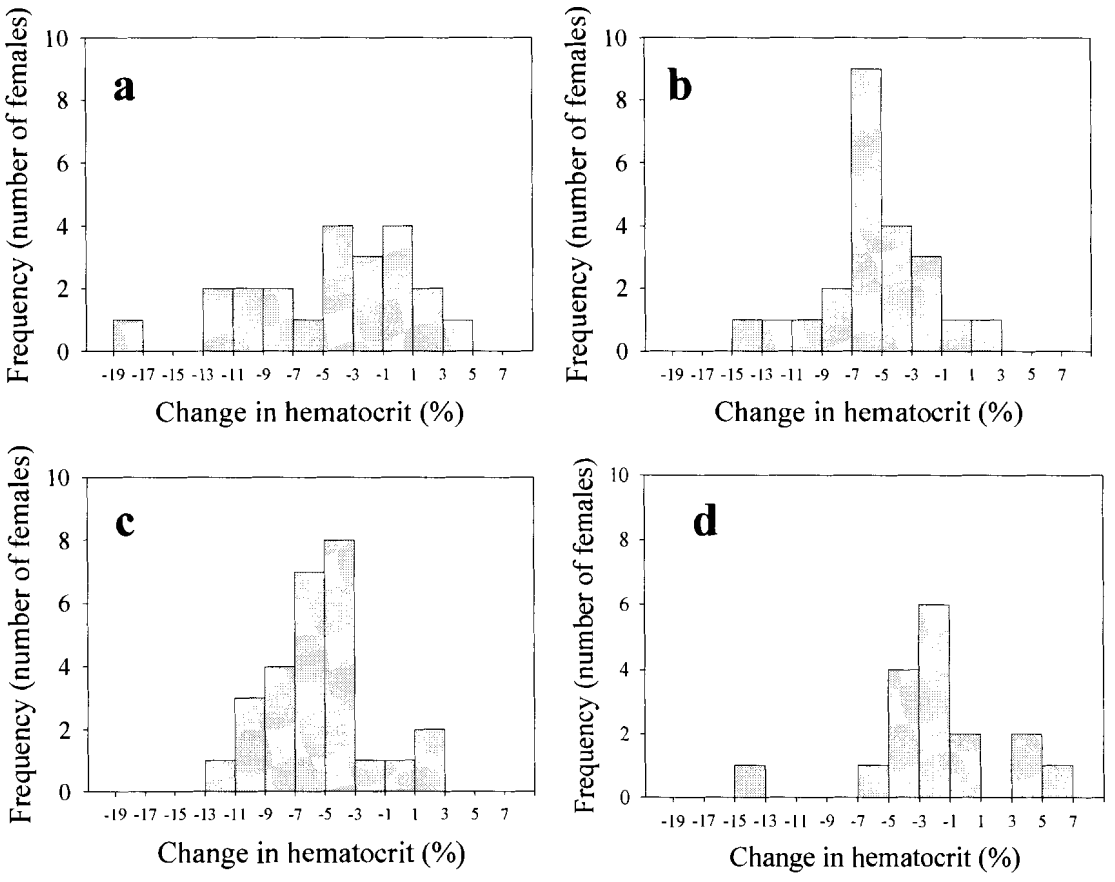

Figure 3.5. The relationship between pre-breeding and 1-egg hematocrit among individual females in the a) unmanipulated trial (n = 22), b) estradiol-treatment (n = 23), c) sham-treatment (n = 26), and d) tamoxifen-treatment (n = 18) experimental trials. Regression lines are presented with 95% confidence intervals (dotted lines).

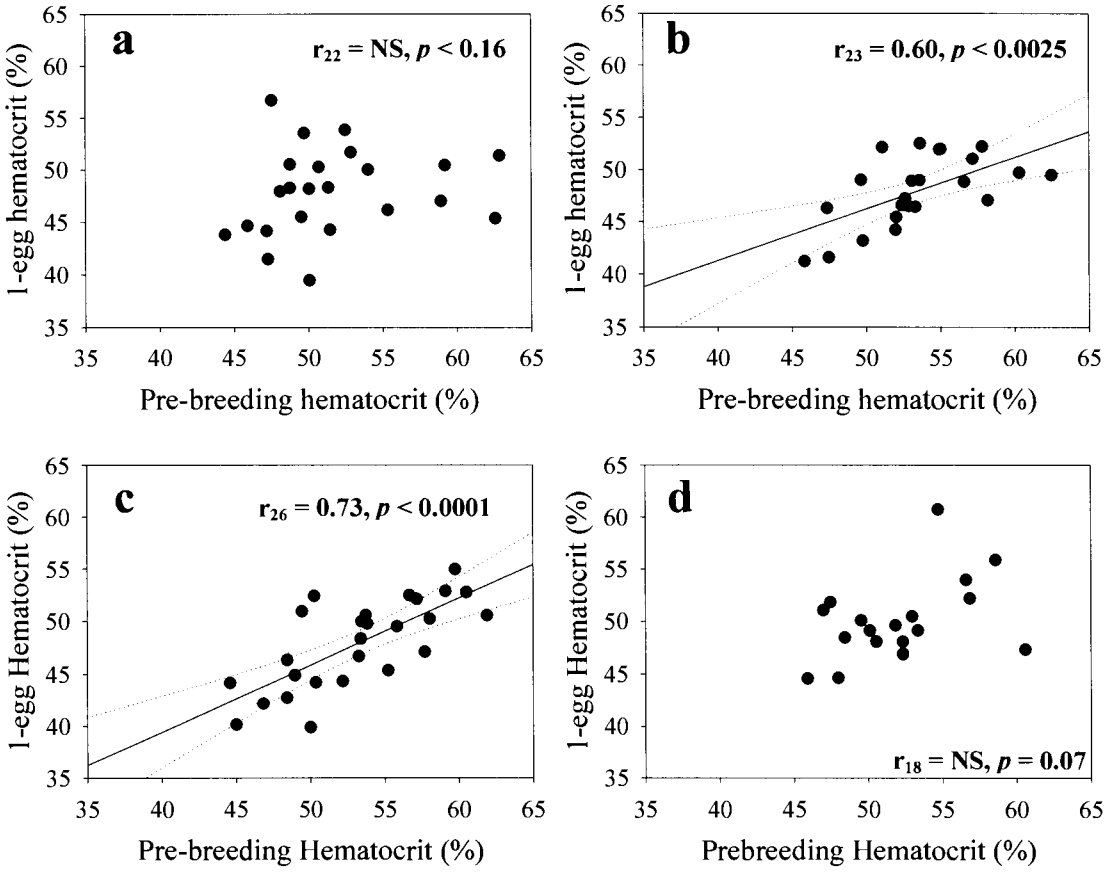

Figure 3.6. The change in hematocrit from pre-breeding to the 1-egg stage as a function of pre-breeding hematocrit for a) unmanipulated (n = 22), b) estradiol (n = 23), c) sham (n = 26), and d) tamoxifen (n = 18) experimental trials. Regression lines are presented with 95% confidence intervals (dotted lines).

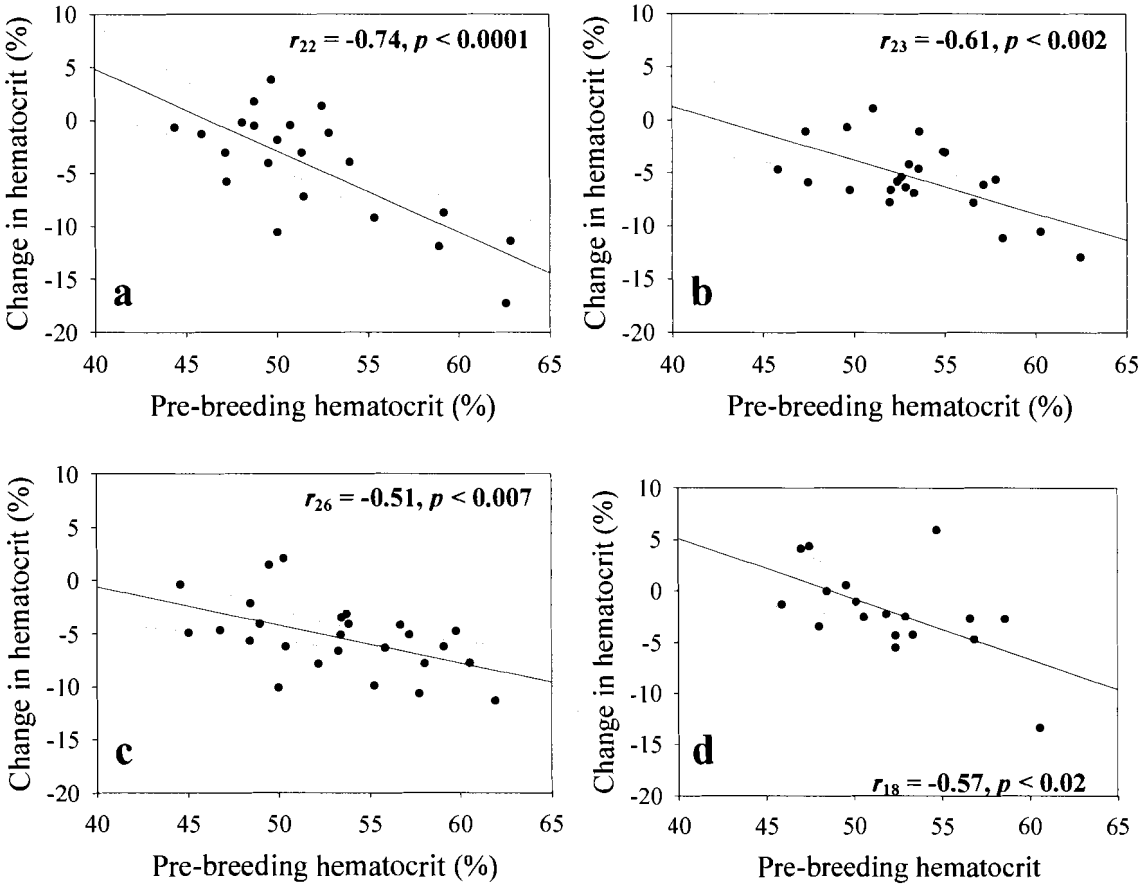

Table 3.1. Sample sizes for hematological analyses at the pre-breeding and 1-egg stages in each experimental trial. Hemoglobin concentration was not measured in the unmanipulated trial.

Trial/Stage	**Unmanipulated (n = 23)**	**Estradiol (n = 24)**	**Sham-treatment (n = 26)**	**Tamoxifen (n = 21)**
Pre-breeding				
Hematocrit	23	23	26	21
Hemoglobin concentration	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 concentration	N/A	19	23	20
Red blood cell number	13	17	13	20
Mean cell volume	13	17	13	18
Estradiol concentration	22	23	26	19

Table 3.2. The effect of experimental treatments on reproductive traits. Clutch size was log-transformed prior to analysis but is presented here unaltered for clarity. Values are least-squares means ± s.e.m. Significant differences in sample means are indicated with * for significance level of $p < 0.05$, and ** for significance level of $p < 0.0001$ (corrected for multiple comparisons using Tukey-Kramer adjustment).

Trait	**Unmanipulated**	**Estradiol**	**Sham-treated**	**Tamoxifen**
Sample size	22	23	26	19
Plasma estradiol concentration (ng/ml)	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

Table 3.3. Temporal variation in hematological variables. 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 (treatment*stage interaction was NS for all, individual was included as a random effect in the mixed-model).

Trait	Prebreeding	1-egg stage	F-statistic	P value
Hematocrit (%)				
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
Hemoglobin (g/dL)				
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**
Red blood cell number (cells*10^6/µL)				
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**
Mean cell volume (fL)				
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

CHAPTER 4: GENERAL SYNTHESIS AND FUTURE DIRECTIONS

Synthesis

The cost of reproduction is a central concept in life history theory, yet we still know very little about the physiological mechanisms underlying this cost. As argued by Harshman and Zera (2007), if we treat a phenomenon such as the cost of reproduction as a 'black box', then the components and processes involved will be reduced to constructs that may have few connections to reality. We cannot fully understand the consequences of the cost of reproduction on ecological and evolutionary processes, or in turn how these processes shape the cost, unless we understand the mechanistic basis (Harshman & Zera 2007). In light of this, we have characterized a specific mechanism, the development of anemia (decreased hematocrit) during egg production in birds, which we believe plays a role in mediating the costs of egg production.

While the traditional 'mechanistic' explanation has been that costs result from preferential allocation of a limited pool of resources towards reproduction (Williams 1966), a more recent approach has focused on reproduction itself and/or the regulatory (physiological) mechanisms underlying reproduction as proximate effectors of such costs (Partridge et al. 2005). In particular, hormones are thought to be key mediators of life-history trade-offs because of their potential for inducing negative effects in non-target tissues (Finch & Rose 1995; Rose & Bradley 1998; Ketterson & Nolan 1999; Williams 2005). In Chapter 2, our objective was to evaluate which explanation better explained the development of anemia during egg production: 1) reallocation of resources to reproduction and away from erythropoiesis, or 2) pleiotropic effects of estrogen (i.e. hemodilution effects and inhibition of red blood cell production). Our results indicated

that the development of anemia during egg production was consistent with transient suppression of erythropoiesis rather than a result of hemodilution effects alone. More importantly, we found limited evidence that anemia during egg production was an energy conservation strategy (e.g. diet*stage effect was significant for hematocrit only), and no evidence of compensation to reduced hematocrit among other variables studied, confirming that anemia could indeed function as a cost.

In Chapter 3, we confirmed that anemia during egg-laying was directly dependent on endogenous estrogens: blocking estrogen receptors using the anti-estrogen tamoxifen reduced the development of anemia in female zebra finches. However, contrary to predictions, administration of exogenous estradiol did not enhance the development of anemia during egg production. Although it has been demonstrated previously that estradiol treatment can induce anemia in a range of avian species (reviewed in Blobel & Orkin 1996), to our knowledge, ours was the first study to investigate effects of estradiol on hematological parameters within the context of reproduction. We discussed several reasons for the seemingly contradictory effects of tamoxifen versus estrogen treatment: 1) that breeding females may be effectively 'maximized' on estradiol in the endogenous form, 2) that homeostatic mechanisms may act to maintain hematocrit at an 'optimum' level, and 3) exogenous estradiol may have affected erythropoiesis in later reproductive stages (i.e. regenerative processes at clutch completion and/or recovery during incubation), but due to our experimental design, this was not assessed. Further research is needed to investigate these issues. Regardless, based on the results of the tamoxifen trial, we can conclude that anemia during egg production is most likely due to pleiotropic

effects of estrogens, and thus remains a strong candidate for a regulatory-network based trade-off.

Future Directions

Physiological compensation for reductions in hematocrit

Although we did not find evidence of compensation to decreased hematocrit among additional hematological parameters studied, there are other compensatory responses that could potentially maintain a stable oxygen carrying capacity during egg production: 1) a reduction in blood oxygen affinity (Riera et al. 1983), and/or 2) an increase in cardiac output (Box et al. 2002). Hemoglobin consists of four globin subunits, each incorporating an iron-containing heme ring where O_2 is bound according to its partial pressure or P_{O2} (Hebert et al. 1997). Avian erythrocytes upregulate synthesis of the organic phosphate inositol pentakisphosphate ($InsP_5$) when tissue oxygen demands increase, for example during flight and diving activity (Riera et al. 1983; Giardina et al. 2004). $InsP_5$ binds to the β-chains of the hemoglobin tetramer, reducing oxygen affinity and facilitating its release to tissues at low to moderate P_{O2} levels (e.g. the 'Bohr effect'; Bohr et al. 1904; Villar et al. 2003). An increase in heart rate and stroke volume (amount of blood pumped per heart beat) has also been reported during activities with high oxygen demand such as sustained flight (Bishop 2005; Peters et al. 2005). It is not known if zebra finches use either of these mechanisms to maintain oxygen delivery to tissues at a steady-state during egg production or chick rearing, but it is an area worth investigating. Methods for measuring cellular InsP5 concentration (Prats & Riera 1994; Casals et al. 2002; Villar et al. 2003), and recording heart rate in zebra finches (Franz & Goller 2002;

Cooper & Goller 2004; Cooper & Goller 2006) have been described previously and could be implemented in future studies.

Hematocrit and aerobic capacity

In order to confirm a functional link between anemia and aerobic capacity, future studies should investigate how intraspecific variation in hematocrit contributes to individual exercise performance in zebra finches. Pierce et al. (2005) described a modified running-wheel respirometer used to measure oxygen consumption and exercise performance of small birds during forced running/hopping activity. A similar apparatus could be developed for use in zebra finches to determine which hematological variables best predict exercise performance (*sensu* Hammond et al. 2000) in non-breeding and breeding birds. In addition, it would also be of interest to examine relationships between variation in hematological parameters and flight performance following protocols established in previous studies of the zebra finch (e.g. Veasey et al. 2001; Kullberg et al. 2005).

Experimental manipulation of hematocrit

We have validated techniques to directly manipulate hematocrit in female zebra finches: stimulating red blood cell production with the hormone erythropoietin (EPO), and inducing moderate anemic states with the xenobiotic agent phenylhydrazine hydrochloride (PHZ). Erythropoietin (EPO) is a hormone normally produced by the kidney in response to hypoxia, and acts to stimulate production of red blood cells from progenitor cells in the bone marrow (Dessypris & Sawyer 2004). Exogenous EPO has been used previously to stimulate erythropoiesis in a wild finch species, the spotted

munia, resulting in a 15% increase in hematocrit and hemoglobin concentration (Thapliyal et al. 1982). Phenylhydrazine (PHZ) is an oxidative compound that has been used to study anemia in several avian species (reviewed in Clark et al. 1988). PHZ treatment induces hemolytic anemia *via* denaturative oxidation of hemoglobin, formation of Heinz bodies, and disruption of the erythrocyte membrane (Riera et al. 1991). Previous studies typically used a single dose of PHZ (1.5-6.0 mg/100g bw) to generate a 20-30% decrease in red blood cell counts and hemoglobin concentration (Ramis & Planas 1982; Datta et al. 1990; Riera et al. 1991).

In non-breeding female zebra finches, we have determined that administration of single 1mg/g dose of PHZ reduces hematocrit by approximately -8%, while administration of 1.5 units of EPO/day for 5 days increases hematocrit by approximately +4% (n = 60, Figure 4.1). These techniques could be used to explicitly test if anemia during egg production is an evolutionary constraint or adaptation *via* direct manipulation of female phenotype (hematocrit) during breeding. If experimentally-manipulated hematocrit values are unrelated to reproductive output, this would suggest that breeding females are able to compensate for the negative pleiotropic effects of estrogen through alternate routes, for example, through behavioural and/or physiological adjustments. However, if a causal relationship was found between these traits, this would confirm that the development of anemia does contribute to the cost of reproduction, a result that may be equally relevant to many other animal species.

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Figure 4.1. Effects of erythropoietin (EPO, n = 30, filled circles) and phenylhydrazine (PHZ, n = 30, open squares) treatment on the change in hematocrit from day 1 of treatment (see text for details of treatment protocols) in non-breeding females. Dashed line represents pre-treatment 'baseline' hematocrit.