The Functional Significance of Variation in Hematological Traits that Determine Aerobic Capacity

by

Raime B. Fronstin

M.Sc., University of North Florida, 2007
B.Sc., University of Florida, 2003

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Name: **Raime B. Fronstin**

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Title: ***The Functional Significance of Variation in Hematological Traits that Determine Aerobic Capacity***

Examining Committee: **Chair:** Harald Hutter
Professor

Julian Christians
Senior Supervisor
Associate Professor

Elizabeth Elle
Supervisor
Professor

John Reynolds
Supervisor
Professor

David Green
Internal Examiner
Professor
Department of Biological Sciences

Charles Thompson
External Examiner
Professor
School of Biological Sciences
Illinois State University

Date Defended/Approved: March 26, 2016

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Abstract

A principal aim of evolutionary physiology is to understand how physiological variation affects the fitness and distribution of organisms. Physiological traits underlie performance, behavior, and life histories and exhibit variation at every taxonomic level. Studies within and between species can reveal whether variation in a physiological trait is currently, and/or was historically, functionally significant. Intraspecific variation is regarded as the raw material on which selection acts, and studying it in relation to measures of fitness can help identify the targets of selection that underlie life history strategies. Comparing interspecific variation with ecological factors can help clarify whether the physiological variation was either shaped by adaptation in response to different selective pressures or influenced differences in habitat and life histories.

Traits related to energy expenditure are believed to play a principal role in shaping the evolution of life histories. Hematocrit, the percent of red blood cells per unit volume of blood is a measure of an individual's oxygen carrying potential and therefore is pivotal in determining endurance. Given that birds show extensive variation in hematocrit within and between species and that flight imposes high energetic costs, variation in hematocrit in birds is likely to be functionally significant.

I used observational, experimental, and comparative approaches to investigate the functional significance of intraspecific and interspecific hematocrit variation in birds. Intraspecific hematocrit variation could influence fitness if it plays a role in regulating eggshell colouration or by limiting physical endurance, thereby shaping reproductive decisions. Experimental manipulation of hematocrit in free-living European starlings provided no evidence that hematocrit variation affects eggshell coloration. However, natural and experimental variation in hematocrit influenced fitness measures, suggesting that hematocrit variation, if heritable, is acted upon by natural selection. Comparative analyses indicated that hematocrit variation across passerines was related to habitat altitude, latitude and migration, suggesting adaptive or exaptive significance. Overall, the results suggest that variation in hematocrit within and between avian species is functionally significant. Such studies can help to understand population dynamics, demography, biodiversity, and responses to climate change.

Dedication

To those without whom I would not be here, my parents
Bernice & Fred Fronstin, my wife Martha Fronstin,
and my Koda Fronstin.

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Preface

Chapters two through four of this thesis are a series of separate journal article manuscripts and are presented as independent, stand-alone chapters. These chapters differ in form because they have been formatted for publication in different journals.

Chapter 1.

General Introduction

Physiological variation is ubiquitous in animals at every taxonomic level. The mechanisms underlying this variation and the factors shaping it are the main focus of evolutionary physiology. Variation among individuals of the same species is commonly regarded as the raw material on which natural selection acts, while the variation among species represents the potential product of past selection. The main goals of evolutionary physiology are to understand 1) the functional and adaptive significance of physiological variation i.e., how do genotype, phenotype, performance, and fitness influence one another, 2) which evolutionary mechanisms have led to and/or maintained the range of physiological diversity existing today and 3) what are the historical, phylogenetic, and ecological patterns of physiological evolution (Garland Jr & Carter, 1994; Feder, Bennett, & Huey, 2000;). Two major approaches have been used to address these questions. One approach addresses the fitness implications of physiological diversity by measuring associations between inter-individual physiological variation and proxies of fitness. A particularly powerful method that can yield insight into mechanistic and causal links between trait variation and performance employs "phenotypic engineering" in which the physiological trait in question is manipulated to observe effects on fitness. For example, a classic study by Andersson (1982) involved artificially shortening and extending tail length of male widowbirds and then monitoring mating success. Although measuring and manipulating phenotypes and fitness proxies is more difficult in natural settings than laboratory settings, studies of natural populations can yield unique insight, as fitness consequences can often be context dependent (see Chapter 3) and selective pressures in the wild cannot be reproduced in laboratory settings. Recent studies have observed fitness associations, in natural free-living populations, with stress responses (Wada et al., 2008); in zebra finches), immune responsiveness (Bowers et al., 2014; Nussey et al., 2014), metabolic rates (Artacho &

Nespolo 2009 in snails; Boratyński & Koteja 2010 in bank voles; see White & Kearney 2013 for review), neonatal condition and hematocrit (Bowers *et al.* 2014 in house wrens) and hemoglobin (Minias *et al.* 2014 in common snipe).

The second approach employed in evolutionary physiology is historically based and focuses on the outcome of previous selective pressures in order to understand current patterns of physiological diversity. This approach typically employs comparisons among species (or higher-level taxa). Recent studies employing the comparative approach have yielded insight into potential selection pressures that have shaped a variety of traits in different organisms. For example, there is evidence that the thermoregulatory pressure imposed by colder climates has shaped metabolic rates in fish (White et al., 2012) and mammals (Raichlen et al., 2010). Results from a study done by Brischoux *et al.,* (2011) suggest that the habitat transition from land to water of some snake species selected for an increase in hematocrit, presumably to increase oxygen reserves enabling prolonged diving times. Physiological traits that relate to energy, such as metabolic rate, hematocrit and hemoglobin, are a focus of many studies because they underlie variation in performance and activity, which selection is considered to affect most directly (Careau & Garland, 2012).

Aerobic capacity sets an upper limit on the intensity of activity that can be sustained (Dlugosz et al., 2013). Therefore, variation in physiological traits that influence aerobic capacity should be particularly susceptible to selection, especially during limiting times (such as reproduction) or in limiting environments. In order to harness the maximum amount of energy animals must catabolize food through aerobic respiration, a process that requires both organic compounds and oxygen to synthesize ATP. Hemoglobin within red blood cells reversibly binds to oxygen, and the primary function of red blood cells is to transport oxygen to tissues throughout the body via the circulatory system. A measure of an individual's oxygen carrying potential is the volume percentage of red blood cells in the blood, a parameter known as hematocrit (measured as % packed cell voume, PCV). Higher hematocrit enables a greater blood oxygen carrying capacity and therefore greater endurance performance (Kanstrup & Ekblom 1984; Ekblom & Berglund 1991, also see chapter four). Hematocrit varies greatly across taxa, being highest in endothermic animals and lowest in poikilotherms. Interestingly,

mammals (Promislow, 1991) and birds (Glomski & Pica, 2011; ~ 75% of bird species range between 41-50% PCV) have both exhibited a mean hematocrit of 44%, while mean hematocrit exhibited by the measured poikilotherms is 27% (Glomski & Pica, 2011). In studies comparing athletic and sedentary mammals, hematocrit levels were found to be 1.8 times greater in athletic species relative to sedentary species (Conley, 1987; Kayar et al., 1994). Conversely, at higher hematocrit levels the rate of blood flow and therefore the speed of oxygen delivery to the tissues is inhibited by an increase in blood viscosity. However, mechanisms that can counteract the negative effects of increased viscosity have been identified. In response to increased viscosity both red blood cells and endothelial cells can release nitric oxide to induce vasodilation and reduce red cell aggregation (Starzyk et al., 1999; Ruschitzka et al., 2000; also reviewed in Harder & Boshkov 2010). In addition, increasing red blood cell flexibility can aid in reducing blood viscosity (Vogel et al., 2003). These mechanisms could facilitate the exploitation of benefits from higher hematocrit. In a transgenic mouse line, generated to overexpress human erythropoietin resulting in very high hematocrit (up to 0.90), blood viscosity was regulated by both nitric oxide mediated vasodilation and increased RBC flexibility, protecting them from acute cardiovascular events (Ruschitzka et al., 2000; Vogel et al., 2003).

Although hematocrit is ultimately bound by lower and upper limits due to a reduction in oxygen carrying capacity from too little hemoglobin and a reduction in the rate of oxygen transport to the tissues from increased blood viscosity, there is still significant variation within and among species. If higher hematocrit denotes greater aerobic capacity then why wouldn't all organisms have hematocrit levels near the upper limit? Variation in a trait can be sustained due to contrasting benefits and costs in different environments. For example, variation in basal metabolic rate (BMR) is likely maintained by balancing costs and benefits such that the higher costs of maintaining a higher BMR may be offset in a situation that requires a maximal rate of energy metabolism, like migration (Speakman et al., 2004). Similarly, a heightened immunity is beneficial for defending against diseases and parasites, however it can also reduce longevity via self-damage (reviewed in Bowers et al., 2014). Variation in hematocrit could be similarly sustained if it offers contrasting benefits and costs in different environments. Many studies in mice and humans demonstrate that increasing

hematocrit (even small increases to ~ 50%) by blood doping or increasing erythropoietin increases maximal metabolic rates (as cited in Weibel et al. 2004), time to exhaustion during exercise and VO_2 max (Ekblom & Berglund 1991; Ekblom 2000; reviewed in Jelkmann & Lundby 2011). However, increases in hematocrit, even within normal ranges, can lead to increases in blood pressure in humans and rats, independent of other risk factors (Letcher et al., 1981; Hart & Kanter, 1990; Göbel et al., 1991; Lowe et al., 1997; Bertinieri et al., 1998; Devereux et al., 2000; Lappin et al., 2002; Locatelli & Del Vecchio, 2003; Atsma et al., 2012; Jae et al., 2014) and increases in blood pressure can be costly. Arterial blood pressure in animals is equal to the rate of blood flow times peripheral resistance (pressure = flow x peripheral resistance), where blood flow is determined by cardiac output and peripheral resistance is determined by viscosity and vessel resistance. Therefore, all else being equal, an increase in hematocrit that results in an increased blood viscosity will increase blood pressure. Prolonged high blood pressure is regarded as the major cause of cardiac failure, stroke, vascular disease, renal failure and cognitive deterioration of vascular origin (reviewed in Kotchen 2011) in humans (Collins et al., 1990; MacMahon et al.,1990; MacMahon & Rodgers, 1993; Black, 1999; Neal & Macmahon, 1999; Neal et al., 2000; Chobanian et al., 2003; World Health Organization, n.d.), rats (reviewed in Amenta et al., 2010; Dornas & Silva 2011), and birds (Merck Veterinary Manual Avian diseases last full review/revision July 2011 by Teresa L. Lightfoot, DVM, DABVP (Avian)). Blood pressure differences of only 2 to 3 mm Hg have been linked to differences in fatal versus nonfatal ischemic heart disease (Staessen et al., 2001; Staessen & Birkenhäger, 2005). High blood pressure promotes the formation of atherosclerotic plaques by damaging the endothelial lining of blood vessels. Over time the plaques develop into scar tissue creating a rough surface that makes the blood susceptible to clotting, which can reduce or block blood flow or break off and cause an embolism. In addition, increased resistance caused by increased pressure forces the cardiac muscle to work harder. Under chronic high pressure the left cardiac ventricle hypertrophies and eventually is pushed beyond its limit. Therefore even minor increases in hematocrit in which viscosity is not mitigated by increased nitric oxide production or increased RBC flexibility could lead to increased blood pressure which if chronic could decrease longevity. In addition, although the transgenic mice that express high hematocrit were protected from high blood pressure related cardiovascular events by high levels of nitric oxide they still suffered endothelial damage and exhibited hepatic,

renal, neuronal, and muscular degeneration as they aged (Ogunshola, 2005; Heinicke et al., 2006), suggesting negative effects of high hematocrit that are independent of blood pressure. Therefore, in demanding environments an increase in aerobic capacity could offset the costs of decreased longevity and maintain the variation observed in hematocrit.

Birds, Passerines in particular, are a good model for studying the functional significance of intraspecific variation in hematocrit as well as past selective pressures that may have shaped the current observed variation between species. First, flying animals have among the highest energetic demands (Maina, 2000) thus it seems likely that avian traits related to aerobic capacity such as hematocrit would have been and could still be under strong selective pressure. Compared to mammalian red blood cells (RBCs) avian RBCs consume 7-10 times more oxygen (Lumeij, 1987; Glomski & Pica, 2011). In addition, compared to mammals and non-Passerines, Passerines have the highest mass-specific metabolic rate (Lasiewski & Dawson, 1967) as well as the largest interspecific variation in hematocrit (Hawkey et al., 1991; Glomski & Pica, 2011) . A large amount of intraspecific variation has been observed among birds as well (Sturkie & Griminger, 1976; Chilgren & deGraw, 1977; Rehder et al., 1982; Ots et al., 1998; Burness et al., 2001), with interspecific variation being greater than intraspecific and intraindividual variation (Hawkey et al., 1991; Glomski & Pica, 2011). Second, birds occupy a wide variety of habitats and a large database of DNA sequences exists from which robust phylogenetic relationships can be deduced, thereby facilitating comparative analyses. Functionally significant variation in hematocrit could be adaptive if it has a genetic basis. Three studies have analyzed the heritability of hematocrit variation in different species of wild birds. Two of the studies, which employed cross-fostering, found significant heritable variation of hematocrit in wild house wrens (Sakaluk et al., 2014) and wild house martins (Christe et al., 2000). Although, no significant heritability could be detected in pied flycatchers, significant repeatability was found (Potti, 2007). In addition, hematocrit variation has been shown to be heritable in captive zebra finches (as cited in Sakaluk et al., 2014), chickens (Shlosberg et al., 1996; Shlosberg et al., 1998) and mammals (Weibust & Schlager, 1968; Stino & Washburn, 1973; Whitfield et al., 1985; Maes et al., 1995; Pravenec et al., 1997; Evans et al., 1999; Foote & Hare, 2001; Lin et al., 2005; Johannes et al., 2006). Furthermore, baseline hematocrit levels

can be modified by direct selection in birds (Washburn, 1967; Shlosberg et al., 1996) and as a correlated response to selection for traits that cause metabolic stress such as rapid growth, cold stress, and high juvenile body weight (Price et al., 1998). In some cases hematocrit has been used as an index for condition in birds (Saino et al., 1997; Ots et al., 1998; Sánchez-Guzmán et al., 2004) though this has been a source of contention (reviewed in Cuervo et al. 2006). While associations between fitness and Hct & Hb have been observed in natural populations (Bowers et al., 2014; Minias et al., 2014), manipulating these traits directly to examine mechanistic and causal links in nature have not been done.

By integrating observational, experimental and comparative analyses I aimed to determine the functional significance of intraspecific hematocrit variation and the potential selective pressures that may have shaped the variation in hematocrit observed among bird species today. In Chapter Two, I used an experimental approach to investigate the role of hematocrit variation in the sexual selection of eggshell color hypothesis (Moreno & Osorno, 2003) in a passerine, the European starling (*Sturnus vulgaris*). The hypothesis aims to explain the persistence of blue-green eggshell pigmentation despite its conspicuousness to predators. Biliverdin, a derivative of hemoglobin, is the blue-green pigment produced by European starlings to color their eggshells. The intensity of color has been hypothesized to be a signal of maternal quality, which may influence paternal investment (Moreno & Osorno, 2003). In order for biliverdin to be an honest signal, it must be a limiting resource. By experimentally manipulating the availability of circulating resources for biliverdin synthesis (Hct & Hb) in free-living female European starlings, I tested whether variation in hematocrit and hemoglobin regulate eggshell color intensity. In addition, I examined whether eggshell color intensity is correlated with female quality, reproductive success, and the level of paternal investment. If eggshell color signals female quality and variation in eggshell color is regulated by circulating resources then variation in hematocrit or hemoglobin could significantly influence fitness.

In Chapter Three, I investigate whether hematocrit and hemoglobin variation during different breeding stages affect reproductive performance. In addition, I address whether a decrease in hematocrit commonly observed during reproduction represents a

cost of reproduction. I explore relationships between natural and experimental variation in post-laying hematocrit and hemoglobin and measures of individual quality (i.e., lay date, egg size, and clutch size), parental provisioning effort, and measures of brood quality (i.e., brood and chick sizes) and fledging success. If inter-individual variability among hematocrit and hemoglobin is adaptive, I would expect reproductive success to be correlated with repeatable hematological parameters and experimental reduction of hematocrit and hemoglobin should decrease reproductive performance.

In Chapter Four, I used phylogenetic analyses to identify ecological factors that may have exerted selective pressure on hematocrit within Passerines. I identified and collected data for 3 major ecological factors that exhibit clear differences in oxygen demands, 1) migratory phenotype, 2) habitat latitude (affecting thermogenic demands), and 3) habitat altitude. Using a phylogenetic tree built from 56 passerine species I tested whether variation in hematocrit is associated with migratory phenotype, habitat latitude or habitat altitude, corrected for phylogenetic relatedness.

Finally, in Chapter Five, I integrate the conclusions drawn from each study and discuss their significance, limitations, and potential future directions.

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Chapter 2.

Effects of manipulating haematocrit on eggshell colouration and sexual signaling in the European starling (*Sturnus vulgaris*)

A modified version of this chapter has been submitted as:

Fronstin RB, Doucet SM, & JK Christians (submitted) Haematocrit, eggshell colouration and sexual signalling in the European starling (*Sturnus vulgaris).* BMC Ecology

2.1. Summary

The hypothesis that blue-green eggshell colour is an honest, sexually selected signal of female quality assumes that eggshell pigmentation carries some cost. One potential cost is that biliverdin, a haem derivative and the pigment responsible for eggshell colouration, is limiting. To assess whether biliverdin is limiting, we manipulated haematocrit and haemoglobin in free-living European starlings (*Sturnus vulgaris* Linnaeus). We also investigated whether egg colour relates to brood quality and whether males use it as a signal to determine their offspring-provisioning rate. Upon collecting unmanipulated first clutches, we treated females with phenylhydrazine (PHZ), a

haemolytic agent, or saline and followed them through the fledging of replacement clutches. We compared eggshell colour, measured by blue-green chroma (BGC), between treatment groups and clutches. Within unmanipulated and control clutches we compared BGC with haematology, measures of maternal and brood quality, and paternal provisioning. Lack of a treatment effect on mean and maximum BGC, and correlations between BGC and haematology, suggest that biliverdin synthesis does not limit eggshell pigmentation. Lower BGC in replacement clutches suggests a possible biliverdin limitation over the season. First clutch BGC was correlated with female mass, consistent with eggshell colour being condition-dependent. BGC was not correlated with other measures of female quality nor any measure of clutch quality within either clutch. Fledglings from eggs with higher BGC had higher haemoglobin levels and longer tarsi, suggesting that eggshell colour could reflect brood quality. BGC was independent of all other measures of brood quality and paternal provisioning effort.

Keywords: eggshell colour, haematocrit, sexual signal

2.2. Introduction

The functions of avian eggshell colour and patterning and the mechanisms underlying variation in these traits remain poorly understood (Gosler et al. 2000; Underwood and Sealy 2002; Maurer et al. 2011). Numerous functions of eggshell pigmentation have been proposed, including crypsis, intra-specific egg recognition, thermal regulation, UV-B protection, strengthening of the shell, and the filtration of light to optimal wavelengths to modulate various aspects of embryonic development (Westmoreland and Kiltie 1996; Underwood and Sealy 2002; Kilner 2006; Lahti 2008; Maurer *et al.* 2011). Given the extensive diversity of avian eggshell patterning and colouration, it is unlikely that a single function can explain all of the observed variation

(Hanley, Cassey & Doucet 2012), though evolutionary history may play a larger role than previously suspected (Cassey *et al.* 2012; Brulez *et al.* 2016).

Blue-green colouration is found in many species (Underwood & Sealy 2002; Kilner 2006) and is conspicuous to some predators, which has incited curiosity into the selective factors that preserve it (Avilés *et al.* 2006; Hanley *et al.* 2008). One hypothesis is that blue-green egg colouration is a sexually selected, condition-dependent signal of the laying female's quality, which males use to determine their own level of post-hatching parental investment (Moreno & Osorno 2003). Indeed, a comparative study found that blue-green pigment concentration was more often associated with species who employed bi-parental provisioning (Cassey *et al.* 2012). Moreno & Osorno's sexual selection hypothesis predicts that blue-green egg colouration should be positively correlated with a) traits that reflect female quality, such as laying date and clutch size, b) male provisioning effort and, consequently, c) the number and/or quality of offspring at fledging. Although some studies have supported these predictions (Moreno *et al.* 2004, 2006b; Morales *et al.* 2008; Soler *et al.* 2008; English & Montgomerie 2010) other studies have found either no support or only mixed support for the sexual signaling hypothesis (Gosler *et al.* 2000; Krist & Grim 2007; López-Rull *et al.* 2007; Hanley & Doucet 2009). For eggshell colour to be a signal, birds would need the visual acuity to differentiate variation in egg colour, particularly within dimly-lit nests (Reynolds *et al.* 2009). While models of avian vision are highly dependent on assumptions (Lind & Kelber 2009; Holveck *et al.* 2010; Avilés *et al.* 2011), behavioral experiments have consistently suggested that birds have the ability to differentiate subtle differences in colour even under low-light conditions (Heeb *et al.* 2003; Jourdie *et al.* 2004; Soler *et al.* 2008; Gomez *et al.* 2014).

One key assumption of this hypothesis is that eggshell colouration carries some cost to the female so that it remains an honest signal (Moreno & Osorno 2003). One potential cost is if the deposition of biliverdin, the pigment responsible for blue-green egg colouration with strong antioxidant activity (Kaur *et al.* 2003), is a limited resource. Biliverdin may reflect maternal antioxidant capacity, and therefore it may be costly for mothers to allocate this pigment to eggshells if this depletes the female's own antioxidant supplies (Moreno & Osorno 2003). Consistent with this hypothesis, (Hanley

et al. 2008) found a positive correlation between blood plasma total antioxidant capacity and blue-green eggshell chroma in Gray Catbirds (*Dumetella carolinensis*). A similar correlation was found in pied flycatchers (*Ficedula hypoleuca*) among birds who were induced to reconstruct their nests (Morales *et al.* 2008) However, few studies have taken a physiological approach to assess the assumption that eggshell biliverdin is a limited resource (Zhao *et al.* 2006; Liu *et al.* 2010; De Coster *et al.* 2012). To our knowledge no studies have experimentally manipulated circulating haematology during egg production.

Biliverdin is derived from haem (Kennedy & Vevers 1973) by the oxidative-stress-inducible haem oxygenase 1 (Maines 1997), and is deposited in the eggshell within the shell gland (Kennedy & Vevers 1976; Burley & Vadehra 1989; Soh *et al.* 1993). The source of eggshell biliverdin is postulated to be from either the circulation (Kennedy and Vevers 1973; Lang and Wells 1987; Zakhary et al 1996) or *de novo* synthesis within the eggshell gland (Zhao *et al.* 2006; Gorchein *et al.* 2011). In either case, biliverdin or the resources necessary for its production (iron, haem or haemoglobin) must come from the circulation. The synthesis of biliverdin for eggshell pigmentation is therefore susceptible to two potential limitations: 1) a limitation in circulating resources and/or 2) a limitation in haem oxygenase 1 expression/activity in the uterus. If the level of circulating resources is the limiting factor for biliverdin synthesis, variation in eggshell pigmentation should reflect the amounts of these circulating resources (i.e., haematocrit or haemoglobin). Alternatively, if circulating resources are available in excess but the conversion of haem to biliverdin by haem oxygenase 1 in the uterus is the limiting step for eggshell pigmentation, variation in eggshell pigmentation should be independent of circulating resources (Wang *et al.* 2011). If the cost of biliverdin maintains the honesty of blue-green egg colouration as a sexual signal (Moreno & Osorno 2003), the circulating resources necessary for biliverdin synthesis should be limiting, such that higher quality females have higher levels of the circulating resource and deposit more biliverdin in eggs.

We used an experimental approach to investigate proximate causes of variation in blue-green eggshell pigmentation in the European starling (*Sturnus vulgaris*). We experimentally manipulated haematocrit and haemoglobin, and therefore the availability of circulating resources for haem or biliverdin synthesis, in free-living female European

starlings using phenylhydrazine (PHZ). PHZ is a xenobiotic oxidant known to modify haem and lyse red blood cells (Shetlar & Hill 1985; Williams *et al.* 2012). Because the necessary materials for biliverdin synthesis must come from circulation regardless of where it is synthesized, we predicted that treatment with PHZ should either 1) decrease the intensity of blue-green colouration by decreasing haemoglobin and the number of erythrocytes available to supply resources for biliverdin synthesis, or 2) increase the intensity of blue-green colouration by increasing the amount of iron available to synthesize heme for biliverdin synthesis. Alternatively, if circulating resources are not limiting we should find no effect of PHZ treatment. We also tested the sexual signaling hypothesis ((Moreno & Osorno 2003) which predicts that, if eggshell colour is a true signal of female quality, a) it should be limiting and therefore decrease from first- to replacement-clutches, and b) variation in eggshell pigmentation should be related to traits that reflect female quality, male provisioning effort, and breeding success (i.e., brood quality and fledging success)

2.3. Materials and Methods

Fieldwork was conducted on a free-living, multiple-brooded, nest box population of European starlings between April – July 2009. The field site is located at the Davistead dairy farm in Langley, British Columbia, Canada (49°10'N, 122°50'W) and consists of ~200 nest boxes mounted on barns and posts throughout the site. Using starling data collected from this site, 13 year mean clutch size is 5.19 ± 0.84 (mean ± SE; Williams *et al.* 2015), incubation length is 10.3 ± 0.1 days, and pre-fledging period is 21 ± 0.6 days (Love & Williams 2008). Nest boxes were monitored daily to establish the date of clutch initiation, laying sequence, egg size, clutch size, and the date of clutch completion for all clutches. Eggs were measured and numbered as they were laid.

2.3.1. Experimental treatment and sample collection

Phenylhydrazine [PHZ (Sigma-Aldrich, Ontario Canada)], a xenobiotic oxidant, was used for our experimental manipulation; PHZ decreases haematocrit and haemoglobin within 24 hours of treatment (Clark *et al.* 1988; Williams *et al.* 2012). After completion of the first clutch, females were captured in their nest boxes just before

dawn, weighed (± 0.01g), banded (permit 10646 K from the Canadian Wildlife Service) and alternately assigned to one of two treatment groups, PHZ (n = 30) or control (n = 33). A pre-treatment blood sample was collected by puncturing the brachial vein with a 26½-gauge needle and collecting blood (<700 µl) into heparinized capillary tubes. A bolus injection of PHZ in saline (1.25 mg / 100 g BW) or saline alone as the control (100 µl injection volume) was injected into the pectoral muscle. In order to induce re-laying, entire first clutches were removed prior to females being returned to their nest boxes. All eggs were stored at -20°C until they could be analyzed for eggshell colouration (see below). Females were returned to the nest box and allowed to lay a replacement clutch without further manipulation. Daily monitoring of nests was continued to collect laying date, egg, and clutch size information for these post-treatment replacement clutches.

To avoid the risk of desertion (which occurs at high frequency in birds captured early in laying), we did not recapture females until one or two days after completion of the replacement clutch, at which time a second blood sample was taken (PHZ, n = 17; control, n = 16). Females were immediately released after blood sampling and replacement nests were monitored through chick fledging. Beginning 9 days after clutch completion, eggs were monitored for signs of hatching (starring or pipping) and when hatching was imminent the entire clutch was removed from the nest and placed in an incubator until hatching (from 1-15 hrs). In order to maintain maternal incubation behavior, removed clutches were replaced with dummy eggs. Hatching eggs in the incubator allowed us to determine brood size at hatching, hatchling mass, hatchling sex (by collecting a drop of blood for molecular sexing, described below), and to collect eggshells from replacement clutches for further colour measurement. Hatchlings were returned to the nest immediately after measurement. During chick rearing, on day 6, 7 and 8 post-hatching, nests were observed for 30 minutes each and maternal and paternal provisioning visits to the nest were recorded. During observations, we would conceal ourselves (e.g., in a car) when possible, but blinds were generally unnecessary as nestboxes were located in active areas on a farm. Rarely, if a bird appeared reluctant to approach the nestbox or made alarm calls, we would move to a different location to ensure that the 30 minute period was undisturbed. Provisioning rates were reported as the mean number of feeding visits per hour over the 3-day period for each parent. To determine maternal condition during provisioning, females were captured and a third

blood sample was taken when chicks were between 11 and 13 days old. In order to determine chick quality just prior to fledging, at 17 days post-hatching we measured chick tarsus length, body mass and collected blood samples to obtain chick haematocrit and haemoglobin levels. Nests were then monitored for fledging and brood size at fledging was recorded.

All procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Simon Fraser University Animal Care Committee (permits 829B-96 and 1018B-96).

2.3.2. Haematocrit and haemoglobin measurement

Whole blood was used to measure pre-treatment (baseline) and post-treatment haematocrit levels and whole blood haemoglobin concentration. Haematocrit levels (Hct) were measured in duplicate following centrifugation at 13,000 g for 3 minutes (Wagner *et al.* 2008). Whole blood haemoglobin (Hb, g/dL) was measured using the modified cyanomethaemoglobin method as described in (Wagner *et al.* 2008). Remaining blood samples were centrifuged at 3,000 rpm for 3 minutes and the supernatant plasma was stored frozen for later analysis.

2.3.3. Eggshell colour measurement

Eggshells were washed with distilled water and the median part of each eggshell was measured using a USB4000 reflectance spectrometer and PX-2 light source (Ocean Optics). We used a reflectance probe tipped with a rubber stopper that maintained the probe 3 mm from and perpendicular to the eggshell surface, and excluded external light. We collected three measurements per egg, lifting and replacing the probe between each measurement, and averaged these measurements to obtain a single reflectance spectrum per egg. To estimate biliverdin content of eggshells, we calculated blue-green chroma (hereafter BGC) as the ratio of the reflectance between 400 and 570 nm to the total reflectance between 300 and 700nm. Among colourimetric variables, chroma should be most indicative of pigment concentration (Andersson & Prager 2006), and our measure of BGC corresponds to the region of greatest reflectance of biliverdin (Falchuk

et al. 2002). In addition, both Moreno et al (2006a) and Morales et al (2013) showed a direct relationship between biliverdin content and BGC in pied flycatcher eggs, and López-Rull et al (2008) found a similar pattern in spotless starlings (Sturnus unicolor). Consequently, we would expect that BGC would be the main cue for male assessment of female quality (Krist & Grim 2007), and the egg trait most closely related to physiological measures of biliverdin availability. We examined mean as well as maximum clutch BGC values when analyzing parental provisioning in consideration of the peak shift phenomenon in which individuals respond to maximal stimuli over average stimuli (Guilford & Dawkins 1993; Moreno et al. 2006a).

2.3.4. Molecular sexing

Egg laying European starling females in poor condition have increased yolk corticosterone, which results in female-biased hatching sex ratios (Love *et al.* 2005). If BGC is correlated to female quality we expect it to be correlated with brood sex ratio as well. A drop of hatchling blood from a small pinprick to the ankle was collected on filter paper and stored at -20^{0}C. DNA was extracted from blood samples using Instagene matrix following the manufacturer's protocol (Bio-Rad Laboratories, cat. No. 732-6030). Nestling sex was determined by polymerase chain reaction using the primers P2 (5'-TCTGCATCGCTAAATCCTTT) and CW (5'-AGAAATCATTCCAGAAGTTCA), based on the protocol of (Griffiths *et al.* 1996), modified by (Love & Williams 2008).

2.3.5. Statistical analyses

All statistical analyses were performed using SAS software version 9.2 (SAS Institute, 2008). Pearson's Product Moment correlations were used to investigate relationships among female characteristics, mean egg colour, parental provisioning, and measures of chick quality. General linear models (GLM procedure) or repeated-measures mixed linear models (MIXED procedure with female identity included as a repeated subject effect) were used to test for effects of treatment, clutch (first vs. replacement) and/or laying order.

2.4. Results

Total haemoglobin was positively correlated to haematocrit in pre-treatment samples (r_{58} = 0.66, P < 0.0001) and post-treatment control samples (r_{15} = 0.59, P = 0.02). No other correlations among haematological variables were observed (P > 0.39 for all). As expected, there was no difference in pre-treatment haematocrit or haemoglobin levels between treatment groups (P > 0.74). We found no difference between treatment groups in replacement clutch post-laying haematocrit levels (P > 0.80) controlling for first clutch post-laying haematocrit, by including this term as a covariate in a general linear model (GLM procedure). In contrast, in a similar analysis of haemoglobin, replacement clutch post-laying haemoglobin was affected by treatment ($F_{1, 23}$ = 6.89, P = 0.01), controlling for first clutch post-laying haemoglobin ($F_{1, 23}$ = 7.17, P = 0.01) with PHZ-treated females having higher replacement clutch haemoglobin than controls, contrary to our expectation. When laying date was added to the model, this term was not significant and the effect of treatment remained significant (data not shown). In a model including clutch (first vs. replacement) and treatment and their interaction as fixed effects, and female identity as a repeated subject effect (MIXED procedure), there was no effect of clutch on haematocrit levels (P > 0.10; Fig 2.1a). In contrast, haemoglobin levels differed between first and replacement clutches ($F_{1, 24}$ = 24.89, P < 0.0001; Fig 2.1b); both treatment groups exhibited higher total haemoglobin levels after laying replacement clutches.

Laying interval, i.e., the time from treatment to initiation of the replacement clutch, was not normally distributed and therefore we used a Kruskal-Wallis test to test for differences in laying interval between treatment groups. Laying interval was significantly greater among PHZ treated individuals (11.00 ± 0.92 days; first egg of replacement clutch was laid 7-18 days after treatment) compared with SAL-treated individuals (7.25 ± 1.08 days; first egg of replacement clutch was laid 6-10 days after treatment: X^2_1 = 10.37, P = 0.0013). Treatment did not affect the mass of the replacement clutch (P > 0.19), controlling for the mass of the first clutch (including this term as a covariate in a general linear model, GLM procedure).

2.4.1. Prediction 1: If eggshell biliverdin is limited by circulating resources, PHZ-treatment should influence eggshell colouration in replacement clutches.

As expected, there was no difference in pre-treatment mean BGC between treatment groups (P > 0.17). Treatment did not affect the mean BGC of replacement clutches (P > 0.33), controlling for first clutch mean BGC when including this term as a covariate in a general linear model (GLM procedure). There was no difference in pre-treatment maximum BGC (i.e., the maximum BGC within a clutch) between treatment groups (P > 0.20). Treatment did not affect replacement clutch maximum BGC ($F_{1, 25}$ = 2.73, P = 0.11), controlling for first clutch maximum BGC (GLM procedure). There was no effect of treatment on the laying sequence of the egg with the highest BGC of the clutch ($F_{1, 23}$ = 0.96, P = 0.34: PHZ, 4.5 ± 0.5; SAL, 3.8 ± 0.5) (GLM procedure). In other words, PHZ treated females did not lay their bluest egg earlier or later in the sequence than controls. Finally, first clutch mean BGC was correlated to replacement clutch mean BGC among control females (r_{15} = 0.57, P = 0.02), and PHZ treated females (r_{13} = 0.64, P = 0.02).

2.4.2. Prediction 2: If eggshell colouration is limited by circulating resources, then eggshell colour should be correlated with female haematology among control females.

In first clutches, both mean and maximum BGC were independent of all post-laying first clutch haematological traits (P > 0.19 for all), controlling for maternal mass (using CORR procedure with a partial statement). Similarly, in replacement clutches of control individuals, there were no correlations between mean or maximum BGC and post-laying haematological variables (P > 0.65 for all), controlling for maternal mass.

2.4.3. Prediction 3: If eggshell colour is an honest signal of female quality, it should be resource-limited, and therefore should decline through the laying sequence and in replacement clutches of control females.

To compare egg colour by clutch number (first vs. replacement) and laying order among control females, a mixed linear model was used with clutch number and laying

order and their interaction as fixed effects and individual female as a repeated subject effect (MIXED procedure). There was no significant clutch by laying order interaction effect on BGC ($F_{5, 40}$ = 1.32, P > 0.27), however there was a significant effect of clutch (first vs. replacement) ($F_{1, 14}$ = 21.17, P = 0.0004) and laying order ($F_{6, 138}$ = 4.23, P = 0.0006; Fig 2.2). BGC increased with laying order and was lower in replacement clutches. In addition, in a repeated measures analysis of control females (MIXED procedure), there was an effect of clutch on maximum BGC, with replacement clutches having a lower maximum BGC ($F_{1, 14}$ = 10.43, P = 0.0061).

2.4.4. Prediction 4: If eggshell colour is a sexual signal, variation in eggshell pigmentation should be correlated with female quality, provisioning effort, breeding success, and chick quality

Post-laying maternal mass was not affected by treatment or the clutch by treatment interaction (P > 0.14 for both); however, it was greater after the replacement clutch ($F_{1, 27}$ = 13.40, P = 0.0011; repeated-measures analysis, MIXED procedure).

In first clutches, mean BGC but not maximum BGC was positively correlated with post-laying body mass (mean BGC: r_{63} = 0.272, P = 0.03; Fig 2.3; max BGC: r_{63} = 0.235, P = 0.06). Both mean and maximum BGC were independent of lay date, clutch size and mean egg mass (P > 0.41 in all cases). Both mean and maximum BGC were independent of post-laying body mass, lay date, and clutch mass within control replacement clutches (P > 0.11 for all).

Provisioning rates were adjusted for brood size by including brood size as a linear covariate in the model. There was no effect of treatment on maternal, paternal, or total provisioning (P > 0.08 for all) when analysed by a general linear model with no covariates (GLM procedure). There were no significant correlations between mean or maximum BGC and provisioning (female, male, or total provisioning rate) within the control treatment (P > 0.39 for all) or among pooled treatment groups (P > 0.09 for all) with treatment as a cofactor (GLM procedure), in replacement clutches.

There was no effect of treatment on brood size at hatch, number of eggs that did not hatch, brood size at fledging or number of chicks that died between hatching and fledging (P > 0.64 for all) when analysed by a general linear model with no covariates (GLM procedure). Likewise, mean and maximum BGC were independent of brood size at hatch, number of eggs that did not hatch, brood size at fledgling and number of chicks that died between hatching and fledging within the control group (P > 0.09 for all) or among pooled treatment groups (P > 0.14) with treatment as a cofactor.

When testing for effects of treatment and mean BGC on nestling traits, laydate and chick sex were included as covariates and removed when they did not significantly contribute to the model. Hatchling mass was not affected by treatment, mean BGC, or the treatment by mean BGC interaction (P > 0.39 for all) in a linear model including these effects as well as laydate, first clutch mean egg mass as a covariate and female identity as a repeated effect (since multiple hatchlings per female were included in the analysis; MIXED procedure). Within the PHZ group, there was a pre-treatment trend towards larger eggs, therefore first clutch mean egg mass was used as a covariate when testing for treatment differences in hatchling mass. Brood sex ratio at hatching was not affected by treatment, mean BGC, or the treatment by mean BGC interaction (P > 0.58 for all). Chick tarsus length was positively associated with mean BGC ($F_{1, 23}$ = 4.50, P = 0.04; Fig 2.4a), but was not affected by treatment or the treatment by mean BGC interaction (P > 0.13 for both), including female identity as a repeated effect (MIXED procedure). In a similar analysis of chick mass at fledging, there was no effect of treatment, mean BGC, or the treatment by mean BGC interaction (P > 0.31 for all). Chick haematocrit at fledging was affected by treatment ($F_{1, 21}$ = 8.45, P = 0.008), but not by mean BGC or treatment by mean BGC interaction (P > 0.22 for both), again including female identity as a repeated effect (MIXED procedure). Chicks hatched from PHZ treated nests had lower haematocrit levels than chicks hatched from control nests (PHZ, 42.43 ± 0.65 %; SAL, 44.96 ± 0.58 %). Finally, chick haemoglobin at fledging was positively correlated with mean BGC ($F_{1, 20}$ = 5.84, P = 0.02; Fig 2.4b) but was not affected by treatment or the treatment by mean BGC interaction (P > 0.62 for both), including female identity as a repeated effect (MIXED procedure).

2.5. Discussion

The sexual selection of eggshell colour hypothesis is predicated on eggshell colouration being costly. One potential cost is that biliverdin, a derivative of haem and the pigment responsible for blue-green egg colour, may be a limited resource, which would maintain the honesty of the signal. We used a physiological manipulation of haematology to test the hypothesis that the availability of circulating resources for biliverdin synthesis limits eggshell colouration. Although haematocrit levels had likely recovered from experimental treatment at the time of eggshell pigmentation (discussed below), haemoglobin levels were increased in the treatment group upon completion of the replacement clutch, and treated females would have been undergoing reticulocytosis during eggshell pigmentation. Therefore, if circulating resources were limiting for biliverdin production for eggshell pigmentation, our experimental perturbation would likely have had some effect on eggshell colouration. However, despite our manipulation of haemoglobin (Williams *et al.* 2012), we detected no effects on eggshell colour. Likewise, we found no correlations between eggshell colour and circulating resources. In addition, the increase in eggshell colour over the laying period and the decrease in eggshell colour across clutches provide equivocal evidence for pigment limitation. The evidence we found to support eggshell colour as a signal of female quality was a correlation between eggshell colour and female mass in first clutches, although such a correlation was not observed in replacement clutches. Eggshell colour was also correlated with some aspects of offspring quality (chick tarsus length and chick haemoglobin levels) but not others (chick mass).

Pigmentation of the eggshell is deposited approximately 4 hours prior to laying (Burley & Vadehra 1989; Soh *et al.* 1993) and prior studies in captive non-breeding European starlings show that haematocrit and haemoglobin return to pre-treatment levels 5 to 10 days post PHZ treatment, respectively (Williams *et al.* 2012), although this recovery might be expected to take longer in free-living birds. The initiation of eggshell pigmentation in the replacement clutch may therefore have begun 5 days after we expected haematocrit levels to return to normal within the treatment group but prior to the normalization of haemoglobin levels. Total haemoglobin was significantly higher among post-treatment PHZ individuals, possibly because of the summation of

hemoglobin from new replaced red blood cells and excessive cell-free plasma hemoglobin released from lysed cells (Rother et al. 2005) or from the high erythropoietin levels needed for recovery. In a previous study (Williams *et al.* 2012), haemoglobin had returned to normal by 10 days after treatment, while in the present study haemoglobin was significantly elevated upon completion of the replacement clutch. This discrepancy may be due to the difference in the physiological and/ or nutritional state of the females, i.e., captive, first year, non-breeding females in the (Williams *et al.* 2012) study vs. free-living, various aged, breeding females in the present study, for whom recovery from PHZ treatment may have been more challenging. While haematocrit levels were likely normalized during eggshell colouration, the composition of red blood cells was expected to be considerably different than normal due to reticulocytosis, which occurs for up to 2-3 weeks after transient anemia (Fernandez & Grindem 2006; Wagner *et al.* 2008). However, despite the increase in circulating haemoglobin and the altered blood composition, eggshell colouration was not affected, which suggests that haem oxygenase 1 activity within the eggshell gland may be the limiting factor in biliverdin production for eggshell pigmentation. Alternatively, females may have maintained eggshell colouration despite biliverdin limitation by sacrificing their own biliverdin reserves, even though post-laying maternal mass and maternal provisioning rates were not affected by treatment. Similarly, the lack of correlations between eggshell colour and female haematology among control females may not reflect a lack of biliverdin limitation, but instead may be the result of females maintaining colouration at some cost to themselves.

Similar to our hypothesis regarding biliverdin eggshell pigmentation, De Coster *et al.* (2012) hypothesized that protoporphyrin eggshell pigmentation might be affected by anaemia. They indirectly manipulated the numbers of immature erythrocytes by infecting nests with hen fleas and found that protoporphyrin colouration of eggs increased less with laying sequence in nests with higher parasite load. However, the increase in eggshell colouration over the laying sequence found in our study does not suggest a within-clutch biliverdin limitation. While the significant decline in eggshell colouration across clutches might reflect pigment limitation over the breeding season, this change in colouration could alternatively be caused by numerous other factors that vary through the season. The observations that very little biliverdin is recycled in the synthesis of new

haemoglobin (London *et al.* 1949), and that biliverdin is excreted in copious amounts (Zhao *et al.* 2006) are consistent with this view. However, due to the large variation in eggshell biliverdin content among different species, up to 125-525 nmol/g in pied flycatchers (*Ficedula hypoleuca*) (Moreno *et al.* 2006a), biliverdin could limit eggshell colouration in some species.

As in previous work (Moreno *et al.* 2005, 2006a), BGC was positively correlated with female mass (a measure of female condition) in unmanipulated first clutches in our study, consistent with the hypothesis that eggshell colour depends on female nutritional state (Moreno *et al.* 2006a). However, eggshell colour was independent of all other measures of female quality or reproductive performance (lay date, clutch size and egg mass). In addition, within the replacement clutch there were no relationships between eggshell colour and any measure of female quality, including mass, in either treatment group.

The differential allocation hypothesis (Burley 1986) predicts that fathers will invest more in higher quality offspring. Studies assessing the relationship between paternal care and eggshell colour have yielded inconsistent results. Several studies have found positive correlations between these traits (Moreno *et al.* 2006b; Hanley *et al.* 2008; Soler *et al.* 2008) while others have not (Krist & Grim 2007; Hanley & Doucet 2009; Morales *et al.* 2009; Stoddard *et al.* 2012) or have found mixed support (Cassey *et al.* 2008). An issue in such studies is that eggshell colour may be confounded with female quality, such that males may respond to female quality rather than eggshell colour *per se*. Of the studies that have attempted to disentangle these effects by cross-fostering eggs, two found no correlation between eggshell colour and paternal provisioning in cross-fostered nests (Krist & Grim 2007; Hanley & Doucet 2009), while a third found a correlation between proportional male provisioning (i.e., the visitation rate of the male divided by the visitation rate of both the male and female combined) and maximum (but not mean) eggshell colour (Moreno *et al.* 2006b). Although we did not perform cross-fostering, when analyzing correlations between eggshell colour and paternal provisioning, we looked at absolute provisioning rates as well as proportional provisioning rates to account for the possibilities that paternal investment is independent of maternal investment, or that males increase or decrease their proportional investment

based on clutch quality (Schwagmeyer *et al.* 2002; Moreno *et al.* 2006b). We found no correlation between mean or maximum BGC and either measure of paternal provisioning effort among either treatment group, suggesting that male European starlings do not use eggshell colour to determine clutch quality and their investment in the offspring.

To our knowledge few studies have investigated links between eggshell BGC and nestling quality, hatching or fledging success. If eggshell BGC reflects maternal or brood quality then there should be a positive relationship between mean BGC and measures of nestling success. While (Hanley & Doucet 2009) found no correlations between eggshell colour and offspring quality, we found that mean eggshell BGC was positively correlated with chick haemoglobin and tarsus length at fledging, but not with measures of hatching or fledging success. Similarly, (Krist & Grim 2007) found a positive effect of eggshell BGC on nestling tarsus length but not on nestling mass or immunity.

In summary, we predicted that if circulating biliverdin or its precursors were a limited resource with respect to eggshell pigmentation, then eggshell colour would be correlated with circulating haematology and would be affected by a manipulation of red blood cells. We found no effect of our manipulation on eggshell colour. In addition, if eggshell colouration were a signal providing fathers a measure of maternal and/or brood quality so that they could adjust their investment, we would expect eggshell colouration to be correlated with measures of maternal quality, brood quality, and paternal provisioning. With the exception of an association between female mass at clutch completion and eggshell colouration in first, unmanipulated clutches, eggshell colour was not correlated with female quality or paternal provisioning measures, although there were associations between eggshell colour and chick haemoglobin and tarsus length at fledging.

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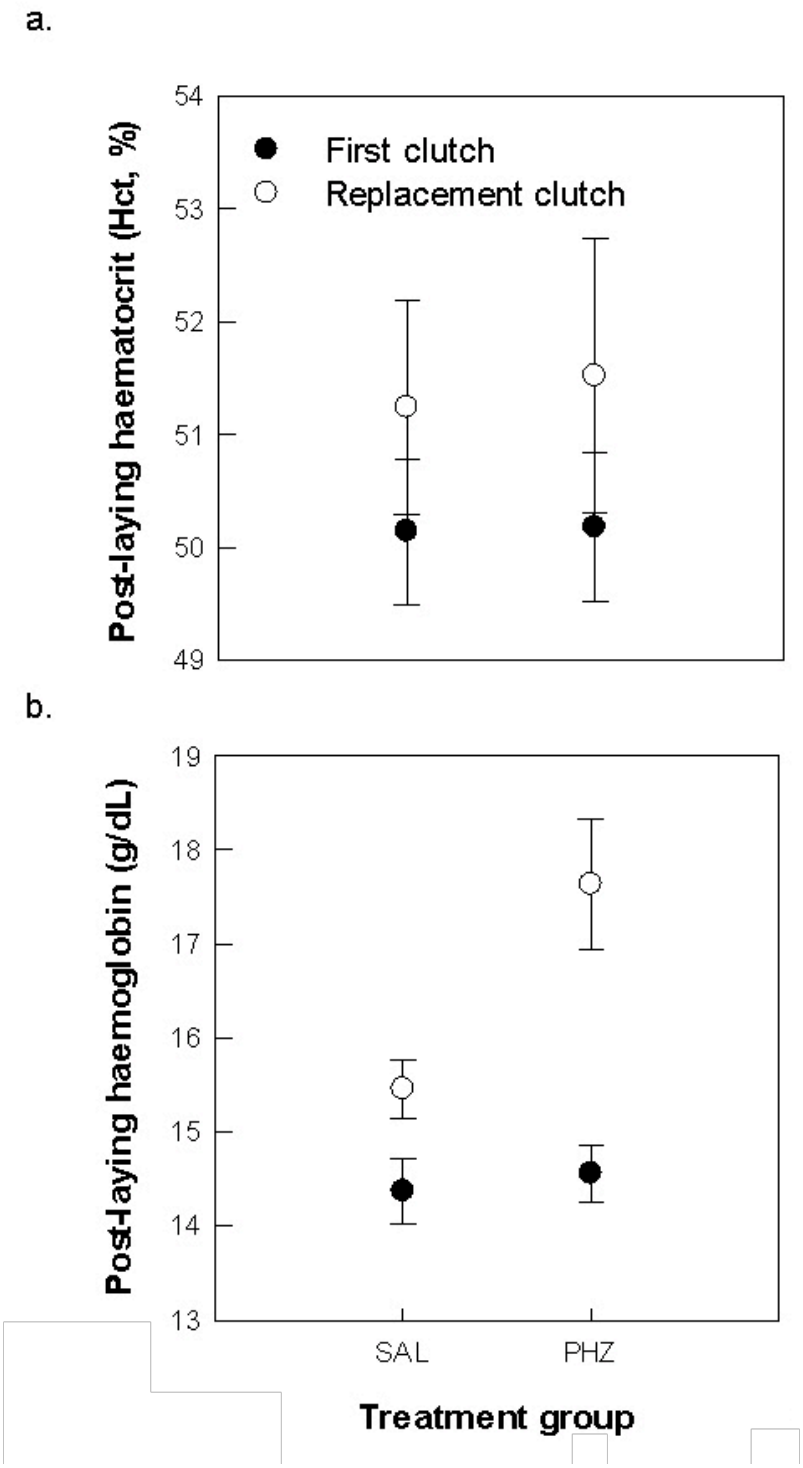

Figure 2.1. Variation in a) haematocrit and b) total blood haemoglobin in relation to clutch number and treatment in European starlings. Haematology was manipulated using phenylhydrazine (PHZ), a xenobiotic oxidant known to lyse red blood cells. Saline (SAL) was used in the control group. The error bars represent one standard error.

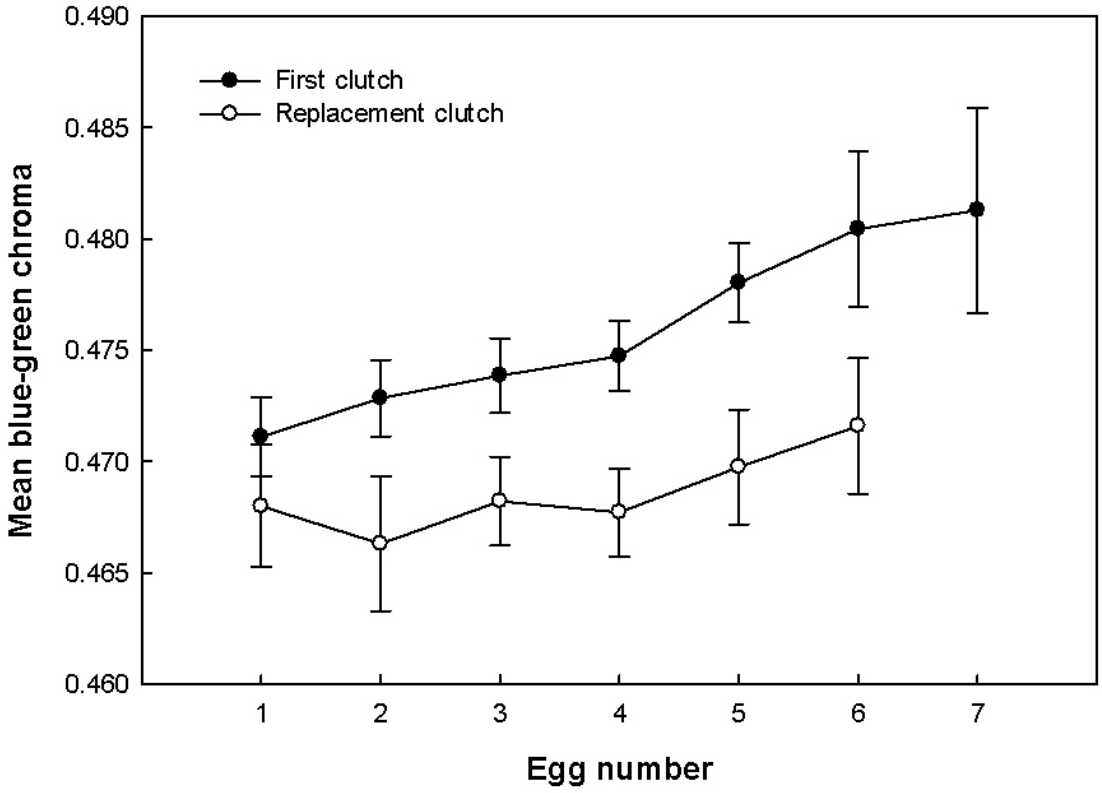

Figure 2.2. **Relationship between mean blue-green chroma and laying sequence in first and replacement clutches within control female European starlings. The error bars represent one standard error.**

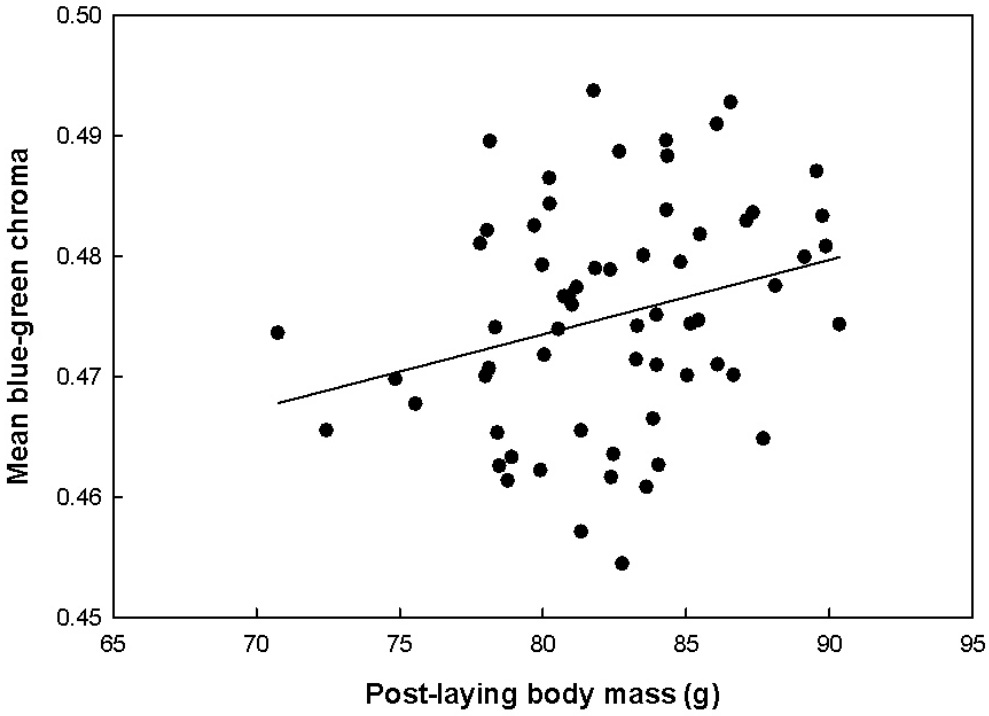

Figure 2.3. **Mean blue-green chroma is positively correlated to female body mass within first (i.e., pre-treatment) clutches in European starlings.**

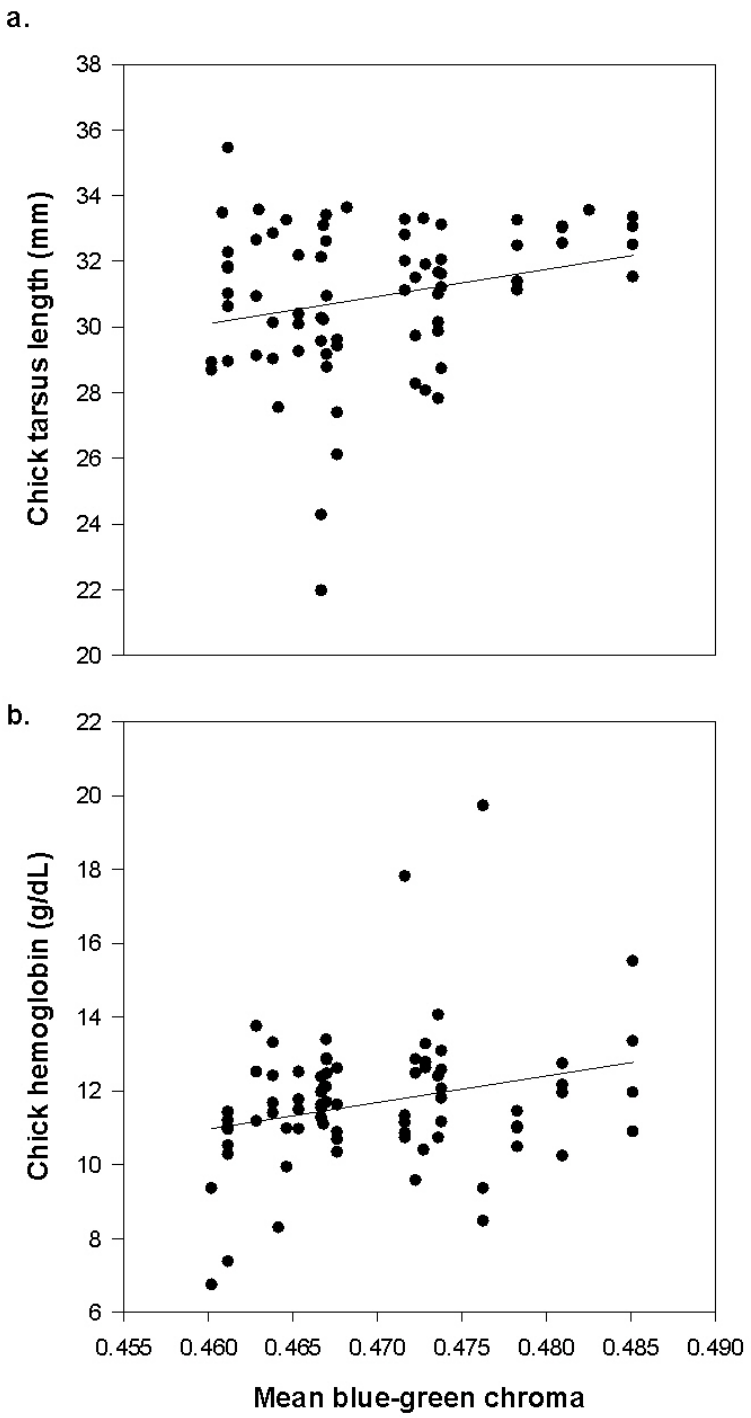

Figure 2.4. Chicks who came from clutches with greater mean blue-green chroma had, a) longer chick tarsus lengths within pooled treatment groups, and b) greater mean chick haemoglobin levels within pooled treatment groups, just prior to fledging (17 days post hatch) in European starlings.

Chapter 3.

Experimental reduction of hematocrit affects reproductive performance in European starlings, *Sturnus vulgaris*

A modified version of this chapter has been published as:

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3.1. Summary

Given the function of hemoglobin and observed increases in hematocrit during periods of increased energetic demands, hematocrit and hemoglobin are assumed to be related to aerobic capacity. Reductions in hematocrit and hemoglobin during reproduction are similar in magnitude to increases associated with aerobically demanding activities and therefore we sought to investigate whether these reductions in hematology have consequences for reproductive performance. We analyzed associations between natural variation in hematology in free-living European starlings (*Sturnus vulgaris*) and reproductive performance. To test whether transient reductions in hematology during different stages of reproduction (egg production and late incubation/early chick rearing) affected measures of reproductive performance, we also manipulated hematology using phenylhydrazine (PHZ), which lyses red blood cells. To

investigate effects of reductions of hematology during egg-laying, we treated females with PHZ or saline (control) upon completion of their unmanipulated first clutch and removed eggs to induce the production and rearing of a replacement clutch. To investigate effects of reductions of hematology during chick rearing, we treated females during incubation of the unmanipulated first clutch and then monitored the subsequent hatching and rearing of the clutch. Individuals with higher hematocrit and hemoglobin initiated nesting earlier. Furthermore, higher hemoglobin levels during incubation were associated with a greater number of chicks fledged. PHZ treatment prior to egg production resulted in a significant delay in the laying of replacement clutches, but had no effect on provisioning rate or the size or number of chicks fledged. PHZ treatment during incubation and early chick rearing resulted in decreased hatchling mass in all years and a decrease in the size and number of fledglings in one of two years. The year that the effect of PHZ was observed appeared to be a particularly difficult year, since hatchling mass, brood size at hatching and at fledging were low among control females compared to other years. Our results suggest that a reduction in hematology during reproduction can be functionally significant, but that these costs are context-dependent.

Key-words: cost of reproduction, hemoglobin, inter-individual variation, reproductive anemia, reproductive success

3.2. Introduction

It is widely assumed that hematocrit (Hct) is positively related to aerobic capacity (Ekblom, Goldbarg & Gullbring 1972; Ekblom 2000; Hammond *et al.* 2000). In birds, this assumption is based largely on the known function of hemoglobin (Hb) and observed increases in Hct (and/or Hb levels) in response to extended periods of increased energetic demands (Palomeque & Planas 1978; Palomeque, Palacios & Planas 1980). Examples of such periods include migration, experimentally increased flight costs, increased flight activity, and increased thermogenic demands (Kubena *et al.* 1972; Carpenter 1975; Carey & Morton 1976; Wingfield & Farner 1976; Banerjee & Banerjee 1977; deGraw, Kern & King 1979; Thapliyal *et al.* 1982; Viscor, Marqués & Palomeque

1985; Clemens 1990; Morton 1994; Piersma, Everaarts & Jukema 1996; Saino *et al.* 1997; Landys-Ciannelli, Jukema & Piersma 2002).

Reproductive anemia is a reduction in Hct, Hb, and red blood cell number that is routinely associated with egg production in female birds (deGraw, Kern & King 1979; Jones 1983; Morton 1994; Gayathri & Hegde 2006; reviewed in Fair, Whitaker & Pearson 2007; Wagner *et al.* 2008a), which can persist after clutch completion into incubation and chick-rearing (Williams *et al.* 2004). The reduction of Hct during egg-production is believed to occur initially via hemodilution (Kern, De Graw & King 1972; Challenger *et al.* 2001) and is prolonged by estrogen's suppression of erythropoiesis (Wagner, Stables & Williams 2008b). The reduction in Hct during egg-laying relative to pre-breeding values ranges from 1.5 % in the great tit (*Parus major*; (Ots, Murumägi & Horak 1998) to 10% in the red-billed quelea (*Quelea quelea*; Jones 1983). The decrease in Hct during reproduction is therefore similar in magnitude to the increases associated with aerobically demanding circumstances such as cold temperatures, migration, and low oxygen partial pressures, i.e., between 1 and 20% (reviewed in Wagner *et al.* 2008b).

During energetically demanding stages of reproduction such as chick provisioning, a decrease in aerobic capacity could negatively affect reproductive success. Hct levels during chick rearing show marked individual variation, e.g., between 40% and 60% in great tits (Ots *et al.* 1998) and between 33% and 54% in tree swallows (Burness, Ydenberg & Hochachka 2001). In addition, Hct levels during chick rearing are repeatable (Potti 2007). The biological significance of this marked inter-individual variation in Hct during an energy-demanding phase of reproduction remains unclear. Previous studies have shown that experimentally increased egg production reduces provisioning of offspring (Heaney & Monaghan 1995; Monaghan, Nager & Houston 1998). Therefore, the estrogen-induced Hct reduction associated with egg production may be a mechanism underlying the trade-off between egg production and provisioning performance and, potentially, between future fecundity and survival (Heaney & Monaghan 1995; Monaghan *et al.* 1998). To our knowledge, no study has experimentally manipulated Hct during chick rearing to investigate the consequences on reproductive success.

The goals of this study were (a) to examine associations between natural variation in hematological parameters in free-living European starlings (*Sturnus vulgaris*) and measures of reproductive performance (i.e. lay date, clutch mass, provisioning rates and breeding success) and (b) because observational data may be confounded by differences in individual quality, to experimentally manipulate Hct and Hb during two different reproductive stages (egg-laying and late incubation/ early chick rearing) to determine whether this reduction affects reproductive performance. We manipulated Hct and Hb using phenylhydrazine (PHZ), a xenobiotic oxidant which causes hemolysis thereby decreasing Hct and Hb in a transient and fully reversible manner (Clark *et al.* 1988; Williams *et al.* 2012). PHZ was administered to females at one of two time-points: (a) upon removal of a first clutch to determine effects of Hct reduction on the ability of females to produce a replacement clutch and (b) during late incubation of a first unmanipulated clutch, to determine whether reproductive Hct reduction, which can last throughout chick rearing (Williams *et al.* 2004), might affect the ability of females to provision their offspring. With respect to natural variation in Hct and Hb, we expected to observe [1] positive correlations between post-laying Hct and Hb and maternal provisioning, i.e., females who maintain a higher Hct during egg production will be able to perform better at subsequent stages of reproduction. In addition, we expected that individuals with lower post-laying Hct would have more female-biased hatchling sex ratios, since European starling females in poor condition have increased yolk corticosterone, which results in male-biased embryonic mortality (Love *et al.* 2005). We also expected [2] individuals treated with PHZ upon removal of a first clutch to have reduced reproductive performance, i.e., an increased interval between treatment and replacement clutch initiation, smaller clutch size, smaller egg size, increased incubation time, fewer chicks develop to hatch, smaller chick size at hatch, more female-biased sex ratio, and, if there are long term effects of Hct and/or Hb reduction during egg-laying, reduced maternal provisioning. Finally, we expected [3] individuals treated with PHZ during late incubation to have reduced Hct during early chick rearing, resulting in reduced maternal provisioning, and, unless males compensate, smaller chicks and fewer chicks fledge.

3.3. Materials and Methods

3.3.1. Study population

Fieldwork was conducted on a free-living, multiple-brooded, nest box population of European starlings (mean ± SE; clutch size: 5.9 ± 0.2 eggs; incubation length: 10.3 ± 0.1 days, measured as the number of days from clutch completion until the day the first chicks hatch; postnatal period: 21 ± 0.6 days; (Love *et al.* 2008)) over 3 years, between April – July, 2009-2011. The field site is located at the Davistead dairy farm in Langley, British Columbia, Canada (49°10'N, 122°50'W) and consists of ~150 nest boxes mounted on barns and posts throughout the site. We monitored nest boxes daily to establish the date of clutch initiation, clutch size, egg size and the date of clutch completion for all clutches. Eggs were measured and numbered as they were laid. Hatch date, brood size at hatch and fledging were also monitored.

3.3.2. Treatment and sample collection common to both experiments

We treated females with phenylhydrazine (PHZ) (Sigma-Aldrich Canada, Oakville, Ontario, Canada) dissolved in saline or vehicle (saline) at one of two time points (described below). For both treatment time points, females were captured in their nest boxes just before dawn, weighed (± 0.01g), banded and assigned PHZ or control (saline) treatment. A bolus injection of PHZ or saline (100 µl injection volume; 1.25 mg / 100 g BW) was injected into the pectoral muscle. According to our previous work (Williams *et al.* 2012), this dosage resulted in a 20% decrease in Hct among non-breeding but photo-stimulated female European starlings. Average pre-treatment Hct was 52% within the PHZ groups therefore we expected treatment to result in an average Hct of 42% which is well within the physiological range of 36-58% observed among egg-laying starlings and just below the range observed in chick-rearing adults (43-59%; TD Williams unpublished data). We cannot rule out the possibility that PHZ has side-effects unrelated to red blood cells. However, we have not found mention of off-target effects in vivo, even though PHZ has been widely used to induce anemia in rodent models (Latunde-Dada, McKie & Simpson 2006). All pre- and post-treatment blood samples

(see Table 1 for sample sizes) were collected by puncturing the brachial vein with a 26½-gauge needle and collecting blood (<700 µl) into heparinized capillary tubes.

3.3.3. Manipulation of Hct and Hb during egg production (2009-2010)

To analyze the effects of Hct reduction on the ability of females to produce a replacement clutch (i.e., the clutch produced following removal of the first clutch), females were caught and treated one to two days following the completion of the first clutch (PHZ, n = 44; control, n = 34). Directly after treatment, all eggs were removed from the nest to induce relaying of a replacement clutch and females were returned to the nest box. Nest boxes were monitored for replacement clutches. There were numerous natural cavities available at our study site, and therefore females who were not observed to produce a replacement clutch in one of our next boxes may have in fact produced one. One to two days after completion of the replacement clutch, females were caught, weighed, blood sampled (PHZ, n = 26; control, n = 16) and released. Replacement clutches were left to hatch. In order to determine maternal Hct and Hb during offspring provisioning, females were trapped in nest boxes and a third blood sample was taken when chicks were between 11 and 13 days old. However, not all females would enter trapped nest boxes and so sample sizes are reduced during maternal provisioning (PHZ, n = 12; control, n= 9). We then followed replacement clutches to fledging. Chick measurements are described below.

3.3.4. Manipulation of Hct and Hb during chick -rearing (2010-2011)

To analyze the effects of reduced Hct on the ability of females to provision their offspring, females were treated during incubation of the first clutch (on average 8 days after the last egg was laid) (PHZ, n = 46; control, n = 46). First clutches were left to hatch and followed to fledging. As above, we attempted to obtain blood samples during maternal provisioning of offspring when chicks were 11 to 13 days old (PHZ, n = 9; control, n = 10). Nest boxes were monitored for second clutches (distinct from replacement clutches in that second clutches follow the natural conclusion of the first clutch) and followed to fledging. Chick measurements are described below.

3.3.5. Offspring characteristics & provisioning rate measurements

Beginning 9 days after clutch completion, all eggs were monitored for signs of hatching (starring or pipping) and when hatching was imminent the entire clutch was removed from the nest and placed in an incubator until hatching (1-15 hrs until the first egg hatched). In order to maintain maternal incubation behavior, removed clutches were replaced with dummy eggs. Hatching eggs in the incubator allowed us to determine brood size at hatching and hatching mass and to obtain blood samples for sex ratio at hatching. Chicks were measured, sampled and returned to the nest as they hatched, leaving unhatched eggs in the incubator until they hatched. No females abandoned at this stage. Sample sizes for hatching weights are substantially smaller than the total number of nests because of space limitations in the incubator. Clutches were moved to the incubator when they appeared about to hatch, and would not be moved if the incubator was already full. This did not create a bias for early- or late-hatching clutches because bottlenecks occurred in the middle of the hatching period, i.e., there was generally space for early- or late-hatching clutches. During chick rearing, on day 6, 7 and 8 post-hatching (based on the day most chicks from the nest hatched), nests were observed for 30 minutes each and maternal and paternal provisioning visits to the nest were recorded. During observations, we would conceal ourselves (e.g., in a car) when possible, but blinds were generally unnecessary as nestboxes were located in active areas on a farm. Rarely, if a bird appeared reluctant to approach the nestbox or made alarm calls, we would move to a different location to ensure that the 30 minute period was undisturbed. Provisioning rates were reported as the mean number of feeding visits per hour over the 3-day period for each parent. All observations took place between 9:00 - 14:30, and the time of observation did not differ between treatments. Provisioning rates did not vary significantly with time of observation (data not shown). At day 17 post-hatching, chicks were blood sampled (manipulation during egg production experiment only) and in all years, mass and tarsus length were measured. Nests were monitored for fledging and brood size at fledging was recorded.

3.3.6. Hematocrit and hemoglobin measurement

Whole blood samples were used to measure pre-treatment and post-treatment Hct levels and Hb concentration. Hct levels (% packed cell volume to total blood ratio) were estimated in duplicate following centrifugation at 13 000 g for 3 minutes (Wagner et al., 2008a). Whole blood Hb (g/dL) was measured using the modified cyanomethemoglobin method as described in Wagner et al., (2008a).

3.3.7. Molecular sexing

A drop of hatchling blood from a small pinprick to the ankle was collected on filter paper and stored at -20^{0}C. DNA was extracted from blood samples using Instagene matrix following the manufacturer's protocol (Bio-Rad Laboratories, cat. No. 732-6030). Nestling sex was determined by polymerase chain reaction amplification using the primers P2 (5'- TCTGCATCGCTAAATCCTTT) and CW (5'- AGAAATCATTCCAGAAGTTCA), based on the protocol of (Griffiths, Daan & Dijkstra 1996; Love & Williams 2008).

3.3.8. Statistical Analysis

All statistical analyses were performed using SAS software version 9.3 (SAS Institute, 2011). Pearson's product-moment correlations or partial correlations were used to test for relationships between Hct and Hb and characteristics of the female, clutch, and chicks. General linear models (GLM procedure) were used to test for treatment effects on post-treatment Hct and Hb and characteristics of the female, clutch, and chicks. To analyse sex ratios, a generalized linear mixed model (GLIMMIX procedure) with a binomial probability distribution (dist=bin) was used. To compare Hct and Hb by reproductive stage (i.e., clutch completion or incubation), clutch number and year, repeated-measures mixed linear models (MIXED procedure) were used. Chi-square tests were used to determine if treatment groups differed in the number of individuals who laid replacement clutches, abandoned nests after treatment, successfully fledged offspring or laid a second clutch. Laying interval i.e., the time from treatment to initiation of the replacement clutch, was not normally distributed and therefore we used a Kruskal-Wallis test to test for differences in laying interval between treatment groups. We also

analysed laying interval using a failure-time analysis (LIFEREG procedure) with a log-logistic distribution, with females that were not observed to produce a replacement clutch included as right-censored observations. For censored observations, we used a laying interval of 6 days (i.e., laying interval was *at least* 6 days), since that is the smallest interval observed among females that did relay. There were numerous natural nesting cavities in the area, and it would be unreasonable to assume that females did not renest elsewhere.

Female body mass, lay-date, year, and treatment * year interaction were included as covariates in all models and removed where they did not contribute significantly to the model (when P > 0.05; see Results). The pdiff option was used to test pairwise differences between least square means for treatment * year effects.

3.4. Results

We pooled the data from pre-treatment first clutch samples collected over all years (2009 – 2011) to analyze partial correlations between Hct and Hb and between Hct, Hb and measures of clutch quality. To control for year and sampling stage, we included partial = sample date - clutch completion date. This resulted in a single partial variable unique to the different combinations of year (because years varied in sample date), and sample stage (because the number of days between the sample date and date of clutch completion differs by stage). Hb was significantly positively correlated to Hct (r_{156} = 0.63, P < 0.0001). Both Hct and Hb were significantly negatively correlated to the day the first egg of the pre-treatment first clutch was laid (Fig. 3.1; Table 3.2), controlling for year and sampling stage. In contrast, Hct and Hb were independent of mean egg mass, clutch size and clutch mass (Table 3.2), controlling for year and sampling stage.

3.4.1. Prediction 1: Females who maintain a higher post-laying Hct will have higher measures of brood quality, provisioning and success among control-treated broods

Hct and Hb at replacement clutch completion (2009-2010): Among control treated females whose first clutch was removed, Hct and Hb upon completion of the replacement clutch were both independent of brood size at hatch, mean hatchling mass, brood sex ratio at hatch, various offspring traits at 17 days (mean chick tarsus length, mass, Hct, Hb), maternal provisioning effort per chick, and number of chicks fledged ($P > 0.05$ in all cases; Table 3.3). In addition, among control females we found no difference in Hct or Hb levels upon completion of the first clutch between individuals who produced a replacement clutch and those who did not (Hct of birds who produced a replacement clutch 49.20 ± 0.90; Hct of birds who did not = 50.89 ± 0.85; Hct, $F_{1, 32} = 1.85$, $P = 0.18$; Hb of birds who produced a replacement clutch = 13.93 ± 0.47 g/dL; Hb of birds who did not = 14.84 ± 0.45 g/dL; Hb, $F_{1, 31} = 1.96$, $P = 0.17$).

Hct and Hb during incubation (2010-2011): Among control birds sampled and treated during incubation, Hct and Hb during incubation of the first clutch was independent of brood size at hatch, mean hatchling mass, brood sex ratio at hatch, mean chick tarsus length at 17 days, mean chick mass at 17 days, and maternal provisioning effort per chick, controlling for year ($P > 0.05$ in all cases; Table 3.4). Maternal Hb during incubation was positively correlated with number of chicks fledged, controlling for year or laying date (Table 3.4). However, the relationship between maternal Hct during incubation and number of chicks fledged was not significant (Table 3.4). Finally, among control females we found no difference in Hct or Hb levels between individuals who produced a second clutch and those who did not (Hct of birds who did = 51.20 ± 0.95; Hct of birds who did not = 50.75 ± 0.87; Hct, $F_{1, 41} = 0.13$, $P = 0.72$; Hb of birds who did = 16.25 ± 0.50; Hb of birds who did not = 16.64 ± 0.48; Hb, $F_{1, 40} = 0.32$, $P = 0.57$), in a general linear model including the effects of year.

3.4.2. Prediction 2: Experimentally reducing Hct during the production of a replacement clutch will delay egg laying and will reduce clutch quality, provisioning effort, and breeding success

The percentage of treated females who laid replacement clutches did not differ between treatments (Table 3.5). Comparing first clutch parameters (Hct, Hb, laying date, clutch size or egg mass) between PHZ and control females that did or did not produce a replacement clutch, there was no interaction between treatment and whether or not a replacement clutch was observed, and no main effect of whether or not a replacement clutch was observed ($P > 0.15$ in all cases). Laying interval (i.e., the time from treatment to initiation of the replacement clutch) was significantly greater among PHZ treated individuals compared with control treated individuals (Kruskal-Wallis test: $\chi^2_1 = 10.37$, $P = 0.0013$; Failure-time analysis: Wald $\chi^2_1 = 7.11$, $P = 0.0077$; Fig. 3.2). At completion of the replacement clutch neither post-treatment Hct, controlling for pre-treatment Hct, nor post-treatment Hb, controlling for pre-treatment Hb, differed among treatments in general linear models (Table 3.5), suggesting rapid recovery from PHZ-induced anemia. However, effects of PHZ on Hct and Hb can last at least 5 days in captive birds fed *ad libitum* (Williams *et al.* 2012), and if anything this recovery time would be expected to be longer in free living, reproducing animals. On average, PHZ treated females laid the first egg of their replacement clutch 11 days post treatment. Given that egg formation is initiated 4-5 days before the first egg is laid (Vezina, Salvante & Williams 2003), the direct effects of PHZ on Hct overlapped with at least part of the egg formation of the replacement clutch in at least 86% of PHZ treated birds. In addition, all PHZ treated females would have developed eggs during reticulocytosis i.e., recovery from a reduction in red blood cell number (Fernandez and Grindem, 2006).

There was no effect of treatment on replacement clutch mass (controlling for first clutch mass), mean egg mass (controlling for first clutch mean egg mass), clutch size, length of incubation, brood size at hatch, mean hatchling mass (controlling for mean egg mass), brood sex ratio, measures of parental provisioning, maternal Hct and Hb during provisioning (controlling for Hct and Hb at completion of replacement clutch), chick Hct, chick Hb, mass or tarsus length at 17 days of age, or brood size at fledging ($P > 0.05$ in all cases; Table 3.5). Due to a trend towards larger pre-treatment eggs within the PHZ

group, first clutch mean egg mass was used as a covariate when testing for treatment differences in mean hatchling mass. Finally, there was no effect of treatment on the proportion of females who successfully fledged chicks in the replacement clutch (Table 3.5).

3.4.3. Prediction 3: Experimentally reducing Hct during late incubation and early chick rearing will reduce provisioning performance, brood quality and breeding success

In the experiment carried out during 2010 and 2011, in which we treated females with PHZ during incubation and did not remove first clutches, the percentage of females who abandoned nests sometime between treatment and fledging chicks did not differ significantly between treatments (Table 3.6). In general linear models, there were no differences between treatments in lay date (controlling for year), maternal mass (controlling for year), mean egg mass of first clutch (controlling for maternal mass), or first clutch mass (controlling for maternal mass) (Table 3.6), confirming that assignment to treatment groups was random.

Chicks hatched on average 3.1 days post treatment. Effects of PHZ on Hct and Hb last at least 5 days post treatment in European starlings (Williams *et al.* 2012), and so would have persisted at least through the provisioning of 1 and 2 day old chicks. In addition, complete recovery from anemia i.e., reticulocytosis, can take 2 to 3 weeks (Fernandez & Grindem 2006). There was no effect of treatment (controlling for year) on duration of incubation (Table 3.6). Likewise, there was no effect of treatment (controlling for maternal mass) on brood size at hatch (Table 3.6). However, mean hatchling mass was significantly affected by treatment and year, controlling for mean egg mass (Table 3.6). Hatchlings from control broods were significantly heavier than those from PHZ treated broods and 2010 hatchlings were significantly lighter than 2011 hatchlings (2010, 4.99 ± 0.08 g; 2011, 5.61 ± 0.10 g). In addition, there was no effect of treatment on hatchling sex ratio (Table 3.6).

Females were blood sampled during provisioning on average 15 days after treatment, at which time there was no effect of treatment on Hct (controlling for Hct during incubation) (Table 3.6). Likewise, treatment had no effect on Hb during

provisioning, controlling for Hb during incubation (Table 3.6). There was no effect of treatment on maternal provisioning per chick (Table 3.6).

Mean chick tarsus length at 17 days of age was significantly affected by treatment ($F_{1, 47}$ = 21.21, P < 0.0001), year ($F_{1, 47}$ = 20.23, P < 0.0001) and by the treatment * year interaction ($F_{1, 47}$ = 18.77, P < 0.0001). PHZ broods had significantly shorter tarsi than broods from control females in 2010 but not in 2011 (Fig 3.3a). There was no overall effect of treatment on mean chick mass at 17 days of age ($F_{1, 47}$ = 3.15, P = 0.08), controlling for year ($F_{1, 47}$ = 12.74, P = 0.0008) . However, there was a significant interaction between treatment and year ($F_{1, 47}$ = 4.98, P = 0.03) on mean chick mass at 17 days of age. PHZ chicks at 17 days were significantly lighter in 2010 with no difference in 2011 (Fig 3.3b). There was no effect of treatment on chick Hct or chick Hb (Table 3.6). In contrast, there was a significant effect of treatment * year ($F_{1, 84}$ = 5.65, P = 0.02) on brood size at fledging controlling for maternal mass ($F_{1, 84}$ = 8.96, P = 0.004; year: $F_{1, 84}$ = 9.57, P = 0.003; treatment: $F_{1, 84}$ = 2.43, P = 0.12). Fewer chicks fledged from PHZ treated broods in 2010, but there was no difference in 2011 (Fig 3.3c). While this analysis included nests in which no eggs hatched, excluding such nests yielded a similar pattern, i.e., a significant effect of treatment * year ($F_{1, 72}$ = 7.63, P = 0.007), on brood size at fledging, controlling for maternal mass, with an effect in 2010 but not 2011. There was no effect of treatment on the proportion of birds who succeeded in fledging chicks from the first clutch (Table 3.6).

Some of the PHZ and control females that successfully reared their first broods produced a second clutch that was not manipulated further. There was no effect of first clutch treatment on the proportion of birds who laid second clutches (Table 3.6). There was no effect of treatment on measures of second clutch quality, second brood quality or parental provisioning of second broods from 2010 (P > 0.05 in all cases; second broods were only measured in 2010; Table 3.6). There was no effect of treatment on the proportion of birds who succeeded in fledging chicks from the second clutch (Table 3.6).

3.5. Discussion

The biological significance of the marked inter-individual variation in Hct during energy demanding stages of reproduction in birds remains unclear. We found that both Hct and Hb were higher in individuals who began laying their first clutch earlier. In addition, higher Hb levels during incubation were correlated with a greater number of chicks fledged. However, we found no evidence that post-laying Hct was correlated with subsequent components of parental care (incubation, provisioning) or breeding productivity (number or size of offspring at hatching or prior to fledging). Because observational data may be confounded by differences in individual quality, we also examined the effect of experimentally induced changes in Hct and Hb on reproductive success. We manipulated Hct and Hb within the physiological range in three years and during two different reproductive stages to test whether transient Hct and/or Hb reduction, as occurs during normal avian reproduction, has negative effects on reproductive performance.

Most treated individuals produced eggs during the direct effects of PHZ, and those that did not would have still developed eggs during reticulocytosis, i.e., recovery from a reduction in red blood cell number (Fernandez & Grindem 2006). This decrease in hematocrit during or prior to egg production resulted in a delay between treatment and laying of the replacement clutch. Among individuals treated with PHZ during late incubation, treatment significantly reduced hatchling size and, in some years, chick size and number, suggesting that the some effects of reduced Hct and Hb are context dependent. Effects of PHZ were likely due to the reduced Hct and Hb per se, rather than a general toxic effect, given that there were no differences between treatment groups in a) female mass post treatment, b) the proportion of individuals who laid a replacement clutch, c) the percentage of females who abandoned nests and d) the proportion of individuals who laid a second clutch.

Consistent with our finding that individuals with naturally lower Hct and Hb began egg laying later, PHZ treatment prior to egg laying increased laying interval (the time from treatment to laying the replacement clutch). Early clutch initiation has generally been considered an indicator of reproductive success (Perrins 1970; Verhulst &

Tinbergen 1991; Brinkhof *et al.* 1993; Blums, Clark & Mednis 2002; Blums *et al.* 2005; Blums & Clark 2004; Verhulst & Nilsson 2008). Although increased laying interval did not result in any differences among pre-fledging measures, the delayed initiation of the replacement clutch among PHZ females could have led to reduced survival of offspring in the post-fledging period (Nilsson 1999; Verhulst & Nilsson 2008; Grüebler & Naef-Daenzer 2008, 2010). There is strong selection for early fledging via increasing fledgling mortality rate later in the season due to increasing predation pressure, decreasing food availability, and greater competition between fledglings (Verhulst & Tinbergen 1991; Verhulst & Nilsson 2008; Grüebler & Naef-Daenzer 2010). In addition, among social birds, individuals who hatch/fledge early are in an advantageous position to establish dominance over later fledglings (Nilsson & Smith 1988; Nilsson 1990), which can impact access to higher quality territories and mates. Therefore, later laying dates within the PHZ treated group would translate into later hatching and fledging dates which could have significant long-term effects on reproductive success. Furthermore, because not all females produced a replacement clutch in one of our nest boxes, it is possible that our manipulation selected for higher-quality females (e.g., better physiological condition, more experienced, and/or access to better territory), and that more substantial treatment effects would have been observed had a replacement clutch been produced by all females, including those of lower quality. However, there was no difference in Hct or Hb among females that did or did not produce a replacement clutch, and similarly no difference in measures reflecting condition, first clutch laying date, clutch size or egg mass.

Among the individuals treated with PHZ during late incubation, we found a greater effect on pre-fledging reproductive success. Despite no difference in mean egg mass or clutch mass, in both years hatchling mass was reduced among offspring of females treated with PHZ during incubation. The timing of PHZ treatment relative to incubation and hatching meant that females would only have been exposed to direct effects of PHZ for the last quarter of incubation (about 3 days on average), assuming an incubation length of 12 days (Ricklefs & Smeraski 1983). Yet, this was associated with a significant decrease in hatching mass, suggesting a negative effect of PHZ on incubation and embryo development. PHZ causes an immediate decrease in hematocrit (within 24 hrs, Williams *et al.* 2012) and we suggest this affected female behavior. We detected no

effect on total incubation duration or the time elapsed between treatment and hatching. However, there can be marked individual variation in the frequency and duration of incubation bouts and off- nest bouts in a range of species, even though these behavioral differences do not explain variation in the total duration of incubation (e.g., Robinson *et al.* 2008; Williams *et al.* 2012). In altricial nestlings embryo mass and growth rate increase continuously throughout incubation so costs for maintenance and synthesis in embryos will increase until hatching (Vleck & Bucher 1998). In addition, small-scale variation in incubation temperature has been reported to have significant effects on embryo development (Hepp, Kennamer & Johnson 2006) and egg temperatures are normally maintained at a higher, and less variable, level closer to hatch (Webb 1987). This involves changes in on-off bouts by females with no increase in the total time incubating, consistent with the hypothesis that embryo thermal tolerances narrow as embryo's age (Cooper & Voss 2013). Potentially, our PHZ treatment interfered with this 'typical' female behaviour towards the end of incubation.

In addition, in one (2010) out of two years we found a reduction in the number and size of 17 day old chicks from PHZ treatment nests. Using 6 seasons of previous data from control females at our field site (unpublished data), average mean brood size at hatching (BSH) is 3.73 and average brood size at fledging (BSF) is 3.10. Relative to the six years of previous data, 2009 (BSH = 4.59, BSF = 3.63) and 2011 (BSH = 3.83, BSF = 3.15) appeared to be above average years for productivity whereas 2010 (BSH = 3.55, BSF = 2.56) had the lowest BSH on record and the second lowest BSF. Likewise, hatchlings from all nests were significantly lighter in 2010 when compared to 2011, suggesting that the 2010 breeding season was more demanding than the 2011 season. In light of this, our data suggest that the effects of reduced hematocrit during incubation are context dependent such that there are greater costs in inferior environmental conditions.

There is substantial evidence for resource-based mechanisms underlying the costs of egg production and incubation in birds (Gustafsson *et al.* 1994; Monaghan, Bolton & Houston 1995; Oppliger, Christe & Richner 1997; Kullberg, Houston & Metcalfe 2002; Martin, Scheuerlein & Wikelski 2003; Veasey, Houston & Metcalfe 2008). Recently, we have proposed that reproductive anemia may function as a non-resource

based cost of egg production (Williams *et al.* 2004; Wagner *et al.* 2008a; b). The suppression of erythropoiesis due to elevated estrogen levels during egg production can result in a prolonged decrease in Hct through incubation and sometimes into chick rearing (Williams *et al.* 2004). By increasing the severity of anemia during egg production, incubation and chick rearing, we tested whether this prolonged reduction in Hct and Hb might pose costs on reproductive performance. Our results, combined with those of (Wagner *et al.* 2008b), provide the first evidence of a hormone-mediated, non-resource based cost of egg production in birds. PHZ treatment during egg production resulted in a significant delay in the timing of reproduction. In addition, a reduction in Hct and Hb during incubation resulted in decreased hatchling mass in all years. However, PHZ treatment during incubation and chick rearing only affected the number of chicks fledged and chick tarsus length at 17 days of age in 2010, a year when reproductive performance was low among control females. These results suggest that costs of anemia may be amplified when conditions are poor, i.e., while the initial cost may be non-resource dependent (due to suppression of erythropoiesis by estrogen), the duration of the cost may be resource dependent, differing between good and bad years.

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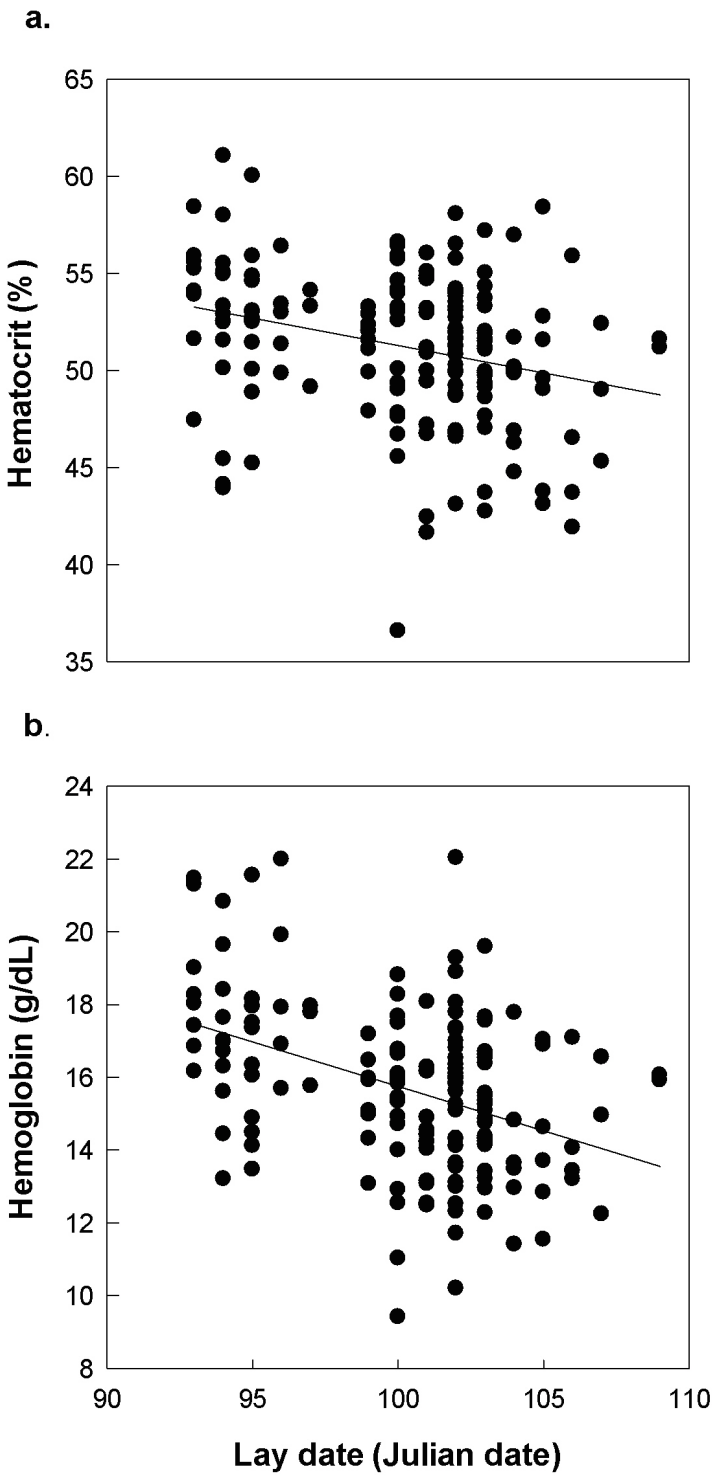

Figure 3.1. Variation in hematocrit and hemoglobin levels with the start date of the first clutch among pooled pre-treatment individuals. To control for variation due to differences in sample timing (i.e., reproductive stage and year) we included one cofactor indicating both the stage (i.e., clutch completion and late incubation) and year that the sample was taken.

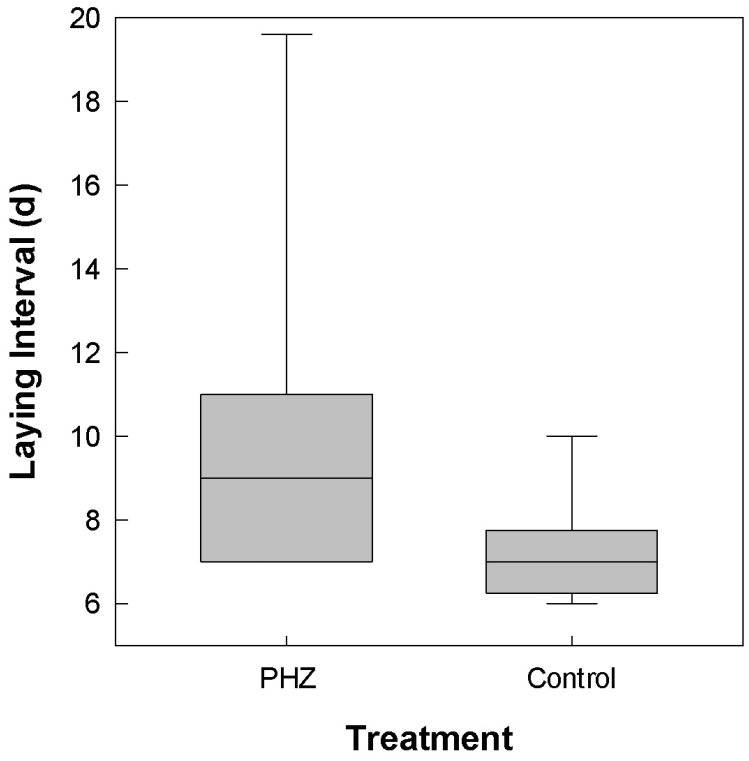

Figure 3.2. **Effect of PHZ treatment on laying interval i.e., the time from treatment to initiation of the replacement clutch. Phenylhydrazine (PHZ) was used to reduce hematocrit upon removal of the first clutch and females were treated with saline in the control group. The lower boundary of the box represents the 25th percentile, upper boundary the 75th percentile and the center line represents the median. The error bars represent one standard error.**

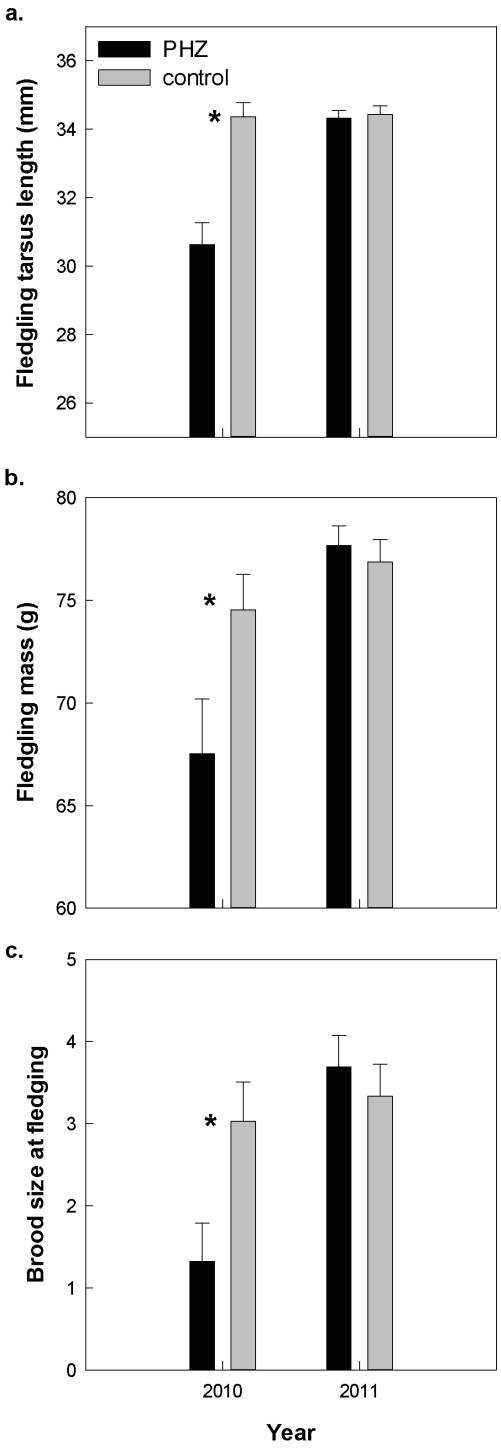

Figure 3.3. Interaction between year and effect of PHZ treatment during incubation on a) mean tarsus length of 17 day old chicks, b) mean mass of 17 day old chicks, and c) mean brood size at fledging. Phenylhydrazine (PHZ) was used to reduce hematocrit during incubation of the first clutch and females were treated with saline in the control group.

Table 3.1. Sample sizes per experiment and treatment group.

	PHZ	Control
Treated one to two days after completion of first unmanipulated clutch, and whose first clutch was removed to induce production of a replacement clutch	44	34
Sampled upon completion of replacement clutch	26	16
Hatched eggs from replacement clutch	25	16
Sampled during provisioning of replacement clutch brood	12	9
Fledged chicks from replacement clutch	21	14
Treated 6 to 9 days after completion of first unmanipulated clutch, and whose first clutch was not removed	46	45
Treated during incubation and sampled during provisioning	9	10
Treated during incubation, did not abandon and hatched chicks from first clutch	41	38
Treated during incubation and fledged chicks from first clutch	31	33
Treated during incubation and produced a second clutch	25	20
Treated during incubation and fledged chicks from second clutch	15	11

Table 3.2. Partial correlations coefficients between Hct and Hb at end of first, pre-treatment clutches and clutch quality, controlling for number of days between clutch completion and sampling.

	Maternal Hct (%)			Maternal Hb (g/dL)		
Trait	**n**	**r**	**P**	**n**	**r**	**P**
Laying date	155	-0.23	0.004	155	-0.40	0.0001
Mean egg mass	155	-0.05	0.53	155	-0.02	0.72
Clutch size	155	0.10	0.22	155	0.13	0.10
Clutch mass	155	0.07	0.38	155	0.10	0.21

Table 3.3. Correlations between maternal hematocrit (Hct) and hemoglobin (Hb) upon completion of the replacement clutch and measures of brood quality among females treated with saline upon removal of a first, unmanipulated clutch.

	Maternal Hct (%)			Maternal Hb (g/dL)		
Trait	**n**	**r**	**P**	**n**	**r**	**P**
Brood size at hatch	27	0.02	0.93	25	-0.26	0.22
Mean hatchling mass (g)	26	0.11	0.60	24	-0.25	0.25
Hatching sex ratio (% males)	22	-0.01	0.96[1]	21	-0.02	0.93[2]
Mean tarsus length of 17 day old chicks (mm)	25	-0.13	0.53	24	-0.04	0.85
Mean mass of 17 day old chicks (g)	25	-0.08	0.71	24	-0.08	0.71
Mean Hct of 17 day old chicks (%)	23	0.14	0.51	21	0.07	0.77
Mean Hb of 17 day old chicks (g/dL)	23	0.14	0.53	21	0.26	0.26
Maternal provisioning (nest visits per chick)	33	0.02	0.89	32	-0.14	0.44
Number of chicks fledged	27	-0.08	0.69	25	-0.15	0.46

[1] $F_{1,20} = 0.00$, P = 0.95 when analyzed using the GLIMMIX procedure.

[2] $F_{1,19} = 0.26$, P = 0.62 when analyzed using the GLIMMIX procedure.

Table 3.4. Partial correlation coefficients between maternal hematocrit (Hct) and hemoglobin (Hb) during incubation and measures of brood quality among females sampled treated with saline during incubation of a first, unmanipulated clutch. Year was included as a partial variable.

	Maternal Hct (%)			Maternal Hb (g/dL)		
Trait	**n**	**r**	**P**	**n**	**r**	**P**
Brood size at hatch	41	0.14	0.40	41	0.21	0.20
Mean hatchling mass (g)	19	-0.25	0.30	19	-0.24	0.34
Hatching sex ratio (% males)	21	-0.14	0.55[1]	21	-0.26	0.27[2]
Mean tarsus length of 17 day old chicks (mm)	24	-0.01	0.95	24	-0.11	0.62
Mean mass of 17 day old chicks (g)	24	-0.31	0.14	24	-0.11	0.62
Maternal provisioning (nest visits per chick)	22	0.19	0.42	22	0.15	0.52
Number of chicks fledged[3]	40	0.28	0.09	40	0.36	0.02

[1] $F_{1,18} = 0.17$, P = 0.68 when analyzed using the GLIMMIX procedure, including year in the model.

[2] $F_{1,18} = 1.98$, P = 0.18 when analyzed using the GLIMMIX procedure, including year in the model.

[3] Including laying date as a partial variable instead of year yielded similar results (Hct: r = 0.26, P = 0.10; Hb: r = 0.33, P = 0.04).

Table 3.5. Effects of PHZ treatment during the laying of a replacement clutch. Except where noted, analyses used general linear models. For analyses of replacement clutch mass, clutch size and egg mass, the values from first, unmanipulated clutches were used as a covariate. For analyses of maternal hematocrit (Hct) and hemoglobin (Hb) during provisioning, Hct or Hb after the completion of the replacement clutch was used as a covariate.

Trait	n	F/ χ^2	df	P	PHZ	Control	Additional terms in model
Proportion of females who produced replacement clutches		1.12[1]	1[1]	0.29[1]	26/44 = 59.1%	16/34 = 47.1%	
Post-treatment Hct, controlling for pre-treatment Hct (%)	34	0.26	1, 31	0.61	50.8 ± 0.9	51.5 ± 0.9	Pre-treatment Hct, $F_{1, 31} = 4.18$, P = 0.05
Post-treatment Hb, controlling for pre-treatment Hb (g/dL)	32	1.24	1, 29	0.27	16.4 ± 0.5	15.6 ± 0.5	Pre-treatment Hb, $F_{1, 29} = 5.32$, P = 0.03
Clutch mass (g)	44	0.16	1, 41	0.69	37.8 ± 1.7	36.7 ± 2.2	Pre-treatment clutch mass, $F_{1, 41} = 0.26$, P = 0.61
Mean egg mass (g)	44	1.71	1, 41	0.20	7.07 ± 0.06	6.94 ± 0.07	Pre-treatment egg mass, $F_{1, 41} = 45.00$, P < 0.0001
Clutch size	44	0.01	1, 41	0.92	5.5 ± 0.2	5.5 ± 0.3	Pre-treatment clutch size, $F_{1, 41} = 0.06$, P = 0.81
Incubation length (days)	38	0.08	1, 36	0.78	9.8 ± 0.4	9.7 ± 0.4	
Brood size at hatch	40	0.04	1, 38	0.84	4.7 ± 0.3	4.6 ± 0.3	
Mean hatchling mass (g)	31	0.41	1, 27	0.53	5.1 ± 0.1	5.1 ± 0.1	Egg mass, $F_{1, 27} = 73.56$, P < 0.0001; Lay date, $F_{1, 27} = 6.82$, P = 0.015
Hatchling sex ratio (% males)	30	0.34	1, 28	0.56	48 ± 6	42 ± 7	
Maternal provisioning (nest visits per chick)	32	2.77	1, 30	0.11	0.9 ± 0.1	0.7 ± 0.1	
Paternal provisioning (nest visits per chick)	32	0.36	1, 30	0.55	0.4 ± 0.1	0.4 ± 0.1	
Total provisioning (nest visits per chick)	32	0.60	1, 30	0.44	1.4 ± 0.1	1.2 ± 0.2	
Proportion of maternal provisioning (%)	32	3.34	1, 30	0.08	66 ± 5	54 ± 5	

Trait	n	F/ χ^2	df	P	PHZ	Control	Additional terms in model
Maternal Hct during provisioning (%)	21	0.01	1, 18	0.91	52 ± 1	52 ± 1	Clutch completion Hct, , $F_{1, 18}$ = 0.00, P = 0.99
Maternal Hb during provisioning (g/dL)	20	0.86	1, 17	0.37	16.3 ± 0.5	17.0 ± 0.6	Clutch completion Hb, $F_{1, 17}$ = 2.53, P = 0.13
Mean Hct of 17 day old chicks (%)	28	3.79	1, 26	0.06	42.2 ± 0.8	44.7 ± 0.9	
Mean Hb of 17 day old chicks (g/dL)	28	2.10	1, 26	0.16	11.3 ± 0.3	11.9 ± 0.4	
Mean tarsus length of 17 day old chicks (mm)	33	0.04	1, 31	0.84	32.3 ± 0.6	32.1 ± 0.7	
Mean mass of 17 day old chicks (g)	33	0.77	1, 31	0.39	76 ± 1	75 ± 1	
Brood size at fledging	40	0.38	1, 38	0.54	3.1 ± 0.4	3.5 ± 0.5	
Proportion of females who fledged chicks in replacement clutch		0.32[1]	1[1]	0.57[1]	21/26 = 80.8%	14/16 = 87.5%	

[1] from Chi-square test

Table 3.6. Effects of PHZ treatment during incubation. Except where noted, analyses used general linear models. Covariates for first clutches are described in the text.

Trait	n	F/ χ^2	Df	P	PHZ	Control	Additional terms in model
First clutches							
Pre-treatment maternal mass (g)	91	1.68	1,89	0.20	81.8 ± 0.5	80.9 ± 0.5	
Pre-treatment laying date (Julian date)	91	0.23	1,89	0.63	98.9 ± 0.6	99.3 ± 0.6	
Pre-treatment first clutch mass (g)	91	0.11	1,89	0.75	37.2 ± 0.8	37.6 ± 0.8	
Pre-treatment mean egg mass (g)	91	0.06	1,89	0.81	7.07 ± 0.07	7.09 ± 0.07	
Pre-treatment clutch size	91	0.01	1,89	0.92	5.3 ± 0.1	5.3 ± 0.1	
Percentage of females who abandoned nest after treatment	91	0.44	1	0.51[1]	5/46 = 10.9%	7/45 = 15.6%	
Total incubation length, including pre-treatment (days)	67	2.34	1, 65	0.13	10.8 ± 0.2	10.5 ± 0.2	
Time between treatment and hatching (days)	67	0.23	1, 65	0.63	3.2 ± 0.2	3.1 ± 0.2	
Brood size at hatch	90	0.12	1,87	0.73	3.8 ± 0.3	3.9 ± 0.3	Female mass, $F_{1, 87} = 6.60$, P = 0.01
Mean hatchling mass (g)	40	13.30	1,36	0.0008	5.1 ± 0.1	5.6 ± 0.1	Egg mass, $F_{1, 36} = 4.39$, P = 0.04; Year, $F_{1, 36} = 21.22$, P < 0.0001
Hatching sex ratio (% males)	41	1.40	1,39	0.24	35 ± 7	46 ± 6	
Maternal Hct during provisioning (%)	18	1.09	1,15	0.31	52 ± 1	51 ± 1	Pre-treatment Hct, $F_{1, 15} = 0.96$, P = 0.34;
Maternal Hb during provisioning (g/dL)	15	1.14	1,12	0.31	17.8 ± 0.5	17.1 ± 0.5	Pre-treatment Hb, $F_{1, 12} = 1.50$, P = 0.24;
Maternal provisioning (nest visits per chick)	40	3.69	1,38	0.06	0.67 ± 0.08	0.47 ± 0.07	
Paternal provisioning (nest visits per chick)	40	0.91	1,38	0.35	0.44 ± 0.09	0.32 ± 0.09	

Trait	n	F/ χ^2	Df	P	PHZ	Control	Additional terms in model
Total provisioning (nest visits per chick)	40	1.47	1, 38	0.23	1.64 ± 0.19	1.34 ± 0.17	
Proportion of maternal provisioning (%)	41	0.67	1, 39	0.42	41 ± 6	35 ± 5	
Mean Hct of 17 day old chicks (%)	10	0.39	1,8	0.54	45 ± 2	47 ± 2	
Mean Hb of 17 day old chicks (g/dL)	10	0.29	1,8	0.61	12.8 ± 0.8	13.3 ± 0.5	
Proportion of females who fledged chicks from first clutch		0.63[1]	1[1]	0.43[1]	31/46 = 67.4%	33/44 = 75.0%	
Second clutches							
Proportion of females who laid second clutches		0.89[1]	1[1]	0.34[1]	25/46 = 54.4%	20/45 = 44.4%	
Clutch mass (g)	46	3.11	1, 43	0.08	35 ± 2	31 ± 2	Year, $F_{1, 43}$ = 5.24, P = 0.03;
Mean egg mass (g)	31	0.94	1, 28	0.34	7.1 ± 0.1	7.3 ± 0.2	Female mass, $F_{1, 28}$ = 5.65, P = 0.02;
Clutch size	30	1.37	1, 27	0.25	4.9 ± 0.2	4.6 ± 0.2	Female mass, $F_{1, 27}$ = 5.61, P = 0.03;
Brood size at hatch	45	0.17	1, 43	0.68	3.0 ± 0.4	2.8 ± 0.5	
Mean hatchling mass (g)	10	0.41	1, 8	0.54	4.9 ± 0.1	5.0 ± 0.2	
Hatching sex ratio (% males)	15	1.08	1, 13	0.32	45 ± 9	60 ± 11	
Maternal provisioning (nest visits per chick)	16	0.09	1, 14	0.77	0.70 ± 0.18	0.62 ± 0.18	
Paternal provisioning (nest visits per chick)	16	0.52	1, 14	0.48	0.40 ± 0.09	0.30 ± 0.09	
Total provisioning (nest visits per chick)	16	0.17	1, 14	0.69	1.29 ± 0.22	1.16 ± 0.22	
Proportion of maternal provisioning (%)	16	0.17	1, 14	0.69	44 ± 11	50 ± 11	
Mean Hct of 17 day old chicks (%)	10	0.02	1, 8	0.89	38 ± 2	38 ± 2	
Mean Hb of 17 day old chicks (g/dL)	10	0.15	1, 8	0.71	10.0 ± 0.8	9.5 ± 0.8	
Mean tarsus length of 17 day old chicks (mm)	10	0.01	1, 8	0.92	32.3 ± 0.8	32.2 ± 0.8	

Trait	n	F/ χ^2	Df	P	PHZ	Control	Additional terms in model
Mean mass of 17 day old chicks (g)	10	0.21	1, 8	0.66	69 ± 1	69 ± 1	
Brood size at fledging	45	1.65	1, 43	0.21	2.3 ± 0.4	1.6 ± 0.4	
Proportion of females who fledged chicks from second clutch		0.11[1]	1[1]	0.74[1]	15/25 = 60.0%	11/20 = 55.0%	

[1] from Chi-square test

Chapter 4.

Hematocrit variation in Passerines is correlated with breeding habitat latitude, altitude, and migratory phenotype

4.1. Summary

Aerobic capacity sets an upper limit on the intensity of activity that can be sustained. Therefore, variation in physiological traits that influence aerobic capacity, such as hematocrit (the primary determinant of blood oxygen carrying capacity), should be particularly susceptible to selective pressures that increase oxygen demands. Birds exhibit extensive variation in hematocrit across species and the reasons for this variation are largely unknown. While it seems likely that there would be the potential for selection to shape hematocrit, there has been little study of ecological factors that might exert selective pressures. There are at least three major avian life history traits that exhibit clear differences in oxygen requirements/availability: 1) Mirgatory phenotype, 2) Habitat latitude (due to cold climates), and 3) Habitat altitude. We used phylogenetically independent contrasts to test whether habitats and life history traits that increase oxygen demands exert selective pressures to increase baseline hematocrit levels within 56 passerine species. Hematocrit was positively associated with 1) migratory prevalence, 2) breeding habitat latitude, and 3) habitat altitude. This suggests that the sustained periods of aerobic metabolism required by migratory flight and by thermogenesis at colder high latitude habitats, particularly during the energy demanding breeding season, have exerted significant selective pressure on hematocrit. Similarly, species that inhabit

higher altitudes have higher hematocrit likely due to the selective pressure imposed by the reduction in oxygen availability.

4.2. Introduction

Hematocrit is a primary determinant of blood oxygen carrying capacity (Hoppeler and Weibel 1998) and varies substantially among populations and species (Dobrowolska 1982; Fullwood et al. 1982; Andersen et al. 1985; Garland Jr and Else 1987; Promislow 1991; Burns and Castellini 1996; Merino and Barbosa 1996; Dawson and Bortolotti 1997; Thompson et al. 1997; Wanless et al. 1997; Peterson 2002; Thirup 2003; Potti 2007; Heatley et al. 2013). The reasons for this variation are largely unknown, although there is believed to be a trade-off between increased blood oxygen capacity and increased blood viscosity associated with increasing hematocrit (Castle and Jandl 1966). Birds show extensive variation in hematocrit (31% in Quail, *Coturnix coturnix* to 61% in Rufous Hummingbirds, *Selasphorus rufus*) (Sturkie and Griminger 1976; Chilgren and deGraw 1977; Rehder et al. 1982; Campbell 1995; Price et al. 1998; Fair et al. 2007). Avian life history traits and habitats are therefore good candidates to identify ecological factors that may have exerted selective pressure on hematocrit.

Differences in hematocrit within avian species are well documented, with hematocrit increasing at high altitude (Jaeger and McGrath 1974; Carey and Morton 1976; Weinstein et al. 1985; Keys et al. 1986; Clemens 1988; Clemens 1990; Morton 1994; Prats et al. 1996; Maginniss et al. 1997; Fair et al. 2007; Borras et al. 2010), in response to thermogenic demands due to cold climates or experimentally reduced temperatures (Kubena et al. 1972; Carey and Morton 1976; Dawson and Carey 1976; Rehder et al. 1982; Rehder and Bird 1983; Clemens 1990; Swanson 1990; Yahav et al. 1997) , and during migration (Banerjee and Banerjee 1977; deGraw et al. 1979; Wingfield and Farner 1980; Thapliyal et al. 1982; Morton 1994; Piersma et al. 1996; Landys-Ciannelli et al. 2002). Despite these intraspecific studies, much less is known about interspecific variation, although this might be expected to follow similar patterns.

Adaptations in hematocrit would require a genetic basis for intraspecific variation, but few studies have assessed the heritability of hematocrit variation in wild birds. Of the two studies that have, Christe et al (2000) found significant additive genetic variance in the house martin (*Delichon urbica)* while Potti (2007) found no significant additive genetic variance in pied flycatchers (*Ficedula hypoleuca)*. However, genetic variance can be obscured by high environmental variation, and Potti (2007) found significant repeatability in hematocrit, which may suggest an upper bound to heritability estimates (Falconer and Mackay 1996). In addition, hematocrit has been shown to be heritable in chickens (Shlosberg et al. 1996; Shlosberg et al. 1998) and mammals (Weibust and Schlager 1968; Stino and Washburn 1973; Whitfield et al. 1985; Maes et al. 1995; Pravenec et al. 1997; Evans et al. 1999; Foote and Hare 2001; Lin et al. 2005; Johannes et al. 2006). Furthermore, baseline hematocrit levels can be modified by direct selection in birds (Washburn 1967; Shlosberg et al. 1996) and as a correlated response to selection for traits that cause metabolic stress (Price et al. 1998).

While it seems likely that there would be the potential for selection to shape hematocrit, there has been little study of ecological factors that might exert selective pressures. There are at least three major avian life history traits that exhibit clear differences in oxygen requirements/availability: 1) Migratory phenotype, i.e., whether a species is migratory. Avian flight requires metabolic rates two times that of similar sized running mammals with periods of activity lasting 50 to 100 hours (Alerstam and Lindström 1990; Butler and Woakes 1990; Butler 1991; Berthold 1996; Guglielmo et al. 2001). 2) Habitat latitude. Latitudes further from the equator are associated with colder habitats (Stevens 1989; Mann et al. 1998) during both winter and breeding seasons (Jetz et al. 2008), requiring increased thermogenesis. Cold-induced metabolic rates exceed basal metabolic rates by 3-9 fold (Saarela et al. 1995; Arens and Cooper 2005) and meeting thermoregulatory demands can represent 45-60% of total daily energy expenditure (Weathers and Sullivan 1993). 3) Habitat altitude. As altitude increases, atmospheric P_{O2} decreases, effectively reducing oxygen availability by decreasing the rate of gas exchange between the environment and the lungs. Theoretical modeling suggests that in moderately hypobaric environments (up to 4500 m elevation) one of the most sensitive steps in the O_2 transport pathway is blood oxygen capacity (Scott 2011).

The passerine order encompasses over 50 percent of all bird species, representing a large fraction of bird diversity (Ehrlich et al. 1988). Passerines are generally small, active birds with higher metabolic rates than non-passerines of the same size (Lasiewski and Dawson 1967), with species occupying a wide variety of habitats, making this group a good candidate for studying adaptations in blood oxygen transport. To our knowledge, there have not been any comparative studies of avian hematocrit that take phylogeny into account. Accounting for phylogeny is important when comparing multiple species because species are differentially related and statistical analyses require independent observations (Felsenstein 1985). We used phylogenetically independent contrasts to assess whether there were ecological factors that may have exerted selective pressure on hematocrit within passerines. Although there are temporal variations in hematocrit due to transitory environmental factors, baseline hematocrit levels could reflect the life history and ecological traits of the species. We hypothesize that habitats and life history traits that increase oxygen demands exert selective pressures to increase baseline hematocrit levels. Therefore, we predict that higher hematocrit will be observed in species 1) who are more migratory (see methods for details), 2) whose habitat is further from the equator, and 3) whose habitat is at higher altitudes.

4.3. Methods

4.3.1. Data collection

A database of hematological parameters and body mass of 56 passerines was collected by searching literature for published hematocrit data. Details regarding the hematocrit sampling environment (i.e., latitude, altitude, migration status) were collected where reported. In an attempt to minimize effects of the sample environment, hematocrit data were only included for wild populations of non-breeding, non-migrating adults living within their native habitat. In addition, all hematocrit samples were measured using the microhematocrit method, which measures the volume percentage of erythrocytes in whole blood by centrifuging whole blood collected in capillary tubes and dividing the packed cell volume by the total volume. This method is considered to be one of the

simplest, most accurate and reproducible laboratory procedures. When more than one reference was found for a given species, we used the mean value. The mean sample size per species for hematocrit data was 13. Sex of the individuals sampled was not determined in all studies, however a meta-analysis found that hematocrit did not differ between sexes (Fair et al. 2007). If body mass was not reported, it was obtained from the CRC book of Avian Body Masses (Dunning 1993). Migratory phenotype data for 56 species, habitat latitude data for 56 species and highest habitat altitude data for 31 species were collected from Handbook Of The Birds Of The World Alive (del Hoyo et al. 2013). Habitat latitude of each species was estimated from the year-round, resident, winter, and breeding distribution maps (del Hoyo et al. 2013). We collected data for central breeding and winter habitat latitudes. Latitude was measured as absolute degrees, i.e., the distance from the equator, as an estimate of thermogenic needs. In order to allow for continuous data analysis, conventionally used migratory phenotypes were classified from lowest to highest migration prevalence as follows: Category 1 included sedentary species in which no significant movement occurs among any populations. Category 2 included species who are irruptive (short distance movements that occurs some years and not others), and species in which some populations are sedentary and others are partial migrants (in which not all individuals within the population migrate). Category 3 included species in which some populations are completely migratory and others are partially migratory. Finally, category 4 included only completely migratory species. Due to the ambiguity of categories 2 and 3, it is possible that the magnitude of the differences between categories was not equivalent. Therefore, to ensure that results were not sensitive to categorization minutiae, migration data were also analyzed with categories 2 and 3 collapsed. Raw data are shown in Table 4.1.

4.3.2. Phylogenetic tree

The phylogenetic tree was built using the protein-coding sequences of mitochondrial cytochrome oxidase I, cytochrome b, ND2, and ND3 and of nuclear RAG-1 sequence (sequence data compiled from Genbank using Geneious and concatenated as described by (Jetz et al. 2012). DNA sequences were aligned in Mesquite 3.00 (Maddison and Maddison 2014). We used a Bayesian analysis of the concatenated mtDNA and nuclear DNA (6990 bp) with MrBayes 3.2 (Ronquist et al. 2012) to calculate

the consensus tree. Because nucleotide substitution rates differ by DNA type and codon position, sequence data were divided into six partitions (one for each of three codon positions, for each of mitochondrial and nuclear DNA). The following procedure was applied to all partitions. To account for uncertainty in the nucleotide substitution model, rather than selecting a substitution model via *a priori* model testing, we used the Bayesian model choice feature in Mr. Bayes 3.2, which samples all 203 possible time-reversible models of DNA substitution (Huelsenbeck et al. 2004; Ronquist et al 2012). We set MrBayes to run 4 simultaneous Markov chain Monte Carlo iterations (three heated and one cold) until convergence (1,550,000 generations) with trees sampled every 100th generation. We assessed chain mixing and convergence (stationarity) by examining the standard deviation of split frequencies, a plot of the log likelihood values per generation, and the Potential Scale Reduction Factor (Gelman and Rubin 1992). After discarding the first 25% of the samples (burn-in), results were combined to obtain a majority-rule consensus tree with the respective posterior probability of each node (Fig. 1). The resulting tree topology was consistent with Barker et al.'s (2002) Passeriformes phylogenetic hypothesis.

4.3.3. Independent contrasts

In order to meet the assumption of independence of statistical tests, species data were converted to phylogenetically independent contrasts (PIC) using the PDAP: PDTREE module version 1.15 (Midford et al. 2009) of Mesquite version 3.00 (Maddison and Maddison 2014). PICs assume that 1) the model of character evolution can be described by Brownian motion, 2) within species variation is negligible, and 3) the phylogenetic topology and branch lengths are known (Felsenstein 1985). According to the Passeriformes phylogenetic tree from (Barker et al. 2002), the yellow-bellied flycatcher (*Empidonax flaviventris*) of the *Tyrannidae* family is the most basal species within our dataset, and was drawn in the tree as such. We exponentially transformed branch lengths to adequately fit them to the character data (Garland et al. 1992). Exponentially transformed branch lengths were confirmed for adequate fit of the tip data (Diaz-Uriarte and Garland 1996; Diaz-Uriarte and Garland 1998) by correlating the absolute values of the standardized contrasts against their standard deviations, for all traits $P > 0.05$ (Garland et al. 1992). To correct for one soft polytomy we set the

corresponding branch length to zero and subtracted one degree of freedom (Purvis and Garland 1993; Garland and Diaz-Uriarte 1999). Analyses were also run on an unrooted tree with exponentially transformed branch lengths to determine whether the results were sensitive to the use of the *Tyrannidae* family as the basal group.

4.3.4. Statistical analyses

All statistical analyses were performed using SAS software version 9.2 (SAS Institute, 2011). Linear regression through the origin was used to analyze the relationship between raw data, phylogenetically independent contrasts (Garland et al. 1992), as well as mass corrected contrasts. Body mass was log transformed for all analyses. We obtained mass-independent residual contrasts by regressing each contrast on contrasts in body mass (PDAP manual).

4.3.5. Testing for phylogenetic signal

In order to determine whether our hematocrit data showed phylogenetic signal i.e., whether the data exhibit a tendency for related species to be more similar, we used the randomization procedure from Blomberg et al. (2003). If our hematocrit data show phylogenetic signal then the variance of the contrasts should be low when compared to the variance after the data have been randomly permuted across the tree. Data were randomized 1000 times across the tips of the tree using PDRANDOM. PDERROR was used to calculate the variance for each permutation and the distribution of the variances was calculated; to infer phylogenetic signal, the observed variance of the data must be less than that of 95% of the permuted datasets. Hematocrit tended to vary with body mass and correction for body mass when testing for phylogenetic signal requires a different approach than the calculation of residuals described above (Blomberg get al. 2003). Standardized phylogenetic contrasts of log(body mass) and hematocrit were regressed through the origin and the slope (*b*) was used to compute size-corrected values for the original trait as: log[hematocrit/(mass^{b})].

4.4. Results

There was no relationship between the hematocrit-sampling altitude and the species' highest habitat altitude (F_{22} = 0.00, P > 0.99). Due to being extreme outliers relative to sampling latitude, the three species who were sampled in Africa were omitted from the following preliminary analyses, comparing sampling latitude to winter and breeding habitat latitude. There was no relationship between hematocrit-sampling latitude and species' winter habitat latitude (F_{45} = 1.35, P > 0.25) or breeding habitat latitude (F_{45} = 0.08, P > 0.77), when omitting the 3 mentioned outliers.

All variables (mass corrected hematocrit, body mass, migratory phenotype, habitat latitudes, and highest habitat altitude) showed significant phylogenetic signal, as the observed variance of each variable was less than that of 95% of permuted datasets. However, for comparison, we also report non-phylogenetically corrected analyses.

Analyses using the unrooted tree produced identical results as the analyses using the *Tyrranidae (Empidonax flaviventris)* rooted tree. There were no associations between contrasts in habitat altitude and habitat latitudes or migratory phenotype (P > 0. 07 for all). Contrasts in migratory phenotype were positively correlated with contrasts in central breeding habitat latitude (n = 55, t = 4.81, P < 0.001; Fig 4.2) i.e., greater migration was associated with breeding farther from the equator. There was no relationship between contrasts in migratory phenotype and central winter habitat latitude (P = 0.25).

All regression statistics between phylogenetically independent contrasts, as well as non-phylogenetically corrected data, are shown in Table 4.2. There was no significant relationship between contrasts in hematocrit and contrasts in body mass, although there was a marginally non-significant negative trend. A similar non-significant trend existed between uncorrected hematocrit and body mass. Because the relationship between hematocrit and body mass was marginally non-significant, we also report regression statistics for mass-corrected variables, the results of which are also shown in Table 4.2. Among phylogenetically corrected contrasts, body mass was independent of migratory phenotype, habitat latitudes, and highest habitat altitude (P > 0.08 for all). However, among non-phylogenetically corrected data, body mass was negatively correlated with

central breeding habitat latitude and migratory phenotype (Fig 4.3). Body mass decreased with central breeding habitat latitude further from the equator and in species with greater migration.

There was a positive relationship between hematocrit and migratory phenotype among both phylogenetically corrected contrasts and non-phylogenetically corrected data, with and without mass correction (Fig 4.4 a, b, c & d). This relationship remained consistent when collapsing migratory categories 2 and 3. As migratory prevalence increases, hematocrit also increases. There was no relationship between hematocrit and central winter habitat latitude among either phylogenetically corrected contrasts or non-phylogenetically corrected data, with and without mass correction. However, there was a positive relationship between hematocrit and central breeding habitat latitude among phylogenetically corrected contrasts (Fig 4.5), with and without mass correction. Among non-phylogenetically corrected data, hematocrit was positively correlated with central breeding habitat latitude without mass correction but not among mass corrected data. There was a positive correlation between hematocrit and highest habitat altitude among phylogenetically corrected contrasts, with and without mass correction (Fig 4.6 a & b), but not among non-phylogenetically corrected data.

Due to the possible confounding relationship between migratory phenotype, central breeding habitat latitude, and hematocrit we included both variables in a linear regression. When regressing the contrast data through the origin, the overall model significantly accounts for some of the variation in hematocrit ($F_{2, 53} = 4.14$, $P = 0.02$), however neither migratory phenotype ($n = 55$, $t = 1.38$, $P = 0.17$) nor central breeding habitat latitude ($n = 55$, $t = 1.37$, $P = 0.17$) contributed significantly when adjusting for the other effect. Similarly, among non-phylogenetically corrected data, the overall model significantly accounts for some of the variation in hematocrit ($F_{2, 53} = 6.68$, $P = 0.003$), however neither migratory phenotype ($n = 56$, $t = 1.95$, $P = 0.06$) nor central breeding habitat latitude ($n = 56$, $t = 1.93$, $P = 0.06$) contributed significantly when adjusting for the other effect.

4.5. Discussion

We used a phylogenetic analysis to test correlations between hematocrit levels and indices of oxygen requirements and availability, i.e., migratory phenotype, seasonal habitat latitudes, and habitat altitude. A significant phylogenetic signal for all variables confirms that correcting for phylogeny was appropriate in this study. We found that hematocrit increased with increasing habitat altitude, increasing migration tendency, and as the distance between breeding habitat boundary and the equator increased.

A previous study suggests that hematocrit does not vary with body mass among mammals (Promislow 1991). However, a phylogenetic study on the allometry of hematological parameters among 124 bird species (E. Wagner unpublished) found a significant negative association between body mass and hematocrit ($P < 0.0012$). Both studies encompass a large range of body masses and therefore may not be representative of relationships among more closely related species or species that are more similar in body mass. Here we found a marginally non-significant relationship between hematocrit and body mass among passerine species, however mass-corrected and mass uncorrected results were similar. Although body mass has been found to strongly correlate with latitude when examined among New World birds (Ramirez et al. 2007) and all bird species (Olson et al. 2009), we did not find correlations between body mass and habitat latitudes. We believe this is due to the reduced variation in body mass among passerines relative to broader groups of birds.

Consistent with our prediction, species that were more migratory had higher hematocrit levels and therefore higher blood oxygen carrying capacity. For example, mean hematocrit for completely migratory species was 51.18 ± 4.1% (mean ± standard deviation; n = 17) while mean hematocrit for sedentary species was 44.80 ± 4.6% (n = 11). These results suggest that the sustained periods of aerobic metabolism required by migratory flight may exert significant selective pressure on hematocrit.

We found that hematocrit was positively related to breeding habitat and not related to winter habitat. This may suggest that migratory phenotype was a stronger selective pressure on hematocrit than habitat latitude due to the confounding relationship between breeding habitat latitude and migratory phenotype. Aptly, species that inhabit

colder breeding habitats (breeding habitats further from the equator) are more migratory. While our study cannot determine if migration or breeding habitat imposes greater selection pressure on hematocrit, a study by Jetz *et al.* (2008) concluded that broad-scale environmental variables, particularly temperature, presented a stronger constraint on avian basal metabolic rate (indicator of minimum maintenance energy requirements) than migratory tendency. Therefore, an alternative explanation could be that breeding habitat latitude has a greater association with hematocrit than winter habitat because breeding habitat latitudes are much further from the equator than winter habitat latitudes. The average central breeding habitat latitude was 51.4° ± 5.3°, while the average central winter habitat latitude was 28.78° ± 9.9°. As Jetz *et al.* (2008) suggested, more extreme climates further from the equator might impose shorter breeding seasons and additional pressures during a time when energetically demanding breeding activities are performed. In this case, migrant species would exhibit higher hematocrit because they breed at higher latitudes than non-migrant species.

As predicted, highest habitat altitude was significantly related to hematocrit, with and without mass correction. Indeed temperature decreases with increasing altitude, which could play a factor in the relationship between hematocrit and altitude. Rather than exerting selection on hematocrit via increasing energy requirements the way that temperature and migratory flight would, altitude may exert selection on hematocrit via decreasing oxygen carrying capacity. As altitude increases, inspired oxygen pressure decreases leading to decreased oxygenation of the blood as it passes through the pulmonary capillaries. At different altitudes the relative influence of each step of the oxygen transport chain varies (Scott 2011). Of the passerine species used in this study, the highest altitude inhabited was 4575 m elevation by the winter wren, *Troglodytes troglodytes* and there were 9 species inhabiting altitudes between 3500 - 4575 m elevation (moderate hypobaric conditions). Circulatory oxygen capacity, which is directly influenced by hematocrit, is theorized to be the most influential step of the oxygen transport chain from sea level to 4500 m (Scott 2011). A significant amount of research has focused on the relationship between hematocrit and altitude, yet the relative roles of phenotypic plasticity versus genetic adaptation remain unclear. Within bird species, hematocrit values generally increase with increasing altitude (Jaeger and McGrath 1974; Carey and Morton 1976; Weinstein et al. 1985; Keys et al. 1986; Clemens 1988;

Clemens 1990; Morton 1994; Prats et al. 1996; Maginniss et al. 1997; Fair et al. 2007; Borras et al. 2010), which could be primarily due to physiological plasticity, as was found among highland and lowland deer mice (*Peromyscus maniculatis*; Tufts et al 2013). However, a slightly increased hematocrit has been described as characteristic of birds and mammals genetically adapted to high altitude (Bullard 1972; Monge and Leon-Velarde 1991).

Higher hematocrit can improve maximal oxygen uptake and endurance performance (reviewed in Calbet et al. 2006). In contrast, a potentially adverse consequence of high hematocrit is increased blood viscosity, which can limit endurance capacity due to higher vascular resistance resulting in a decrease in the rate of blood flow (Crowell and Smith 1967). Adaptive mechanisms to offset the increased viscosity associated with chronically high hematocrit have been identified and include vasodilation (Ruschitzka et al. 2000; also reviewed in Harder and Boshkov 2010) and increased red blood cell flexibility (Vogel et al. 2003). Decreased blood flow resistance, has been associated with genetic variation found in high altitude dogs (Gou et al. 2014). Although it may be more energetically efficient to only increase hematocrit just prior to and during times of increased energetic demand, maintaining higher baseline hematocrit could be advantageous. In mice, due to the adaptive mechanisms that counteract increased viscosity, individuals with chronically excessive erythrocytosis acclimate better to different hematocrit levels than mice with acute increases in hematocrit (Schuler et al. 2010). Therefore, birds that experience periods of differing oxygen requirements/availability, such as during migration or when residing in cold habitats or at high altitudes, may be better able to exploit the benefits of increased hematocrit by maintaining higher baseline hematocrit.

Many factors are involved in the oxygen transport cascade. Consequently, there are multiple ways to adapt to environments that increase demand on this pathway, even among different populations of the same species (Beall 2007). The goal of this study was not to identify the primary adaptations that allow certain animals to live in extreme conditions, but rather to identify selective pressures that might have contributed to the existing variation in hematocrit between species.

Due to the phenotypic plasticity of hematocrit and the nature of correlative studies, we cannot determine how much of the observed variation in hematocrit associated with migration, habitat latitude and altitude is due to adaptation versus acclimatization. However, it has been suggested that when shifting comparisons from inter-individual variation to inter-specific variation the role of acclimatization decreases as the role of adaptation increases (Spicer 1999, pg. 188). In addition, there were no relationships between hematocrit-sampling environment and habitat variables, which should reduce some of the concern regarding confounding environmental effects. In order to confirm adaptations comparative analyses must be complemented with other approaches, such as quantitative genetic analyses, measurements of selection and studies of individual variation (Rezende and Diniz-Filho 2012). In addition, studies that employ geographical ranges are complex due to unequal abundance distributions and intra-range biotic and abiotic variation, which presents the need for further large-scale observational studies to assess spatial patterns of individuals and populations (see Sagarin et al. 2006). We feel that this model system offers promise for such studies and can yield insight into furthering our understanding of physiological adaptation.

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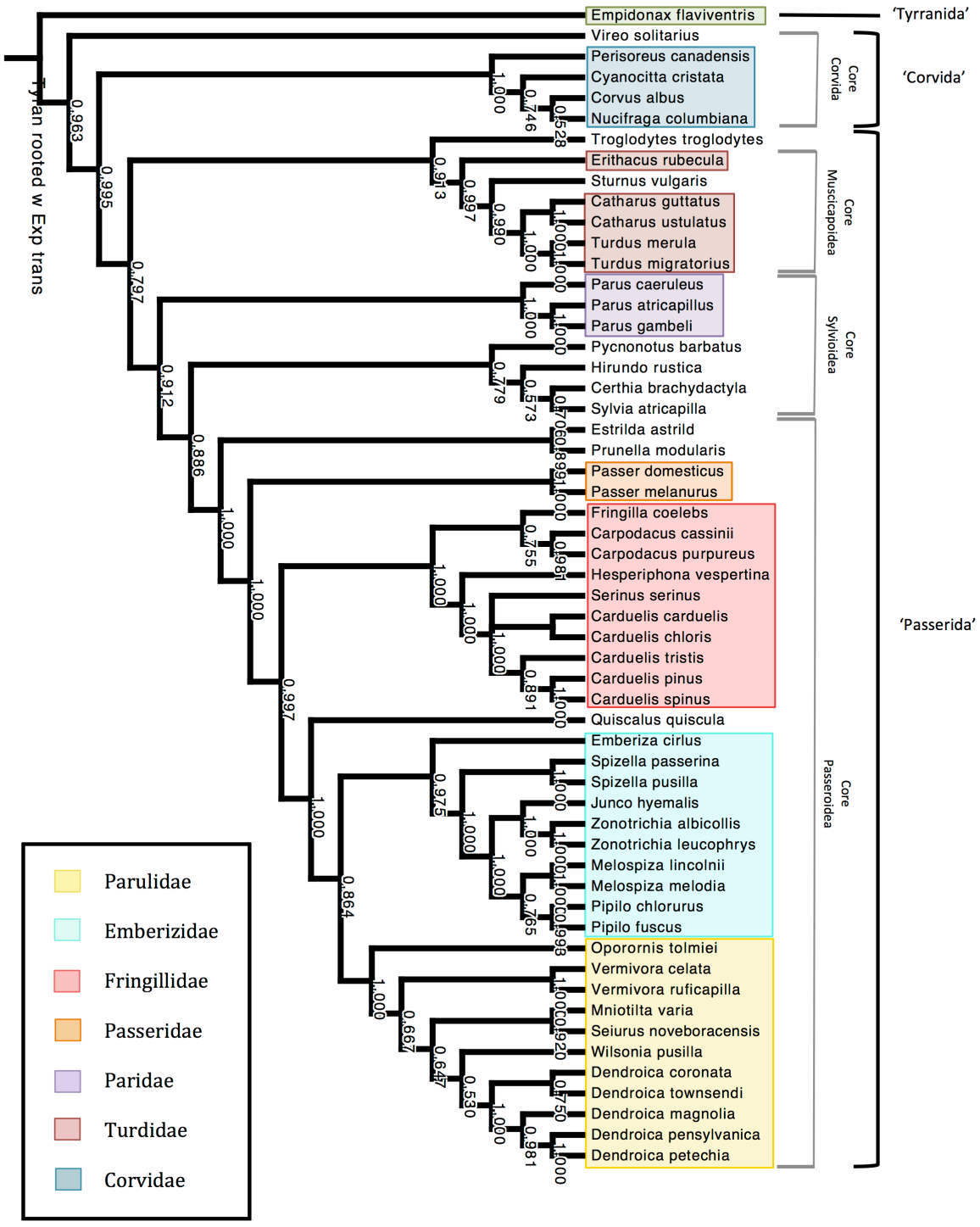

Figure 4.1. Phylogeny for 56 species of passerines estimated by Bayesian Inference in Mr.Bayes 3.2. This is a majority-rule consensus tree with the respective posterior probability of each node, obtained by analysis of combined protein-coding sequences of mitochondrial cytochrome oxidase I, cytochrome b, ND2, and ND3 and of nuclear RAG-1 sequence data.

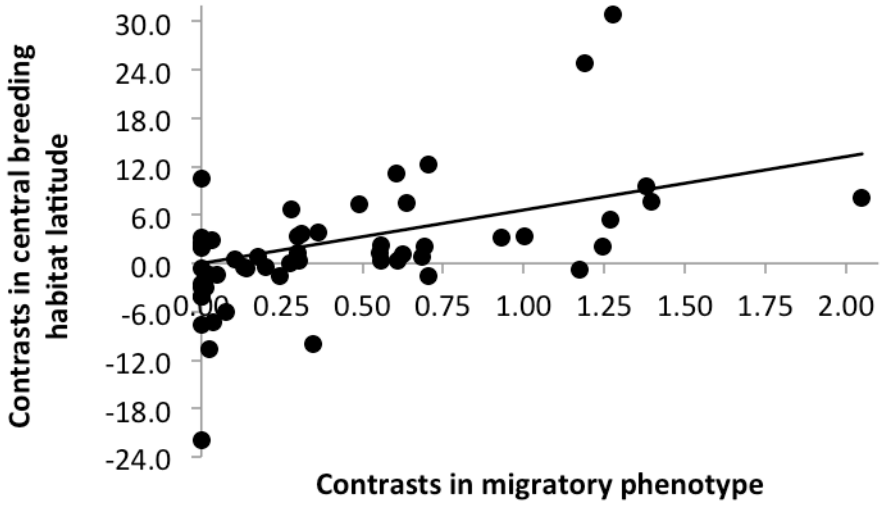

Figure 4.2. **Regression (through the origin) of phylogenetically independent contrasts in migratory phenotype on contrasts in central breeding habitat latitude across 56 passerine species. In order to allow for continuous data analysis, migratory phenotype was classified from lowest to highest selective pressure as follows: sedentary = 1, partial migrant = 2, populations of partial and complete = 3, and complete migrant = 4.**

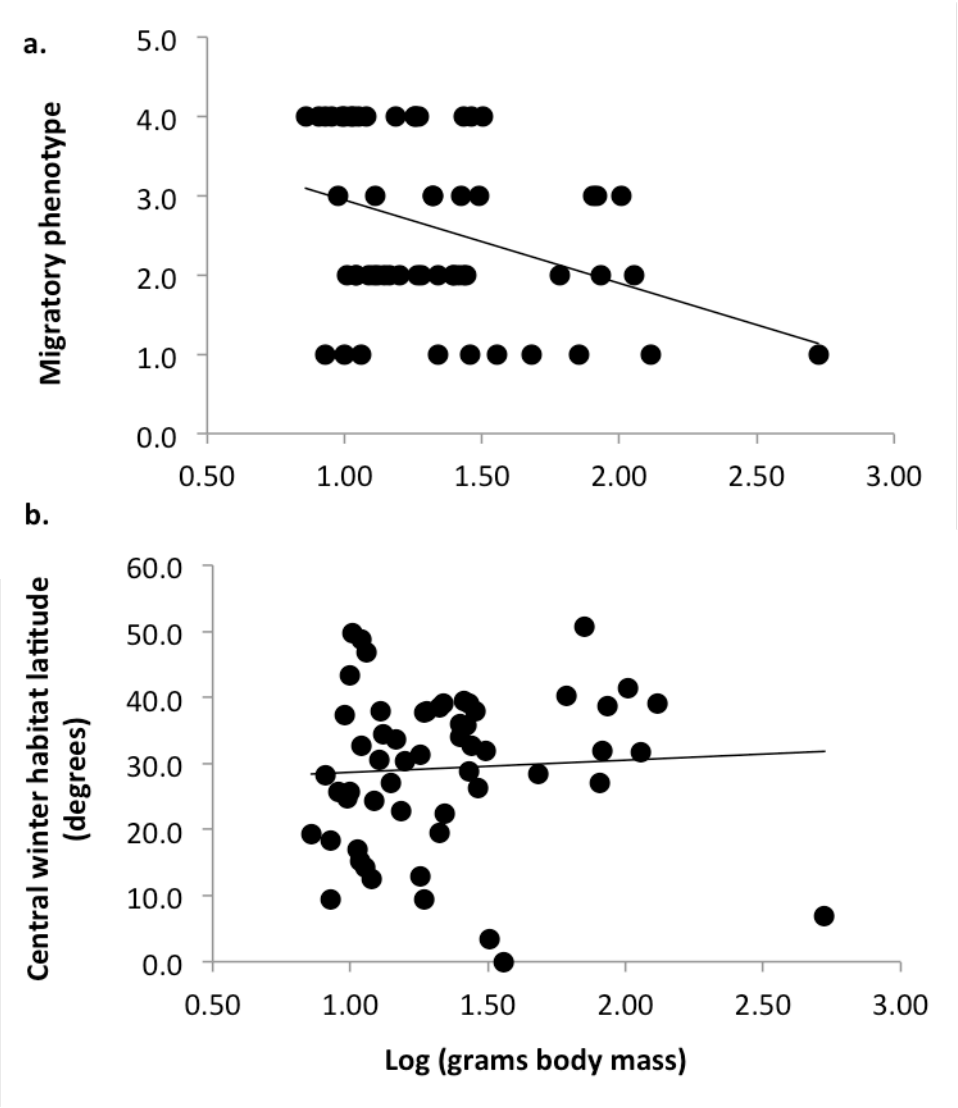

Figure 4.3. **Regression of log (body mass) on a) migratory phenotype and b) central winter habitat latitude, across 56 passerine species. In order to allow for continuous data analysis, migratory phenotype was classified from lowest to highest selective pressure as follows: sedentary = 1, partial migrant = 2, populations of partial and complete = 3, and complete migrant = 4.**

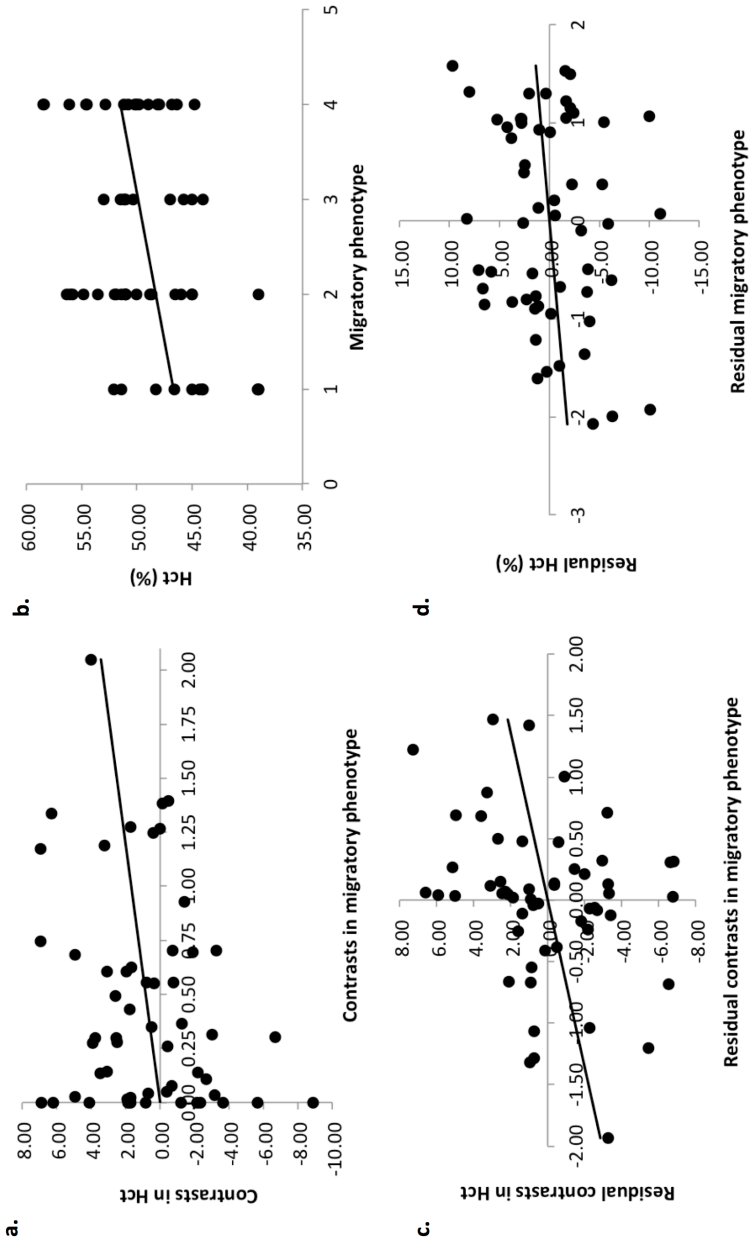

Figure 4.4. Regressions of a) phylogenetically independent contrasts in migratory phenotype on contrasts in hematocrit (Hct) through the origin, b) migratory phenotype on hematocrit, c) residuals from regressions of contrasts in migratory phenotype on contrasts in body mass and the residuals from hematocrit on contrasts in body mass through the origin, and d) residuals from regressions of migratory phenotype on body mass and the residuals from hematocrit on body mass, across 56 passerine species. In order to allow for continuous data analysis, migratory phenotype was classified from lowest to highest selective pressure as follows: sedentary = 1, partial migrant = 2, populations of partial and complete = 3, and complete migrant = 4.

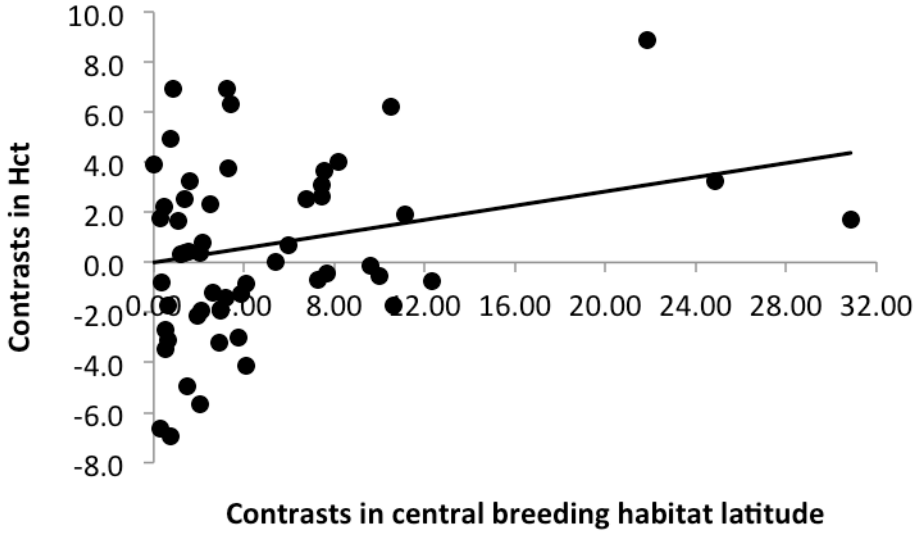

Figure 4.5. **Regressions (through the origin) of phylogenetically independent contrasts in central breeding habitat latitude on contrasts in hematocrit (Hct), across 56 passerine species.**

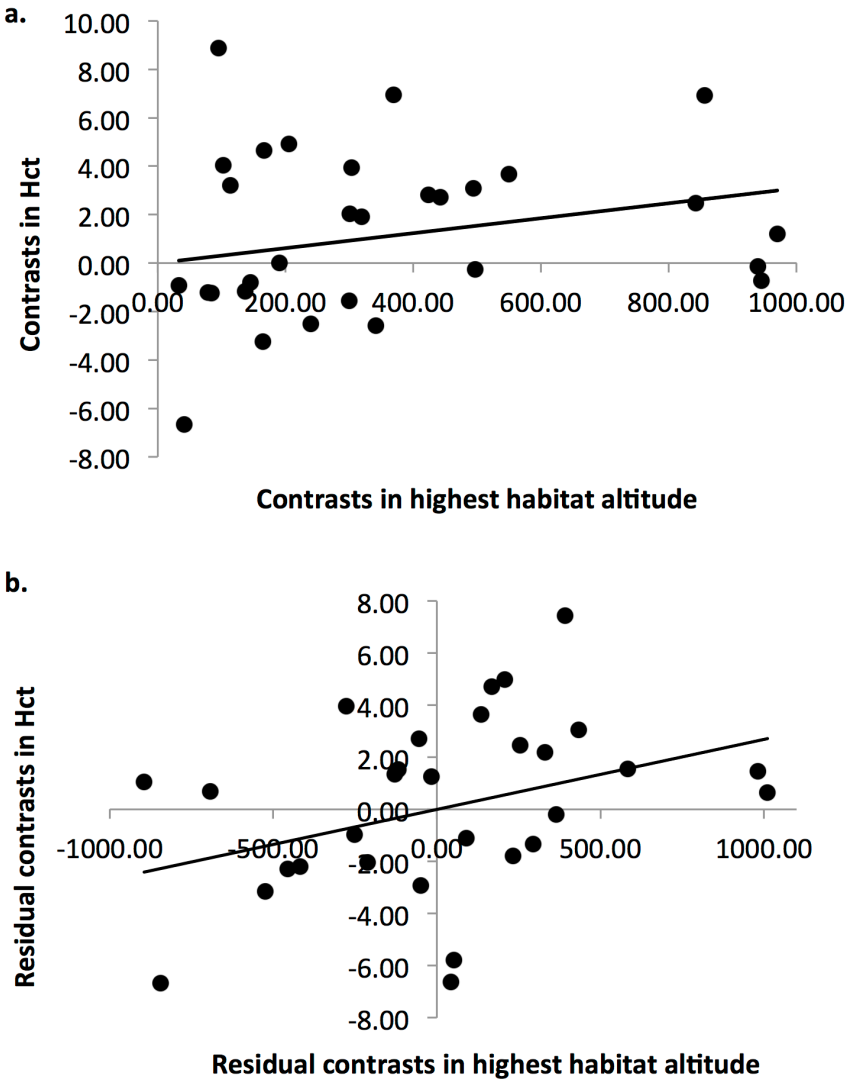

Figure 4.6. **Regressions through the origin of a) phylogenetically independent contrasts in hematocrit (Hct) on contrasts in highest habitat altitude b) residuals from regressions of contrasts in hematocrit on contrasts in body mass and the residuals from highest habitat altitude on contrasts in body mass, across 31 passerine species.**

Table 4.1. Raw dataset for 56 passerine species.

Genus species	Common name	Log (mass)	Hct %	Migratory Phenotype Code	Central winter habitat latitude (abs deg)	Central breeding habitat latitude (abs deg)	Highest Habitat Altitude (m)
Carduelis carduelis	European goldfinch	1.20	51.00	2	30.38	61.50	.
Carduelis chloris	European greenfinch	1.44	45.00	2	32.75	62.50	.
Carduelis pinus	Pine Siskin	1.16	52.04	2	33.75	56.00	.
Carduelis spinus	Eurasian Siskin	1.11	51.00	3	38.00	56.75	.
Carduelis tristis	American Goldfinch	1.12	56.35	2	34.38	42.00	.
Carpodacus cassinii	Cassin's Finch	1.43	56.05	2	28.88	50.25	.
Carpodacus purpureus	Purple Finch	1.41	54.83	2	39.50	54.75	.
Catharus guttatus	Hermit Thrush	1.49	51.20	3	31.88	49.75	3500
Catharus ustulatus	Swainson's Thrush	1.51	58.40	4	3.38	50.88	3800
Certhia brachydactyla	Short-toed Treecreeper	1.00	44.00	1	43.25	43.25	2500
Corvus albus	Pied Crow	2.72	39.00	1	6.88	6.88	3700
Cyanistes caeruleus	Common Blue Tit	1.04	39.00	2	48.75	48.75	3500
Cyanocitta cristata	Blue Jay	1.93	48.80	2	38.75	41.50	.
Dendroica coronata	Yellow-rumped Warbler	1.09	50.99	2	24.38	49.38	1140
Dendroica magnolia	Magniola Warbler	0.93	48.10	4	18.25	50.25	1500
Dendroica pensylvanica	Chestnut-sided Warbler	1.08	52.85	4	12.50	45.00	.
Dendroica petechia	Yellow Warbler	0.99	50.20	4	24.75	49.25	3200
Dendroica townsendi	Townsend's Warbler	0.91	50.80	4	28.25	55.25	2500
Emberiza cirlus	Cirl Bunting	1.40	39.00	2	36.00	40.75	.
Empidonax flaviventris	Yellow-bellied Flycatcher	1.05	49.00	4	14.25	50.75	1500

Genus species	Common name	Log (mass)	Hct %	Migratory Phenotype Code	Central winter habitat latitude (abs deg)	Central breeding habitat latitude (abs deg)	Highest Habitat Altitude (m)
Erithacus rubecula	European Robin	1.28	45.00	2	37.88	56.75	.
Estrilda astrild	Common Waxbill	0.93	44.30	1	9.38	9.38	.
Fringilla coelebs	Common Chaffinch	1.34	46.00	2	39.00	57.00	.
Hesperiphona vespertinus	Evening Grosbeak	1.79	55.85	2	40.25	40.25	2695
Hirundo rustica	Barn Swallow	1.27	56.10	4	9.50	46.00	3000
Junco hyemalis hyemalis	Dark-eyed Junco	1.27	51.43	2	37.75	57.50	3775
Melospiza lincolnii	Lincoln's Sparrow	1.26	51.17	4	31.38	52.00	.
Melospiza melodia	Song Sparrow	1.40	51.83	2	34.00	53.00	.
Mniotilta varia	Black-and-white Warbler	1.03	46.40	4	15.25	49.25	.
Nucifraga Columbiana	Clark's Nutcracker	2.11	52.05	1	39.00	39.00	3840
Oporornis tolmiei	MacGillivray's Warbler	1.03	58.45	4	17.00	46.75	3000
Passer domesticus	House Sparrow	1.45	48.25	1	38.00	38.00	.
Passer melanurus	Cape Sparrow	1.34	39.10	1	22.50	22.50	.
Perisoreus canadensis	Grey Jay	1.85	46.60	1	50.75	50.75	.
Pipilo chlorurus	Green-tailed Towhee	1.46	50.00	4	26.25	40.50	3200
Piplio/Pyrgisoma fuscum	Canyon Towhee	1.68	44.10	1	28.50	28.50	3050
Poecile atricapillus	Black-capped Chickadee	1.01	48.63	2	49.75	49.75	3200
Poecile gambeli	Mountain Chickadee	1.06	51.40	1	46.75	46.75	3660
Prunella modularis	Dunnock	1.32	47.00	3	38.50	57.75	2600
Pycnonotus barbatus	Common Bulbul	1.56	45.00	1	0.00	0.00	3000
Quiscalus quiscula	Common Grackle	2.05	46.50	2	31.75	49.50	.

Genus species	Common name	Log (mass)	Hct %	Migratory Phenotype Code	Central winter habitat latitude (abs deg)	Central breeding habitat latitude (abs deg)	Highest Habitat Altitude (m)
Seiurus noveboracensis	Northern Waterthrush	1.26	49.80	4	13.00	52.88	3000
Serinus serinus	European Serin	1.04	50.00	2	32.75	53.88	.
Spizella passerina	Chipping Sparrow	1.15	53.50	2	27.00	49.75	.
Spizella pusilla	Field Sparrow	1.11	51.00	2	30.50	43.13	.
Sturnus vulgaris	European Starling	1.92	45.78	3	32.00	51.38	2500
Sylvia atricapilla	Blackcap	1.32	44.00	3	19.50	51.75	2200
Troglodytes troglodytes	Northern Wren	0.98	53.00	3	37.25	55.75	4575
Turdus merula	Common Blackbird	2.01	45.00	3	41.50	44.75	2700
Turdus migratorius	American Robin	1.91	50.34	3	27.00	55.75	3500
Vermivora celata	Orange-crowned Warbler	0.95	54.63	4	25.75	50.38	3350
Vermivora ruficapilla	Nashville Warbler	1.00	44.80	4	25.75	45.75	2134
Vireo solitarius	Blue-headed Vireo	1.19	48.00	4	22.75	48.75	2700
Wilsonia pusilla	Wilson's Warbler	0.86	54.53	4	19.25	51.00	2700
Zonotrichia albicollis	White-throated Sparrow	1.43	46.83	4	39.00	53.50	.
Zonotrichia leucophrys	White-crowned Sparrow	1.42	51.47	3	35.88	55.75	.

Table 4.2. **Regressions of mass, hematocrit (Hct) and mass-corrected hematocrit [Hct (mc)] with seasonal habitat latitude (absolute degrees), highest habitat altitude and migratory phenotype, corrected and uncorrected for phylogeny, across 56 passerine species. Regressions of phylogenetically independent contrasts were forced through the origin. In order to allow for continuous data analysis, migratory phenotype was classified from lowest to highest selective pressure as follows: sedentary = 1, partial migrant = 2, populations of partial and complete = 3, and complete migrant = 4. Significant *P*-values are in bold.**

		Raw data			Phylogenetically independent contrasts		
		Mass	**Hct**	**Hct (mc)**	**Mass**	**Hct**	**Hct (mc)**
Mass (g)	n		56			55	
	r^2		0.06			0.065	
	t		-1.87			-1.94	
	P		0.07			0.06	
Central winter habitat (absolute degrees latitude)	n	56	56	56	55	55	56
	r^2	0.003	0.005	0.003	0.009	0.004	0.004
	t	-0.41	0.50	0.41	-0.72	-0.46	-0.44
	P	0.69	0.62	0.68	0.48	0.64	0.66
Central breeding habitat (absolute degrees latitude)	n	56	56	56	55	55	55
	r^2	0.09	0.12	0.0006	0.06	0.10	0.08
	t	-2.36	2.77	-0.18	-1.80	2.50	2.13
	P	**0.02**	**0.008**	0.86	0.08	**0.015**	**0.04**
Highest habitat altitude (m)	n	31	31	31	30	30	30
	r^2	0.08	0.03	0.08	0.02	0.15	0.13
	t	1.56	0.98	1.57	-0.81	2.28	2.10
	P	0.13	0.33	0.13	0.42	**0.031**	**0.04**
Migratory phenotype	n	56	56	56	55	55	55
	r^2	0.14	0.86	0.08	0.04	0.10	0.08
	t	-3.01	18.53	2.15	-1.53	2.51	2.19
	P	**0.004**	**<0.0001**	**0.04**	0.11	**0.01**	**0.03**

Chapter 5.

General Conclusions

5.1. Introduction

Traits related to energy expenditure are believed to be primary components in the evolution of life history traits (Careau & Garland 2012). This is because selection acts most directly on variation at the level of the whole organism i.e., behavioral and performance traits (often used as proxies of fitness) and behavioral and performance traits are bound by energetic constraints and traits that limit physical activity (see Fig. 1). Given the role of hematocrit in oxygen delivery and the fact that the oxygen carrying capacity of the blood is a limiting factor for the intensity of physical activity that can be sustained (reviewed in Jelkmann & Lundby 2011; Dlugosz *et al.* 2013), intraspecific hematocrit variation plays a potential role in influencing traits that are associated with fitness. For the same reason, interspecific hematocrit variation is a potential product of past selective pressures. The principal goals of this thesis were to determine the functional significance of intraspecific hematocrit variation and the potential selective pressures that may have shaped the variation in hematocrit observed among bird species today.

5.1.1. General limitations

Measuring fitness, or lifetime reproductive success, in natural populations is exceedingly difficult; therefore, traits that are considered to contribute to fitness are used

as proxies. However, this limits the inferences we can draw. The principal life history traits measured here [amongst others outlined by Stearns (1992)] which include reproductive schedule, size at birth, and number, size and sex ratio of offspring, are used to estimate fitness in population demographic growth models and are therefore considered to be directly related to fitness.

5.1.2. Inferring the functional significance of intraspecific hematocrit variation

Intraspecific hematocrit variation could influence fitness indirectly if it plays a role in regulating eggshell coloration or directly by limiting physical endurance and in so doing shaping reproductive decisions.

5.2. Eggshell color

The preservation of conspicuously colored blue-green eggshells alludes to having some adaptive function. Although I found some evidence to suggest that the magnitude of blue-green eggshell color may reflect brood quality, there was little to suggest that hematocrit or hemoglobin was involved in determining the magnitude of eggshell color.

The sexual selection of eggshell color hypothesis posits that biliverdin, the pigment responsible for blue-green eggshell color, is a limiting resource constraining eggshell coloration and thereby ensuring an honest signal of quality (Moreno & Osorno 2003). Biliverdin is derived from the decomposition of hemoglobin via HO-1, however the origin of eggshell biliverdin and its constituents is still unknown. Results from chapter two provide no evidence that circulating hemoglobin or its constituents limit eggshell coloration. This conclusion is consistent with previous research exhibiting that biliverdin is excreted in copious amounts (Zhao *et al.* 2006) and very little is recycled in the synthesis of new hemoglobin (London *et al.* 1949). The lack of correlation found between circulating resources and eggshell colour suggests that HO-1 activity within the eggshell gland (Wang *et al.* 2011) or constituents involved in the deposition of biliverdin from the shell gland to the egg (Sparks 2011) may be the limiting factor in eggshell

pigmentation. Previous research has found greater HO-1 mRNA expression and activity in blue-shelled chickens when compared to brown-shelled chickens (Wang *et al.* 2010, 2011), although no difference in HO-1 activity was found between blue-shelled ducks and white-shelled ducks (Liu *et al.* 2010). Therefore, future research could determine if differences in HO-1 activity explains individual variation in eggshell color. In addition, future research should aim to clarify the components involved in the transfer of pigments from shell gland to eggshell and whether this is a limiting step in pigmentation.

An unexpected positive correlation was observed between eggshell color and chick hemoglobin. In order for avian embryos to synthesize hemoglobin, they must have access to enough iron in the egg. The embryo's major source of iron is deposited in the egg yolk, however the shell was recently found to be a minor source of iron for developing chicks (Yair & Uni 2011). In fact, 99% of the iron deposited in the shell is released to the embryo during incubation (Yair & Uni 2011). Although the contribution of iron from the shell is minor relative to the yolk, iron has been considered a limiting mineral and nearly all of the iron content in the egg is required by the developing embryo (Elvehjem *et al.* 1929). Considering that the products of heme degradation are biliverdin and iron, further investigation into the relationship between eggshell color, egg iron content, and chick hemoglobin could yield interesting results, particularly in light of the fact that neonatal hematocrit has recently been found to predict longevity in a wild population of house wrens (*Troglodytes aedon*; Bowers *et al.* 2014).

5.3. Reproductive success

This research provides several lines of evidence, which indicate that individual variation in hematocrit could lead to differences in fitness, reflecting functional significance. Both naturally and experimentally low hematocrit and hemoglobin resulted in a delay in the timing of reproduction, which has significant negative consequences for offspring survival and long-term success (Perrins 1970; Nilsson & Smith 1988; Nilsson 1990, 1999; Verhulst & Tinbergen 1991; Brinkhof *et al.* 1993; Blums *et al.* 2002, 2005; Blums & Clark 2004; Verhulst & Nilsson 2008; Grüebler & Naef-Daenzer 2008, 2010) . In addition, individuals with naturally higher hemoglobin levels during incubation fledged a greater number of chicks. Likewise, a reduction in Hct and Hb during incubation 1)

decreased hatchling size in all years and, 2) decreased chick size and number during one year in which environmental conditions appeared inferior. This context dependence suggests that effects of reduced Hct and Hb may be amplified when conditions are poor. Similarly, reproductive costs have been found to differ in magnitude in different environments, often exhibiting fitness effects or trade-offs only under stressful conditions (Tuomi *et al.* 1983; Reznick 1985; Stearns 1989; Marden *et al.* 2003; Fricke *et al.* 2009; Fricke *et al.* 2010; Cornwallis & Uller 2010; Careau & Garland 2012). The influence of hematocrit on reproductive success identified by this thesis is corroborated by studies in which maternal hematocrit in humans was negatively associated with low birth weight and premature births (Lieberman *et al.* 1988; Levy *et al.* 2005).

Future research should aim to identify the mechanisms by which hematocrit influences reproductive timing and success. For instance, with regard to the delay in egg laying, we know that hematology changes during egg production in captive birds (Wagner *et al.* 2008a; Wagner *et al.* 2008b) but it is currently unknown how hematocrit may influence pre-laying foraging or aspects of egg production, such as vitellogenesis. With regard to how hematocrit affects chick hatching and rearing, studies should focus on the influence of hematocrit on foraging behavior during incubation and on the duration of incubation bouts. This and total provisioning throughout chick rearing could be studied in depth by using nest box cameras. This would enable all provisioning attempts to be recorded as well as enabling a non-invasive estimate of feeding quantity and quality. Subsequent research should incorporate various ecological conditions and use free-living populations as much as possible. In addition, as data on post-fledging success are difficult to obtain, using species that have high recruitment would offer better estimates of fitness and, combined with cross-fostering, better estimates of heritability. Finally, future studies could experimentally manipulate hematocrit and test for differences in flight performance using a wind tunnel.

5.4. Selective pressures that may have shaped interspecific hematocrit variation

If variation in hematocrit had functional significance in the past, we should expect to find relationships between species-specific hematocrit and ecological factors. The

results from chapter four suggest that habitat altitude, migration phenotype and breeding habitat latitude were past selective pressures that shaped the current variation in hematocrit among passerines species. This suggests that the sustained periods of aerobic metabolism required by migratory flight and by thermogenesis at colder high latitude habitats, particularly during the energy demanding breeding season, have exerted significant selective pressure on hematocrit. Similarly, species that inhabit higher altitudes have higher hematocrit likely due to the selective pressure imposed by the reduction in oxygen availability.

The significance of avian hematocrit supported by this thesis is corroborated in bats in which it is believed that high hematocrit is an adaptation to the energetic constraints of flight (reviewed in Maina 2000). In fact, there are several species of bats that can only attain flight once their hematocrit levels reach adult status (Bassett & Wiederhielm 1984; but reviewed in Maina 2000). In addition, it has been proposed that relative to birds, the geographic distribution of bats has been largely limited to the warm tropical and subtropical regions as a result of the high demands for oxygen and relatively less efficient mammalian lung relative to the avian lung (reviewed in Maina 2000).

The phenotypic plasticity of hematocrit makes it difficult to determine whether the variation in hematocrit correlated with habitat latitude, habitat altitude, and migration is a result of adaptation or acclimatization. Future studies incorporating species-specific magnitudes of phenotypic plasticity (e.g., reaction norms) would be helpful (see Stearns 2000). Although I attempted to control for the influence of acclimatization when comparing species, acclimatory responses may play an important role by facilitating adaptation as proposed by the Baldwin Effect (see Milo *et al.* 2007). Several mechanisms for the Baldwin Effect have been suggested. For instance, acclimatory changes may enable individuals to survive a change in environment long enough for selection to yield a beneficial adaptation. Alternatively, the mechanistic parameters that facilitate acclimation can act as a platform for genetic assimilation (Waddington 1957; West-Eberhard 2003; Bateson 2014; Noble *et al.* 2014), a concept related to the fact that evolution modifies existing traits. An empirical example of this was demonstrated in the house finch (*Carpodacus mexicanus*) populations that extended their range north (Badyaev 2009). Initially, incubation behavior and an ovulation order sex bias were

dependent upon ambient temperature cues. This enabled populations to take advantage of colder northern habitats. Following their move, the northern house finch populations spontaneously began to express these traits without the need for external temperature cues (Badyaev & Uller 2009).

While we did identify correlations between species-specific hematocrit and relevant ecological factors, this analysis is unable to differentiate between adaptation and exaptation. Therefore, rather than interpreting high hematocrit as being an adaptive response to selective pressures, it could be interpreted as being a pre-existing exaptation by which high hematocrit in some species facilitated the exploitation of habitats and activities that were inaccessible to those species with lower hematocrit. By estimating and tracing ancestral trait values and corresponding selective regimes, further studies could help identify whether current observed interspecific hematocrit variation is the cause for or the result of these lifestyle & habitat differences among Passerines.

Finally, studies that employ geographical ranges are complex due to unequal abundance distributions and intra-range biotic and abiotic variation, which presents the need for further large-scale observational studies to assess spatial patterns of individuals and populations (see (Sagarin *et al.* 2006). Greater distribution accuracy would enable greater accuracy of the average estimates of relevant ecological factors.

5.5. Summary and broad implications

The results presented in this thesis suggest that intraspecific hematocrit variation is currently functionally significant and appears to have been functionally significant in the past. The importance of hematocrit being functionally significant is that it confirms two of the three requirements of natural selection, first that there is existing phenotypic trait variation, and second, differential fitness. Heritability of this variation is the third requirement, and previous studies suggest that hematocrit variation is heritable in birds (see chapter four for review). However, exciting new evidence is challenging our traditional concept of heritability, i.e., the genetic inheritance of phenotypic differences (reviewed in Bateson 2014; Danchin & Pocheville 2014; Noble *et al.* 2014). The new emergent concept of heritability is a more inclusive heritability (Danchin & Wagner 2010;

Danchin *et al.* 2011) and proposes that acclimation (a.k.a. accommodation) which represents the phenotypic plasticity of a trait, plays an important role in evolution such as in the Baldwin Effect discussed previously. This presents a plethora of questions including how the magnitude of plasticity in hematocrit influences factors such as adaptation and selection for inherited epigenetic changes (see West-Eberhard 2003; Noble *et al.* 2014).

Finally, the integration of such research on physiological variation is now being used 1) to bridge gaps between performance, energy, behavior, ecological context, and fitness (Careau & Garland 2012) as well as 2) to understand population dynamics, demography, biodiversity, and responses to climate change (Stearns 2000; Chown *et al.* 2010; Moran, Hartig & Bell 2015).

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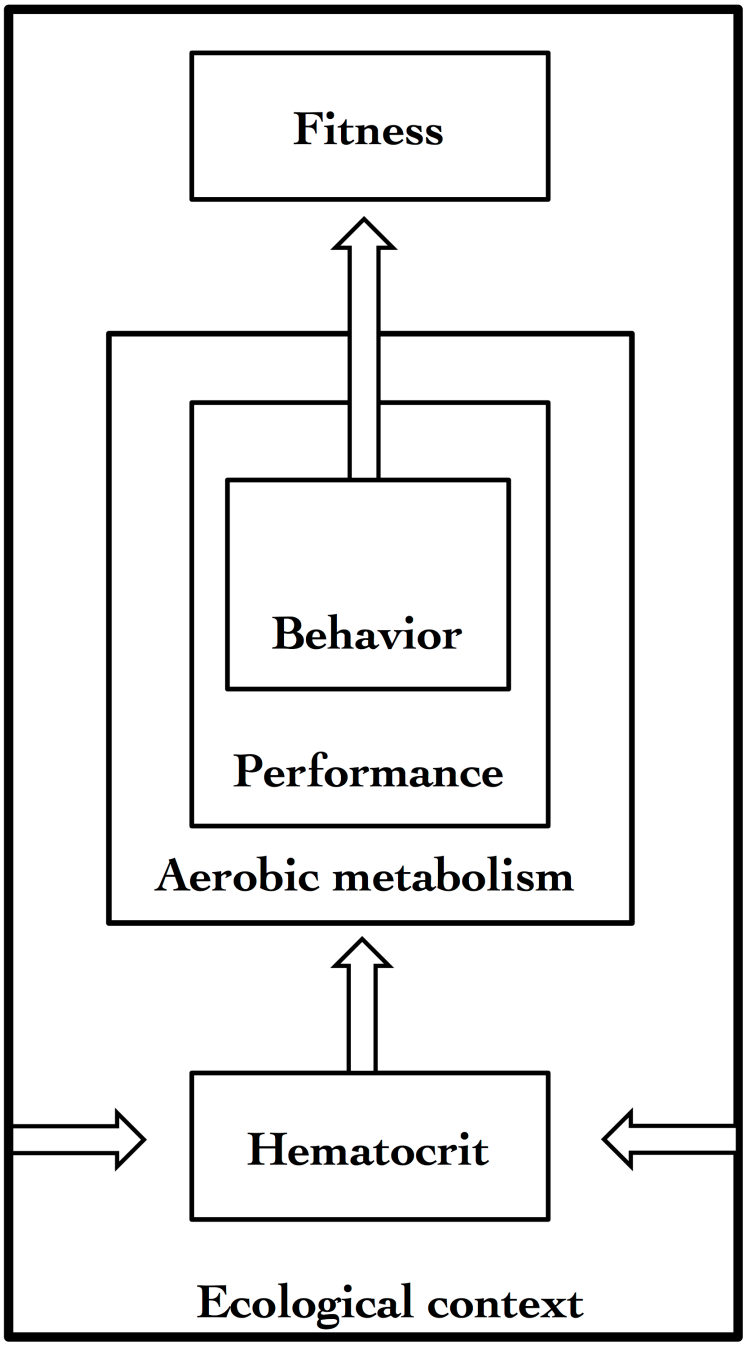

Figure 5.1. **Hematocrit-performance-behavior-fitness framework, highlights the conceptual and functional links between physiology (identified as hematocrit here), performance, behavior, and fitness as well as its context dependence. Performance and behaviour are bound by aerobic capacity or traits that limit physical activity and the entire framework is enveloped by the ecological context, which can affect the relationships among all of the parameters.**