Physiological Basis of Aerobic Capacity and Workload Ability in Birds

by

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B.A., University of British Columbia, 2013

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY
Summer 2018

Approval

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Abstract

Many behaviours exhibited by free-living animals that are crucial for survival and reproduction involve elevated levels of activity or “workload”. Individuals with higher workload ability should be able to cope with the high metabolic demands imposed by these behaviours better and consequently would have higher fitness. High workload could also result in costs such as impaired reproduction or reduced survival. However, the underlying physiological mechanisms that allow individuals to have higher workload ability, and the mechanisms underlying costs of high workload remain poorly understood. This thesis took an exercise perspective and investigated the physiological basis of aerobic capacity and workload ability in birds, using both a comparative, phylogenetic approach, as well as various laboratory-based experimental approaches. In surveying the literature, we identified several potential common physiological markers underlying individual variation in exercise performance and costs of exercise. We also found that hematological traits co-vary with life-history variables, and to a certain extent, energy metabolism in birds at the interspecific level. Additionally, we provided experimental evidence for physiological responses to flight at high attitude and showed that the relationship between hematocrit and flight performance is dependent on altitude. Lastly, we provided experimental evidence for behavioural and physiological adjustments to high workload and demonstrated that physiological adjustments to high workload can negatively impact reproduction. Taken together, this thesis uncovered several physiological mechanisms underlying workload ability and costs of high workload in birds. Future work should consider and integrate multiple physiological systems when studying the physiological basis of workload ability, and more generally, life-history trade-offs in animals. Ultimately, we hope that the knowledge we gain from this thesis can be used to complement studies in free living animals and aid in the design of field experiments.

Keywords: Exercise physiology, life-history trade-offs, reproduction, energy metabolism, training

This thesis is dedicated to my beloved grandmother, who taught me the meaning of unconditional love. And to Tony D. Williams, who doesn't get enough credit for how inspiring and amazing he is as a mentor and as an individual.

Acknowledgements

I would like to give a huge thank you to the best supervisor and mentor anyone could ever ask for, Tony Williams. Thank you for being so supportive over the years, for always having my back when things didn't go well, and most importantly, for making me the scientist I am today. I would also like to thank my undergraduate mentor, Dr. Nora Prior for introducing me to biological research when I was a naïve undergraduate.

Thank you to my dissertation committee members, Dr. Julian Christians and Dr. Doug Altshuler, who have been very accommodating and generous with their time. I am grateful for their constructive feedback on experimental design and various drafts of my manuscripts. I also appreciate their continuous effort in pushing me to think critically about both the big picture questions, as well as the details of my thesis. I would also like to thank Dr. Gordon Rintoul and Dr. Scott McWilliams for serving as the internal and external examiners, and Dr. Jenny Cory as chair for my thesis defence.

I would also like to acknowledge my collaborators Dr. David Swanson, Dr. Yufeng Zhang, Dr. Don Powers, and Dr. Doug Altshuler and his lab group for contributing to my research work. Special thanks to Dr. Christopher Guglielmo and the Advanced Facilities for Avian Research for hosting me and allowing me to use their hypobaric wind tunnel to conduct my experiment for Chapter 4 of this thesis. Dr. Arne Mooers and Dr. Bernie Crespi provided invaluable guidance with statistical analysis for Chapter 3 of this thesis.

Graduate school would not have been nearly as fun without my amazing lab mates. Thank you to Dr. Melinda Fowler, Dr. Margaret Eng, Dr. Allison Cornell, Mitchell Serota, Olga Lansdorp, Chloe Boynton, Maria Yu, Spencer Morran, Cybele Heddle, Mason King, Elizabeth Ruberg, Victor Fuhrer, and Chloris Maury for putting up with my boring presentations, endless rants, and more importantly for all the moral and emotional support over the years. Shout out to Allison Cornell for being the best desk mate for most of my PhD. Special thanks to the 'big sisters' of the Williams Lab, Drs. Eunice Chin and Katrina Salvante for their friendship and wise advice. I would also like to acknowledge members of the Guglielmo lab, specifically Dr. Morag Dick, and members of the McWilliams lab for their hospitality at AFAR.

This work would not have been possible without my undergraduate assistants and animal care staff at both SFU and AFAR. Thank you to all the work study students and animal care staff for keeping the birds happy and healthy. I am indebted to Karilyn Harris, Oh Run Kim, Alex Macmillan, Sarosha Ali, Melissa Vermette, and Olivia Tsai, whom I consider myself extremely lucky to mentor, for their significant contribution to my thesis work, as well as for their emotional support and continued friendship.

Despite not knowing much about my thesis research, my family have been my biggest supporters by far. I am thankful to my parents for allowing me to freely explore different avenues in life while being supportive along the way, and to my brother for providing the much needed warmth of home in a foreign country. Thank you to the Vung family, the Gui family, and my ex-housemates at 5829 for giving me a home away from home. Thank you to my tennis buddy Tay and the SFU badminton group, specifically Emelia Kirkwood, for providing pleasant distractions during stressful phases of my thesis.

All the staff members and technicians in the biology department have been incredibly helpful with administrative work and logistics. I have lost count of how many times I ran up to their offices and pester them with stupid questions. Special thanks to Dr. Carl Lowenberger and Dr. Bernie Crespi, who despite not being directly involved in my thesis work, have always given me helpful academic and career advice.

Throughout my time at SFU, I have been extremely fortunate to be able to work with and TA for some incredible instructors. Peter Hollman, Joan Sharp, Mala Fernando, Dr. Tony Williams, and Dr. Gordon Rintoul have opened my eyes to the world of university teaching. I also had a great experience working with the STEPS program, particularly Jasleen Arora during my first TA appointment. My teaching skills have improved tremendously since the beginning because of them.

This work was funded by the Natural Sciences and Engineering Research Council (NSERC), the Canada Foundation for Innovation (CFI), and multiple graduate fellowships and travel grants awarded by SFU.

Table of Contents

List of Tables

List of Figures

Chapter 1.

Introduction

Exercise Physiology in Birds

Many behaviours exhibited by free-living animals such as migration, foraging, escaping from predators, and thermoregulation involve elevated levels of activity or "workload" (Halsey, 2016; Killen et al., 2017; Sinclair et al., 2014; Yap et al., 2017). These energetically demanding behaviours are often crucial for survival and successful reproduction of animals. It is often assumed that individuals with higher aerobic capacity should be able to cope with the high metabolic demands imposed by these behaviours better and consequently would have higher reproductive success than other individuals. High workload could also result in negative consequences or costs such as impaired reproduction or reduced survival (Briga et al., 2017; Daan et al., 1996; Husak and Lailvaux, 2017; Veasey et al., 2001; Wiersma, 2005). However, the underlying physiological mechanisms that allow individuals to have higher workload ability, as well as the mechanisms underlying costs of high workload, remain poorly understood (Harshman and Zera, 2007; Husak and Lailvaux, 2017; Killen et al., 2017; Piersma and van Gils, 2011; Williams, 2012).

Furthermore, different behaviours or activities vary in duration, intensity, and aerobic scope, and thus might require different physiological responses (Piersma, 2011; Piersma and van Gils, 2011). While the physiology of some high intensity behaviours like migration (Guglielmo, 2010; Guglielmo, 2018; Pierce and McWilliams, 2014) and thermoregulation (Bicego et al., 2007) are relatively well-studied, the physiology of other sustained, the physiology of lower intensity behaviours such as foraging (Maurer, 1996) and parental care (Clutton-Brock, 1991) remain largely unstudied. Additionally, studies investigating physiology in animals are often conducted in conditions very different from that free-living animals experience, divorced from the critical relationship in free-living animals between exercise and acquisition of resources (Fonseca et al., 2014; Yap et al., 2017).

Exercise can be broadly defined as any behaviour that elevates the level of intensity of activity or workload (Booth et al., 2012; Halsey, 2016; Sinclair et al., 2014), often in response to an ecological demand for increased performance. Therefore, given the high activity level and metabolic demand associated with many of the aforementioned behaviours (Piersma, 2011), and the positive relationship between performance and fitness (Irschick, D. J., & Higham, 2016; Lailvaux, S. P., & Husak, 2014), it might be valuable to apply an exercise perspective on the physiology underlying these behaviours.

The overarching goal of this thesis is to investigate the physiological basis of aerobic capacity and workload ability in animals, using birds as our model species. Chapter 2 reviews laboratory-based model systems for exercise to ask if they can inform us about common physiological markers underlying high exercise capacity or the physiological costs of exercise. Chapter 3 considers how interspecific variation in two of the physiological markers- hematocrit and hemoglobin relate to life-history traits and energy metabolism. Chapter 4 focuses on how manipulation of hematocrit can affect flight performance. Chapter 5 and chapter 6 focus on physiological responses of exercise training and effects of training on reproduction.

Study species

Yellow-rumped warbler (*Setophaga coronate*) is a small migratory songbird that exists throughout North and Central America. Yellow-rumped warblers winter across and southern US and migrates to northern US and Canada in the spring and summer to breed (Rodewald, 2015). They have a very diverse diet, consuming mainly insects in the summer, but switching to fruits in the winter and during migration. The yellow-rumped warblers can be brought into captivity relatively easily and their flight is agile and swift (Rodewald, 2015), making them an ideal model system for laboratory-based wind tunnel studies (Guglielmo, 2018; Ma et al., 2018; Marshall et al., 2016).

Zebra finch (*Taeniopygia guttata*) is a songbird native to Australia, as well as parts of East Timor and Indonesia. They are gregarious and form a life-long monogamous pair bond (Zann, 1994). They can also be domesticated relatively easily

and they breed readily in captive condition (Griffith et al., 2017). More importantly, they can be manipulated relatively easily in experimental settings. This, together with the wealth of knowledge we possess about the zebra finch genome (Backström et al., 2010; Griffith and Buchanan, 2010), life-history (Zann, 1994), and reproductive performance (Griffith et al., 2017), make the zebra finch arguably the most important avian model species for research across a broad range of fields (Griffith et al., 2017). Zebra finches typically live for about one year in the wild but can live up to about eight years in captivity (Zann, 1994; Zann, 1996). Although there is considerable variation in reproductive success across individuals in nature and laboratory settings (Griffith et al., 2017; Zann, 1994), zebra finches typically lay two to eight eggs per clutch, depending on environmental and/or experimental conditions (Griffith et al., 2017; Zann, 1996).

Objectives and Content of Thesis

Chapter 2: The Physiology of Exercise in Free-Living Vertebrates: What Can We Learn from Current Model Systems?

Many behaviours exhibited by free-living animals involve exercise, which can be broadly defined as physical activity that involves movement supported by sustained locomotor performance, increased cardiac output, and increases in energy expenditure above basal levels (Booth et al., 2012; Halsey, 2016; Sinclair et al., 2014). Although there has been longstanding interest and research into the physiological mechanisms underlying variation in exercise performance, most of the work to date has been conducted in laboratory settings that are often quite removed from the animal's ecology (Fonseca et al., 2014). Chapter 2 reviews current laboratory-based model systems for exercise to 1) identify common physiological markers that underpin individual variation in exercise performance and costs of exercise, 2) investigate whether physiological responses can be influenced by the nature of activity and resource acquisition, and 3) consider the evidence that physiological adjustments to exercise directly affect survival and reproduction.

Chapter 3: Phylogenetic comparative analysis of the relationship between hematology, life-history variables and energy metabolism

Aerobic capacity is assumed to be a main predictor of endurance capacity or the ability to sustain high workload in animals (Lourdais et al., 2014; Wagner, 1996). Due to the role of hematocrit and hemoglobin in oxygen transport and delivery, these traits have been proposed as key determinants of aerobic performance (Butler, 2016; Calbet et al., 2006; Hammond et al., 2000). Hematocrit and hemoglobin also vary significantly among individuals in free-living birds throughout the annual cycle (Fair et al., 2007; Krause et al., 2016; Swanson, 1990). Together with evidence of increased hematocrit and hemoglobin in response to experimentally increased flight costs (Hõrak et al., 1998), cold acclimation (Petit and Vezina, 2014), and altitude acclimation (Borras et al., 2010), the variation of these traits likely reflects adaptive modulation of haematological traits to meet seasonal changes in energy demands. Although there have been many studies looking at intraspecific variation of hematocrit and hemoglobin (Fair et al., 2007; Minias, 2015), to our knowledge there has been no comprehensive, phylogenetically-controlled test of hypotheses for interspecific variation in hematological traits or the relationship with energy expenditure in general. Chapter 3 takes a comparative, phylogenetic approach to rigorously test several hypotheses for adaptive variation in hematocrit and hemoglobin in relation to various life-history traits and energy expenditure in birds.

Chapter 4: Effects of experimental manipulation of hematocrit on avian flight performance

Hematocrit and hemoglobin are widely assumed to be two of the key determinants of aerobic capacity and exercise performance (Böning et al., 2011; Calbet et al., 2006; Carpenter, 1975; Hammond et al., 2000). However, most studies that investigated this relationship to date have been correlational and this relationship has not always been tested experimentally and rigorously in birds. For instance, it has also been shown that hematocrit is positively correlated with exercise-induced maximal metabolic rate (VO_2max) (Hammond et al., 2000) and cold-induced summit metabolic rate (Petit and Vézina, 2014) in birds. Furthermore, unlike animals like dogs and horses, which increase hematocrit and hemoglobin in response to exercise (Wu et al., 1996),

birds decrease hematocrit in response to exercise (Jenni et al., 2006). These findings suggest that the relationships between hematocrit, aerobic capacity, and exercise performance may not be straightforward and might be taxa- or activity-specific. Chapter 4 examines how experimental manipulation of hematocrit affects flight performance at low and high altitudes using a hypobaric wind tunnel.

Chapter 5 and 6: Effects of physiological adjustments to high foraging effort on reproduction

Foraging to obtain food and to provision offspring is an important behaviour that can determine reproductive success in free-living animals (Clutton-Brock, 1991). Given that foraging at elevated rates to provision offspring is energetically expensive (Caro et al., 2016; Maurer, 1996; Piersma, 2011) and involves increased levels of activity or "workload", it seems intuitive that animals would exhibit a suite of behavioural and physiological adjustments in order to cope with the high workload (Halsey, 2016; Sinclair et al., 2014; Yap et al., 2017). For example, endurance training in lizards resulted in an increase in hematocrit and larger fast glycolytic muscle fibres (Husak et al., 2015). In birds, exercise training caused increases in pectoralis muscle citrate synthase activity and fatty acid transporters (Zhang et al., 2015). However, most studies to date only investigated transient, short-term effects of exercise training. It is unclear whether similar physiological responses will occur with long-term exercise training.

Furthermore, it has also been suggested that there are potentially costs associated with working hard to provision offspring (Yap et al., 2017). For instance, it has been shown that experimentally increased foraging and parental effort resulted in poorer body condition (Veasey et al., 2001; Wiersma, 2005), decreased survival (Briga et al., 2017; Daan et al., 1996), and impaired subsequent reproduction (Deerenberg and Overkamp, 1999; Simons et al., 2014). However, many studies also failed to find evidence for costs of reproduction (Santos and Nakagawa, 2012; Williams, 2012; Zhang and Hood, 2016), perhaps because these studies only investigated short-term costs and ignored the fact that costs can be deferred to later life-stages (Harrison et al., 2011). Furthermore, the physiological mechanisms underlying costs associated with high workload remain poorly understood at present. Chapter 5 and chapter 6 examine

physiological adjustments to high workload associated with experimentally increased foraging effort, as well as how these physiological adjustments affect subsequent reproductive performance.

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Chapter 2.
The Physiology of Exercise in Free-Living Vertebrates: What Can We Learn from Current Model Systems?

Abstract

Many behaviors crucial for survival and reproductive success in free-living animals, including migration, foraging, and escaping from predators, involve elevated levels of physical activity. However, although there has been considerable interest in the physiological and biomechanical mechanisms that underpin individual variation in exercise performance, to date, much work on the physiology of exercise has been conducted in laboratory settings that are often quite removed from the animal's ecology. Here we review current, laboratory-based model systems for exercise (wind or swim tunnels for migration studies in birds and fishes, manipulation of exercise associated with non-migratory activity in birds, locomotion in lizards, and wheel running in rodents) to identify common physiological markers of individual variation in exercise capacity and/or costs of increased activity. Secondly, we consider how physiological responses to exercise might be influenced by (1) the nature of the activity (i.e., voluntary or involuntary, intensity, and duration), and (2) resource acquisition and food availability, in the context of routine activities in free-living animals. Finally, we consider evidence that the physiological effects of experimentally-elevated activity directly affect components of fitness such as reproduction and survival. We suggest that developing more ecologically realistic laboratory systems, incorporating resource-acquisition, functional studies across multiple physiological systems, and a life-history framework, with reproduction and survival end-points, will help reveal the mechanisms underlying the consequences of exercise, and will complement studies in free-living animals taking advantage of new developments in wildlife-tracking.

Originally published as Yap, K. N., Serota, M. W., & Williams, T. D. (2017). The physiology of exercise in free-living vertebrates: what can we learn from current model systems? *Integrative and comparative biology*, 57(2), 195-206.

Introduction

Exercise can be defined as physical activity that involves movement supported by sustained locomotor performance, increased cardiac output, and increases in energy expenditure above basal levels (Piersma 2011; Booth et al. 2012). As a consequence, the term can theoretically be applied to most activities or behaviors exhibited by free-living animals, for instance, migration, foraging, finding mates and escaping predators. All of these behaviors are essential for successful reproduction and survival. Thus, it would be predicted that individuals with higher "exercise capacity," or ability to cope with the costs of exercise (i.e., reduced fecundity or survivorship due to exercise), would have higher fitness. Furthermore, there has been a long-standing interest in the physiological and biomechanical mechanisms that underpin individual variation in exercise performance (Arnold 1983; Lailvaux and Husak 2014; Storz et al. 2015). Nevertheless, to date, much work on physiology of exercise has been conducted using captive animals in laboratory settings that are often quite different from the animal's ecology, for example, wheel-running in mice, wind tunnel flights in birds, and treadmill endurance tests in lizards (see below for references). Recent technological advances in methods for wildlife tracking or bio-logging (Wilmers et al. 2015) are giving biologists an unprecedented ability to track the behavior of free-living animals 24/7. However, this technology also provides physiologists and endocrinologists with an unprecedented opportunity to investigate the physiological mechanisms underlying individual variation in movement behavior, activity, or performance in free-living animals. This raises the question of how we should approach the integration of physiology and behaviour in the context of movement in free-living animals, for example, what physiological traits should we measure? Here we review current, laboratory-based, model systems for exercise or activity to ask if these can inform us about common physiological markers underlying high exercise capacity (Halsey 2016) or the physiological costs underlying reduced fecundity and/or survival due to exercise, that might provide relevant "targets" for mechanistic analysis of routine activities in free-living animals. We review studies using wind or swim tunnels to mimic migration in birds and fishes, manipulation of exercise associated with non-migratory activity in birds, locomotion in lizards and wheel running in rodents. Our main objectives are first to identify common physiological signatures of individual variation in exercise capacity and/or costs of exercise, focusing on suites of functionally-related traits, for example, those underpinning aerobic or metabolic capacity,

intermediary metabolism or energy supply, oxidative stress, and immune function. A table summarizing a list of common physiological markers will be provided at the end of this review (Table 1). Second, we consider how physiological responses to exercise or activity might be influenced by (1) the nature of the exercise (i.e., voluntary or involuntary, intensity, duration, aerobic scope of activity, and recovery) performed by animals in each model system and (2) resource acquisition and food availability, in the context of routine activities in free-living animals. Finally, we consider evidence that the physiological effects of experimentally-elevated activity affect components of fitness such as reproduction and survival.

Migration in birds and fishes

Migration is ubiquitous across many taxonomic groups, including birds, fish, reptiles, insects, and mammals (Bowlin et al. 2010), although much of what we know about migration comes from migratory birds and fish. Large-scale migrations often involve long-distance movements lasting from a few days to weeks (Bowlin et al. 2010; Guglielmo 2010) requiring metabolic scopes of 8 × basal metabolic rate (BMR) in birds (Piersma 2011) and 7-15 × standard metabolic rate (SMR) in salmonids (Morash et al. 2013). Recent technological advancements, for example, small data loggers, digital telemetry, wind tunnel, and swim tunnel respirometry, have advanced the study of physiological aspects of animal migration not only in the field, but also under controlled laboratory conditions. Though there are some conflicting results between laboratory- and field based studies, laboratory based migration studies have largely been successful at providing a parallel for long-distance migration in natural settings (Bowlin et al. 2010), for example, energy expenditure of birds flying in wind tunnels is consistent with data gathered from transoceanic flights by shorebirds (Piersma 2011).

Migration can therefore be extremely energetically demanding compared to other activities such as lactation and thermoregulation (in terms of metabolic scope) and it is not surprising that a range of physiological changes associated with aerobic capacity and fuel metabolism sometimes result from this high-intensity exercise (Palstra and Planas 2011; Piersma 2011; Pierce and McWilliams 2014; Butler 2016). Given that considerable variation exists within and among species in migratory behavior and distance, we might expect to see some differences in physiological traits mediating these aspects of migration (Terrill 1990). Nevertheless, many migratory birds and fish appear

to up-regulate a common suite of physiological traits associated with aerobic and metabolic capacity in preparation for and during migration, increasing hematocrit, muscle myoglobin, and activity of metabolic enzymes (Palacios et al. 1984; Wingfield et al. 1990; Morash et al. 2013). Laboratory studies that simulate migration in fish using a swim tunnel have confirmed some of the field observations with increased citrate synthase (CS) activity (Morash et al. 2014), increased vascularization and angiogenesis, as well as myogenesis (Palstra et al. 2014). Fuel use or metabolism is probably the most researched aspect of migration physiology and field data suggest that most migratory birds and salmonids use predominantly lipids to fuel migration (Weber 2009; Guglielmo 2010; Butler 2016). Migrants up-regulate lipoproteins such as muscle fatty-acid binding proteins to increase transport of fuel to working muscles to sustain the exercise necessary for migration (Weber 2009; Guglielmo 2010). Again, similar findings have been reported in laboratory studies, for example, fish show an increase in lipoprotein lipase activity in response to endurance training in a swim tunnel (Weber et al. 2016).

Are there common physiological markers of “costs” associated with the high metabolic demand imposed by migration (i.e., exercising at or close to maximal aerobic capacity for days and sometimes weeks) (Piersma 2011)? European starlings, *Sturnus vulgaris*, showed a decrease in constitutive immune function immediately after an endurance flight in a wind tunnel experiment (Nebel et al. 2012). Similarly, Chinook salmon, *Oncorhynchus tshawytscha*, suffer from loss of immune functions during spawning migration, possibly due to diminished energy reserves or hormonal changes associated with spawning (Dolan et al. 2016). Wilson et al. (2014) found that spawning migration causes decreased antioxidant capacity and higher oxidative DNA damage in various tissues in pink salmon. However, although it has been documented that exercise associated with migration causes reduced spawning success and increased mortality in salmon (Berman and Quinn 1991) and birds (Baduini et al. 2001), more research is needed to causally link observed mortality to markers of physiological costs such as oxidative damage and immune suppression.

With the exceptions of migratory flights of short hop migrant species, almost all long-distance migratory flights and spawning migrations are associated with fasting (Dickhoff et al. 1997; Guglielmo 2010; Piersma 2011; Eliason and Farrell 2016). Thus, some physiological adjustments observed in preparation for, and during migration, could be due to food deprivation, changes in activity level and intensity, or a combination of

both factors. For instance, several long-jump migrant species exhibit facultative reduction of the digestive organs as an energy saving mechanism (Piersma et al. 1993, 1999) though this physiological adjustment could also be due to lack of food ingestion per se. Conversely, the observed increase in fatty-acid binding protein levels in migratory birds (Weber 2009; Guglielmo 2010) and up-regulation of plasma lipoproteins in salmonids (Magnoni et al. 2006) are most likely a direct response of increased activity level. However, changes in fuel selection in migratory birds and fish, probably involve demands of both exercise and osmoregulation: for example, Guglielmo (2010) suggested that while birds catabolize proteins and lipids in muscle during migration (Jenni and Jenni-Eiermann 1998), they also generate metabolic water under dehydrating conditions. It is currently unclear whether birds that engage in activity such as foraging for food, exercising at lower-intensity but for longer duration than migrating birds (Drent and Daan 1980), use the same fuel mixture for exercise, or upregulate lipid metabolism enzymes (e.g., lipoprotein lipase).

Despite the high metabolic demand of migration, associated with a wide range of physiological changes, evidence of “cost” is scarce, mainly because lab studies have not often investigated “fitness” endpoints such as reproduction and survival. To address this particular caveat, researchers could design lab experiments that investigate carry-over effects (processes in one season affecting processes in subsequent season) of migration on reproduction, for example, simulating migratory flight in wind tunnels (for birds) and spawning migration in swim tunnels (for fishes) and then allowing individuals to breed to assess reproductive success. Findings from these lab studies can be used to complement field studies that are often limited by researchers’ ability to obtain detailed physiological measurements from large samples of animals.

Manipulation of exercise associated with non-migratory activity in birds in a laboratory setting

Birds exhibit a wide range of non-migratory behaviors that impose elevated metabolic demand at a lower (3-4 × BMR) but more sustained level (3-4weeks) than during migration (e.g., foraging, chick provisioning during parental care; Piersma 2011). One method used in early studies to manipulate foraging effort involved increasing chaff/seed ratio so birds had to work harder or longer to maintain intake rates (Deerenberg and Overkamp 1999; Lemon 1993, Wiersma and Verhulst 2005), though

this method had problems—birds continuously increase the chaff : seed ratio by consuming seeds and dropping chaff in the mix, making it a challenge to maintain the experimentally-targeted food ratio (Koetsier and Verhulst 2011). More recently, a number of automated systems have been developed to manipulate flight activity in captive birds (Nudds and Bryant 2000; Koetsier and Verhulst 2011;Costantini et al. 2012). Energy expenditure of birds subjected to increased exercise using these techniques generally resemble those of “hard working” birds feeding chicks in the field (Wiersma et al. 2005). Other lab studies confirm that birds trained to work harder show significantly higher exercise capacity post-training and compared to sedentary controls (Butler and Turner 1987; Zhang et al. 2015b).

Is there evidence from laboratory studies in birds that sustained, but lower-intensity exercise (3-4 × BMR) is associated with up-regulation of physiological traits, and are these the same traits that are modified during high-intensity exercise (e.g., migration)? In relation to traits associated with aerobic capacity, pectoralis muscle CS activity, an indicator of cellular aerobic capacity, increases in birds that are trained to fly non-stop between two perches located 6 m apart for 45 min each day over 30 days (Zhang et al. 2015a). This finding is consistent with changes in free-living birds preparing for migration (Guglielmo et al. 2002) and cold-acclimatized birds in winter (Vézina and Williams 2005). In addition, similar to migratory birds, captive birds that are exercise trained also up-regulate a suite of physiological traits associated with fatty-acids transport and oxidation, for example, carnitine palmitoyl transferase (CPT), β-hydroxyacyl CoA-dehydrogenase (HOAD) and fatty-acid binding proteins (Zhang et al. 2015b). Hence, sustained work at lower intensity but for longer duration appears to involve similar physiological changes to those associated with higher aerobic scope activities such as migration and cold-acclimatization.

Does sustained, lower-intensity exercise generate physiological costs? Traditionally, many studies have focused on the energetic costs of exercise (but see Nilsson 2002; Veasey et al. 2001; Simons et al. 2014) although the idea of non-energy based mediators of carry-over effects has been increasingly recognised (Zera and Harshman 2001; Williams 2012). For example, Costantini et al. (2012) showed that increased flight activity (distance: 165.8 m × 3 days) in zebra finches, *Taeniopygia guttata*, leads to increased plasma oxidative damage and decreased thiol concentration in red blood cells. Costantini et al. (2013) then investigated biochemical integration of the

blood redox system (i.e., how different antioxidants interact synergistically and/or competitively and how antioxidants respond to changes in levels of oxidative damage). They found that increased flight activity caused a reduction in biochemical integration among different components of blood antioxidant defences. However, contrary to the findings by Costantini et al. (2012), Larcombe et al. (2010) found that levels of malondialdehyde (MDA) in budgerigars, *Melopsittacus undulates*, decreased after 9 weeks of escape flight training (flown six times on 1 day per week), suggesting that the type of exercise training and the duration of training play important roles in mediating exercise induced oxidative stress. Hard work may depress the immune system in mammals (Pedersen and Hoffman-Goetz 2000; Nieman 2000), but the idea has yet to be tested experimentally for sustained, low-intensity exercise in birds in a laboratory setting.

Are the physiological correlates and costs of sustained, low-intensity exercise observed in lab-based studies ecologically relevant? Furthermore, are there discrepancies among different lab-based studies utilizing similar technique to manipulate exercise? Despite the fact that researchers studying birds in lab setting managed to simulate exercise intensity and duration typical of free-living birds (3-4 × BMR) we need to be cautious about extrapolating these findings to “hard-working” free-living birds (e.g., brood provisioning birds) (Drent and Daan 1980). First, while some lab based studies in zebra finch have found an increase in daily energy expenditure (DEE) with increased exercise (Wiersma and Verhulst 2005), other studies using the same species and European Starling either found no change in DEE (Lemon 1993), or a decrease in DEE (Bautista et al. 1998) respectively, with increased exercise. Wiersma and Verhulst (2005) suggested that these discrepancies might be due to the design of the reward schedule, which ties into the animal’s foraging motivation. Furthermore, many of the studies that utilize automated systems to manipulate exercise in birds (e.g., Costantini et al. 2012; Nudds and Bryant 2000; Zhang et al. 2015b) did not include elements of resource acquisition and food availability, and often involved forced exercise (see Fonseca et al. 2014). The techniques for manipulation of foraging effort with resource acquisition developed by Koetsier and Verhulst (2011) and Wiersma et al. (2005) overcome this issue and could prove valuable in future studies.

Physiological costs incurred due to sustained, low intensity exercise in laboratory systems can lead to downstream effects of reduced survival and/or reproduction.

Increased exercise intensity at 2-3 × BMR for 2-5 weeks can delay onset of reproduction, though other metrics of reproductive output such as clutch size, brood size, egg mass, and fledgling number do not seem to be affected (Deerenberg and Overkamp 1999; Wiersma and Verhulst 2005; Simons et al. 2014). However, these studies did not comprehensively assess physiological response to increased foraging effort, making it difficult to causally link indicators of physiological costs to delayed reproduction. Wiersma and Verhulst (2005) found that birds exposed to increased foraging cost invested less in somatic maintenance though it was not shown that this lead to decreased survival. Reichert et al. (2014) looked at effects of brood size manipulation on telomere dynamics and survival in captive zebra finch. Although enlarged brood size led to greater telomere loss in parents, they did not detect any effects of brood size manipulation on short-term survival (Reichert et al. 2014). Reichert et al. (2014) speculated that the ad libitum feeding conditions might have prevented detection of a significant negative effect on adult survival, again highlighting the importance of considering resource acquisition as a component of lab studies. Briga et al. (2017) addressed the issue of resource acquisition by directly manipulating foraging effort in zebra finch using the technique developed by Koetsier and Verhulst (2011) and found that birds raised in larger brood size suffered higher mortality when subjected to increased exercise in adulthood, although actuarial senescence did not seem to be affected.

Endurance and sprint speed in lizards

Locomotion as a form of exercise has been studied extensively in lizards (Irschick and Losos 1998; Le Galliard et al. 2004; Husak et al. 2006), and is viewed as an integral contributor to reproductive success and survival (Irschick and Garland 2001; Lailvaux and Husak 2014). In terms of intensity of exercise, many lizard studies distinguish two components of locomotion: sprint speed and endurance, both of which are ecologically relevant. Lizards may need a high sprint speed in order to escape predators (Irschick and Losos 1998), or in some cases to forage, but lizards may also require endurance to patrol their territory or to forage more widely (Le Galliard et al. 2013). Thus, sit-and-wait predators can sprint after their prey at a high-intensity for a short period of time (10 × SMR), while widely foraging lizards will work at a lower metabolic scope for a longer period of time (Nagy et al. 1984). Although maximal aerobic

capacity might also play a more important role in foraging and patrolling a territory given these activities involve intermittent bouts of intense movement (John-Alder 1984; John-Alder et al. 2009), the research effort on maximal aerobic capacity has been limited to date for these types of behavior. Hence, we will focus on sprint speed and endurance for the purpose of this review. Perhaps surprisingly, early studies of exercise training in lizards, including training for treadmill endurance, maximum run time, and maximum burst speed often failed to lead to an increase in running performance or changes in metabolic correlates or performance (Gleeson 1979; Garland et al. 1987; O'Connor et al. 2011). Husak et al. (2015) suggested that this lack of response could be due to (1) an insufficiently intense training regimen, (2) a training regimen too intense resulting in skeletal joint degradation, and (3) a lack of specialized exercise, that is, endurance versus speed. Using a training regimen specific for either endurance or sprinting, Husak et al. (2015) found that endurance, but not sprint speed, did improve and that this was associated with metabolic changes (increased hematocrit and increased size of fast glycolytic muscle fibers). Another possible reason for inconsistent results on effects of training on performance and physiology is that many studies used different species, sometimes distantly related, the discrepancies regarding physiological and performance can sometimes be confounded by phylogeny (Huey et al. 2001).

Does exercising for short bursts at a high-intensity (i.e., sprinting) or running at a lower-intensity for a longer period of time (i.e., endurance) induce physiological costs in lizards, and are the same traits affected? Much research on lizard locomotion has focused on morphology, rather than physiology per se. Vanhooydonck et al. (2015) found a trade-off between power output and fatigue resistance using data from isolated muscle tissue in 17 lacertid lizard species, though this trade-off was not apparent at the whole organism level. At the intraspecific level too, effects of exercise training on morphology and physiology has produced equivocal results. After training for submaximal exercise at 1 km/h on a motorized treadmill for 5 days a week and 8weeks in total, *Amphibolurus nuchalis* showed decreased heart and thigh muscle mass, but increased liver mass, hematocrit, liver pyruvate kinase, and heart CS activity (Garland and Else 1987;Garland et al. 1987). Meylan et al. (2013) found that *Zootoca vivipara,* when mounting an immune response did not compromise their treadmill endurance. However, Garland et al. (1987) observed that some lizards exhibit swelling and partial immobilization of hind limbs and muscle fiber necrosis in response to endurance training,

potentially due to depression of immune system. Furthermore, green anole lizards, *Anolis carolinensis*, when subject to endurance training for 2 days a week and 9 weeks in total, suppressed reproduction and immune function, and immune suppression was associated with increased corticosterone (Husak et al. 2016).

Are laboratory-based systems used to investigate exercise in lizards, and the physiological changes revealed by these techniques, ecologically relevant? Given that the vast majority of lizard locomotion research uses treadmills and circular racetracks, we must first consider whether varying environments affect performance (Irschick and Garland 2001). Lizards in nature may choose to vary their speed and duration of activity to increase their efficiency (Christian et al. 1997; Irschick and Jayne 1999). In addition, differing substrates might alter the performance of lizards (Sathe and Husak 2015; Vanhooydonck et al. 2015). In a field experiment, *Uma scoparia* moved about their habitat at two preferred speeds depending on the environment and incline of the surface (Jayne and Irschick 2000). Moreover, while sprint speed may be important for escaping predators and obtaining prey, there may be a trade-off between speed and maneuverability which may affect the lizard's fitness (Wynn et al. 2015). Therefore, one could postulate that the use of forced exercise on treadmills and circular tracks in laboratory studies may inaccurately represent individual performance of free-living animals (Irschick and Garland 2001).

Finally, do the physiological costs associated with endurance and sprint training in lizards lead to negative effects on fitness? Endurance in juvenile *Lacerta vivipara* predicted survival (Le Galliard et al. 2004) and Clobert et al. (2000) found that individual *Lacerta vivipara* with low endurance at birth tended to have reduced activity and growth rate, and a higher parasite load, although they experienced lower predation risk as assessed by tail loss. Conversely, individuals with high endurance at birth had higher activity and growth rates, lower parasite load, but higher incidence of tail loss though endurance at birth was not correlated with survivorship. In some lizards, sprint speed predicts juvenile survival (Miles 2004; Husak 2006), though not adult survival (Husak 2006; Irschick et al. 2008). Sprint speed also predicts mating success and the number of offspring in some lizards (Husak et al. 2006). Nevertheless, given that increasing aerobic performance through training in the laboratory has proven to be difficult, data on exactly how physiological changes mediate the effect of exercise on reproduction and/or survival in lizards are largely lacking (Husak et al. 2015). It is also important to note that it is

unclear if lizards undergo training-like situations in the wild. If most of the within-individual variation in locomotor performances in the wild are caused by hormonal fluctuations, for example, then the effect of training over fitness is not important ecologically (John-Alder et al. 2009).

Wheel-running activity in rodents

Wheel-running in captive rodents is perhaps the most well-studied model system for exercise in non-human animals (Sherwin 1998; Garland et al. 2011; Meijer and Robbers 2014; Mason and Wurbel 2016) utilizing two main experimental approaches: endurance training (voluntary and involuntary) and artificial selection on voluntary wheel-running activity. Involuntary endurance training in rodents often involves using electrical stimulation on treadmills to force animals to exercise (Even et al. 1998; Chappell et al. 2007). In contrast, training paradigms for voluntary exercise involves giving animals access to a running wheel and allow them to run willingly (Sherwin 1998; Novak et al. 2012). Artificial selection experiments have identified changes in suites of morphological and physiological traits, as well as the underlying neuroendocrine mechanisms, associated with increased exercise performance (Garland 2003; Rhodes et al. 2005; Rhodes and Kawecki 2009; Swallow et al. 2009). These varied experimental approaches result in considerable variability in metabolic scope associated with wheel running exercise but this generally ranges from 2 to 6 × BMR (Even et al. 1998; Koteja et al. 1999; Garland et al. 2011) so this would represent sustained, relatively low intensity activity (cf. migration).

Most studies of rodents, using either endurance training or artificial selection, have shown an increase in exercise capacity (Conley et al. 1985; Willis et al. 1988; Swallow et al. 1998; Hoydal et al. 2007; de Araujo et al. 2016), so do we see up-regulation of similar physiological traits underlying this increased exercise? Once again traits underlying aerobic capacity such as CS activity, hematocrit, muscle capillarity, mitochondrial volume, and number, and exercise-induced vascular endothelial growth factor expression were up-regulated in response to both endurance training (Holloszy and Coyle 1984; Fentz et al. 2015; Hedrick et al. 2015;) and artificial selection for voluntary wheel running (Houle-Leroy et al. 2000; Garland 2003; Swallow et al. 2005; Audet et al. 2011) (Table 1). In contrast, aside from higher insulin stimulated glucose uptake by muscles (Dumke et al. 2001; Garland 2003), there were few adjustments in

fuel metabolism in mice selected for high voluntary wheel running activity. However, endurance trained rats and mice do show increased muscle and liver glycogen levels (de Araujo et al. 2016), decreased glucose oxidation (Even et al. 1998), and increased plasma membrane fatty-acid binding protein (Fentz et al. 2015). Thus, despite the common response of increasing exercise performance, physiological traits underlying high activity level seemed to be modulated differently in endurance training versus artificial selection.

Does a higher activity level, due either to endurance training or selection for high voluntary wheel running activity, generate physiological costs in rodents? As with metabolic changes (above), responses in immune function and oxidative state seem to be different depending on the specific training paradigm. Female mice selected for voluntary wheel running exhibit reduced activities of the antioxidant enzymes superoxide-dismutase-2 and catalase, compared to control lines (Thomson et al. 2002). Similarly, Downs et al. (2013) found that mice selected for high mass-independent metabolic rate have suppressed immune function, as measured by cytokine production in response to injection with lipopolysaccharide. Conversely, most studies that employ an endurance training approach have found beneficial effects of exercise on oxidative stress: decreased lipid peroxidation (Costa et al. 2014), decreased thiobarbituric acid-reactive substances (TBARS; Boveris and Navarro 2008; Oharomari et al. 2015), and decreased protein carbonyls (Oharomari et al. 2015). Gholamnezhad et al. (2014) also found that moderate exercise training results in a decrease in susceptibility to viral infection, that is a positive effect on immune function, but that “overtraining” caused immune suppression, characterized by prolonged IL-6 and TNFα elevation (Gholamnezhad et al. 2014). This again highlights the fact that the nature, intensity and duration of the training paradigm need to be considered in interpreting physiological correlates of exercise in these laboratory systems.

Given the centrality of wheel-running to mammalian models of exercise there has been debate about whether wheel-running behaviour in the lab reflects locomotion of free-living animals in the wild, that is whether this behavior is ecologically relevant. Some studies suggested that domestication of house mice has involved minor differences in overall physiology and behavior (Dohm et al. 1994; Richardson et al. 1994; Garland 2003; Rezende et al. 2005). Indeed, Meijers and Robbers (2014) placed running wheels in animals’ natural habitat and found that free-living wild mice, shrews, rats (as well as

snails, slugs and frogs) used the running wheels. However, the act of wheel running itself is not a goal-oriented activity and can become a self-perpetuating behavior that has the capacity to reach obsessive levels (Novak et al. 2012) and could be considered an "abnormal," maladaptive behavior (Sherwin 1998). The neurobiological profile of mice selected for wheel running does share features of Attention Deficit Hyperactivity Disorder (ADHD) indicating, perhaps, that mice become "addicted" to exercise (Rhodes et al. 2005; Garland et al. 2011; Kolb et al. 2013). Fonseca et al. (2014) showed that rats subjected to exercise contingent training protocol, where individuals need to perform exercise to acquire food, expressed significantly different physiological and morphological changes (e.g., lower adiposity, reduction in body mass, smaller liver, and heart masses) compared to mice subjected to non-exercise-contingent training protocol. Careful experimental design that addresses food-exercise contingency is needed in studies linking exercise, food, and physiology going forward.

Despite the vast body of literature on wheel running activity in rodents, relatively few studies have investigated fitness consequences of wheel running activity, despite some reported effects on the reproductive axis (e.g., Klomberg et al. 2002). Female golden hamsters housed with functional running wheels had significantly larger litters than those housed with non-functional wheels and Gebhardt-Henrich et al. (2005) attributed this to "improved well-being" due to the presence of functional wheels, for example less stereotypical bar-mouthing behavior. Endurance training, either forced or voluntary, improved survival in both rats and mice (Holloszy 1993; Boveris and Navarro 2008) but although exercise increased average longevity in rats, maximum lifespan was unaltered (Holloszy 1993). Given the role of oxidative stress in ageing and senescence (Selman et al. 2012) and findings that showed attenuation of oxidative stress response due to exercise (see above), it is conceivable that exercise could actually have positive effects on reproduction and survival in free-living animals.

Conclusion

A multitude of laboratory-based, model systems have been used to investigate "exercise" encompassing a wide range of intensities of activity. Nevertheless, our review indicates that there are some common physiological markers of increased exercise across systems, such as upregulation of traits underlying aerobic capacity (hematocrit, metabolic enzyme activity) in response to increased activity level (Table 1). However,

specific changes in traits associated with fuel metabolism through training seem to be dependent on the intensity of the exercise and study system. In terms of costs of exercise, increased oxidative stress (DNA damage, lipid peroxidation, suppressed antioxidant enzyme activity) appears to be a common response (Table 1), though seemingly with the exception of training in rodents, and a decline in innate immune function (though not adaptive immune function) occurs in response to increased exercise across study systems. Nevertheless, we suggest that more ecologically relevant study systems to understand exercise in free-living animals should be developed. In the wild, free-living animals may choose to vary their speed and duration of activity to increase efficiency (e.g., Irschick and Jayne 1999). Moreover, resource acquisition is often the main goal of activity in the wild, but this is often absent from lab-based systems (Fonseca et al. 2014). As mentioned before, physiological adjustments observed in exercising animals could occur in response to food deprivation (fasting), changes in activity level and intensity, or a combination of both factors. Finally, few laboratory studies directly link physiological correlates of high activity levels with downstream effects of reduced survival and reproduction; though these links are often assumed. Therefore, developing more ecological relevant laboratory systems (e.g., Fonseca et al. 2014; Briga et al. 2017), explicitly incorporating both functional studies across multiple physiological systems and a life-history framework, with reproduction and survival end-points, will help reveal the mechanisms underlying the consequences of exercise. The knowledge we gain from these laboratory based studies can then be used to complement studies in free living animals. In other words, new ground and space-based tracking can be used to field test what is learned from captive animals.

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Tables

Table 2-1 Summary of physiological markers associated with exercise in different model systems in relation to intensity and duration of exercise.

Model systems	Exercise intensity	Exercise duration	Physiological markers			
			Aerobic capacity	Fuel metabolism	Oxidative stress	Immune function
Migration in birds and fish	8 × BMR (birds) 7-15 × SMR (fish)	Days	↑ Hematocrit ↑ Myoglobin ↑ Metabolic enzyme activity	↑ Fatty-acid binding proteins ↑ lipoprotein ↑ lipoprotein lipase activity	↓ Anti-oxidant capacity ↑ DNA damage	↓ Constitutive immune function
Exercise associated with non-migratory activity in birds	3-4 × BMR	Weeks	↑ Muscle CS activity	↑ CPT ↑ HOAD ↑ Fatty-acid binding proteins	↑ plasma oxidative damage ↓ thiol concentration ↓ biochemical integration of antioxidant defenses ↓ MDA[a]	No data
Endurance and sprint speed in lizards	10 × SMR	Seconds to minutes	↑ Hematocrit ↑ Heart CS activity ↑ Liver pyruvate kinase	No data	No data	↓ Immune function
Wheel-running activity in rodents	2-6 × BMR	Days to weeks depending on training regime	↑ Hematocrit ↑ CS activity ↑ Muscle capillarity ↑ Mitochondrial volume and number ↑ Vascular endothelial growth factor	↑ Insulin-stimulated glucose uptake[b] ↑ Liver and muscle glycogen level[c] ↓ Glucose oxidation[c] ↑ Plasma membrane fatty-acid binding protein[c]	↓ Superoxide-dismutase-2 activity[b] ↓ Catalase activity[b] ↓ Lipid peroxidation[c] ↓ Protein carbonyls[c] ↓ TBARS[c]	↓ Immune function[b] Dose-dependent effect on immune function[c]

[a] Only observed under escape flight training regime.
[b] Observed in artificially selected mice.
[c] Observed in endurance exercise trained mice.

Chapter 3.
Haematological traits co-vary with migratory status, altitude and energy expenditure: a phylogenetic, comparative analysis

Abstract

Aerobic capacity is assumed to be a main predictor of workload ability and haematocrit (Hct) and haemoglobin (Hb) have been suggested as key determinants of aerobic performance. Intraspecific studies have reported increases in Hct and Hb in response to increased workload. Furthermore, Hct and Hb vary markedly among individuals and throughout the annual cycle in free-living birds and it has been suggested that this variation reflects adaptive modulation of these traits to meet seasonal changes in energy demands. We used a comparative dataset of haematological traits, measures of metabolic rate, and life-history traits (160 bird species) to test several hypotheses for adaptive variation in haematology in relation to migration and altitude. Although Hct and Hb are only two of the components of the complex machinery underpinning metabolic rate, we extended these general ideas to test relationships between Hct and basal metabolic rate, daily energy expenditure and activity energy expenditure for 66 species. We found that migratory status and altitude predicted interspecific variation in haematology and that Hct is positively associated with activity energy expenditure, suggesting that haematological traits could be adaptively modulated based on life-history traits and that Hct is a potential physiological mediator of energetic constraint.

This chapter will be submitted to *Journal of Comparative Physiology B* as Yap, K. N., Tsai. O., & Williams, T. D. (2018). Haematological traits co-vary with migratory status, altitude and energy expenditure: a phylogenetic, comparative analysis.

Introduction

Whole-organism aerobic capacity is assumed to be one of the main predictors of endurance, or the ability to sustain a high workload in a wide range of animals (Lourdais et al., 2014; Wagner, 1996), and haematocrit (Hct) and haemoglobin (Hb) concentration have been proposed as key determinants of aerobic or metabolic performance through their role in oxygen transport and delivery (Butler, 2016; Calbet, Lundby, Koskolou, & Boushel, 2006; Hammond, Chappell, Cardullo, Lin, & Johnsen, 2000; Theunis Piersma & van Gils, 2011). 'Blood doping' increases Hct and Hb, resulting in an increase in aerobic capacity in humans (Böning et al., 2011; Calbet et al., 2006). Furthermore, increases in Hct and Hb in response to increased workload (i.e. exercise training) have been reported in mice (Fentz et al., 2015), lizards (Husak et al., 2015), and fish (Morash et al., 2014) (reviewed in Yap, Serota, & Williams (2017)). In birds, Hct, and to some extent Hb, increase in response to experimentally increased flight costs (Hõrak et al., 1998), thermogenic demands (Petit and Vezina, 2014), or altitude acclimatization (Borras et al., 2010). Hct and Hb also vary markedly among individuals and through the annual cycle in free-living birds and it has been suggested that this variation reflects adaptive modulation of haematological traits to meet seasonal changes in energy demands, e.g. during migration (Butler, 2016; Fudickar et al., 2016; Krause et al., 2016; Wingfield et al., 1990) or winter acclimatization (Clemens, 1990; Swanson, 1990). For example, Barve, Dhondt, Mathur, & Cheviron (2016) and Clemens (1990) found that birds that reside at high altitude year round have high Hct and Hb relative to lowland birds, and that Hb is correlated with altitude in both high altitude residents and elevational migrants. Furthermore, Hct and Hb are also highly repeatable within individuals and within species (Hatch and Smith, 2010; Wagner et al., 2008). From a life-history point of view, in the right environmental context (e.g. during breeding or migratory seasons), individuals with higher Hct and Hb could "perform better", e.g. rearing chicks or migrating to breeding grounds faster, and thus will have higher reproductive success and fitness (Velmala et al., 2015). In other words, modulation of Hct and Hb could be key adaptations for variable, but sometimes high-intensity, workload (Williams 2012).

More generally, although Hct and Hb are clearly only two components of the complex machinery underpinning energy metabolism in animals (Butler, 2016), they might be functionally significant in determining how animals manage and allocate

energy to cope with environmental challenges (Clarke and Johnston, 1999; Gavrilov, 2014; Hayssen and Lacy, 1985; Mathot and Dingemanse, 2015a; Portugal et al., 2016; Ricklefs et al., 1996). For instance, when exposed to cold environment, a bird with high Hct and Hb would be able to utilize more energy for shivering thermogenesis, as a result of its higher thermogenic capacity (Petit and Vezina, 2014), compared to a bird with low Hct and Hb. Common measures of energy expenditure include basal or resting metabolic rate (BMR: the minimum energy required for self-maintenance), field metabolic rate (FMR: the total energy expenditure of an unrestrained animal over the course of 24 h), and activity energy expenditure (AEE: the amount of energy available to fuel behavior, i.e. FMR – BMR) (Mathot and Dingemanse, 2015a; Portugal et al., 2016). Recently, Mathot & Dingemanse (2015) and Portugal et al. (2016) summarized three different models of energy management:

1) The 'performance' model, which assumes that the capacity to expend energy at a high rate during activity requires greater maintenance costs (e.g. long distance flight requires maintenance of big flight muscles), and that higher BMR is predicted to be positively related to FMR with a slope > 1.

2) The 'allocation' model, which assumes that there is an energetic ceiling, above which animals would suffer from increased risk of mortality due to physical fatigue, predation or infection (Piersma, 2011). This model sets FMR as a fixed amount, thus, it does not vary with BMR, while AEE decreases with increasing BMR. In this case, the predicted slope of FMR-BMR is zero.

3) The 'independent' model assumes that there is no energetic ceiling and that the relationship between BMR and AEE are uncoupled. In other words, BMR and AEE can be adjusted independently. However, since BMR is still a component of FMR, higher BMR is predicted to be positively related to FMR, albeit with a slope < 1.

From these models, they suggested that AEE might be a more valid proxy for energetic constraints (i.e. the total amount of energy that can be expended and/or assimilated during a given period of time) (Portugal et al., 2016, but see Careau and Garland, 2015; Mathot and Dingemanse, 2015b).

However, although progress has been made in terms of understanding the relationship between BMR, AEE and FMR (Portugal et al., 2016), surprisingly little is

known about the physiological basis of the observed relationship, as well as the physiology underpinning energetic constraints in general (Mathot and Dingemanse, 2015a)). Fair, Whitaker, & Pearson (2007) concluded that there are conflicting data concerning changes in Hct in relation to energy expenditure, with studies reporting increases, no change, and decreases in Hct with increased energy expenditure. Similarly, a review by Minias (2015) suggested that Hb is positively correlated with a suite of fitness related traits that might reflect energetic constraints, such as egg size and developmental stability, as well as life-history stages associated with high energy demand (e.g. migration), but the relationships were not always consistent between species. Nevertheless, Hct and Hb could still be functionally important in determining how animals manage their energy budget.

To our knowledge there has been no comprehensive, phylogenetically-controlled test of hypotheses for inter-specific variation in hematological traits (Hct and Hb) or the relationship with energy expenditure in general. Furthermore, there has been a number of studies investigating scaling relationships between body mass and traits like energy expenditure (Clarke and Johnston, 1999; Clarke et al., 2010; Nagy, 2005; Nevill et al., 1992), organ sizes (Howland et al., 2004), and mitochondrial volume (Mathieu et al., 1981) just to name a few, no studies to date have investigated how Hct and Hb scale with body mass at the interspecific level. Fair et al. (2007) and Minias (2015) reviewed sources of variation in Hct and Hb in birds and found that these traits either increased or were not affected by altitude, and that the relationship between Hct and energy expenditure was inconsistent. They also discussed how Hct and Hb vary with body mass and attributed the changes observed in hematological traits to variation in body condition (Fair et al., 2007; Minias, 2015), rather than a scaling relationship per se. However, neither review used a phylogenetic framework. Therefore, here we took a comparative, phylogenetic approach to rigorously test several hypotheses for adaptive variation in Hct and Hb. Specifically, based on findings from previous studies (summarized by Fair et al. (2007) and Minias (2015)), we hypothesized that 1) Hct and Hb would scale positively with body mass, 2) migratory birds will have the highest Hct and Hb levels, followed by partially migratory birds and non-migratory birds, and 3) birds found in higher altitude will have higher Hct and Hb levels than birds found in lower altitude. We then extended these general ideas to test relationships between Hct and BMR, FMR and AEE, in the context of different models of energy management. If Hct is

indeed a physiological mediator of energetic constraints more generally (Mathot and Dingemanse, 2015a; Portugal et al., 2016), we would predict that regardless of life-history traits, Hct will be positively associated with AEE but not associated with FMR and BMR.

Materials and Methods

Data collection

The literature (Web of Science and Google Scholar) was surveyed for studies reporting energetics, body mass and haematology, using the search terms 'basal metabolic rate', 'field metabolic rate', 'daily energy expenditure', 'body mass', 'haemoglobin' and 'haematocrit'. Additional Hct and Hb data were obtained from "The Avian Erythrocyte: Its Phylogenetic Odyssey" (Glomski and Pica, 2011). Information on migratory status and mean altitude were obtained from the "Birds of North America" (Rodewald, 2015), Penguin World (Davis, 2018), and Bird Life International (Bird Life International, 2018). Mean altitude is estimated for Important Bird and Biodiversity Areas (IBAs) for a particular species when data is not available; sites selected based on knowledge of "presence and abundance of species that occur there, year round or seasonally" (Bird Life International, 2018). A total of 160 species were used in the analysis of the relationship between life-history traits and haematology and a total of 57 species were used in the analysis of energetics and Hct. Body mass was either obtained from the same study, or from the "CRC book of Avian Body Masses" (Dunning Jr., 2008). Only non-experimentally manipulated adult populations were considered. Since Hct and Hb are highly correlated between males and females, data for different sexes were pooled. In all studies, BMR was measured using flow-through respirometry within the animals' thermoneutral zone, whereas FMR was either measured using doubly-labelled water technique or estimated using time-energy budget method (it has been demonstrated that both techniques yield reasonable and consistent results (Williams and Nagy, 1984)). In all cases, Hct was measured using microhaematocrit centrifugation and Hb was measured using the cyanomethemoglobin method. Units were converted to grams (g) for body mass, percentage for Hct, and Watts (W) for BMR and FMR. AEE was calculated by subtracting BMR from FMR. Data and references for haematology

and energetics are provided in tables in Dryad Digital Repository (doi will be provided once received).

Phylogenetic Tree Construction

Using the website BirdTree.org, we obtained 500 phylogenetic trees of all species considered in both sets of analyses using the Ericson Sequenced Species backbone posterior distribution from a global phylogeny of birds (Jetz et al., 2012). Briefly, the tree construction approach combines relaxed clock molecular trees of well-supported avian clades with a fossil calibrated backbone with representatives from each clade. To obtain the phylogenetic trees for the species considered in our analyses, the global phylogeny was first trimmed to a subset, and 500 trees were randomly sampled from a chosen pseudo-posterior distribution (see Jetz et al. (2012) for detailed methods). The consensus trees for both sets of analyses are provided in electronic supplementary material (S3-1 and S3-2).

Statistical Analyses

Analyses were carried out using R version 0.99.467 (R Core Team 2013) and the packages “ape”, “geiger”, “nlme”, “phytools” and “visreg”. Data were first examined for normality using Shapiro-Wilk test. Body mass, Hb, BMR, FMR and AEE were log transformed, whereas data for mean altitude was square-root transformed prior to analysis to improve their distributions. Linear regressions were computed using phylogenetic generalized least squares (PGLS) in which the residuals are modeled as having evolved via a Brownian Motion process (Symonds and Blomberg, 2014). PGLS was conducted in lieu of an ordinary least squares model (OLS) because OLS assumes that each independent data point contributes equally to the estimation of the regression line, whereas PGLS ‘downweights’ points in proportion to the degree of shared phylogenetic history (Symonds and Blomberg, 2014).

We first tested whether Hct and Hb scale with body mass by including body mass as a predictor of Hct and Hb in our regression models. We also tested whether Hct and Hb are correlated at the interspecific level by using Hct as a predictor and Hb as a response variable in our PGLS regression model. To test the hypotheses for adaptive variation in Hct and Hb, altitude was included in the second regression models first as a

predictor of Hct and Hb, and subsequently as a covariate along with body mass, when testing for the relationships between migratory status and Hct and Hb. To investigate how Hb varies with migratory status independent of Hct, we also ran a separate regression model using migratory status as a predictor and altitude, body mass, and Hct and covariates. Tukey's HSD (package multcomp, (Hothorn et al., 2008)) was used to evaluate pairwise comparisons between migratory status following a significant PGLS model.

For energetic traits, in light of previous studies showing positive scaling of body mass to BMR and FMR (Clarke and Johnston, 1999; Clarke et al., 2010; Portugal et al., 2016), we tested the effect of body mass on measures of energy expenditure, using body mass as a predictor of BMR, FMR and AEE in our PGLS regression models. To test the hypotheses regarding different models of energy management, we tested the relationship between BMR and FMR, BMR and AEE. Finally, to test relationships between Hct and energy expenditure, we included Hct as a predictor of BMR, AEE and FMR in our regression models. To account for multiple test correction, likelihood ratio tests were conducted to compare models using only body mass as a predictor of energetics measures and models using Hct as predictor of energetic measures. Due to insufficient data for Hb, we could not rigorously test the relationships between Hb and measures of energy expenditure. Degrees of freedom of the residuals, slope, intercept, R-squared values, and p-values were reported for regressions with continuous predictors, while F- and Z-statistics were reported for regressions with categorical variables. Additionally, estimates of the phylogenetic signal associated with all regressions (Pagel’s λ), which indicates the extent to which closely related species tend to resemble each other, were reported as well (Symonds and Blomberg, 2014). The variables used and all PGLS models are provided in a table in electronic supplementary material (Table S3-3).

Results

Do migratory status and altitude predict variation in haematocrit and haemoglobin?

Hct and Hb were significantly positively associated across species (*df* = 158, y = 0.007x + 0.85, p < 0.01, R^2 = 0.07, Pagel's λ = 0.81), but both Hct (*df* = 158, y = -1.53x + 49.90, p = 0.29, R^2 < 0.01, Pagel's λ = 0.62) and Hb (*df* = 158, y = -0.01x + 1.23, p = 0.40, R^2 = 0.02, Pagel's λ = 0.79) were independent of body mass.

There was a significant positive association between migratory status and Hct ($F_{2, 155}$ = 4.95, p < 0.01, Pagel's λ = 0.62, Fig. 1A), where full migrants have significantly higher Hct than both partial migrants (*Z* = 2.79, p = 0.01) and non-migrants (Z = 2.65, p = 0.02). A similar pattern was found between migratory status and Hb ($F_{2, 155}$ = 4.31, p = 0.015, Pagel's λ = 0.82, Fig. 1B), where full migrants have significantly higher Hb than both partial migrants (Z = 4.31, p < 0.01) and non-migrants (Z = 2.33, p = 0.05). The relationship between migratory status and Hb holds true even after Hct was included in the regression model as a covariate ($F_{2, 154}$ = 12.80, p < 0.0001, Pagel's λ = 0.83). Contrary to our initial prediction, Hct was independent of altitude (*df* = 157, y = 0.03x + 49.35, p = 0.22, R^2 < 0.01, Pagel's λ = 0.67, Fig. 1C). However, a significant positive relationship was found between altitude and Hb (*df* = 157, y = 0.001x + 1.21, Pagel's λ = 0.82, R^2 = 0.09, p < 0.01, Fig. 1D).

Does variation in haematocrit predict variation in basal metabolic rate, field metabolic rate and activity energy expenditure?

PGLS indicated a significant positive association between body mass and FMR (*df* = 55, y = 0.62x – 1.93, p < 0.01, R^2 = 0.64, Pagel's λ = 0.58), between body mass and BMR (*df* = 55, y = 0.67x – 3.33, p < 0.01, R^2 = 0.73, Pagel's λ = 0.001), and between body mass and AEE (*df* = 55, y = 0.56x – 2.11, p < 0.01, R^2 = 0.37, Pagel's λ = 0.61). There was a significant positive relationship between BMR and AEE (*df* = 54, y = 3.22x – 1.17, p < 0.01, R^2 = 0.57, Pagel's λ = 0.06), and between BMR and FMR (*df* = 54, y = 3.13x + 0.89, p < 0.01, R^2 = 0.72, Pagel's λ = 0.06).

A non-significant relationship was found between Hct and BMR (*df* = 54, y = -0.02x – 2.40, p = 0.06, R^2 = 0.09, Pagel's λ = 0.13, Fig. 2A) and a marginally significant

positive relationship was found between Hct and FMR (df = 54, y = 0.02x – 3.16, p = 0.05, R^2 < 0.01, Pagel's λ = 0.58, Fig. 2B). In contrast, a stronger positive relationship was found between Hct and AEE (df = 54, y = 0.06x – 5.28, p < 0.01, R^2 = 0.05, Pagel's λ = 0.70, Fig. 2C). Likelihood ratio tests indicated that when predicting interspecific variation in BMR, model was marginally significantly improved when Hct was compared to model based on body mass alone (Chisq = 0.63, p = 0.05). When predicting interspecific variation in FMR and AEE, models were significantly improved when Hct was incorporated into models based on body mass alone (FMR: Chisq = 4.22, p = 0.04; AEE: Chisq = 12.80, p < 0.01).

Discussion

We took a comparative, phylogenetic approach to rigorously test the hypotheses that 1) Hct and Hb would scale positively with body mass, 2) migratory birds will have the highest Hct and Hb levels, followed by partially migratory birds and non-migratory birds, and 3) birds found in higher altitude will have higher Hct and Hb levels than birds found in lower altitude. With the exception of the regressions for body mass and BMR and for BMR and FMR, most PGLS regressions indicated moderate to strong phylogenetic signal (i.e. Pagels' λ of 0.5 to 0.9). PGLS was used in all of our regressions because it can explicitly take into account phylogenetic signal and control for it appropriately (Symonds and Blomberg, 2014). Consistent with findings from intraspecific studies in endothermic animals (i.e. birds and mammals) (Glomski and Pica, 2011), we showed that Hct and Hb are positively correlated at the interspecific level. We also showed that full migrants have higher Hct and Hb than partial migrants and non-migrants, largely consistent with common assumptions in the literature (Fair et al., 2007; Minias, 2015). This result is also consistent with previous intraspecific studies looking at partial migrants (Chapman et al., 2011), where some individuals of the population that migrate have either increased erythropoiesis or Hct, whereas the others that stay as residents maintain low levels of erythropoiesis and consequently low Hct (Fudickar et al., 2016; Krause et al., 2016). Interestingly, the relationship between migratory status and Hb holds true even after Hct has been included as a covariate, suggesting that migrants do not only have more erythrocytes per unit of blood in their circulatory system, but they also have higher mean cell haemoglobin content. Contrary to our initial prediction, birds found at higher altitude do not have higher Hct than birds found at lower altitude but they

do have higher Hb. Increases in Hct and Hb as a means to increase blood oxygen carrying capacity is a well-documented acclimatization response to hypoxia in many vertebrates (Barve et al., 2016; Borras et al., 2010; Fair et al., 2007; Moore et al., 1998; Storz et al., 2010; Zubieta-Calleja et al., 2007). However, an increase in Hct also results in an exponential increase in viscosity, thus hindering blood oxygen transport (Birchard, 1997; Schuler et al., 2010). By having high mean cell Hb concentration and relatively low Hct, animals can have low blood viscosity without compromising oxygen carrying capacity. This is consistent with findings from Barve et al. (2016), who found that birds with different migration patterns (e.g. elevational migrants vs. residents) appear to adopt alternative physiological strategies to regulate blood oxygen carrying capacity, suggesting that this phenomenon is perhaps an adaptation for animals that experience fluctuating oxygen demand regularly.

Interspecific variation in energy expenditure was partly explained by variation in body mass: all measures of energy expenditure (BMR, AEE and FMR) scale positively with body mass, consistent with findings from other studies (Clarke et al., 2010; Gavrilov, 2014; Hayssen and Lacy, 1985; Portugal et al., 2016; Ricklefs et al., 1996). In terms of how different measures of energy expenditure relate to each other, our study showed that there were positive correlations between BMR and AEE, and BMR and FMR with a slope > 1. This is contrary to findings of other intraspecific studies that looked at associations between measures of energy expenditure, which found that most bird species employ the independent model of energy management (Portugal et al., 2016; Ricklefs et al., 1996). Our study indicated that birds tend to employ the performance model of energy management. In other words, it appears that variation in basic energy requirements (BMR) predicts the capacity to expend energy at high rate during activity (AEE), as well as the total energy expenditure (FMR). The coupled relationship between BMR and AEE makes biological sense when we consider the fact that the capacity to expend energy at a high rate during activity requires greater maintenance costs. However, one should be cautious about distinguishing among energy management models based on the relationship between BMR and FMR alone since trade-offs can occur at either the among- or within-species level (Careau and Garland, 2015).

We sought to investigate the potential physiological basis of interspecific variation in energy expenditure and tested the relationships between Hct and BMR, FMR and AEE. In support of our initial prediction, we found that Hct is positively and more

strongly related to AEE than to BMR and FMR at the interspecific level (based on slope of regression). As mentioned before, AEE, as opposed to BMR and FMR, has been suggested to be a more valid proxy for energetic constraints as it is a measure of how much energy can be spent specifically on energetically costly activities (Mathot and Dingemanse, 2015a; Portugal et al., 2016). Although, many studies have found positive relationships between body mass and measures of energy expenditure (Portugal et al., 2007; Portugal et al., 2016; Ricklefs et al., 1996; Williams et al., 2001), few studies have explored the physiology underpinning energetics. To the best of our knowledge, our study is one of the first to explore the physiological basis of energetic constraints from an interspecific perspective. Our findings that interspecific variation in AEE can be explained by variation in Hct, and that variation in Hct can help explain some of the residual variation in the relationships between body mass and measures of energy expenditure, suggested that perhaps Hct is a mediator of energetic constraint. It should be noted that some of the significant models (e.g. altitude and Hb, Hct and AEE) had relatively small R^2 values and the slope of the regressions are relatively shallow, suggesting that perhaps there is very little biological relevance despite the observed statistical significance. However, we need to be caution about interpreting R^2 values obtained from PGLS since they are not comparable with R^2 values obtained from ordinary least squares (OLS) models. Residuals calculated from PGLS are not orthogonal and therefore, it is difficult to ascribe portions of the explained variation to independent variables (Lavin et al., 2008; Symonds and Blomberg, 2014). In light of this finding, future studies should look at how manipulation of Hct can affect the way animals allocate and manage energy.

In summary, our study has shown that interspecific variation in Hct and Hb can be explained by altitude and migratory status of birds, and that Hct is a potential physiological mediator of energetic constraints and trade-offs in birds. We know that there are interspecific variations in reproductive effort and output in animals (Christians, 2000; Glazier, 1999; Martin, 2000; Warne and Charnov, 2008), both of which are key determinants of Darwinian fitness and life-history trade-offs. Given the potential role of Hct in mediating energetic constraints, it remains to be determined if interspecific variation in reproductive effort and output such as clutch size and egg size can be explained by variation in Hct.

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Figures

Figure 3-1 Relationship between A) Hct and migratory status, B) Hb and migratory status, C) Hct and altitude, and D) Hb and altitude. Data shown in 1A and 1B are individual species data and means. Different letters denote statistical significance. Data shown in 1C and 1D are individual species data and PGLS regression line.

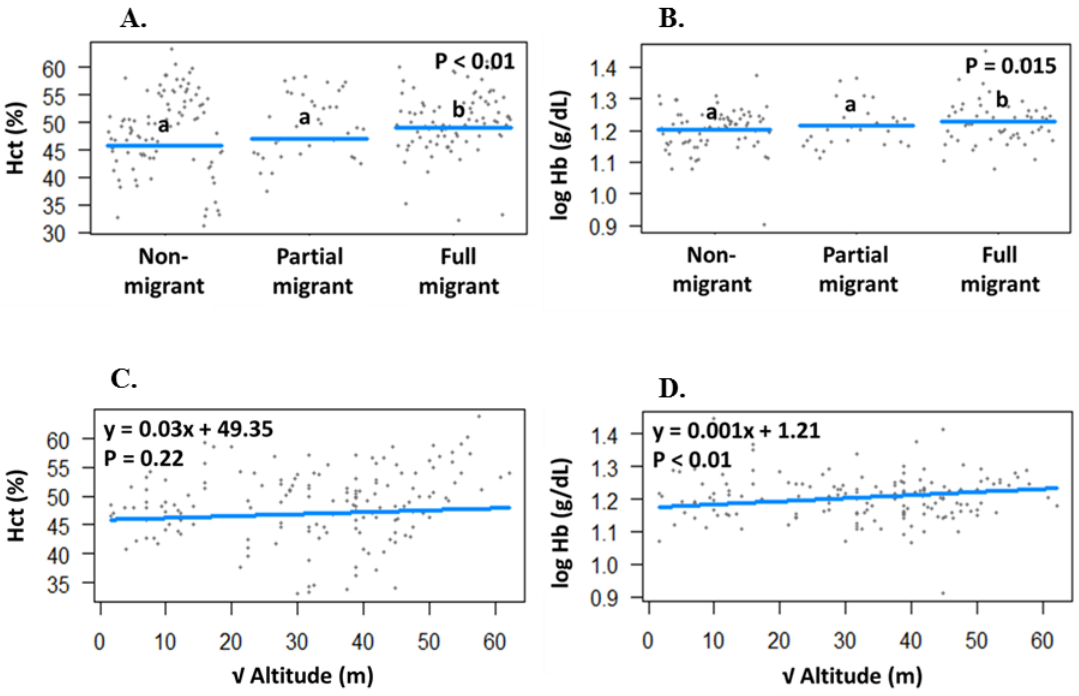
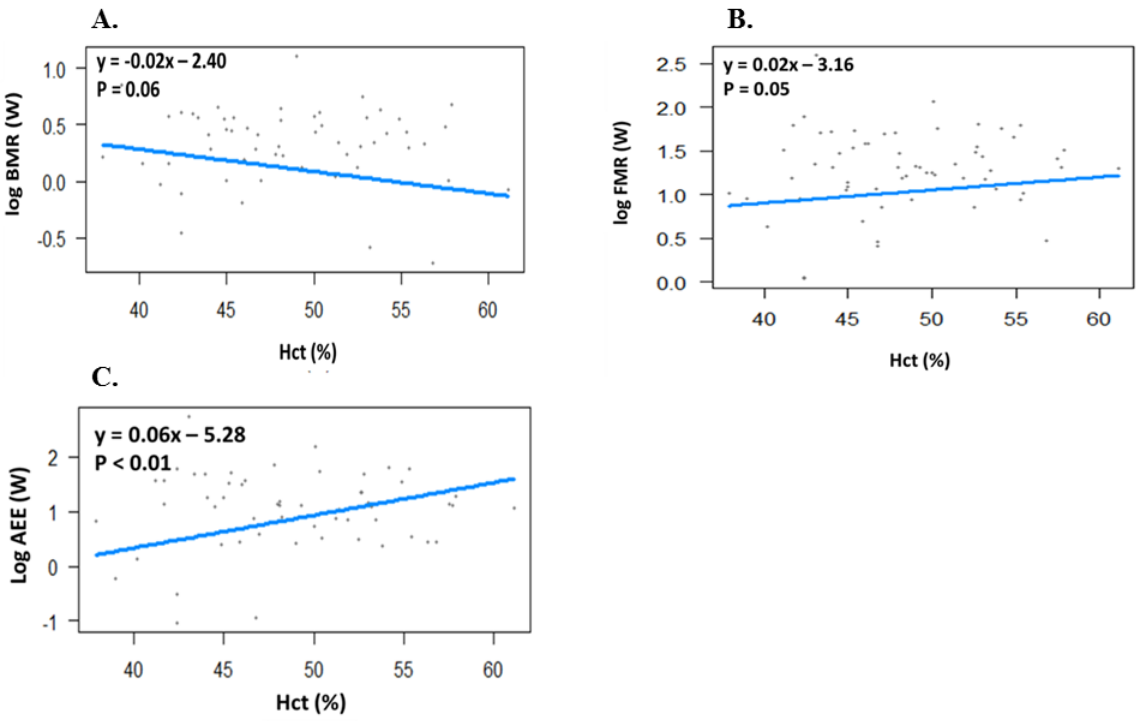


Figure 3-2 Relationship between A) Hct and BMR, B) Hct and FMR, and C) Hct and AEE. Data shown are individual species data and PGLS regression line.



Chapter 4.
Effects of experimental manipulation of hematocrit on avian flight performance

Abstract

Despite widely held assumptions that hematocrit (Hct) is a key determinant of aerobic capacity and exercise performance, this relationship has not often been tested rigorously in birds and results to date are mixed. Migration in birds involves high intensity exercise for long durations at various altitudes. Therefore, it provides a good model system to examine the effect of Hct on flight performance and physiological responses to exercise at high altitude. We treated yellow-rumped warblers (*Setophaga coronata*) with avian erythropoietin (EPO) and anti-EPO to experimentally manipulate Hct and assessed flight performance at low and high altitudes using a hypobaric wind tunnel. We showed that anti-EPO treated birds had lower Hct than vehicle and EPO treated birds post-treatment. Anti-EPO treated birds also had marginally lower exercise performance at low altitude, committing a higher number of strikes (mistakes) in the first 30 min of flight. However, anti-EPO treated birds performed significantly better at high altitude, attaining a higher altitude in a ramped altitude challenge to 3000 m equivalent altitude, and with longer duration of flight at high altitude. Birds exercising at high altitude condition over short duration of time, decreased Hct, increased glucose mobilization, and decreased antioxidant capacity, regardless of treatment. In summary, we provide experimental evidence that the relationship between Hct and exercise performance is dependent on altitude. Future studies should investigate whether free-living birds adaptively modulate their Hct based on the altitude they fly at during migratory flight.

This chapter is in review in *Journal of Experimental Biology*. Originally submitted as Yap, K. N., Dick, M. F., Guglielmo, C. G., & Williams, T. D. (2017). Effects of experimental manipulation of hematocrit on avian flight performance in high and low altitude conditions. *Journal of Experimental Biology. In Review.*

Introduction

Hematocrit (Hct) and hemoglobin (Hb) are widely assumed to be key determinants of aerobic capacity and exercise performance (Böning et al., 2011; Calbet et al., 2006; Carpenter, 1975; Hammond et al., 2000; Piersma and van Gils, 2011), however this functional relationship has not often been tested rigorously and results to date are mixed. For instance, studies in migratory birds show that birds up-regulate Hct and Hb in preparation for and during migration, presumably to sustain the high metabolic demand imposed by long migratory flights (Fair et al., 2007; Hahn et al., 2018; Krause et al., 2016). It has also been shown that Hct is positively correlated with exercise-induced maximal metabolic rate (VO_2max) (Hammond et al., 2000) and cold-induced summit metabolic rate in birds (Petit and Vezina, 2014). However, although many studies on human athletes reported improved aerobic capacity as a result of increases in Hct and Hb due to treatment with recombinant human erythropoietin (EPO) or erythropoiesis-stimulating agents (Böning et al., 2011; Lundby et al., 2007; Thomsen et al., 2007), other studies have found minimal or no effects of EPO treatment on exercise performance in human athletes (Heuberger et al., 2017; Sgrò et al., 2018). In addition, unlike mammals such as horses and dogs, which up-regulate Hct and Hb in response to exercise (Wu et al., 1996), birds decrease Hct during exercise due to hemodilution (Jenni et al., 2006). These findings suggest that the relationships between Hct, Hb, aerobic capacity, and exercise performance may not be straightforward and might be taxa- or activity-specific. Therefore, experimental manipulations of Hct and Hb should be conducted to confirm their role in sustaining energetically demanding exercise (Petit and Vezina, 2014; Yap et al., 2017a).

Migration in birds provides a good model system to examine the relationship between Hct and aerobic capacity (Yap et al., 2017a). Bird migration often covers long distances and spans days to weeks of activity (Guglielmo, 2010; Piersma, 2011b; Scott et al., 2015; Yap et al., 2017a). Furthermore, migratory birds operate at 8 × BMR (or 90% VO_2max) during long migratory flights (Guglielmo, 2010; Piersma, 2011b; Yap et al., 2017a). Although most migratory passerines spend the majority of time flying at low altitude (< 800m) they do sometimes fly at higher altitudes depending on winds and weather (up to 4000m) (Dokter et al., 2013; Kemp et al., 2013; Scott, 2011). However, most studies investigating physiology of migration have been conducted at low altitude

and have largely ignored this transient but potentially important altitude component. Outside of a few species adapted to high altitude (Barve et al., 2016; Fedde et al., 1989; Lague et al., 2016; Scott and Dawson, 2017; Scott et al., 2015), physiological responses to exercise at high altitude in many passerines are still poorly understood. Borras et al. (2010) found that citril finches (*Carduelis citronella*) increased Hct in response to increases in altitude, but these birds were transported passively to high elevation by the researcher. Therefore, it is unclear whether birds that are actively exercising would experience the same physiological response when exposed to high altitude condition. With regard to fuel metabolism, carbohydrate (glucose) has been thought to be the preferred fuel during exercise at high altitude (Hochachka et al., 1991), and at high exercise intensity (McClelland et al., 1998) in mammals. Given that migratory birds often fly at 70-90% VO_2max with fatty acids as the predominant fuel, at least at low altitude (Guglielmo, 2010), it is not known whether we would see similar changes in fuel selection (increased glucose metabolism) in birds exercising at high altitude. In addition, whereas it has been shown that endurance flight can generate a physiological cost, in terms of increased oxidative stress in migratory birds (Jenni-Eiermann et al., 2014), the synergistic effect of exercise and acute hypoxia exposure at high altitude on oxidative stress remains poorly understood at present.

Here we investigate the effects of experimental manipulation of hematocrit and hemoglobin on flight performance and the physiological response to exercise at low and high altitude in a migratory songbird. We treated yellow-rumped warblers (*Setophaga coronata*) with avian erythropoietin (EPO), erythropoietin antibody (Anti-EPO), and vehicle (Veh) and assessed flight performance using a hypobaric wind tunnel (Gerson and Guglielmo, 2011; Hedenström and Lindström, 2017). We predicted that 1) EPO treated birds would have the highest Hct/Hb level, followed by Veh treated birds and Anti-EPO treated birds, 2) EPO treated birds would have the best exercise performance during wind tunnel flight at both low and high altitude, followed by Veh treated birds and Anti-EPO treated birds, and 3) regardless of treatment, all birds would increase Hct/Hb and glucose mobilization during flight at high altitude. We also predicted that birds dosed with Anti-EPO would have increased flight-induced oxidative stress compared to Veh and EPO dosed birds, due to compromised oxygen carrying capacity, and hence decreased endurance capacity and increased difficulty flying in the wind tunnel (Jenni-Eiermann et al., 2014).

Materials and Methods

Animals and husbandry

Yellow-rumped warblers (n = 53) were captured using mist nets at Long Point, Ontario, Canada between 13-16 October 2015, and transported to the Advanced Facility for Avian Research (AFAR) at the University of Western Ontario, London, Canada by animal carriers in a vehicle. Birds were initially housed in cages (2-3 birds per cage) from 13 October 2015 to 22 February 2016. They were transferred to indoor aviaries (2.3W × 2.4H × 3.5L m) on 26 February 2016 and were kept in aviaries until the end of the experiment when all birds were released. Birds were kept in constant room temperature (~20^{o}C), with *ad libitum* access to a synthetic agar-based mash diet (60.2% carbohydrate, 13.4% protein, and 10.7% lipid (Marshall et al., 2016)). Light cycles were set to mimic seasonal overwintering and migratory conditions in the wild. Birds were housed on a 12L:12D light cycle (to simulate fall migration photoperiod) for 2 months upon being brought into captivity. They were switched to an 8L:16D light cycle (to simulate overwintering photoperiod) from 22 December 2015 to 17 March 2016, prior to the beginning of experiment. All birds were collected under a scientific collection permit from the Canadian Wildlife Service (CA-0256), and under Animal Ethics Protocol 2010-216 from the University of Western Ontario Animal Care Committee.

Dosing pilot: Validation of the effects of EPO and anti-EPO on Hct and Hb

Birds were given 2 weeks to acclimate to captivity after being captured during their fall migratory season, prior to the start of dosing pilot, and n = 30 birds were randomly chosen for the dosing pilot. At the start of the pilot, a blood sample was taken from all birds to obtain baseline measurements of Hct and Hb. Birds were then given 7 days to recover from the initial blood sampling, at the end of which they were randomly assigned into 3 treatment groups (n = 10 per treatment): EPO, Anti-EPO, and Veh. Cross use between mammalian and avian EPO is ineffective (Rosse and Waldmann, 1966). Therefore EPO, anti-EPO, and anti-EPO diluent were acquired from chicken EPO ELISA kits (MyBioSource Inc.). Birds in the EPO group were injected intramuscularly (IM; right pectoral muscle) with 100µL of 8000pg/mL purified chicken EPO; Birds in the Anti-EPO group were injected IM with 50µL of diluted chicken anti-EPO; Birds in the Veh

group were injected IM with 50µL of anti-EPO diluent. These volumes and concentrations were chosen based on a previous dosing pilot conducted in captive zebra finches (*Taeniopygia guttata*) at Simon Fraser University, BC, Canada (O. Kim et al. *unpublished data*). A second blood sample was taken 3 days after injections to assess changes in Hct and Hb.

Hypobaric climatic wind tunnel and screening

The hypobaric climatic wind tunnel used in this experiment is housed in the AFAR. It is capable of simulating high altitude conditions with low turbulence air flow at various speeds. It has been used routinely to study flight in birds and birds do exhibit natural flight behaviour in the tunnel (Ma et al., 2018; Maggini et al., 2017). To familiarize birds with the wind tunnel and to screen for birds that would fly voluntarily and reliably for long periods in the wind tunnel, individuals were flown at 15^{o}C, 70% relative humidity, and between 7 and 8 m.s^{-1} two weeks prior to the start of the experiment. Birds were only flown once for a maximum of 10 minutes during the screening phase, and would not have any more exposure to the wind tunnel until pre-treatment flight (see below).

Experimental timeline and wind tunnel endurance flight

Starting in March, birds were transferred to a separate indoor aviary with long day photoperiod (16L:8D) three weeks before the pre-treatment wind tunnel endurance flight (Day -21), to be photo-stimulated into a spring migratory condition. To ensure that all birds are in similar migratory state, we staggered our photo-stimulation treatment so that every bird had been exposed to long day photoperiod for exactly 21 days prior to pre-treatment flight. On Day 0, birds were fasted for one hour before the start of pre-treatment flight, after which their body mass was measured (±0.01 g) while fat and lean mass were measured using a quantitative magnetic resonance body composition analyzer (QMR; Echo Medical Systems) (Guglielmo et al., 2011). Birds were flown at 15^{o}C, 70% relative humidity, ground level (low altitude: approximately 182 m a.s.l.) and 8 m.s^{-1} equivalent wind speed for 120 min. If a bird failed to maintain continuous flight for 5 min after the initial 30 min adjustment period, the bird was removed and the flight terminated. To determine pre-treatment (Day 0) flight energetics and physiology (Hct, Hb, oxidative stress), all birds were weighed, scanned by QMR, and blood sampled immediately following flight. Birds were then returned to their respective aviaries and

allowed to rest and recover for 7 days. On day 7, birds were randomly assigned into one of three treatment groups and injected either with EPO (n=7), Anti-EPO (n=8), or Veh (n=7), by an experimenter blind to pre-treatment flight performance. Three days after treatment (day 10), prior to post-treatment flight, birds were fasted, weighed, and scanned by QMR. During the first 105 min of post-treatment flight, birds were flown at low altitude and in the same wind speed and environmental conditions as the pre-treatment flight. We then decreased air pressure to simulate an increase in altitude at 5 $m.s^{-1}$ up to a maximum of 3000m a.s.l. equivalent altitude, while maintaining temperature and humidity constant and the equivalent wind speed at 8 $m.s^{-1}$ (i.e. true wind speed was increased to maintain the same dynamic pressure and thus mechanical flight power (Pennycuick, 2008). Birds were kept flying in the wind tunnel at 3000m for 5 min. 3000m was chosen because it is at the upper limit of altitude at which most passerines migrate (Scott, 2011).In total, birds spent the same amount of time (120 min) flying in the wind tunnel during the post-treatment flight as the pre-treatment flight, with the last 15 min being an altitude challenge. However, due to logistical constraints, only 18 birds of the original 22 were subjected to altitude challenge, whereas the remaining 4 were flown at low altitude for the entire duration (i.e. 120 min). Of the 18 birds that were subjected to altitude challenge post-treatment, all 6 Anti-EPO treated birds, 4 of 6 Veh treated birds, and 1 of 6 EPO treated birds attained the 3000 m simulated altitude. To determine post-treatment flight energetics and physiology, all birds were weighed, scanned by QMR, and blood sampled immediately following flight. Blood was not collected before flight to avoid any effect of blood sampling on flight performance (i.e. puncture wound, dehydration due to blood loss, anemia, etc.). A summary of the experimental timeline is provided in Fig. 1.

Behavioural observation and determination of flight performance

Behavioural observations of flying birds were conducted by an independent observer blind to dosing treatments. Four different metrics were used to assess flight performance during both pre-treatment and post-treatment flights: 1) number of strikes in the first 105 min, 2) number of strikes in the first 30 min, 3) energy expenditure, and 4) total duration of flight. Strikes were defined as mistakes that individuals make during flight (i.e. when a bird landed or was blown to the back net of the wind tunnel; (Maggini et al., 2017)). Total energy expenditure was determined using fat and lean mass lost

during flight as quantified by the QMR scans pre- and post-flight (E = Δ fat mass × 39.6 kJ g^{-1} + Δ lean mass × 5.3 kJ g^{-1}; (Gerson and Guglielmo, 2011)). We also calculated both cost of transport (COT = E / (wind speed × flight duration)) and flight power (E / flight duration). We chose to assess number of strikes in the first 30 min because it has been shown that first 30 min were the most challenging time as birds adjust to conditions in the wind tunnel (Ma et al., 2018), possibly because birds made a suite of physiological adjustments during the onset of flight (Gerson and Guglielmo, 2013; Jenni et al., 2006). For post-treatment flights, in addition to the four performance metrics above, we also included three other performance metrics: 1) Altitude (the maximum altitude attained by the bird at the end of flight test period, or the altitude at which the bird failed to maintain continuous flight for 1 min), and 2) Alt. Duration (the total duration of flight at 3000m), and 3) Alt. Strikes (the number of strikes at 3000m).

Physiological measurements and assays

Pre-treatment (Day 0) and post-treatment (Day 10) bloods samples were obtained from the brachial vein following puncture with a 26G needle, and blood was collected using heparinized microhaematocrit tubes. Hct (percent packed cell volume) was measured with digital callipers (±0.01 mm) following centrifugation of whole blood for 5 min at 10,000 g (Haematokrit 210, Hettich). Hb (g dL^{-1}) was measured using the cyanomethaemoglobin method (Drabkin and Austin, 1932) modified for use with a microplate spectrophotometer (BioTek Powerwave 340; BioTek Instruments, Winooski, VT, USA), using 5 µl whole blood diluted in 1.25 ml Drabkin's reagent (D5941; Sigma-Aldrich Canada, Oakville, Ontario, Canada) with absorbance measured at 540 nm. Intra-assay coefficient was 2.51%. Blood glucose was also measured in individuals at the time of blood sampling using a glucose meter (Accu-Chek Aviva; Roche Diagnostics, Mannheim, Germany). Blood samples from both time points were also assayed for total antioxidant capacity (µmol HClO L^{-1}; OXY) and reactive oxygen metabolites (mg H_2O_2 dL^{-1}; dROMs). All plasma samples were analyzed using a microplate spectrophotometer (BioTek Powerwave 340) and 96-well microplates. Analyses of oxidative stress were carried out according to established protocols as described in Costantini et al. (Costantini et al., 2008; Costantini et al., 2011), with slight modification. Specifically, we measured dROMs and OXY using the commercial kits dROMs and OXY-Adsorbent Test (Diacron International, Grosseto, Italy), respectively. Inter- (n=4)

and intra- (n=3) assay coefficients for OXY were 5.95% and 5.41%, respectively. Inter- (n=2) and intra- (n=2) assay coefficients for dROMs were 0.27% and 3.56%, respectively.

Statistical analyses

Analyses were carried out using R version 0.99.467 (R Core Team 2013). Data were first examined for normality using Shapiro-Wilk test and data were either transformed prior to analysis or analyzed using a non-parametric test (Kruskal–Wallis test). For the dosing pilot, we tested the effect of EPO, Anti-EPO, and Veh on Hct and Hb using general linear model (GLM), with treatment as main effect, and body mass and pre-treatment Hct/Hb as covariates. We also reported the least-squared means and standard errors of pre-treatment Hct and Hb and post-treatment Hct and Hb in a separate table (Table 1). For the wind tunnel flight experiment, physiological metrics (glucose, Hct, Hb, OXY, dROMs) were analyzed using repeated measure with time and treatment as main effects, and individual bird ID as a random factor. Body mass was included in all models as a covariate. In addition, to investigate the effect of flight at high altitude on Hct and Hb, we analyzed Hct and Hb using repeated measure with flight and time as main effects, and individual bird ID as a random factor. All treatment groups were pooled in this particular model because we did not detect any main effects of treatment, nor interactions between treatment and other variables ($P>0.1$ in all cases). We did not test for the effect of flight at high altitude on other physiological measures because of we only had enough plasma samples from 1 bird (out of 4) that were flown at low altitude for both pre- and post-treatment flight. *F* statistics and *P* values were generated using the lmerTest package (Kuznetsova et al., 2014) and Tukey's HSD (package multcomp, (Hothorn et al., 2008)) was used to evaluate pairwise comparisons between treatments and time points following a significant mixed model.

To look at change in performance between pre-treatment and post-treatment flights, we subtracted pre-treatment values of strikes (Δ Strikes for first 105 min and Δ 30-Strikes for first 30 min), cost of transport (Δ COT), flight power (Δ Power), and flight duration (Δ Duration) from their respective post-treatment values. We then used a general linear model (GLM) to test for the effect of treatment on Δ Strikes, Δ 30-Strikes, Δ COT, Δ Power, and Δ Duration. We also reported the least-squared means and standard errors of the same performance metrics for both pre-treatment and post-

treatment flights in a separate table (Table 2). Altitude attained (Altitude), flight duration at altitude (Alt. Duration), and number of strikes at 3000m (Alt. Strikes) were analyzed using Kruskal–Wallis test, followed by multiple comparisons using LSD-test if the model was significant. We report the *H*-, *F*-, and *Z*- statistics and the associated *P* values.

Final sample sizes for all behavioural and physiological metrics are listed in table S 4-1.

Results

Dosing pilot: Effects of avian EPO and anti-EPO on Hct and Hb

There was a marginally significant effect of treatment on Hct ($F_{2,27}$ = 3.28, P = 0.05; Fig. 2A): birds dosed with Anti-EPO had lower Hct than birds dosed with EPO (pairwise contrast, Tukey HSD, Z = -3.56, P < 0.01), and birds dosed with Veh (Z = -3.26, P < 0.01). Although Hct of the EPO group tended to be higher than Veh group, the effect was not significant (Z = -0.57, P = 0.83). There was no significant effect of treatment on Hb ($F_{2,28}$ = 2.32, P = 0.11; Fig. 2B). or on body mass ($F_{2,28}$ = 1.05, P = 0.36).

Effects of EPO and anti-EPO on flight performance at low altitude

There was a marginally significant effect of treatment on the change in number of strikes in the first 30 min of flight (Δ 30-Strikes; $F_{2,19}$ = 3.12, P = 0.06; Fig. 3A). Anti-EPO treated birds performed worse than EPO birds during the post-treatment flight (pairwise contrast, Tukey HSD, Z = 2.51, P = 0.03). There was no significant difference between Anti-EPO treated birds and Vehicle treated birds (Z = 1.01, P = 0.57) and between EPO treated birds and Veh treated birds (Z = -1.44, P = 0.32) in terms of Δ 30-Strikes. There was no significant effect of treatment on the change in number of strikes in the first 105 min of flight (Δ Strikes; $F_{2,19}$ = 2.03, P = 0.15; Fig. 3B), change in flight duration (Δ Duration; ($F_{2,19}$ = 0.17, P = 0.84; Fig. 3C), change in cost of transport (Δ COT; $F_{2,19}$ = 1.18, P = 0.33; Fig. 3D), or change in flight power (Δ Power; $F_{2,19}$ = 1.18, P = 0.33; Fig. 3E).

Effects of EPO and anti-EPO on flight performance at high altitude

There was a significant effect of treatment on altitude attained (Altitude; $H = 7.37$, $df = 2$, $P = 0.02$, Fig. 4A): birds dosed with Anti-EPO attained significantly higher altitude than EPO dosed birds (LSD test, $Z = 3.06$, $P < 0.01$), but similar altitude to birds dosed with Veh ($Z = 1.43$, $P = 0.15$). Altitude attained by EPO dosed birds tended to be lower than Veh dosed birds but the difference was not significant ($Z = -1.62$, $P = 0.10$). Similarly, there was a significant effect of treatment on flight duration at altitude (Alt. Duration; $H = 7.24$, $df = 2$, $P = 0.02$, Fig. 4B), where both Anti-EPO dosed birds and Veh dosed birds had significantly greater Alt. Duration than EPO treated birds ($Z = 3.33$, $P < 0.01$ and $Z = -2.06$, $P = 0.03$ respectively). The Anti-EPO group and Veh group had similar Alt. Duration ($Z = 1.27$, $P = 0.20$). There was no significant difference in number of strikes at 3000m between the 3 different treatment groups (Alt. Strikes; $H = 4.30$, $df = 2$, $P = 0.11$) for birds that attained this altitude.

Physiological responses to flight at high altitude

All 3 treatment groups were pooled for analyses of Hct, glucose, total antioxidant capacity (OXY) as there was no significant treatment effect or treatment × time interaction ($P > 0.1$ in all cases). There was a significant flight × time interaction for Hct ($F_{1,18} = 4.87$, P = 0.04; Fig. 5A). Regardless of treatment, birds that were flown at high altitude post-treatment had significantly lower Hct post-flight than when they were flown at low altitude pre-treatment ($t_{18} = 3.62$, P < 0.01), while birds that were flown at low altitude post-treatment had similar Hct post-flight as pre-treatment ($t_{18} = -0.88$, P = 0.81). Hb was independent of the flight × time interaction ($F_{1,18} = 0.18$, P = 0.67). We did not examine flight × time interaction for glucose, OXY and dROMs because we only had enough plasma samples from one bird (out of four) that were flown at low altitude for both pre- and post-treatment flight. However, for the one bird that we do have data for, blood glucose were 16.5 mmmol/L and 15.9 mmol/L for pre-treatment and post-treatment flights respectively. Regardless of treatment, all birds that were exposed to altitude challenge during the post-treatment flight had significantly lower Hct ($F_{1,16} = 12.59$, $P < 0.01$; Fig. 5A), higher blood glucose ($F_{1,16} = 7.40$, $P = 0.01$; Fig. 5B), and lower OXY ($F_{1,16} = 10.36$, $P < 0.01$; Fig. 5C) in response to flight at high altitude. There were neither main effects of treatment and time, nor treatment × time interaction for Hb

($F_{2,14}$ = 0.48, P = 0.62) and reactive oxygen metabolites production (dROMs; $F_{2,14}$ = 2.79, P = 0.09; Fig 5D).

Discussion

We manipulated Hct and Hb of yellow-rumped warblers experimentally using EPO and Anti-EPO and investigated flight performance at low and high altitude. Our experiment showed that compared to birds treated with EPO, birds treated with Anti-EPO had significantly lower Hct and more difficulty flying in the wind tunnel (i.e. lower exercise performance) at the low altitude condition. However, Anti-EPO treated birds performed significantly better than EPO treated birds in high altitude conditions. All birds appeared to experience similar physiological responses to exercise at high altitude regardless of treatment. When exercising at high altitude condition, birds decreased Hct but not Hb. There is also some evidence for increased glucose mobilization and decreased antioxidant capacity in response to flight at high altitude, although more data is required to justify this conclusion.

Migratory birds up-regulate the rate of red blood cell production and Hct prior to and during migratory season to fuel energetically demanding (but presumably low altitude) migratory flights (Fudickar et al., 2016; Krause et al., 2016), potentially through action of the hypoxia-inducible factor (HIF) signalling pathway and downstream production of the hormone EPO (Fudickar et al., 2016; Jelkmann, 2011). Although we did observe a significant decrease in Hct in birds treated with Anti-EPO, consistent with findings from other taxa (Schooley and Mahlmann, 1971; Wolf et al., 2001), we did not observe the anticipated increase in Hct in EPO treated birds. Behavioural and physiological effects of hormones often involve more than just circulating level of hormones, but rather a complex mechanism that includes components such as receptors, enzymes, and binding globulins (Williams, 2008). It is possible that EPO receptors, rather than plasma EPO level, are the limiting factor in our study system. In other words, EPO receptors could have already been saturated at the time of the study due to the migratory status of the birds and thus further augmentation of the ligand via exogenous method (i.e. injections) was not able to elicit a further increase in the rate of erythropoiesis. Our birds were still in migratory condition at the time of the dosing study, as they were brought into captivity during the fall migration season and were still being exposed to long day length in captivity. Anti-EPO did not produce the same anticipated

effect on Hb in the dosing pilot, suggesting that Hb is regulated somewhat independently of Hct in birds (Wagner et al., 2008; Williams et al., 2012). It should also be noted that although we found an effect of Anti-EPO on Hct in the dosing pilot, a treatment effect was not observed in the wind tunnel endurance flight experiment. However, the two findings are not directly comparable because post-treatment blood samples were collected under resting condition in the dosing pilot whereas in the flight experiment, post-treatment blood samples were collected after flight at high altitude. Additionally, the values of Hct are higher in the wind tunnel endurance flight experiment than in the dosing pilot perhaps because different individuals were used in the pilot and the flight experiment (screening of birds was only performed after the dosing pilot; see Materials and Methods for details) and differences in time of the year (fall migration vs. spring migration).

Consistent with our initial prediction, as well as the physiological response observed in the dosing study, we found that birds dosed with Anti-EPO had lower flight performance (i.e. higher number of strikes in the first 30 min of flight) in the wind tunnel in low altitude conditions. However, it should be noted that Anti-EPO birds were committing higher number of strikes in the first 30 min in the pre-treatment flight relative to EPO and Veh birds (Table 2), hence “regression toward the mean” phenomenon (i.e. if a variable is extreme on its first measurement, it will tend to be closer to the average on its second measurement) (Nesselroade et al., 1980) could not be ruled out completely. We also did not find a significant treatment effect for either measure of flight energy expenditure. In other words, the finding of low exercise performance in Anti-EPO treated birds was made based only on number of strikes in the first 30 min of flight, among other metrics but this metric has been proven to be a valid measurement of flight performance in other wind tunnel studies (Ma et al., 2018; Maggini et al., 2017). As discussed before, migratory endurance flight is an energetically demanding activity (Piersma, 2011b), and thus, high oxygen carrying capacity is essential for maintenance of the intense exercise. The higher number of strikes in the first 30 min of post-treatment flight exhibited by Anti-EPO dosed birds could be explained by impaired oxygen carrying capacity due to an Anti-EPO induced decrease in Hct. Our study suggests that birds with lower Hct and reduced aerobic capacity may have increased risk of mortality during migration due to more erratic flight behaviour (i.e. higher number of strikes).

Contrary to our initial prediction, we found that birds dosed with Anti-EPO had better exercise performance at high altitude: they attained significantly higher altitude and had significantly longer flight duration at high altitude. We did not detect a treatment effect on the number of strikes at 3000m (as at low altitude) but this was because all but one EPO-dosed birds failed to attain an altitude of 3000m, when number of strikes was quantified. These findings stand in contrast with findings from other studies that manipulated Hct and investigated performance (Fronstin et al., 2016; Schuler et al., 2010), which generally found impaired performance with reductions in Hct. However, it is widely accepted that the relationship between Hct and oxygen carrying capacity is not linear, but rather parabolic in shape (Birchard, 1997; Petit and Vezina, 2014; Schuler et al., 2010). An increase in Hct can lead to a linear increase in blood oxygen carrying capacity, but also an exponential increase in blood viscosity (Fig. 6), which would hinder transport of oxygen to active tissues (Birchard, 1997). This suggests that the higher performance of Anti-EPO treated birds at high altitude might be due to lower blood viscosity, particularly since birds had very little time to adjust to the increase in altitude in this experiment; to address this possibility we need to first consider our data on the physiological response of exercise at high altitude.

Our study showed that yellow-rumped warblers decreased Hct when exercising at altitude, regardless of treatment a result which is contrary to our initial prediction, as well as other studies of the effects of altitude on Hct (Borras et al., 2010; Jelkmann, 2003; Jelkmann, 2011). It has been shown that birds decrease Hct as a result of hemodilution in response to endurance flight (Jenni et al., 2006). Jenni et al. (2006) suggested that hemodilution is an adaptive response to endurance exercise in birds because it lowers blood viscosity and thereby lowering heart energy expenditure, as well as increases delivery of fuel in circulation. Given that hemodilution is an adaptive response to exercise at low altitude condition, and given that birds presumably have to work harder to extract oxygen from the environment at high altitude condition, perhaps it was not surprising to see further hemodilution with high altitude exposure. With further hemodilution, it seems intuitive that the optimal hematocrit for exercise at high altitude would be lower than the optimal hematocrit for exercise at low altitude, in other words, a left shift of the optimal hematocrit curve (Birchard, 1997) with increasing altitude (Fig. 6). If this is the case we suggest a potential explanation for the seemingly paradoxical findings of flight performance in Anti-EPO vs. EPO dosed birds in low and high altitude

conditions. At the low altitude condition, Anti-EPO birds would have lower performance due to lower than optimal Hct values and consequently lower oxygen carrying capacity, but at high altitude condition, they would have higher flight performance because their initially low Hct would now be closer to a new “optimal Hct” for exercise due to lower blood viscosity (Fig. 6), at least over the short term measured in our study.

There is some evidence for increased glucose mobilization in response to high intensity exercise at high altitude in birds subjected to altitude challenge, regardless of treatment. However, a weakness of the study is that we did not include a low-altitude control group, which limits our ability to specifically pinpoint the effects of high altitude exposure on blood glucose and oxidative stress. Nevertheless, the finding of increased glucose mobilization at high altitude is consistent with our initial prediction, as well as findings from mammalian studies (Hochachka, 1985; McClelland et al., 1998; Schippers et al., 2012). Although birds use predominantly fatty acids to fuel endurance flight, due to the fact that lipid is the most energy dense metabolic fuel (Guglielmo, 2010), oxidizing lipid becomes increasingly challenging as altitude increases and partial pressure of oxygen decreases (Melzer, 2011). Therefore, birds likely altered their fuel selection and relied more on glucose as an oxygen-saving strategy during hypoxia (i.e. high altitude) exposure (Melzer, 2011). Contrary to our initial prediction, we did not find a treatment effect of EPO and Anti-EPO on oxidative stress. However, we did observe a decrease in total anti-oxidant capacity and hence, increased oxidative stress with exercise at high altitude. This suggests that flying at high altitude for long period of time can be detrimental to the birds’ physical condition, and could potentially explain why most birds spend the majority of time flying at low altitude during migratory flight (Scott, 2011). As mentioned before, the finding of increased oxidative stress at high altitude should be taken with a grain of salt due to a lack of low-altitude control. Additionally, although we saw an overall increase in oxidative stress, similar to the general finding of Jenni-Eiermann et al. (2014), the increased oxidative stress observed in our study was mainly due to a decrease in antioxidant capacity and not an increase in reactive oxygen metabolite production, whereas Jenni-Eiermann et al. (2014) found increases in both antioxidant capacity and oxidative damage. This difference could either be due to inherent species difference or slight difference in migration strategy or behaviour. It should also be noted that increased oxidative stress does not always result in negative

outcome as it is widely known that ROS production is commonly used for signaling mechanisms (Dzal et al., 2015; Metcalfe and Alonso-Alvarez, 2010).

In summary, we provide experimental evidence that the relationship between Hct and exercise performance is dependent on altitude, with low Hct being detrimental at low altitude, potentially due to low oxygen carrying capacity, but advantageous at high altitude, potentially due to low blood viscosity. Free-living birds likely climb at slower speeds and thus adjust their physiology better to the changing altitude. Therefore, determining whether free-living migratory birds adaptively modulate their Hct level based on the altitude they fly at during long distance migratory flight requires further work. Given the rapid advancement of technology associated with remote tracking of wild animals such as biosensors and radio telemetry (Gumus et al., 2014; Gumus et al., 2015; Killen et al., 2017), future studies should manipulate Hct in the field and investigate migratory capacity of free-living migratory birds (Yap et al., 2017a).

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Figures and Tables

Figure 4-1 Experimental timeline

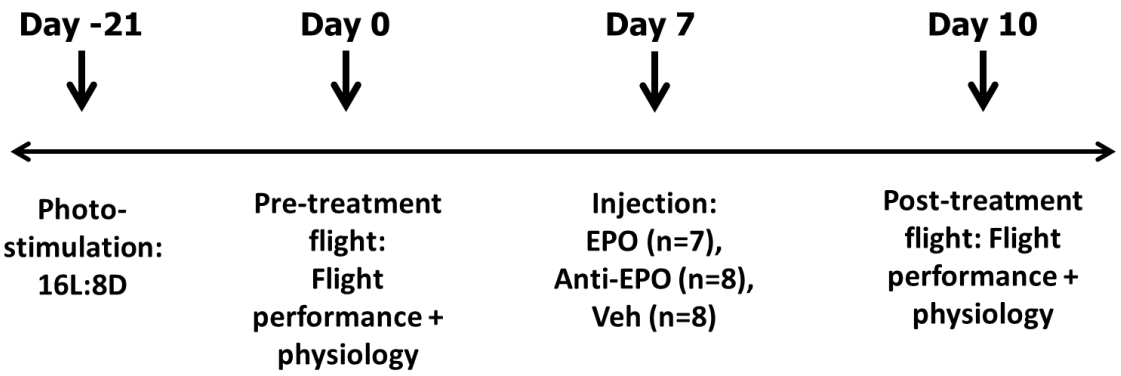

Figure 4-2 Effects of avian EPO and anti-EPO on A) Hct and B) Hb. Data shown are least-squared means ± s.e.

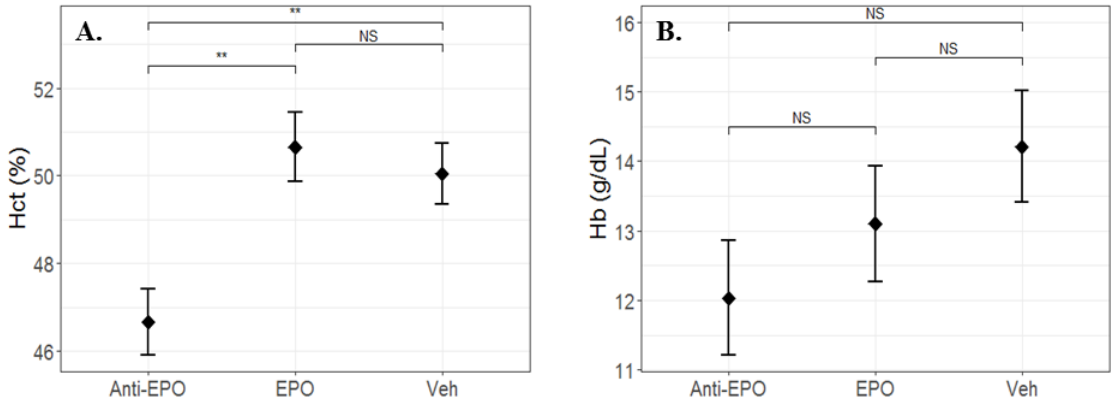

Figure 4-3 **Effects of EPO and anti-EPO on A) Δ 30-Strikes, B) Δ Strikes, C) Δ Duration, D) Δ COT, and E) Δ Power. Data shown are least-squared means ± s.e.**

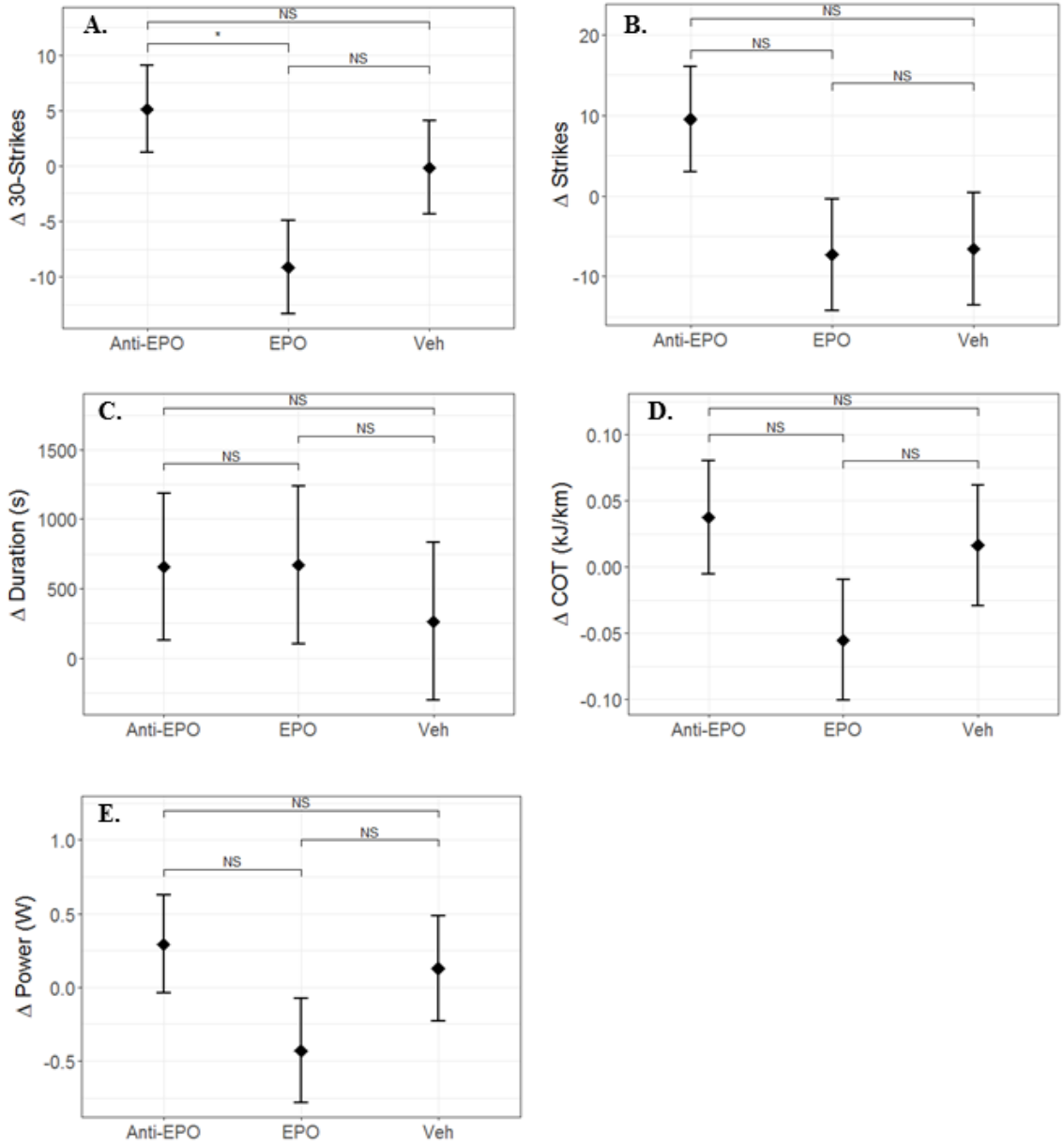

Figure 4-4 **Effects of EPO and anti-EPO on A) Altitude and B) Alt. Duration. Data shown are least-squared means ± s.e.**

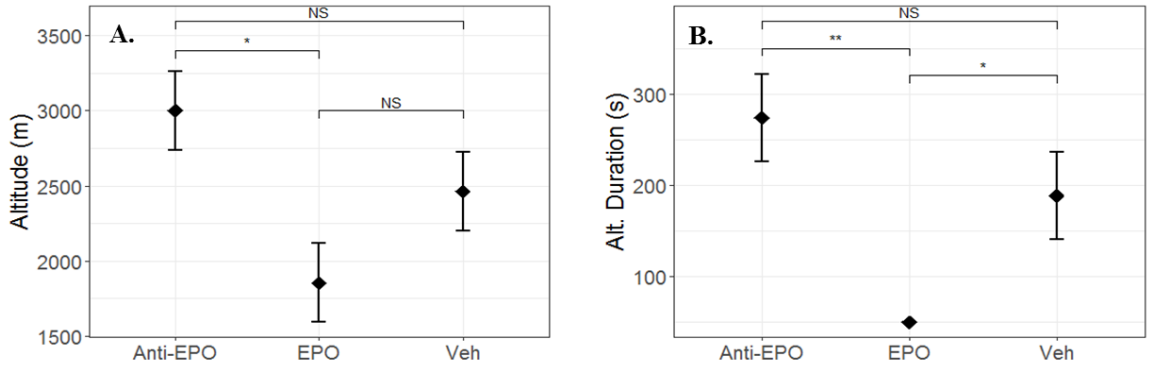

Figure 4-5 **Effects of exercise at high altitude on A) Hct, B) Glucose, C) OXY, and D) dROMs. Data shown are least-squared means ± s.e. Treatments groups pooled for A, B, and C.**

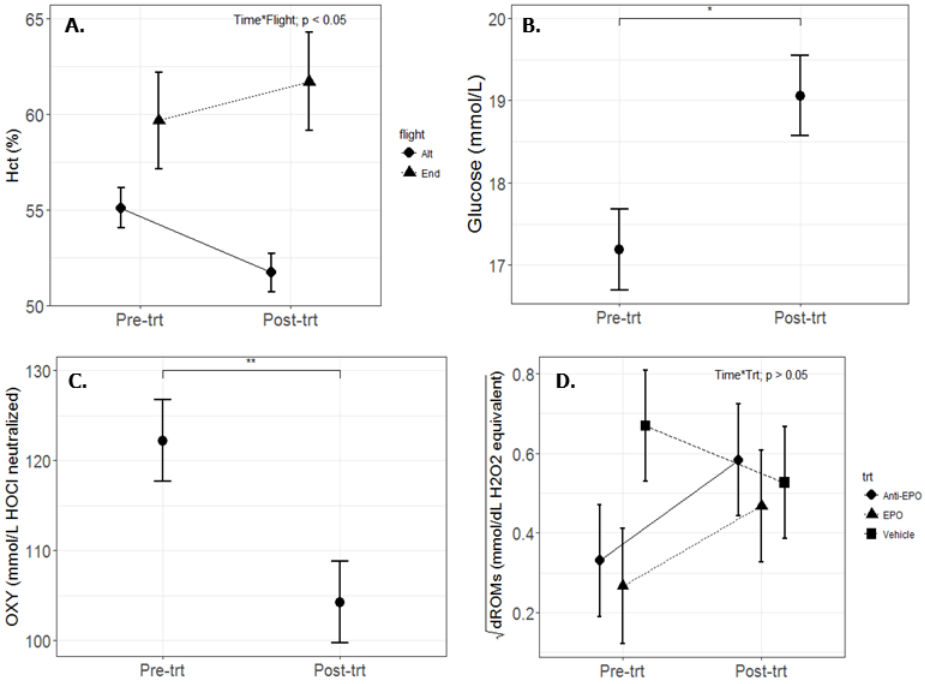

Figure 4-6 **Hypothetical post-hoc rationale for the effects of Anti-EPO on flight performance at low and high altitudes. At the low altitude condition, Anti-EPO birds had lower performance due to lower than optimal Hct values and consequently lower oxygen carrying capacity, but at high altitude condition, they had higher flight performance because their initially low Hct was now closer to a new "optimal Hct" for exercise due to lower blood viscosity, at least over the short term measured in our study.**

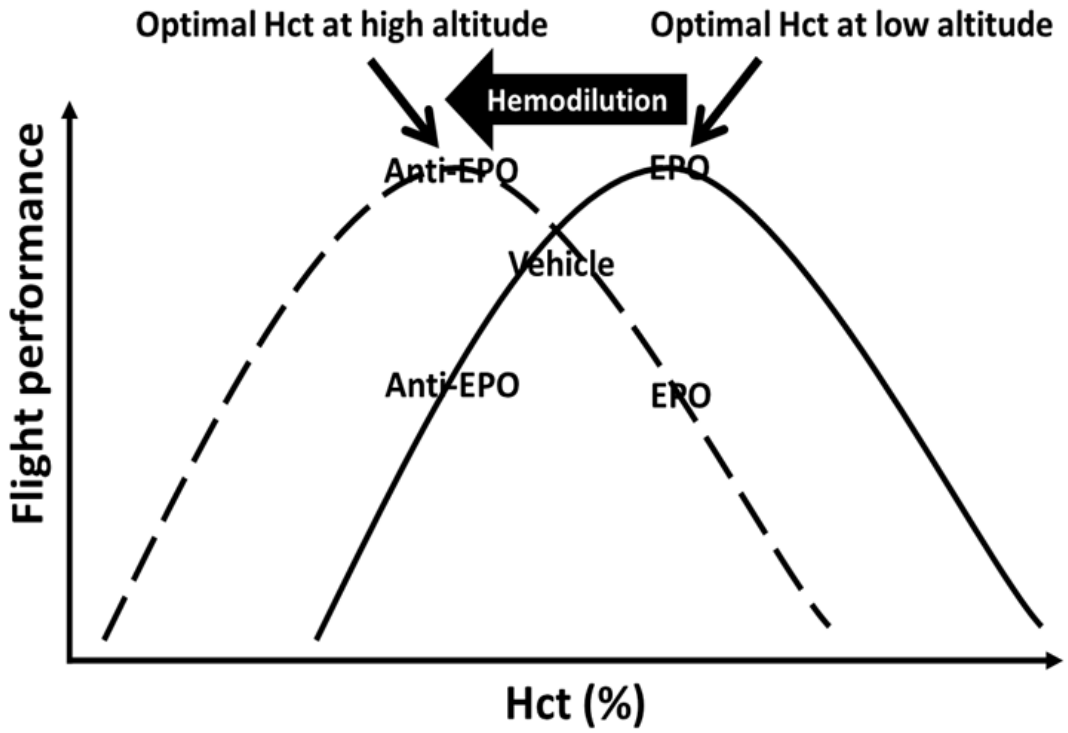

Table 4-1 Pre-treatment and post-treatment values for Hct and Hb during dosing pilot. Data shown are least-squared means ± s.e.

	Pre-treatment			Post-treatment		
	Anti-EPO	**EPO**	**Veh**	**Anti-EPO**	**EPO**	**Veh**
Hct (%)	49.49 ± 1.13	47.96 ± 1.12	48.28 ± 1.07	47.33 ± 1.13	50.28 ± 1.17	49.98 ± 1.07
Hb (g/dL)	13.14 ± 0.77	12.78 ± 0.76	14.32 ± 0.73	11.93 ± 0.76	13.01 ± 0.77	14.54 ± 0.73

Table 4-2 **Pre-treatment and post-treatment values for Strikes, 30-strikes, COT, Power, and Duration during wind tunnel endurance flight. Data shown are least-squared means ± s.e.**

	Pre-treatment			Post-treatment		
	Anti-EPO	EPO	Veh	Anti-EPO	EPO	Veh
Strikes	11.13 ± 6.99	20.14 ± 7.47	9.43 ± 7.47	20.63 ± 6.99	12.86 ± 7.47	2.86 ± 7.47
30-Strikes	6.25 ± 4.21	11.43 ± 4.50	2.28 ± 4.50	11.38 ± 4.21	2.28 ± 4.51	2.14 ± 4.50
COT (kJ/km)	0.24 ± 0.03	0.26 ± 0.04	0.25 ± 0.04	0.28 ± 0.03	0.20 ± 0.04	0.27 ± 0.04
Power (W)	1.90 ± 0.26	2.03 ± 0.28	1.95 ± 0.28	2.19 ± 0.26	1.60 ± 0.28	2.08 ± 0.28
Duration (s)	4999.00 ± 478.00	5572.85 ± 511.00	6004.29 ± |511.00	5655.75 ± 478.00	6241.14 ±| 511.00	6267.86 ± 511.00

Chapter 5.
Physiological effects of increased foraging effort in a small passerine

Abstract

Foraging to obtain food, either for self-maintenance or at presumably elevated rates to provide for offspring, is thought to be an energetically demanding activity but one that is essential for fitness (higher reproductive success and survival). Nevertheless, the physiological mechanisms that allow some individuals to support higher foraging performance, and the mechanisms underlying costs of high workload, remain poorly understood. We experimentally manipulated foraging behaviour in zebra finches (*Taeniopygia guttata*) using the technique described by Koetsier and Verhulst (2011). Birds in the 'high foraging effort' (HF) group had to obtain food either while flying/hovering or by making repeated hops or jumps from the ground up to the feeder, behaviour typical of the extremely energetically expensive foraging mode observed in many free-living small passerines. HF birds made significantly more trips to the feeder per 10 min, whereas control birds spent more time (perched) at the feeder. Despite this marked change in foraging behaviour, we documented few short- or long-term effects of 'training' (3 days and 90 days of 'training', respectively) and some of these effects were sex specific. There were no effects of treatment on basal metabolic rate, haematocrit, haemoglobin or plasma glycerol, triglyceride and glucose levels, and masses of kidney, crop, large intestine, small intestine, gizzard and liver. HF females had higher masses of flight muscle, leg muscle, heart and lung compared with controls. In contrast, HF males had lower heart mass than controls and there were no differences for other organs. When both sexes were pooled, there were no effects of treatment on body composition. Finally, birds in the HF treatment group had higher levels of reactive oxygen metabolites (dROMs) and, consequently, although treatment did not affect total anti-oxidant capacity, birds in the HF treatment group had higher oxidative stress.

Originally published as Yap, K. N., Kim, O. R., Harris, K. C., & Williams, T. D. (2017). Physiological effects of increased foraging effort in a small passerine. *Journal of Experimental Biology*, 220(22), 4282-4291.

Introduction

Foraging to obtain food is essential for successful reproduction and survival. However, foraging in many animals, either for self-maintenance or at presumably elevated rates to provide for offspring, is thought to be an energetically demanding activity that should select for high workload ability (Bryant and Tatner, 1991; Maurer, 1996; Piersma and van Gils, 2011). Strong selection would be expected to decrease variation in traits underpinning foraging but we see considerable individual variation in foraging and provisioning effort (Royle et al., 2014; Fowler and Williams, 2015). This suggests that although some individuals might have higher foraging ability the high workload associated with foraging and provisioning is costly, which would oppose directional selection. In support of this view, Mariette et al. (2011) found that wild breeding zebra finches (*Taeniopygia guttata* Reichenbach 1862) covered an average of 6.4 km daily to forage for food, but some individuals travelled up to 19.4 km and these 'hard-working' individuals appeared to pay a cost in that they took longer to re-nest after a successful breeding attempt. Although there is some experimental evidence from studies directly manipulating foraging costs, or demand via brood size manipulation, that increased workload leads to reduced fecundity (Veasey et al., 2001; Simons et al., 2014) and increased mortality (Daan et al., 1996), the physiological mechanisms that allow some individuals to support higher foraging performance, and the mechanisms underlying costs of high workload, remain poorly understood.

Exercise can be broadly defined as any behaviour that elevates the level of intensity of activity or workload, in response to an ecological demand for increased performance (Booth et al., 2012; Halsey, 2016; Irschick and Higham, 2016). Hence, given the high activity level and metabolic demand associated with foraging flights (Maurer, 1996), and the intuitive, positive relationship between foraging performance and fitness during chick-rearing, it might be valuable to apply an exercise perspective on workload during foraging and parental care (Williams and Fowler, 2015). The physiology of exercise has been investigated in many model systems, e.g. migratory birds flying in wind tunnels (Guglielmo, 2010; Price et al., 2010) and exercise training in captive birds using automated systems (Nudds and Bryant, 2000; Costantini et al., 2012; Zhang et al., 2015). Although these model systems might provide a good starting point for understanding physiological adaptations of aerobic capacity associated with exercise or

workload, the critical relationship in free-living animals between exercise and acquisition of resources is often ignored in these studies, many of which also use using forced exercise protocols. Specifically, in relation to foraging it is of great importance to adopt an exercise contingent method, where animals have to work for food, because the physiological effects of voluntary exercise with access to resources might be very different from those induced by forced exercise in less ecologically relevant contexts (Fonseca et al., 2014; Irschick and Higham, 2016). For instance, Fonseca et al. (2014) found that when acquisition of food was contingent upon the distance rats need to run, adipose tissue was significantly decreased, compared with rats in which food acquisition was not dependent upon running distance. It is also important to consider the relative energetic cost of different types of flight and foraging mode. For instance, some birds use more energetically expensive flapping/ hovering flights during foraging, while others use less energetically costly soaring flights during foraging (Norberg, 1996). Small passerines search for, and capture, insects during short flights or quick hovers, which has been suggested to be an extremely energetically expensive foraging mode [with a scaling exponent of daily energy expenditure (DEE)=mass$^{1.99}$, as opposed to scaling exponents of DEE=mass$^{0.66–0.75}$, in birds that do not engage in this kind of foraging mode; Tinbergen and Dietz, 1994]. Furthermore, the duration of exercise training can also influence the physiological response of exercise. Most studies only looked at acute physiological effects of exercise, and long-term physiological adjustments have rarely been considered. Koetsier and Verhulst (2011) and Simons et al. (2014) addressed this issue of the influence of food availability on exercise and workload by using a technique to manipulate foraging effort in birds. Their technique forces birds to hop to and hover briefly in front of the feeder to obtain seeds, mimicking the energetically expensive foraging mode of small passerines described above (Tinbergen and Dietz, 1994).

Koetsier and Verhulst (2011; see also Simons et al., 2014; Briga et al., 2017) showed that experimental manipulation of foraging costs affected energy expenditure, survival (individuals reared in experimentally enlarged brood only), and reproduction, but the physiological basis of these effects remains unknown. The objective of our study was therefore to investigate physiological effects of training for increased foraging effort. As animals appear to be able to regulate individual components of their physiology independently (Buehler et al., 2012; Williams and Fowler, 2015), we measured multiple physiological traits: basal metabolic rate (BMR), haematocrit (Hct), haemoglobin (Hb),

body composition, glucose, glycerol, triglyceride and oxidative stress. We predicted that in response to high foraging effort treatment, birds would: (1) adopt an energetically costly foraging mode, have higher flight activity and decrease BMR (Koetsier and Verhulst, 2011), (2) elevate Hct and Hb (Fair et al., 2007) in the short term but decrease Hct and Hb eventually when foraging costs become too high and maintaining energy balance becomes more difficult, (3) have enlarged metabolic machinery organs and food processing organs (Swallow et al., 2010) despite an overall decrease in energy expenditure (Westerterp et al., 1994; Wiersma and Verhulst, 2005; but see Williams and Vézina, 2001; Zhang et al., 2015), (4) show increases in markers of energy supply such as triglyceride (Kern et al., 2005), but also (5) show increased levels of oxidative stress (Costantini et al., 2012; Jenni-Eiermann et al., 2014).

Materials and Methods

Animal husbandry

Zebra finches were maintained in controlled environmental conditions (temperature 19–23°C; humidity 35–55%; constant light schedule, 14 h:10 h light:dark, lights on at 07:00 h). All birds were provided with a mixed seed diet [Panicum and white millet (1:3), 11.7% protein, 0.6% lipid and 84.3% carbohydrate by dry mass], water, grit (coral sand) and cuttlefish bone (calcium) *ad libitum*, and received a multi-vitamin supplement in the drinking water once per week. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 1074B-94), in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

Experimental manipulation of foraging costs

Foraging costs were experimentally manipulated in a ‘high foraging effort’ (HF) group using the technique described by Koetsier and Verhulst (2011). Food (mixed seed) was provided in transparent Plexiglas containers (length×width×height: 40×10×13 cm) suspended from the roof of the cage (length×width×height: 122×46×41 cm),with feeding holes low on the front panel to allow access to seeds. Perches made of wooden pencils (diameter 0.8 cm) were fitted adjacent to feeding holes to allow birds to perch while foraging for 21 days prior to the start of the experiment (similar to standard feeders

in control cages). We also measured BMR and collected blood samples during the 21-day period, prior to shortening the perches. Over a 14-day period, perches were gradually shortened (0.5 cm every 2 days) and eventually removed completely to train birds to modify their foraging behaviour and obtain seeds in the high foraging cost condition. As the perches became shorter the birds were unable to perch and had to obtain seeds either while flying/hovering in front of the suspended feeder, or by making repeated hops or jumps from the ground up to the feeder (the vertical distance between the cage floor and the feeding holes was ~30 cm). To prevent birds from eating seeds spilled on the cage floor, the metal tray was removed from the bottom of all HF cages, so that seeds fell through the cage bottom. In lieu of the metal tray, small resting platforms made of egg carton were secured to each side of the cage to allow birds to rest when not foraging. Birds in control foraging conditions (CTR) were given standard feeders (seed fountains) with perches adjacent to them throughout the experiment. A total of four HF cages and four CTR cages were used for the experiment and both HF and CTR conditions were offered simultaneously during the experiment. A picture of the set-up of the HF cage is provided (Fig.S5-1). Several notable differences between the set-up of this experiment and the set-up in Koetsier and Verhulst (2011) include: (1) the size of the cage is smaller than the aviaries used by Koetsier and Verhulst (2011), and as a consequence the distance birds had to fly for food is presumably smaller, and (2) the aviaries used by Koetsier and Verhulst (2011) were outdoor, i.e. at lower and fluctuating ambient temperatures, while birds in this experiment were housed in temperature-controlled indoor facilities.

Experimental timeline

Male and female birds were housed in groups of eight, in single-sex cages during the experiment and were kept in their respective foraging condition (HF and CTR) for 90 days. To ensure sufficient sample size, the main experiment was repeated over two trials: trial 1 (summer 2014) and trial 2 (spring 2015) with all birds exposed to the same experimental conditions and protocols, as well as environmental conditions in both trials. Birds were randomly assigned to HF and CTR conditions. Specifically, birds from the same home cages were distributed across both treatments, and each treatment consisted of more than one cage. For example, the first bird caught was placed in a HF cage, the second bird caught was placed in a CTR cage, the third bird caught in another

HF cage, and so on. Hence, both high- and low-quality birds should be at least somewhat evenly distributed across both treatment groups. We measured BMR and collected blood samples at three time points: (1) prior to the start of the 14-day perch shortening period (pre-treatment), (2) ~3 days after complete removal of perches (day 3) and (3) ~60 days after complete removal of perches (day 60) to assess both short- and long-term responses to change in activity level. Birds were kept in their respective foraging conditions for an additional 30 days after the last BMR measurement, at the end of which they were killed and tissues and blood samples were collected for further analysis (day 90). A summary of the experimental timeline is provided in Fig. 1.

Behavioural observations

After completion of all BMR measurements at day 60 (see below), we video-recorded behaviour of birds in each treatment cage for a total duration of 30 min between 09:00 h and 15:00 h. Individual birds could be identified using a unique combination of colour leg bands. Behaviours quantified during the entire 30 min duration include total time spent foraging, resting and engaging in other physical activities (e.g. preening, perch hop, displacement behaviour, etc.). In addition, similar to Koetsier and Verhulst (2011), foraging flight activity (trips to feeder) was scored for individual birds for a period of 10 min. All behaviour was scored by a single researcher.

Basal metabolic rate measurement

All BMR measurements were conducted using a flow-through respirometry system (Sable Systems International, Las Vegas, NV, USA) similar to that described in Salvante et al. (2010). O_2 and CO_2 analyzers (Sable Systems International FC-1 and CA-1, respectively) were calibrated every day using standard air containing 20.8% O2 and 1.10% CO2. To ensure post-absorptive state at the time of BMR measurement, individuals undergoing metabolic measurement that night were fasted for 3 h before entering the metabolic chambers (Secor, 2009; Salvante et al., 2010). Birds were taken from their cages at 21:00 h and placed in one of four metabolic chambers (1.5-litre stainless-steel coffee canisters, Great Canadian Superstore, Coquitlam, BC, Canada) for 2 h prior to the beginning of measurements. The system was checked for leaks before each round of metabolic rate measurement. All metabolic chambers were placed in an incubator (PTC-1 Peltier effect temperature-controlled portable cabinet, Sable Systems

International) maintained at 36°C for the entire duration of BMR measurement, within the thermoneutral zone of the zebra finch (Marchall and Prinzinger, 1991). Each metabolic chamber continuously received ~500 ml min^{-1} of dry air (using magnesium perchlorate as scrubber). Each of the three metabolic chambers containing a bird and an empty chamber sampling baseline ambient air were sampled for 10 min by a multiplexer (TR-TM4, Sable Systems International) every 40 min, allowing a total of 100 min of recording per chamber over 7 h. BMR calculations were done based on the lowest averaged 5 min of oxygen consumption per measurement sequence according to Lighton's eqns 10.6 and 10.7 (Lighton, 2008) with ExpeData software, version 1.2.6 (Sable Systems International). Birds were weighed immediately before and after measurement and the average of the two masses was used in BMR analysis. Birds were taken out of the metabolic chambers at 06.00 h the next morning.

Physiological measurements and assays

Pre-treatment, day 3 and day 60 blood samples (~100 µl) were obtained from the brachial vein following puncture with a 26G needle, and blood was collected using a 75 µl microhaematocrit tube. Hct (percent packed cell volume) was measured with digital callipers (±0.01 mm) following centrifugation of whole blood for 3 min at 13,700 g (Autocrit Ultra 3; BD Diagnostic Systems, Sparks, MD, USA). Hb (g dl^{-1} whole blood) was measured using the cyanomethaemoglobin method (Drabkin and Austin, 1932) modified for use with a microplate spectrophotometer (BioTek Powerwave 340; BioTek Instruments, Winooski, VT, USA), using 5 µl whole blood diluted in 1.25 ml Drabkin's reagent (D5941; Sigma-Aldrich Canada, Oakville, Ontario, Canada) with absorbance measured at 540 nm. Intra- and inter-assay coefficients were 3.1 and 3.8%, respectively. Blood glucose was also measured in individuals at the time of blood sampling using a glucose meter (Accu-Chek Aviva; Roche Diagnostics, Mannheim, Germany).

Blood samples collected at day 90 were assayed for total antioxidant capacity (µmol HClO ml^{-1}; OXY), reactive oxygen metabolites (mg H_2O_2 dl^{-1}; dROMs), and plasma glycerol and triglyceride, in addition to Hct, Hb and glucose. Not all samples were assayed for all measures owing to insufficient plasma volumes, and haemolyzed and lipolyzed plasma samples were excluded (final sample sizes are listed in Table S5-2). All plasma samples were analyzed using a microplate spectrophotometer (BioTek Powerwave 340) and 96-well microplates. Free glycerol and total glycerol were assayed

via sequential colour end-point assay (Sigma-Aldrich Canada), using 5 µl of plasma with 240 and 60 µl of glycerol reagent and triglyceride reagent, respectively, with a reading taken at 540 nm after 10 min of incubation at 37°C after the addition of each reagent. Plasma triglyceride concentration was calculated by subtracting free glycerol from total glycerol. The intra-assay coefficient of variation was 4.8%. Analyses of oxidative stress were carried out according to established protocols as described in Costantini et al. (2011), with slight modification. Specifically, we measured dROMs and OXY using the commercial kits dROMs and OXY-Adsorbent Test (Diacron International, Grosseto, Italy), respectively. Intra-assay coefficients for OXY and dROMs were 3.8 and 2.4%, respectively.

Determination of immediate food consumption, dissection and body composition analysis

At 90 days birds were killed by exsanguination under anaesthesia (0.05 ml of 20 mg ml^{-1} xylazine and 0.05 ml of 100 mg ml^{-1} ketamine) and tissues were collected for further analysis. To determine immediate food consumption, we collected and weighed seeds from each bird's oesophagus at the time of tissue collection. After dissection, a sample of the right pectoralis muscle was immediately removed and weighed to be used as part of another study. The rest of the carcass was stored at −20°C until all the birds had been killed for further processing. The following tissues were dissected out from each bird: flight muscle (including the supracoracoideus and left pectoral muscle), leg muscle, crop, large intestine, small intestine, gizzard, heart, lungs, liver, kidney and reproductive organs (testes from males; ovary, ovarian follicles and oviduct from females). The presence of yolky follicles allowed us to determine the reproductive state of birds, and birds that were found to be in breeding condition (six females in trial 1, and seven females in trial 2) were excluded from subsequent analysis. Tissues were dried at 60°C for 24 h, weighed (mg, ±0.0001), and the final mass is reported as dry mass.

Statistical analyses

Analyses were carried out using R version 0.99.467 (R Core Team, 2013). Data were first examined for normality using the Shapiro–Wilk test, and data were either transformed prior to analysis or analyzed using a non-parametric test (independent two-group Mann–Whitney U-test). For repeated measures analysis (body mass, BMR, Hct,

Hb and glucose), we used the lme4 package (Bates et al., 2013) with sex, time and treatment as main effects, and individual bird identity (ID) as a random factor. Trial was initially included in all models but was taken out because we did not detect any main effects of trial, nor interactions between trial and other variables (P>0.1 in all cases). F-statistics and P-values were generated using the lmerTest package (Kuznetsova et al., 2013). Tukey's honest significant difference test (package multcomp; Hothorn et al., 2008) was used to evaluate pairwise comparisons between treatments and time points following a significant mixed model. Additionally, we also ran the repeated measures analysis (body mass, BMR, Hct, Hb and glucose) with day 3 and day 60 time points and treatment as main effects, pre-treatment values as covariate, and induvial bird ID as a random factor. For body composition, OXY, dROMs, triglyceride and glycerol analyses, we used a general linear model (GLM) testing for the effects of sex, treatment, and sex×treatment. To control for the effect of body mass on tissue mass, we used non-reproductive dry body mass (total dry body mass minus dry masses of reproductive organs) as a covariate. In addition, to account for part-whole correlation (Christians, 1999), we subtracted the mass of the tissue used as the dependent variable from the covariate. For instance, the model for testing the effect of treatment on heart mass would read 'heart mass~treatment+(body mass – heart mass)'. Furthermore, to investigate whether there was a treatment effect on dROMs after controlling for total anti-oxidant capacity, we conducted additional analysis by including OXY as a covariate in the model. We report the Z-statistics and the associated P-values. A summary of all data and statistical analyses is provided in Table S5-3.

Results

Effects of foraging treatment on behaviour and food consumption

When comparing foraging flight activity, HF birds made significantly more trips to the feeder per 10 min (W_{54} = 215, P < 0.01, Mann–Whitney–Wilcoxon rank sum test, Cohen's d = 1.05; Fig. 2A). Conversely, CTR birds spent more time (perched) at the feeder than HF birds (W_{54} = 452.5, P < 0.01, Mann–Whitney–Wilcoxon rank sum test, Cohen's d=0.74; Fig. 2B). There was no significant treatment effect for time spent resting (Z_{54} = 1.10, P = 0.27) or time spent engaging in other activities (Z_{54} = −1.48, P = 0.14). HF birds had ~50% more seeds in their oesophagus at the time of tissue collection than

CTR birds (W_{54} = 214, P < 0.01, Mann–Whitney–Wilcoxon rank sum test, Cohen's d = 0.30; Fig. 2C). It should be noted that the order in which birds were sacrificed was randomised and hence approximately the same number of birds in each treatment group was killed in the morning and in the afternoon.

Effects of foraging treatment on body mass, BMR and hematology

Sex was not included in the overall model as there was no significant sex effect or sex × treatment interaction for body mass, BMR and hematology. There was a significant treatment × time interaction for body mass ($F_{2,108}$ = 4.50, P = 0.01) (Fig 3A). Body masses of HF birds were significantly lower than CTR birds at day 3 (t_{41} = 2.23, P = 0.03), but in HF birds there was only a marginally significant decrease in body mass between pre-treatment and day 3 time points (P = 0.07; Fig.3A). There was no treatment × time interaction for BMR ($F_{2,107}$ = 0.14, P = 0.87) (Fig. 3B), Hct ($F_{2,107}$ = 1.16, P = 0.31) (Fig. 3C) and Hb ($F_{2,107}$ = 1.09, P = 0.34) (Fig. 3D) and no main effect of treatment. It should also be noted that there appears to be small to moderate differences between pre-experimental values between treatments (Cohen's d ranges from 0.04 to 0.49), although none of the differences were significant (P > 0.05 in all cases). None of the differences between pre-experimental values between treatments observed could be attributed to sex differences (sex × treatment interactions, P > 0.05 in all cases). Similar results were found even when the models were ran using Day 3 and Day 60 timepoints and treatment as main effects, pre-treatment values as covariate, and bird ID as a random factor (Fig. S5-4).

Effects of foraging treatment on body composition

When both sexes were pooled, there was no significant treatment effect at day 90 for dry mass of organs related to aerobic and metabolic capacity: flight muscle (Z_{52} = -1.59, P = 0.11, Cohen's d = 0.35), leg muscle (Z_{52} = -7.93, P = 0.43, Cohen's d = 0.29), heart (Z_{52} = 0.41, P = 0.68, Cohen's d = 0.12), and lungs (Z_{52} = -1.59, P = 0.11, Cohen's d = 0.44). However, there was a significant sex × treatment interaction at day 90 for dry mass of organs related to aerobic and metabolic capacity: flight muscle (t = -1.80, P = 0.05), leg muscle (t = -2.40, P = 0.02), heart (t = -2.58, P = 0.01) and lungs (t = -2.61, P = 0.01). HF females had higher flight muscle mass (Z_{52} = -3.26, P < 0.01, Cohen's d = 1.06; Fig. 4A), leg muscle mass (Z_{52} = -2.38, P = 0.02, Cohen's d = 1.32; Fig. 4B), lung

mass (Z_{52} = -3.15, P < 0.01, Cohen's d = 1.30; Fig. 4C), and heart mass (Z_{52} = -0.20, P = 0.05, Cohen's d = 0.74; Fig.4D) compared to controls. In contrast, HF males had lower heart mass (Z_{52} = 2.02, P = 0.04, Cohen's d = 0.50; Fig. 4D) than controls and there were no differences for other organs (P > 0.05 in all cases). Dry mass of kidneys (t = 0.73, P = 0.47) and food processing organs: crop (T = -0.54, P = 0.60), large intestine (t = -0.59, P = 0.56), small intestine (t = -1.09, P = 0.28), gizzard (t = 0.10, P = 0.92), and liver (t = -1.02, P = 0.31) were not affected by HF treatment in either sex.

Effects of foraging treatment/effort on plasma metabolites and oxidative stress

HF treatment did not influence levels of blood glucose ($F_{2,53}$ = 2.22, P = 0.11; Fig. 5A), plasma glycerol (Z_{35} = -0.57, P = 0.57; Fig 5B) and triglyceride (Z_{35} = 1.79, P = 0.86; Fig. 5C). OXY did not differ significantly between treatment groups (Z_{46} = 0.70, P = 0.48; Fig. 5D). However, HF treatment induced significantly higher dROMs (Z_{38} = -2.06, P = 0.04, Cohen's d = 0.60; Fig. 5E) than CTR treatment, even after controlling for OXY (Z_{38} = -2.11, P = 0.03, Cohen's d = 0.64).

Discussion

We used the technique of Koetsier and Verhulst (2011) to experimentally manipulate foraging behaviour in zebra finches and investigate physiological correlates of 'exercise' (*sensu* Halsey, 2016) and increased foraging effort. Birds in the experimental 'high foraging cost' group (HF) dramatically changed their foraging behaviour upon removal of perches: they made repeated, short (30 cm) vertical flights from the cage bottom to the feeder, or hovered at the feeder, whereas controls obtained seeds by perching on the feeder for more prolonged periods. HF birds made significantly more trips to the feeder per unit time but spent less total time at the feeder than control birds. This is probably due to differences in foraging behaviour between the two treatment groups, as well as the way we scored foraging behaviour, where any time spent at or near the feeder was included. To illustrate the differences in foraging behaviour, HF birds had to hop to and hover briefly in front of the feeder placed ~30 cm above the cage floor multiple times in order to obtain seeds, while control birds sat and perched on the feeder while they fed. This foraging mode in HF zebra finches mimics the energetically costly foraging typical of small free-living passerines (Tinbergen and Dietz,

1994). Furthermore, the effect of increased foraging effort on number of foraging trips to the feeder is comparable in magnitude to Koetsier and Verhulst (2011). Despite this marked change in foraging behaviour we documented few short- or long-term effects of 'training', and some of these effects were sex specific. There was a transient decrease in body mass in HF birds immediately after removal of perches, but body mass recovered to pre-treatment levels subsequent to a short term drop. This finding differs somewhat from the findings of Briga and Verhulst (2017), where birds subjected to high foraging cost weighed on average 4% less than control birds. There was no effect of foraging treatment on BMR, Hct, Hb or plasma glucose, glycerol and triglyceride levels. HF females had higher flight muscle, leg muscle and heart mass compared with controls, but HF males had lower heart mass than controls, and there was no effect of treatment on kidney and digestive organs. Finally, HF birds had a higher level of oxidative stress, with higher levels of reactive oxygen metabolites (dROMS) but similar anti-oxidant (OXY) levels. It should be noted that body composition measurements were carried out at a different time point relative to metabolic rate and physiology measurements. Therefore, the possibility of temporal variation in body composition in relation to training could not be ruled out.

Zebra finches in our high foraging cost treatment obtained seeds by making repeated, short (30 cm) vertical flights from the cage bottom to the feeder, or by hovering at the feeder. Tinbergen and Dietz (1994) showed that great tits (Parus major) spent less than 20% of their total time budget flying, while foraging for food to feed their chicks, yet their daily energy expenditure increased with body mass with an exponent of 1.99 (cf. exponent = 0.657 for the interspecific relationship between DEE and mass; Daan et al., 1991). They suggested that the high energetic cost of small jumps and hovers was more mass dependent than longer, sustained flight, due to low flight costs and frequent accelerations. In captivity, zebra finches feed throughout daylight hours, with some diurnal variation. Foraging distance of our captive birds calculated using data collected from our behavioural observations yielded ~0.65 km day^{-1}, within the range of foraging distance in free-living zebra finches (Mariette et al., 2011). Although we did not measure DEE in our study, we found no effect of treatment on BMR, contrary to the findings of Koetsier and Verhulst (2011) and Briga and Verhulst (2017). However, the possibility of undetected energy savings could not be ruled out because it has been found that experimental effects of increased foraging costs on metabolic rate were stronger with

decreasing temperature (Briga and Verhulst, 2017). Mathot and Dingemanse (2015) suggested that BMR and DEE can be related to each other in different ways. The 'independent allocation model' proposed that the amount of energy available above basic maintenance costs is independent of maintenance metabolic rate (i.e. BMR), and hence individuals can increase DEE independent of BMR (Mathot and Dingemanse, 2015; Portugal et al., 2016). Furthermore, behavioural observations suggested that they did increase workload in response to HF treatment. Birds in the HF group were also found to have more seeds in their oesophagus at the time of tissue collection, suggesting that food intake was higher in HF birds. However, the possibility of a treatment effect on total food intake being an artefact of treatment effect on temporal food intake patterns (i.e. foraging bouts being more spread out throughout the day in HF birds) could not be ruled out.

Even though HF birds markedly changed their foraging behaviour they were apparently able to maintain food intake and energy balance as their body mass was not different from pre-treatment mass even after 90 days. In other studies that employed exercise training in birds, Costantini et al. (2012) and Briga and Verhulst (2017) reported a decrease in body mass in exercise trained birds, whereas Zhang et al. (2015) reported an increase in body mass in exercise-trained birds. Similar to Costantini et al. (2012), our study found that HF birds showed a slight (but not statistically significant) initial decrease in body mass at day 3 but then recovered to pre-treatment mass at day 60, but unlike Zhang et al. (2015) we did not detect a subsequent increase in body mass. Many other studies in birds also found either no change or a decrease in body mass when exposed to increased foraging cost (summarized in Wiersma and Verhulst, 2005). This discrepancy could be due to differences in training method (e.g. food availability) or length of training period (~60 days in our study versus 24 days in Zhang et al., 2015). Taken together, it appears that HF birds expended more energy and consumed more energy in response to increased foraging effort.

Despite evidence for higher instantaneous food intake in HF birds compared with controls (based on higher crop contents), we did not detect any changes in digestive organs in HF birds. To address the potential issue of low sample size and to obtain an indication of an upper limit of body composition effects that could have gone undetected, a *post hoc* power analysis was conducted with both sexes pooled. The analysis suggested that with 90% power, we could have established an effect size of 0.89,

suggesting that undetected effects were smaller than 0.89. It should also be noted that our finding of higher instantaneous food intake in response to increased foraging cost contrasts with most studies that manipulated foraging effort in birds (summarized in Wiersma and Verhulst, 2005), but is consistent with findings by Wiersma et al. (2005). However, for organs related to aerobic and metabolic capacity (i.e. exercise organs), male and female birds appeared to adopt different strategies in response to increased foraging costs. Whereas HF females upregulated a suite of exercise organs such as flight muscle, leg muscle, heart and lungs presumably to cope with the high workload, HF males decreased their heart mass and did not change other organs. A number of studies investigating the relationship between exercise and body composition in mammals and lizards suggested that exercise performance generally exhibits weak positive correlations with organ masses (Chappell et al., 2007) and that level of workload or endurance training usually elicits changes in body composition (Garland et al., 1987; Swallow et al., 2010), although the direction and magnitude of changes are rather inconsistent among taxa and specific studies, presumably due in part to training regime and food availability. Nevertheless, findings from these studies, together with studies on migratory birds (Piersma, 1998; Guglielmo and Williams, 2003) suggested that birds that are trained to work harder should upregulate both exercise and digestive organs (Swallow et al., 2010) to cope with the increased workload. Increased workload corresponds to increased food consumption based on data from our study as well as studies in mice (e.g. Copes et al., 2015). Studies have also shown a positive correlation between food consumption and gut size (Mathot et al., 2017), presumably because a larger gut allows animals to eat more and be more efficient at processing food. Alternatively, birds exposed to increased workload could downregulate metabolically expensive organs as an energy-saving mechanism to avoid exceeding the 'metabolic ceiling' and face increases in mortality risk (Piersma, 2011). Similar to the physiological changes observed in migratory birds preparing for long-distance flight (Piersma, 1998; Guglielmo and Williams, 2003), HF females increased mass of organs associated with metabolic and aerobic capacity. In contrast, HF males decreased their heart mass, possibly as an energy-saving mechanism. This particular finding is consistent with other studies in mammals and lizards (Scheuer and Tipton, 1977; Garland et al., 1987), which also report a decrease in heart mass in response to endurance training. Furthermore, the sex-specific adjustments observed in our study could also be attributed to differences in wing length between male and female birds. Like many other passerine

species, female zebra finches have shorter wings, and thus higher wing loading than male zebra finches (Yap, unpublished data). Therefore, it is plausible that females have to upregulate mass of organs associated with metabolic and aerobic capacity in response to increased workload, as a means to compensate for the comparatively higher wing loading.

Birds in the HF treatment did not show any adjustments in other traits associated with aerobic capacity (Hct and Hb) compared with controls. This finding is inconsistent with the widely established positive correlation between energy expenditure or workload and Hct or Hb in interspecific studies (Fair et al., 2007; Lourdais et al., 2014), but consistent with the findings of some intraspecific studies, which found no effects of workload on Hct or Hb (Burness et al., 2001; Schumacher et al., 2002).

We found no evidence for adjustment of traits associated with fuel use or energy supply (glycerol, triglyceride, glucose) despite the observed changes in foraging behaviour. It should be noted that the order in which birds are being sampled was randomized, and hence approximately the same number of birds in each treatment group was sampled in the morning and in the afternoon. Studies on migratory birds exercising at high intensity for long durations indicated that they use predominantly lipids to fuel energetically demanding migratory flight (Piersma, 1990; Piersma and Jukema, 1990; Egeler and Williams, 2000). Glucose is known to be an important fuel for 'fast-twitch' muscle fibres responsible for a sudden burst of activity (Hultman, 1995; Weber and Haman, 2004; Melendez-Morales et al., 2009) such as take-off flight in birds. Given that foraging flight in most passerines often involves landing and take-offs interspersed between multiple sustained flights, we had expected that birds that forage more (i.e. HF trained birds) would have higher levels of triglyceride and glucose compared with controls. The lack of adjustment in lipid and glucose metabolism in HF birds is perhaps not unsurprising considering that birds exercise at lower intensity during foraging compared with migration (Piersma, 2011). Although there is evidence from studies of migratory birds suggesting that glucose level decreases in response to exercise (Hullár et al., 2008; Gerson and Guglielmo, 2013), we are not aware of any studies that investigated the effects of long-term endurance training on glucose in birds.

Although we found little evidence for physiological adjustments to support increased workload, we did find evidence that the high foraging costs treatment

generated a potential physiological cost. Although HF and control birds did not differ in their total antioxidant capacity, HF birds had higher plasma levels of reactive oxygen metabolites, which suggests that increased foraging cost causing increased oxidative stress (i.e. a cost of high workload) (Stier et al., 2012), consistent with other studies showing a link between high levels of ROS production and exercise (Costantini et al., 2008; Alan and McWilliams, 2013; Jenni-Eiermann et al., 2014). Taken together, these findings indicate that working hard does perhaps come at a cost in the form of increased oxidative stress.

In summary, our study has shown that despite the significant behavioural adjustment observed in birds that were made to 'work harder', surprisingly few physiological adjustments were observed, especially in the case of male birds. However, given the relationship between increased workload and increased oxidative stress, an obvious next step is to investigate fitness consequences of high foraging costs. Briga et al. (2017) found that birds reared in harsh environmental conditions had a shorter lifespan when subjected to increased foraging cost. Simons et al. (2014) found that increased foraging cost during reproduction can negatively affect breeding success. However, the physiological link between increased foraging effort and reduced reproductive fitness has not been established. We know that the physiological costs of activity can often be deferred from one life-history stage to a later stage, i.e. there can be carry-over effects (Harrison et al., 2011; Williams and Fowler, 2015). Future studies could repeat the training protocol described above and investigate the link between training, physiology and reproduction. Whether the higher oxidative stress caused by increased foraging costs would reduce reproductive success remains to be determined.

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Figures

Figure 5-1 Experimental timeline

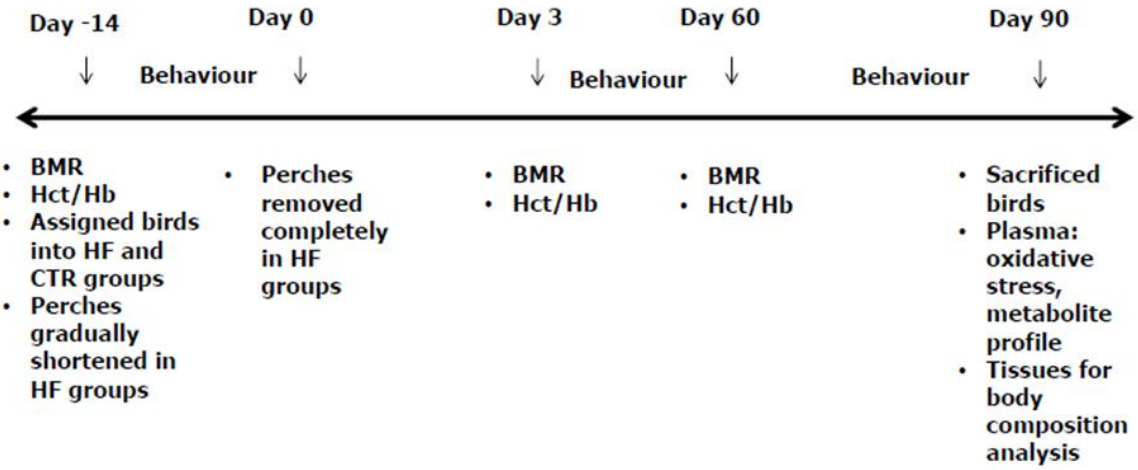


Figure 5-2 **The high foraging cost treatment significantly increased (A) the number of trips birds made to the feeder and (C) immediate food consumption (i.e. dry mass of seeds in the birds' esophagus at the time of tissue collection), but decreased (B) time spent at feeder (per 1800 seconds). Data shown are least-squared means ± s.e.**

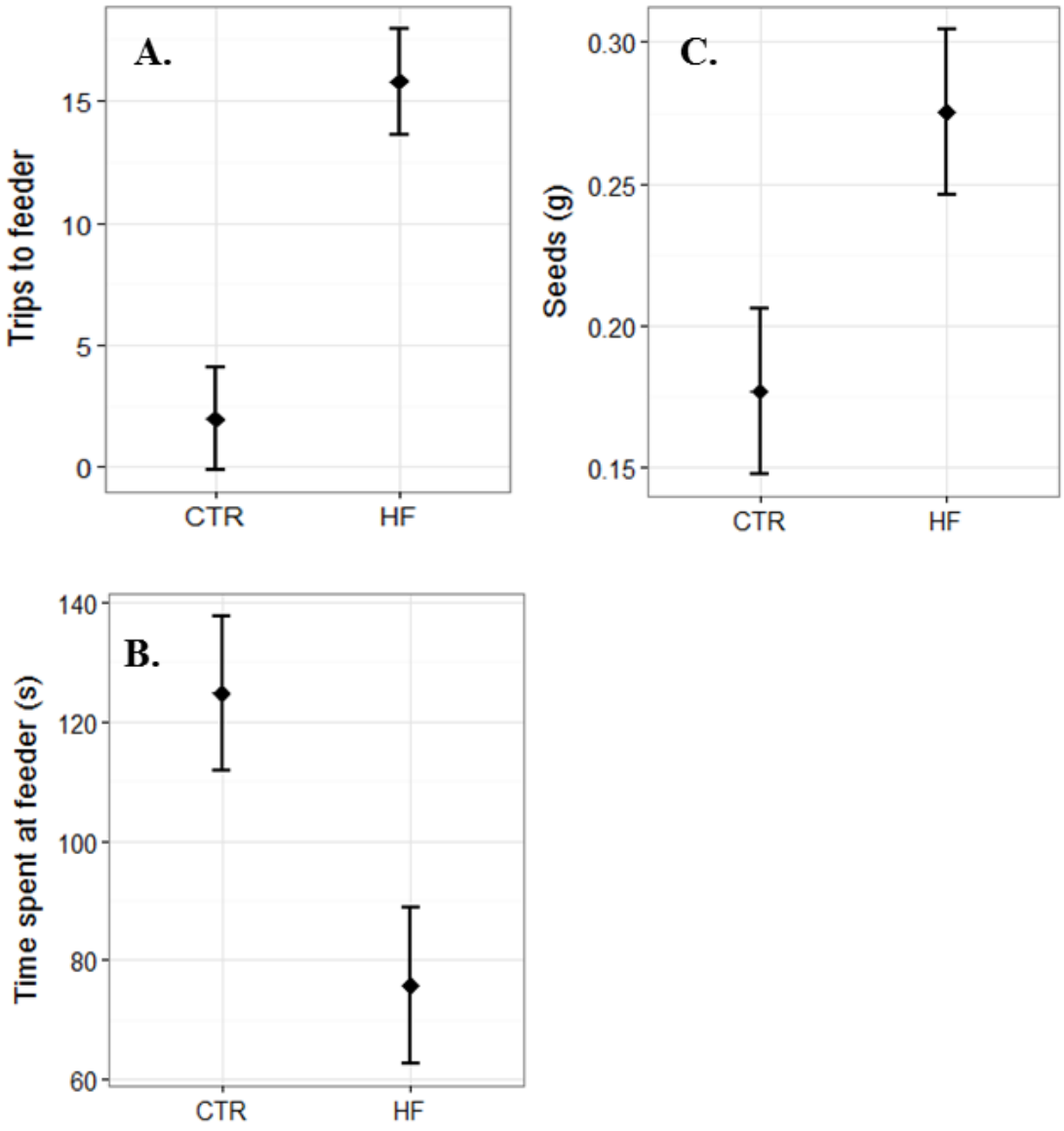


Figure 5-3 **The high foraging cost treatment did not affect (A) body mass, (B) basal metabolic rate (BMR), (C) hematocrit (Hct) and (D) hemoglobin (Hb). Filled circles and solid lines represent CTR birds; Filled triangles and dashed lines represent HF birds. Data shown are least-squared means ± s.e.**

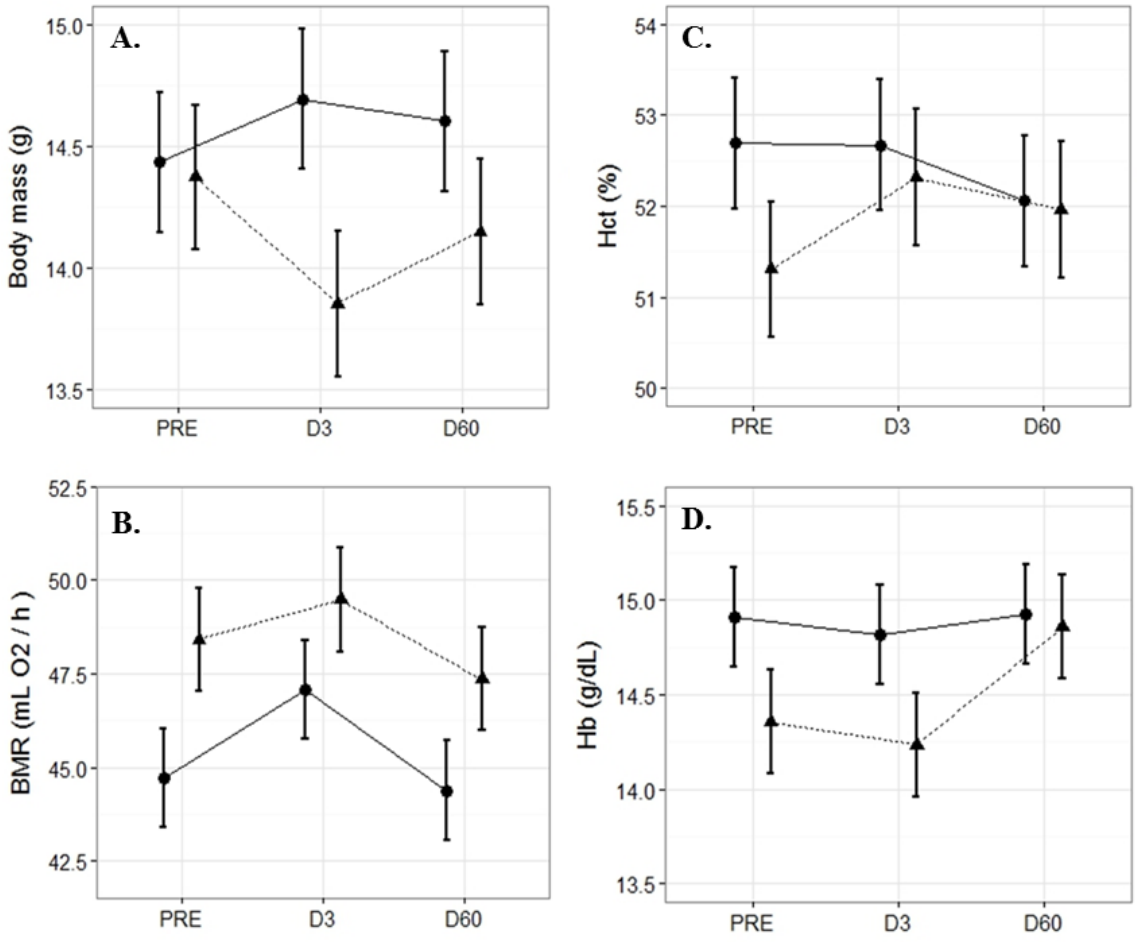


Figure 5-4 **The high foraging cost treatment significantly increased (A) Flight muscle mass, (B) leg muscle mass, (C) lung mass, and (D) heart mass in females (circles) but decreased heart mass in males (triangles). Data shown are least-squared means ± s.e.**

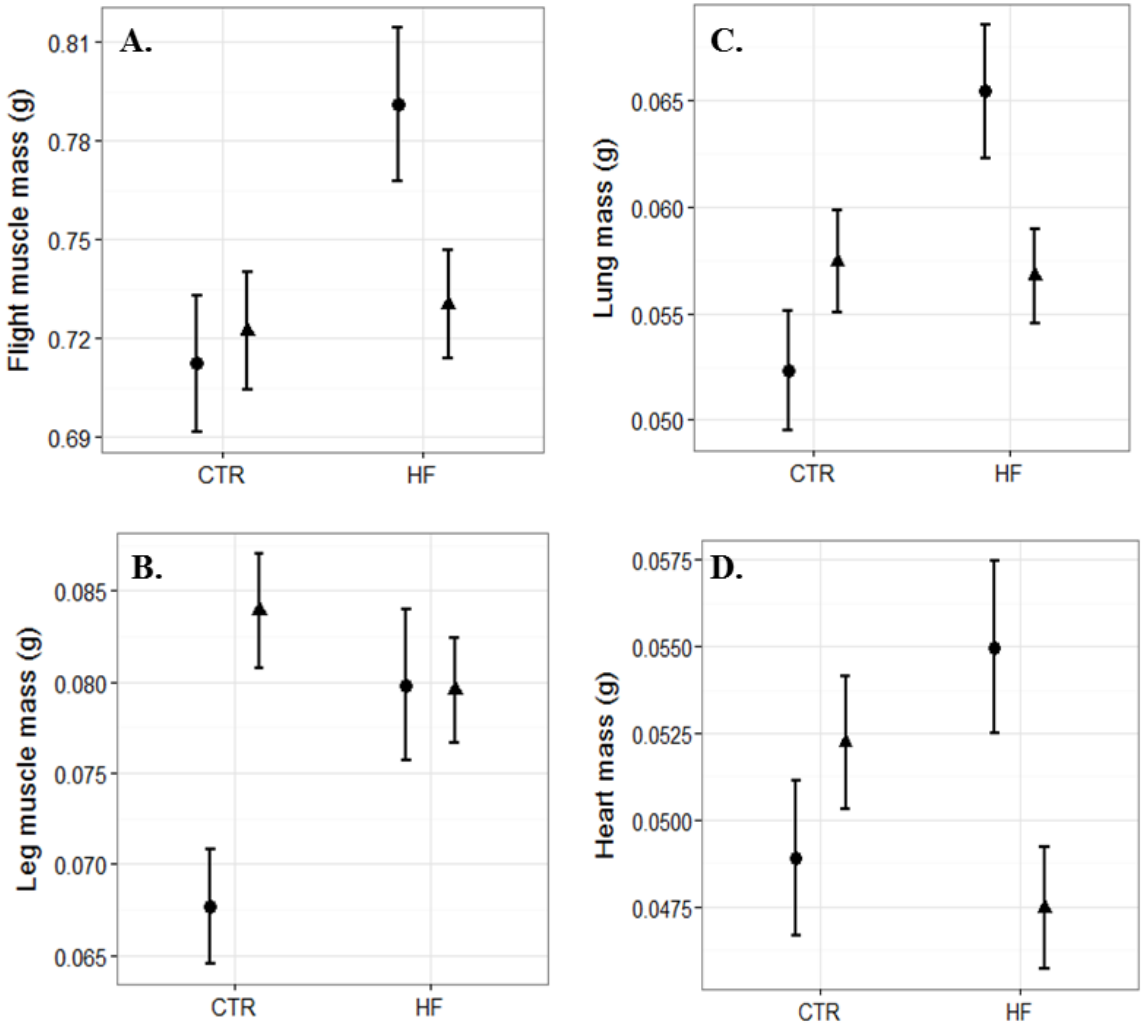
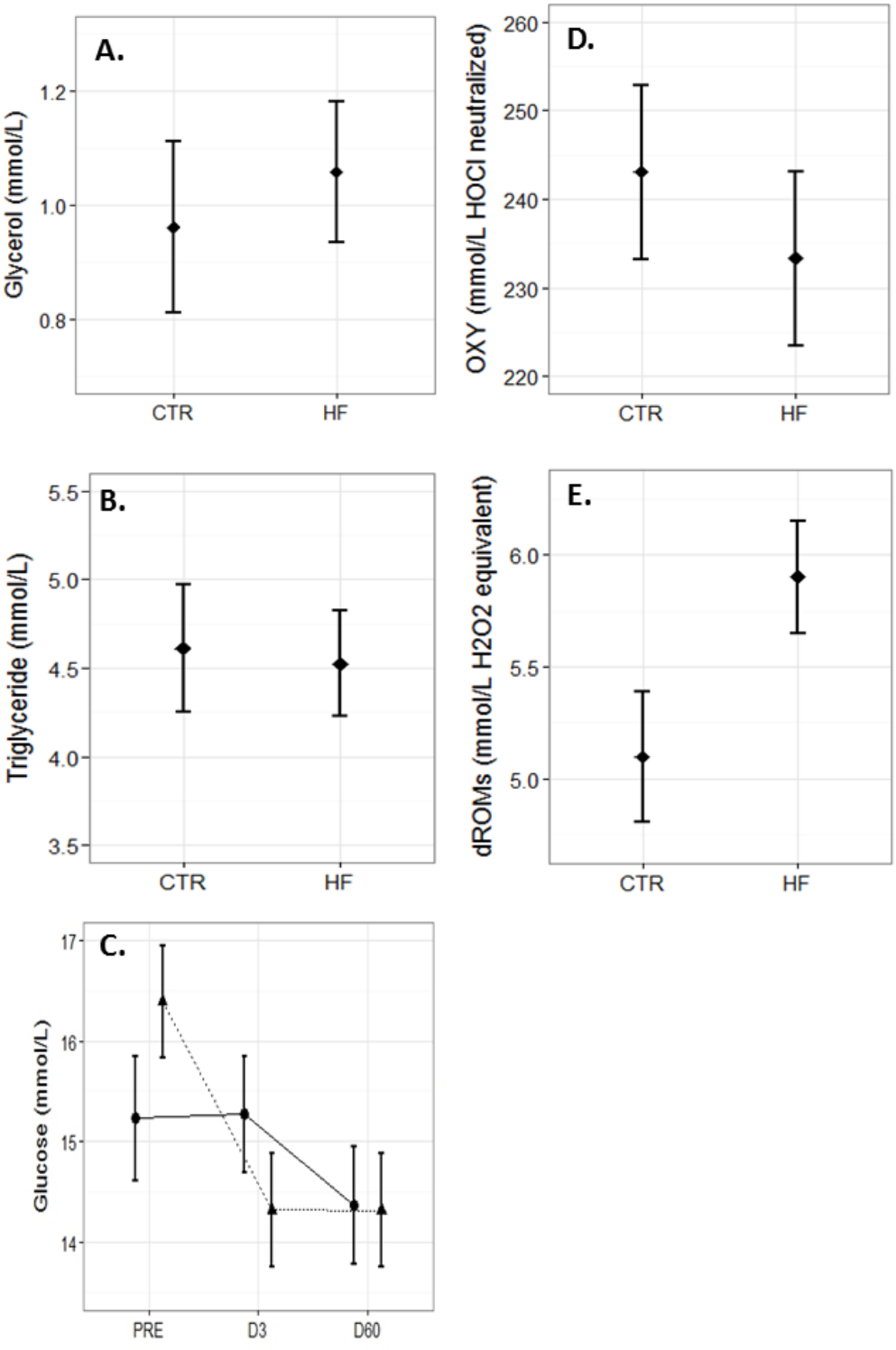


Figure 5-5 **The high foraging cost treatment did not affect (A) glycerol, (B) triglyceride, and (C) blood glucose (D) total antioxidant capacity (OXY), but significantly increased (E) reactive oxygen metabolites production (dROMs). Filled circles and solid lines represent CTR birds; Filled triangles and dashed lines represent HF birds. Data shown are least-squared means ± s.e.**



Chapter 6.
Do physiological adjustments to high foraging effort affect reproduction?

Abstract

Foraging at elevated rates to provision offspring is thought to be an energetically costly activity and it has been suggested that there are potentially costs associated with the high workload involved. However, for the most part evidence for costs of increased foraging and/or reproductive effort is weak. Furthermore, despite having some experimental evidence demonstrating negative effects of increased foraging and parental effort, the physiological mechanisms underlying costs associated with high workload remain poorly understood. To examine how high workload affects hematology, oxidative stress and reproductive output, we experimentally manipulated foraging effort in captive zebra finches, *Taeniopygia guttata*, using a previously described technique, and allowed individuals to breed first in low foraging effort condition, and then in high foraging effort condition. We found that birds up-regulated hematocrit and hemoglobin in response to training. Birds subjected to increased workload during reproduction had lower fecundity, although final reproductive output was not significantly different than that of controls. Offspring of parents subjected to high workload during reproduction also had higher oxidative stress when they were 90 d of age. Findings regarding total antioxidant capacity and reactive oxygen metabolites were different in the two breeding attempts, but we did detect an overall increase in oxidative stress in response to training in both attempts, which could explain the lower fecundity observed in birds subjected to increased workload during reproduction.

This chapter will be submitted to *Journal of Experimental Biology* as Yap, K. N., Powers, D. R., Vermette, M. L., & Williams, T. D. (2017). Do physiological adjustments to high foraging effort affect reproduction?

Introduction

Many behaviours crucial for survival and reproductive success in free-living animals such as foraging and parental care involve elevated levels of activity or "workload" (Halsey, 2016; Sinclair et al., 2014; Yap et al., 2017a). Foraging at elevated rates to provision offspring is thought to be an energetically costly activity (Caro et al., 2016; Maurer, 1996; Piersma, 2011b) and it has been suggested that there are potentially costs associated with the high workload involved (Yap et al., 2017a). Indeed, there is some evidence suggesting that experimentally increased foraging and parental effort (e.g. via increased brood size, clutch size, etc.) adversely affect body condition (Veasey et al., 2001; Wiersma, 2005), survival (Briga et al., 2017; Daan et al., 1996), and reproduction (Deerenberg and Overkamp, 1999; Simons et al., 2014). However, for the most part evidence for costs of increased foraging and/or reproductive effort is weak (Santos and Nakagawa, 2012; Williams, 2012; Zhang and Hood, 2016), one potential reason being these studies have only looked at short-term costs and ignored the fact that costs can be deferred to later life-stages. Furthermore, despite some experimental evidence demonstrating negative effects of increased foraging and parental effort, the physiological mechanisms underlying costs associated with high workload remain poorly understood. A number of studies suggest that the carry-over effect of hard work on reproduction might not be purely energetic and that other "hidden costs" (i.e. physiology) might be involved (Harrison et al., 2011; Nilsson, 2002; Simons et al., 2014; Veasey et al., 2001). Alternatively, increased workload could lead to increased reproductive performance due to improved physiological functions (Zhang and Hood, 2016; Zhang et al., 2018a).

Given that foraging and provisioning offspring involves significantly elevated level of activity for extended period of time (Drent and Daan, 1980; Piersma, 2011b), it is possible that animals would exhibit a suite of behavioural and physiological adjustments in order to cope with the high workload (Sinclair et al., 2014; Yap et al., 2017a). For instance, Husak et al. (2015) found that green anole lizards (*Anolis carolinensis*) that were trained using an endurance training regime had higher hematocrit (Hct) and larger fast glycolytic muscle fibres. Similarly, pectoralis muscle citrate synthase activity and fatty acid transporters increase in response to exercise training in house sparrows (*Passer domesticus*) (Zhang et al., 2015). However, most of the aforementioned studies

only investigated transient, short-term effects of exercise training. It is possible that animals are capable of upregulating physiology (e.g. metabolic enzyme activity, Hct, muscle mass) in the short term but would also downregulate physiology eventually when the costs of activity become too high and maintaining energy balance becomes more difficult. The subsequent downregulation of physiology could be interpreted as physiological costs of activity. Therefore, it is important to consider both short and long term physiological adjustments to increased foraging effort.

Although drastic changes in behaviour in response to increased foraging effort have been reported in a model passerine (zebra finch, *Taeniopygia guttata*), these studies found few physiological adjustments to training except an increase in masses of metabolic organs such as the flight muscle in females (Yap et al., 2017b; Zhang et al., 2018b), and a decrease in basal metabolic rate (Koetsier and Verhulst, 2011). Oxidative stress has been proposed as a general physiological mechanism mediating a diverse range of life-history trade-offs, such as survival and reproduction (Monaghan et al., 2009; Selman et al., 2012; Speakman and Garratt, 2014; Stier et al., 2012; Yap et al., 2017a; Zhang and Hood, 2016). Some studies have shown that high workload (e.g. forced exercise training, increased foraging effort, increased flight costs) increases oxidative stress (Costantini et al., 2012; Fowler and Williams, 2017; Skrip et al., 2016; Yap et al., 2017b). In contrast, other studies that manipulated workload during reproduction (e.g. brood manipulation and wing clipping studies) had mixed findings, with some studies showing no change in oxidative stress (Wegmann et al., 2015a; Wegmann et al., 2015b), and others showing an increase in oxidative stress (Christe et al., 2012; Losdat et al., 2011; Wiersma et al., 2004) in response to experimentally increased workload. These seemingly paradoxical findings could potentially be attributed to either differences in life-history strategies (Harrison et al., 2011; Zhang and Hood, 2016) or oxidative shielding during reproduction (Blount et al., 2016; Naviaux, 2012; Viblanc et al., 2018). However, many of these studies also failed to tease apart the effects of increased workload and the effects of reproduction.

To examine how physiological adjustments to high workload affect subsequent reproductive output, we experimentally manipulated foraging effort in captive zebra finches, *Taeniopygia guttata*, using a previously described technique (Koetsier and Verhulst, 2011; Yap et al., 2017b), and allowed individuals to breed first in low foraging effort condition, and then in high foraging effort condition. We predicted that individuals

subjected to experimentally increased foraging effort will have 1) higher Hct and Hb in the short term but decrease Hct and Hb eventually when foraging costs become too high and maintaining energy balance becomes more difficult, 2) lower anti-oxidant defense and higher reactive oxygen metabolite production (i.e. higher oxidative stress overall), and 3) lower reproductive performance. We also predicted that amongst the individuals subjected to experimentally increased foraging effort, oxidative stress will be higher in the second (high foraging effort) breeding attempt compared to their oxidative stress in the first (low foraging effort) breeding attempt. Consequently, reproductive performance will also be lower in the second breeding attempt compared to the first breeding attempt in birds subjected to increased foraging effort.

Materials and Methods

Animal husbandry

Zebra finches were maintained in controlled environmental conditions (temperature 19–23 °C; humidity 35–55%; constant light schedule, 14L:10D, lights on at 7:00h). All birds were provided with a mixed seed diet (Panicum and white millet, 1:3, 11.7% protein, 0.6% lipid and 84.3% carbohydrate by dry mass), water, grit (coral sand) and cuttlefish bone (calcium) ad libitum, and received a multi-vitamin supplement in the drinking water once per week. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (No. 1231B-94), in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

Experimental timeline and protocol

A total of 36 male and 36 female zebra finches were randomly selected from our colony at Simon Fraser University to be included in the experiment. Half of the birds from each sex were randomly assigned to either a high foraging effort group (HF) or control group (CTR). For the HF group, foraging effort was experimentally manipulated using a previously described training protocol (Koetsier and Verhulst, 2011; Yap et al., 2017b; Zhang et al., 2018a). Briefly, food (mixed seed) was provided in transparent Plexiglas containers (L×W×H: 40×10×13cm) suspended from the roof of the cage (L×W×H: 122×46×41 cm), with feeding holes low on the front panel to allow access to seeds. Perches made of wooden pencil (diameter 0.8cm) were fitted adjacent to feeding holes

to allow birds to perch while foraging for 21 days prior to the start of the experiment (similar to standard feeders in control cages). Over a 14-day period perches were gradually shortened (0.5cm every 2 days) and eventually removed completely to train birds to modify their foraging behaviour and obtain seeds in the high foraging cost condition. Birds in control foraging condition (CTR) were given standard feeders (seed fountains) with perches adjacent to them throughout the experiment. All birds were kept in their respective foraging condition for a further 21 days until the end of the training period. Previous studies using this technique have found that HF birds adjusted their foraging behaviour and made significantly more trips to the feeder (Koetsier and Verhulst, 2011; Yap et al., 2017b).

To investigate the effect of training on subsequent reproduction, at the end of the training period, HF birds were switched to CTR condition and paired for breeding (HF males with HF females, CTR males with CTR females) in common garden, low foraging effort breeding condition (i.e. all HF and CTR birds were given regular feeders). The breeding protocol followed previously described methods (Tissier et al., 2014; Yu et al., 2016), with slight modification. Briefly, birds were paired and housed in individual breeding cages (51 cm × 39 cm × 43 cm), each with an external nest box (14 cm × 14.5 cm × 20 cm). During the first breeding attempt, to avoid washing out the effects of high foraging effort, egg food supplement (eggs, bread crumbs and cornmeal: 20.3% protein, 6.6% lipid) was only provided until the first egg was laid, after which only mixed seed diet was provided to all breeding pairs. Nest boxes were monitored daily between 8:00 AM and 2:00 PM for eggs laid, and new eggs were weighed (0.01 g) and numbered in consecutive order. Nest boxes were continued to be monitored after clutch completion until all the eggs have hatched. On fledge day (21 d of age), chicks were weighed (0.01g) and tarsus and wing length were measured. Chicks were reared by parents until they reached 30 d of age, at which time they were separated and maintained in nonbreeding juvenile groups. Once birds could be sexed by the appearance of bill color and sexually dimorphic plumage, they were separated into sex-specific groups. Meanwhile, parents were returned to single-sex cages with regular feeders and were allowed to rest for 60 days, at the end of which they were subjected to the same training protocol again before making a second breeding attempt with the same partner. During the second breeding attempt, instead of breeding in common garden, low foraging effort condition like in the first attempt, birds were paired and bred

in their respective foraging treatment (i.e. HF birds breeding in HF condition, CTR birds breeding in CTR condition). Birds were subjected to training and reproduction simultaneously in the second breeding attempt because we wanted to investigate additive or synergistic effects of training and breeding on reproductive output and physiology. Due to logistical constraints, we did not provide egg food supplement during the second breeding attempt.

We collected blood samples for physiological measurements at six time points throughout the experiment: : (1) prior to the start of the 14-day perch shortening period before the first breeding attempt (pre-train 1), (2) 14 days after complete removal of perches before the first breeding attempt (post-train 1), (3) when the chicks were 21 d of age during the first breeding attempt (fledge 1), (4) prior to the start of the 14-day perch shortening period before the second breeding attempt (pre-train 2), (5) 14 days after complete removal of perches before the first breeding attempt (post-train 2) and (6) when the chicks were 21 d of age during the second breeding attempt (fledge 2). We also measured daily energy expenditure using doubly-labeled water method (see detailed methods below) at four time points throughout the experiment: (1) prior to the start of the 14-day perch shortening period before the first breeding attempt (pre-train 1), (2) 14 days after complete removal of perches before the first breeding attempt (post-train 1), (3) when the chicks were 6 d of age during the first breeding attempt (chick-rearing 1), and (4) when the chicks were 6 d of age during the second breeding attempt (chick-rearing 2). All blood samples were taken between 8am and 12pm during each of the six time points from both male and female birds. Physiological measurements and energy expenditure measurements were carried out at slightly different time points due to logistical issues (e.g. amount of blood per sample, amount of doubly labeled water, etc.). A summary of the experimental timeline is provided in Figure 1.

Reproductive success and offspring effect

For both breeding attempts, laying interval (days to lay first egg), clutch size, egg mass, hatching success, fledging success, brood size at hatch (BSH), and brood size at fledge (BSF) were monitored. Additionally, for both breeding attempts when the chicks were 21 d of age, we also measured their body mass, wing length, and tarsus. Then, when the chicks were 90 d of age, we collected blood samples from them for physiological measurements, in addition to morphological traits measurements. Blood

samples were collected between 8am and 12pm from all chicks when they were 90 d of age.

Physiological measurements and assays

All blood samples (~100 µL) were obtained from the brachial vein following puncture with a 26G needle and blood was collected using a 75- µL microhematocrit tube. Hematocrit (% packed cell volume) was measured with digital callipers (± 0.01 mm) following centrifugation of whole blood for 3 min at 13 700 g (Autocrit Ultra 3; BD Diagnostic Systems, Sparks, MD, USA). Hemoglobin (Hb, g⁄dL whole blood) was measured using the cyanomethaemoglobin method (Drabkin and Austin 1932) modified for use with a microplate spectrophotometer (BioTek Powerwave 340; Bio-Tek Instruments, Winooski, VT, USA), using 5 µL whole blood diluted in 1.25 mL Drabkin's reagent (Sigma-Aldrich Canada, Oakville, Ontario, D5941) with absorbance measured at 540 nm. Intra- and inter-assay coefficients were 4.0% (triplicate) and 3.8% (n = 8), respectively.

Blood samples were also assayed for total antioxidant capacity (µmol HClO ml^{-1}, OXY) and reactive oxygen metabolites (mg H_2O_2 dl^{-1}; ROMs). All plasma samples were analyzed using a microplate spectrophotometer (BioTek Powerwave X340, Bio-Tek Instruments, Inc., Winooski, VT, USA) and 96-well microplates. Analyses of oxidative stress were carried out according to established protocols as described in Costantini et al. (2011), with slight modification. Specifically, we measured ROMs and OXY using the commercial kits dROMs and OXY Adsorbent Test (Diacron International, Grosseto, Italy) respectively. Intra-assay coefficient for OXY and dROMs were 6.2% (triplicate) and 8.5% (duplicate), respectively. Inter-assay coefficient for OXY and dROMs were 5.9% (n = 11) and 6.8% (n = 10), respectively. In addition, we calculated an index of overall oxidative stress (OS) by taking the ratio between ROMs and OXY and multiplying it by 1000 (OS = ROMs/OXY × 1000) (Costantini et al., 2008; Costantini et al., 2011).

Statistical Analyses

Analyses were carried out using R version 0.99.467 (R Core Team 2013). Data were first examined for normality using Shapiro-Wilk test and non-normally distributed data were log-transformed prior to analysis (but plotted using untransformed values in

figures). Proportion measures of reproductive performance (i.e., hatching success, fledging success) were modeled with generalized linear mixed models using a binomial distribution and a logit link and female ID as a random factor. Hatching success was scored as ratio of eggs to hatch, and fledging success was scored as ratio of chicks to fledge. All measures of reproductive success (i.e. laying interval, clutch size, egg mass, brood size at hatch, brood size at fledge, offspring morphology at 21 d and 90 day of age, and physiological measures of offspring at 90 d of age) were analyzed using repeated measure with breeding attempt and treatment as main effects, and individual female ID as a random factor. To link physiological measures of parents to the observed reproductive performance in each attempt, breeding attempt 1 and breeding attempt 2 were analyzed separately. First, to look at physiological responses to increased foraging effort (i.e. post-train 1 and 2), we tested the effect of treatment on body mass and all physiological metrics (i.e. Hct, Hb, OXY, ROMs, and OS) using general linear model (GLM), with treatment as main effect, and pre-training values as covariates. To investigate how physiology responds from pre-breeding stage (i.e. post-train 1 and 2) to fledging (fledge 1 and 2), all physiological metrics were analyzed using repeated measure with time and treatment as main effects, body mass and pre-training values as covariates, and individual bird ID as a random factor. Sex was initially included in all models but was taken out because we did not detect any main effects of sex nor interactions between sex and other variables ($P > 0.1$ in all cases). *F*- and *t*-statistics and *P* values were generated using the lmerTest package (Kuznetsova et al., 2014) and and Tukey's HSD (package multcomp, (Hothorn et al., 2008)) was used to evaluate pairwise comparisons between treatments and breeding attempts following a significant mixed model. We also reported the least-squared means and standard errors of all physiological metrics from all time points in a separate table (Table 1).

Results

Effects of increased foraging effort (training) on body mass and physiology (Pre-train 1 and 2 to Post-train 1)

Body mass was independent of treatment in both the first (t_{39} = -1.04, P = 0.31; Figure 2A) and second breeding attempt (t_{50} = -0.94, P = 0.35; Figure 2B) during post-treatment. There was a significant treatment effect for Hct, with HF birds having higher Hct than CTR birds in both the first (t_{39} = 3.15, $P < 0.01$; Figure 2C) and second breeding

attempt (t_{47} = 2.44, P = 0.02; Figure 2D) during post-treatment. Similarly, there was a significant treatment effect for Hb, with HF birds having higher Hb than CTR birds in both the first (t_{30} = 2.73, P = 0.01; Figure 2E) and second breeding attempt (t_{49} = 3.35, P < 0.01; Figure 2F) during post-treatment.

OXY was independent of treatment in the first breeding attempt (t_{38} = 0.35, P = 0.72; Figure 2G) but there was a significant treatment effect for OXY in the second breeding attempt, with HF birds having lower OXY than CTR birds (t_{46} = -2.24, P = 0.03; Figure 2H). There was a significant treatment effect for ROMs in the first breeding attempt, with HF birds having higher ROMs than CTR birds (t_{25} = 3.06, P < 0.01; Figure 2I), but ROMs was independent of treatment in the second breeding attempt (t_{29} = 1.32, P = 0.20; Figure 2J). There was a significant treatment effect for OS, with HF birds having higher OS than CTR birds in both the first (t_{24} = 2.44, P = 0.02; Figure 2K) and second breeding attempt (t_{25} = 2.27, P = 0.03; Figure 2L).

Variation in body mass and physiology during post-training phase and reproduction (Post-train 1 and 2 to Fledge 1 and 2)

Body mass was independent of the treatment*time interaction in both the first ($F_{1,20}$ = 0.60, P = 0.44) and the second breeding attempt ($F_{1,11}$ = 4.01, P = 0.07). However, there was a main effect of time on body mass in both the first ($F_{1,20}$ = 48.83, P < 0.01; Figure 3A) and second breeding attempt ($F_{1,11}$ = 22.77, P < 0.01; Figure 3B): regardless of treatment, body masses of birds decreased significantly from post-training to fledging in both the first (t_{27} = 8.97, P < 0.01) and second breeding attempt (t_{29}= 3.42, P < 0.01).

There was a significant treatment*time interaction for Hct in the first breeding attempt ($F_{1,19}$ = 7.84, P = 0.01; Figure 3C): HF birds had higher post-training Hct compared with controls (t_{50} = -2.98, P < 0.01) but similar Hct at fledging (t_{58} = 0.47, P = 0.64). In contrast, Hct was independent of the treatment*time interaction in the second breeding attempt ($F_{1,10}$ = 1.71, P = 0.22; Figure 3D). Although the overall model showed a main effect of treatment on Hct in the second breeding attempt ($F_{1,46}$ = 4.55, P = 0.04), pairwise comparisons after tukey adjustments suggested that Hct was only higher in HF birds at post-training (t_{54} = -2.13, P < 0.03) but not fledging (t_{45} = 0.24, P = 0.81).

There was a significant treatment*time interaction for Hb in the first breeding attempt ($F_{1,14}$ = 6.15, P = 0.02; Figure 3E): HF birds had higher post-training Hb (t_{33} = -3.21, P < 0.01) but similar Hb at fledging (t_{43} = -0.21, P = 0.84). There was a marginally significant treatment*time interaction for Hb in the second breeding attempt ($F_{1,10}$ = 4.93, P = 0.05; Figure 3F): HF birds had higher post-training Hb (t_{52} = -3.24, P = 0.01) but similar Hb at fledging (t_{31} = 0.24, P = 0.99).

OXY was independent of the treatment*time interaction ($F_{1,16}$ = 1.45, P = 0.25; Figure 3G), as well as the main effects of time ($F_{1,16}$ = 1.18, P = 0.29) and treatment ($F_{1,38}$ = 0.05, P = 0.83) in the first breeding attempt. OXY was also independent of the treatment*time interaction in the second breeding attempt ($F_{1,10}$ = 0.04, P = 0.85; Figure 3H). However, there was a main effect of treatment on OXY in the second breeding attempt ($F_{1,45}$ = 4.69, P = 0.03): HF birds had lower OXY than CTR (t_{42} = 2.49, P = 0.02).

There was a significant treatment*time interaction for ROMs in the first breeding attempt ($F_{1,10}$ = 6.15, P = 0.03; Figure 3I): HF birds had higher post-training ROMs (t_{31} = -2.62, P = 0.01) but similar ROMs at fledging (t_{37} = 0.21, P = 0.84). Similarly, there was a marginally significant treatment*time interaction for ROMs in the second breeding attempt ($F_{1,2}$ = 17.95, P = 0.05; Figure 3J): HF birds had higher post-training ROMs (t_{35} = -2.10, P = 0.04) but similar ROMs at fledging (t_{14} = -2.06, P = 0.06).

OS was independent of the treatment*time interaction ($F_{1,7}$ = 4.54, P = 0.07; Figure 3K), as well as the main effects of time ($F_{1,7}$ = 1.82, P = 0.22) and treatment ($F_{1,26}$ = 3.61, P = 0.07) in the first breeding attempt. In contrast, there was a significant treatment*time interaction for OS in the second breeding attempt ($F_{1,2}$ =29.60, P = 0.03; Figure 3L): HF birds had higher post-training OS (t_{31} = -3.25, P < 0.01) but similar OS at fledging (t_{20} = 1.52, P = 0.14).

Effects of increased foraging effort on reproductive output

Hatching success was independent of the attempt*treatment interaction ($F_{1,284}$ = 2.63, P = 0.12; Figure 4A), and the main effect of treatment ($F_{1,284}$ = 0.19, P = 0.93). However, there was a significant main effect of attempt ($F_{1,284}$ = 27.90, P < 0.01). Hatching success was 53.75% and 51.85% for CTR and HF respectively in the first breeding attempt, but decreased to 18.06% and 25.00% for CTR and HF respectively in

the second breeding attempt. Similarly, fledging success was independent of the attempt*treatment interaction ($F_{1,118}$ = 1.79, P = 0.21; Figure 4B), and the main effects of treatment ($F_{1,118}$ = 2.30, P = 0.19), and attempt ($F_{1,118}$ = 0.0006, P = 0.22). Fledging success was 58.14% and 78.57% for CTR and HF respectively in the first breeding attempt, and was 61.54% and 66.67% for CTR and HF respectively in the second breeding attempt.

Laying interval was independent of the attempt*treatment interaction ($F_{1,9}$ = 3.90, P = 0.08; Figure 4C), as well as the main effects of treatment ($F_{1,34}$ = 0.84, P = 0.36) and attempt ($F_{1,9}$ = 0.13, P = 0.72). There was a significant attempt*treatment interaction for egg mass ($F_{1,244}$ = 5.38, P = 0.02; Figure 4D): HF birds laid significantly smaller eggs in the second breeding attempt compared to the first attempt (t_{144} = 4.16, P < 0.01). There was also a significant attempt*treatment interaction for clutch size ($F_{1,8}$ = 11.82, P = 0.01; Figure 4E): HF birds laid significantly smaller clutch in the second breeding attempt (t_{30} = 3.01, P = 0.02).

Brood size at hatch (BSH) was independent of the attempt*treatment interaction ($F_{1,8}$ = 0.25, P = 0.63; Figure 4F), the main effects of treatment ($F_{1,33}$ = 0.34, P = 0.56) and attempt ($F_{1,8}$ = 4.42, P = 0.07). Similarly, brood size at fledge (BSF) was independent of the attempt*treatment interaction ($F_{1,2}$ = 0.10, P = 0.78; Figure 4G) and the main effects of treatment ($F_{1,22}$ = 0.19, P = 0.67) and attempt ($F_{1,2}$ = 4.33, P = 0.17).

Effects of increased foraging effort on offspring morphology and physiology

Offspring mass at 21 d of age was independent of the attempt*treatment interaction ($F_{1,54}$ = 0.42, P = 0.52; Figure 5A), as well as the main effects of treatment ($F_{1,22}$ = 0.18, P = 0.67) and attempt ($F_{1,54}$ = 1.27, P = 0.27). Similarly, offspring tarsus at 21 d of age was independent of the attempt*treatment interaction ($F_{1,53}$ = 0.12, P = 0.73; Figure 5B), as well as main effects of treatment ($F_{1,22}$ = 0.01, P = 0.91) and attempt ($F_{1,53}$ = 0.91, P = 0.35). Offspring wing length at 21 d of age was independent of the attempt*treatment interaction ($F_{1,53}$ = 0.31, P = 0.58; Figure 5C) and the main effect of attempt ($F_{1,53}$ = 0.23, P = 0.64). However, there was a significant main effect of treatment on offspring wing length ($F_{1,22}$ = 5.79, P = 0.02), where offspring from HF parents had shorter wing at 21 d of age (t_{20} = 2.67, P = 0.01).

Offspring mass at 90 d of age was independent of the attempt*treatment interaction ($F_{1,32}$ = 1.17, P = 0.29; Figure 5D), as well as the main effects of treatment ($F_{1,21}$ = 1.51, P = 0.23) and attempt ($F_{1,32}$ = 0.42, P = 0.52). Offspring tarsus at 90 d of age was independent of the attempt*treatment interaction ($F_{1,31}$= 0.06, P = 0.81; Figure 5E) and the main effect of treatment ($F_{1,21}$ = 0.02, P = 0.88). However, there was a significant main effect of attempt ($F_{1,32}$ = 4.65, P = 0.04) on offspring tarsus at 90 d of age: regardless of experimental treatment of parents, offspring from the second breeding attempt had significantly smaller tarsus than offspring from the first attempt at 90 day of age (t_{50} = 2.16, P = 0.03). Offspring wing length at 90 d of age was independent of the attempt*treatment interaction ($F_{1,32}$ = 0.04, P = 0.85; Figure 5F), as well as the main effects of treatment ($F_{1,21}$ = 1.67, P = 0.21) and attempt ($F_{1,32}$ = 2.23, P = 0.15).

Offspring Hct at 90 d of age was independent of the attempt*treatment interaction ($F_{1,30}$ = 3.39, P = 0.07; Figure 5G), as well as the main effects of treatment ($F_{1,21}$ = 0.10, P = 0.75) and attempt ($F_{1,30}$ = 0.83, P = 0.37). Offspring Hb at 90 d of age was independent of the attempt*treatment interaction ($F_{1,31}$= 0.28, P = 0.60; Figure 5H) and the main effect of treatment ($F_{1,21}$ = 0.72, P = 0.41). However, there was a significant main effect of attempt ($F_{1,32}$ = 4.41, P = 0.04) on offspring Hb at 90 d of age: offspring from the second breeding attempt had significantly lower Hb than offspring from the first attempt at 90 day of age (t_{47} = 2.10, P = 0.04). Offspring OXY at 90 d of age was independent of the attempt*treatment interaction ($F_{1,24}$ = 1.67, P = 0.21; Figure 5I), as well as the main effects of treatment ($F_{1,15}$ = 0.98, P = 0.34) and attempt ($F_{1,24}$ = 0.17, P = 0.68). There was a significant attempt*treatment interaction ($F_{1,24}$ = 9.52, P < 0.01; Figure 5J) for offspring ROMs at 90 d of age: offspring from HF parents had higher ROMs than offspring from CTR parents in the second breeding attempt (t_{45} = -2.46, P = 0.02), as well as than offspring from HF parents in the first breeding attempt (t_{43} = -2.48, P = 0.02). Similarly, there was a significant attempt*treatment interaction ($F_{1,19}$ = 7.95, P = 0.01) for offspring OS at 90 d of age: offspring from HF parents had significantly higher OS than offspring from CTR parents in the second breeding attempt (t_{33} = -2.25, P = 0.03), as well as than offspring from HF parents in the first breeding attempt (t_{32} = -2.15, P = 0.04).

Discussion

We experimentally manipulated foraging behaviour and workload in zebra finches using a previously described technique (Koetsier and Verhulst, 2011; Yap et al., 2017b) and investigated how physiological adjustments to 'exercise' (*sensu* Halsey, 2016) affected subsequent reproductive performance and offspring quality. We trained birds to high foraging effort twice, with an intervening reproductive attempt prior to the second training period. Training had a consistent effect on traits reflecting aerobic capacity: birds up-regulated Hct and Hb during both the first and second training phase, but there was no effect of experimental treatment on body masses. Training did not affect total antioxidant capacity of birds prior to their first breeding attempt but HF birds had significantly lower post-training total antioxidant capacity prior to their second breeding attempt. In contrast, plasma reactive oxygen metabolites were higher when birds were subjected to training prior to their first breeding attempt but did not change in response to a second bout of training prior to their second breeding attempt. Therefore, overall, oxidative stress was consistently higher in birds subjected to training prior to both breeding attempts but for different reasons.

Having established that training for high foraging effort did affect physiological state we then asked how post-training state was affected by subsequent reproduction. Regardless of experimental treatment, birds decreased body mass from post-training to chick fledging. Although training increased Hct and Hb, these traits returned to pre-training levels at the end of both reproductive attempts, even when HF birds were maintained in HF conditions during breeding in the second breeding attempt. Total antioxidant capacity was not affected by reproduction in the first breeding attempt. Training resulted in lower total antioxidant capacity and total antioxidant capacity stayed low until the end of reproduction in the second breeding attempt. Although training increased ROMs, ROMs returned to pre-training levels at the end of both reproductive attempts, again even when HF birds were maintained in HF conditions during breeding in the second breeding attempt. Overall oxidative stress remained the same from post-training to the end of reproductive bout in the first attempt. However, overall oxidative stress was higher in HF birds at post-training but returned to pre-training level at the end of the second reproductive attempt, even though they were still being kept in HF condition during breeding.

Finally, we asked how post-training physiological state affected reproductive performance under 'common garden' low foraging effort conditions and high foraging effort conditions. Hatching success was significantly lower in the second breeding attempt but fledging success did not differ between different treatments and different breeding attempts. When birds were trained and bred in HF conditions, they had lower fecundity (smaller clutch size and egg mass) but final reproductive output (BSH and BSF) was not affected by training for increased foraging effort. We did not detect any difference in offspring morphology and physiology, except shorter wing length in chicks from HF parents at 21 d of age in both breeding attempts, as well as higher reactive oxygen metabolites production in chicks from HF parents in at 90 d of age in the second breeding attempt.

We found no effect of training on body mass confirming results of a previous study (Yap et al., 2017b; but see Briga et al., 2017). Unlike Yap et al. (2017b), we found that birds increased Hct and Hb in response to training, providing evidence for physiological responses to training. The discrepancies in findings despite both studies employing identical training technique could be due to a difference in timing of Hct and Hb measurements- day 3 post-training in Yap et al. (2017b) vs. day 14 post-training in the current study. This suggests that 3 days of training might not be sufficient to cause upregulation of Hct and Hb, as physiological processes such as erythropoiesis typically take place over several days (Rosse and Waldmann, 1966; Williams et al., 2012). Training affected antioxidant capacity and reactive oxygen metabolites production differently in the two attempts, although overall oxidative stress was consistently higher in trained birds prior to both breeding attempts, similar to finding by Yap et al. (2017b). Previous studies suggested that antioxidant capacity and reactive oxygen metabolite production are not necessarily coupled (Costantini and Verhulst, 2009; Skrip and Mcwilliams, 2016), and that there is not always repeatability of antioxidant capacity over time, although there is substantial repeatability in overall oxidative stress (Beamonte-Barrientos and Verhulst, 2013). These findings together indicated that it is important to look at both antioxidant capacity and reactive oxygen metabolites together when evaluating oxidative stress.

Although post-training Hct and Hb were higher in HF birds, these traits returned to pre-training level at fledging in the first breeding attempt, when birds were bred in low foraging effort conditions. This suggests that high Hct and Hb were required to sustain

the high workload of increased foraging costs, but the high levels were no longer maintained when foraging conditions become easier. However, Hct and Hb returned to pre-training level at fledging in the second attempt as well, even though birds were still being kept at HF condition. Downregulation of Hct and Hb in this case possibly represents a cost of high workload, where birds could no longer maintain high Hct and Hb when the combined effort of high foraging costs and parental care became too high and maintaining energy balance became more difficult. The effect of training on antioxidant capacity persisted until the chicks fledged. Reproduction did not affect total antioxidant capacity in the first attempt, likely because birds were breeding in relatively easy (i.e. low foraging effort) conditions. It should be noted that although HF birds had lower antioxidant capacity relative to CTR birds in the second breeding attempt, this was mostly due to increased antioxidant capacity in CTR birds. This suggests that the observed higher antioxidant capacity in CTR birds was due to the effects of oxidative shielding (Blount et al., 2016; Naviaux, 2012; Viblanc et al., 2018) in the first attempt being carried over to the second attempt. HF birds were not able to maintain high antioxidant capacity because they were being kept in hard (i.e. high foraging effort) condition throughout the second attempt. Similar to other studies that demonstrated that animals tend to upregulate antioxidant defense during reproduction to minimize oxidative stress (i.e. oxidative shielding) (Blount et al., 2016; Naviaux, 2012; Viblanc et al., 2018), our study showed that oxidative stress was not affected by reproduction or the combined effort of training and reproduction in the case of the second breeding attempt.

Training for increased foraging effort did not affect subsequent reproduction in the first breeding attempt. Although some studies investigating increased the effects of increased workload on reproduction generally found a delay in the timing of reproduction (Deerenberg and Overkamp, 1999; Simons et al., 2014; Wiersma, 2005), many other studies failed to find evidence of impaired reproduction due to increased workload (Schmidt-Wellenburg et al., 2008; Tomotani et al., 2017). A few studies in rodents even found positive effects of high activity level on reproduction (Vega et al., 2015; Zhang et al., 2018a), possibly due to mitochondrial hormesis (Zhang and Hood, 2016; Zhang et al., 2017). The absence of a treatment (i.e. training) effect on reproductive output in the first breeding attempt is not surprising considering that most of the time, tradeoffs and “costs of reproduction” can only be detected when environmental conditions are poor

(Stearns, 1989; Stearns, 1992), and in some cases, training might even be beneficial for reproduction (Zhang et al., 2018a).

Contrary to the findings in the first breeding attempt, and unlike other studies that showed no effects of increased workload on reproduction (Schmidt-Wellenburg et al., 2008; Tomotani et al., 2017), findings from the second breeding attempt of the current experiment showed reduced fecundity (i.e. smaller clutch size and egg mass) when birds were subjected to increased foraging effort and breeding at the same time. Although we cannot completely rule out the possibility of diet (i.e. egg food supplement in first breeding attempt vs. no egg food supplement in second breeding attempt) being a potential confound, we do not think that diet caused the decrease in fecundity observed in HF birds in the second breeding attempt because CTR birds were given the same diet and yet we did not observe a similar decrease in clutch size and egg mass. Despite finding lower fecundity in birds subjected to increased foraging effort and breeding at the same time, we did not find any evidence for a reduction in final reproductive output. This could be explained by individual parents optimizing reproductive investment- investing less resource in egg production and more resource for chick rearing (Linhares et al., 2014; Schwarzkopf and Andrews, 2012; Williams, 2012). However, we did have some evidence that offspring produced by HF parents in the second breeding attempt were of lower quality, as indicated by the higher oxidative stress observed when they were 90 d of age, although other indicators of quality including multiple morphological traits and hematology were not significantly different between offspring of HF parents and CTR parents. It should be noted that many of the metrics for reproductive success such as BSH, chick mass, and BSF are lower than previously reported in the same species from the same colony (Tissier et al., 2014; Yu et al., 2016), likely due to a lack of egg food supplementation during chick-rearing. However, the values observed in our study are similar to other studies that had captive zebra finches reproduce under poor diet quality conditions (i.e. mixed seed diet only) (Criscuolo et al., 2011; Griffith et al., 2017).

In summary, our study has shown that birds exhibited consistent physiological adjustments to training (e.g. increased Hct and Hb), but these physiological responses were subsequently affected by reproduction (e.g. decreased Hct and Hb), even when birds were maintained in high foraging effort conditions. Findings from our study also suggested that experimentally increased workload during reproduction can lead to physiological costs in the form of increased oxidative stress, potentially to a high enough

level to negatively affect reproductive performance, as evident from the lower fecundity observed in HF birds in the second breeding attempt, as well as the poorer offspring quality produced by HF parents in the second breeding attempt. It is unclear whether reproduction can modulate any subsequent response to training. Some studies have suggested that moderate increases in ROS due to increased activity level should lead to increased respiratory capacity of tissues and physiological functions, and consequently improved reproductive performance (Zhang and Hood, 2016; Zhang et al., 2017; Zhang et al., 2018a). Although our study did not document any positive effects of training on reproduction, future studies should repeat the training and breeding protocol described above and investigate whether different levels of activity (i.e. shorter duration, lower training intensity) would affect subsequent reproduction differently, as well as whether reproduction can in turn modulate any subsequent physiological responses to training. An alternative explanation for the finding of low fecundity in birds subjected to increased workload during reproduction is energy allocation trade-off- perhaps HF birds spent more time and energy foraging for self-maintenance and less time and energy developing reproductive machinery and forming eggs. Future studies should employ accelerometry or measure both BMR and DEE to examine the issue of energy allocation trade-off in birds subjected to increased foraging effort during reproduction.

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Figures and Tables

Figure 6-1 Summary of the experimental timeline.

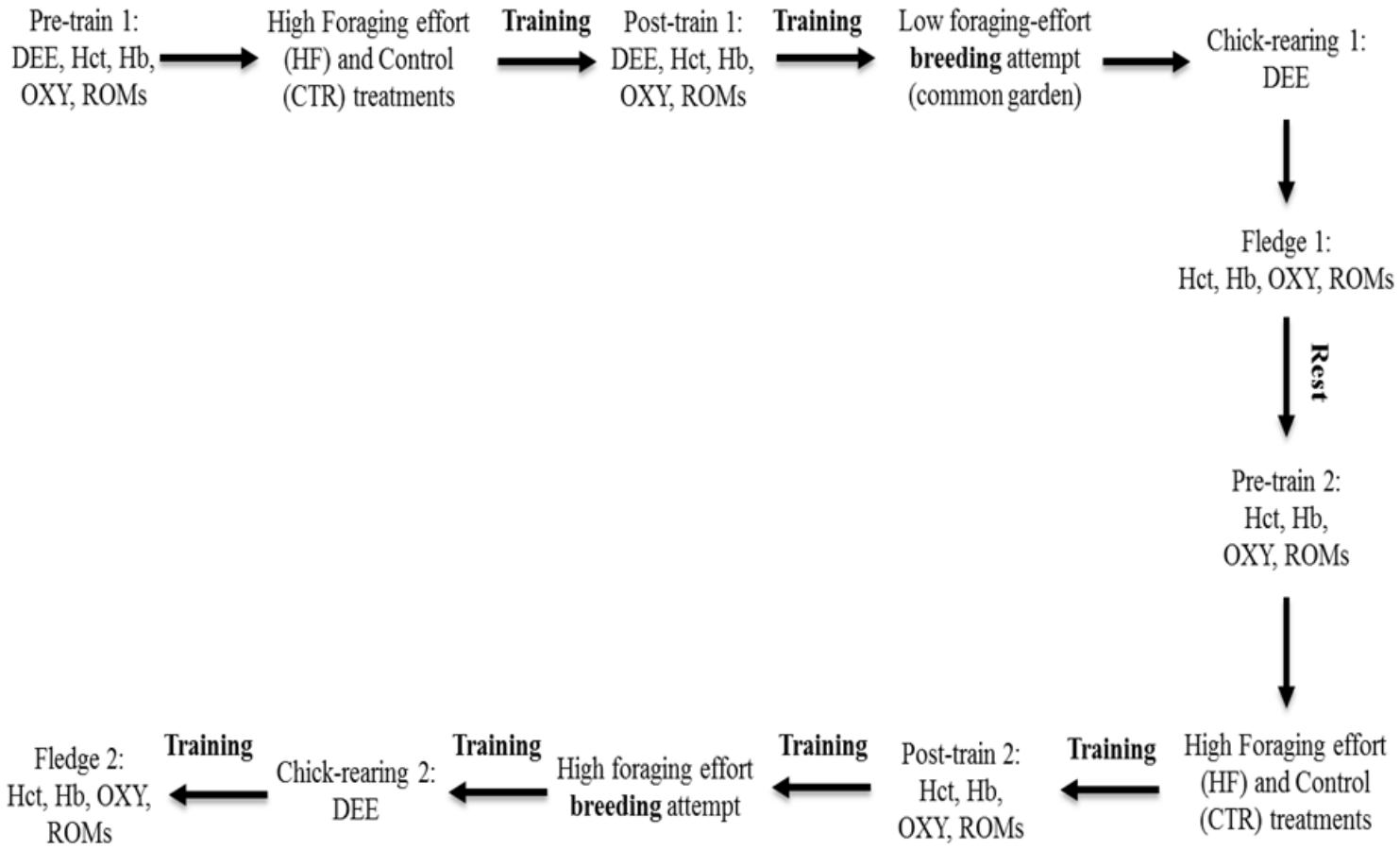
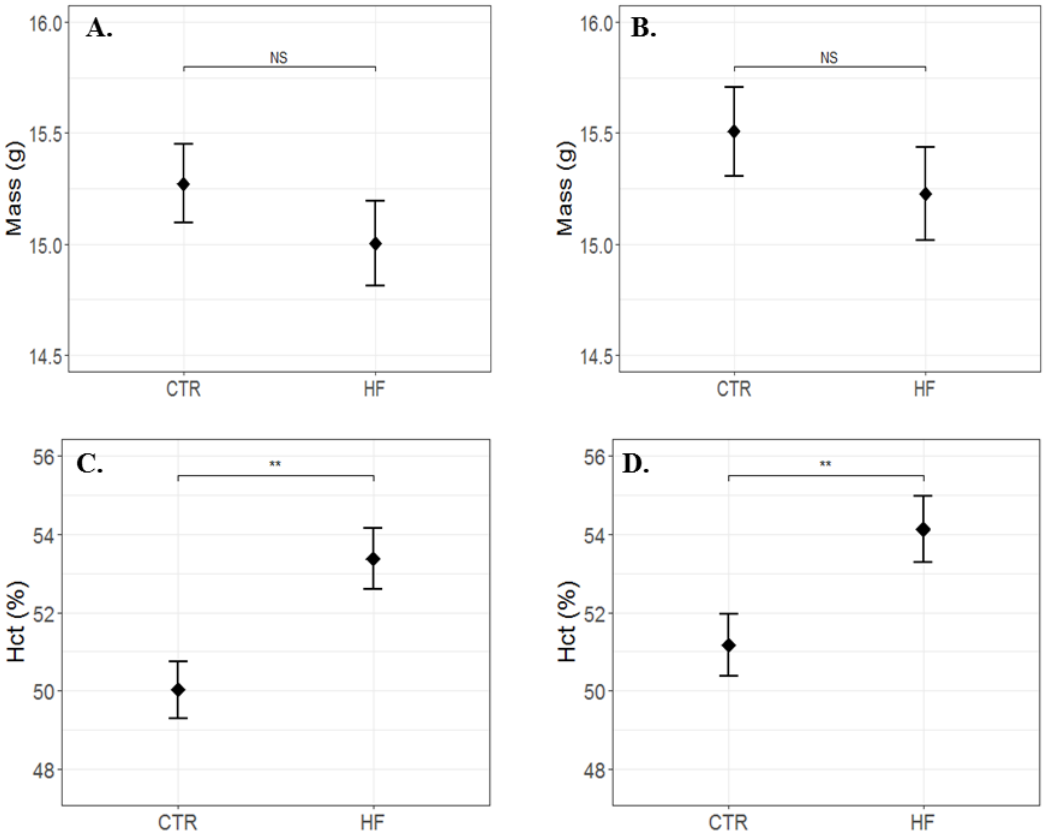


Figure 6-2 **Effects of increased foraging effort on A) body mass in the first breeding attempt, B) body mass in the second breeding attempt, C) Hct in the first breeding attempt, D) Hct in the second breeding attempt, E) Hb in the first breeding attempt, F) Hb in the second breeding attempt, G) OXY in the first breeding attempt, H) OXY in the second breeding attempt, I) ROMs in the first breeding attempt, J) ROMs in the second breeding attempt, K) OS in the first breeding attempt, and L) OS in the second breeding attempt. Data shown are least-squared means ± s.e.**



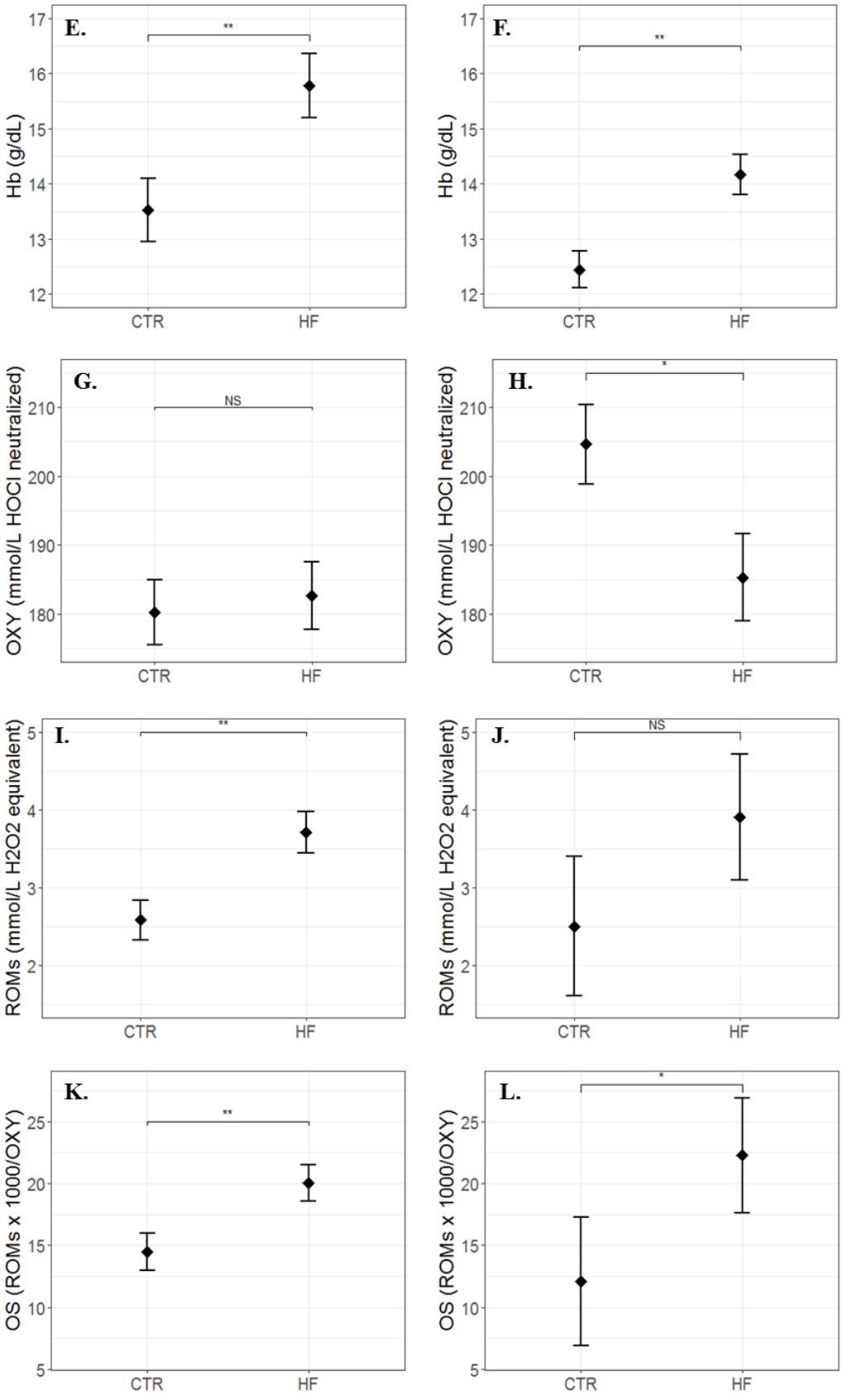
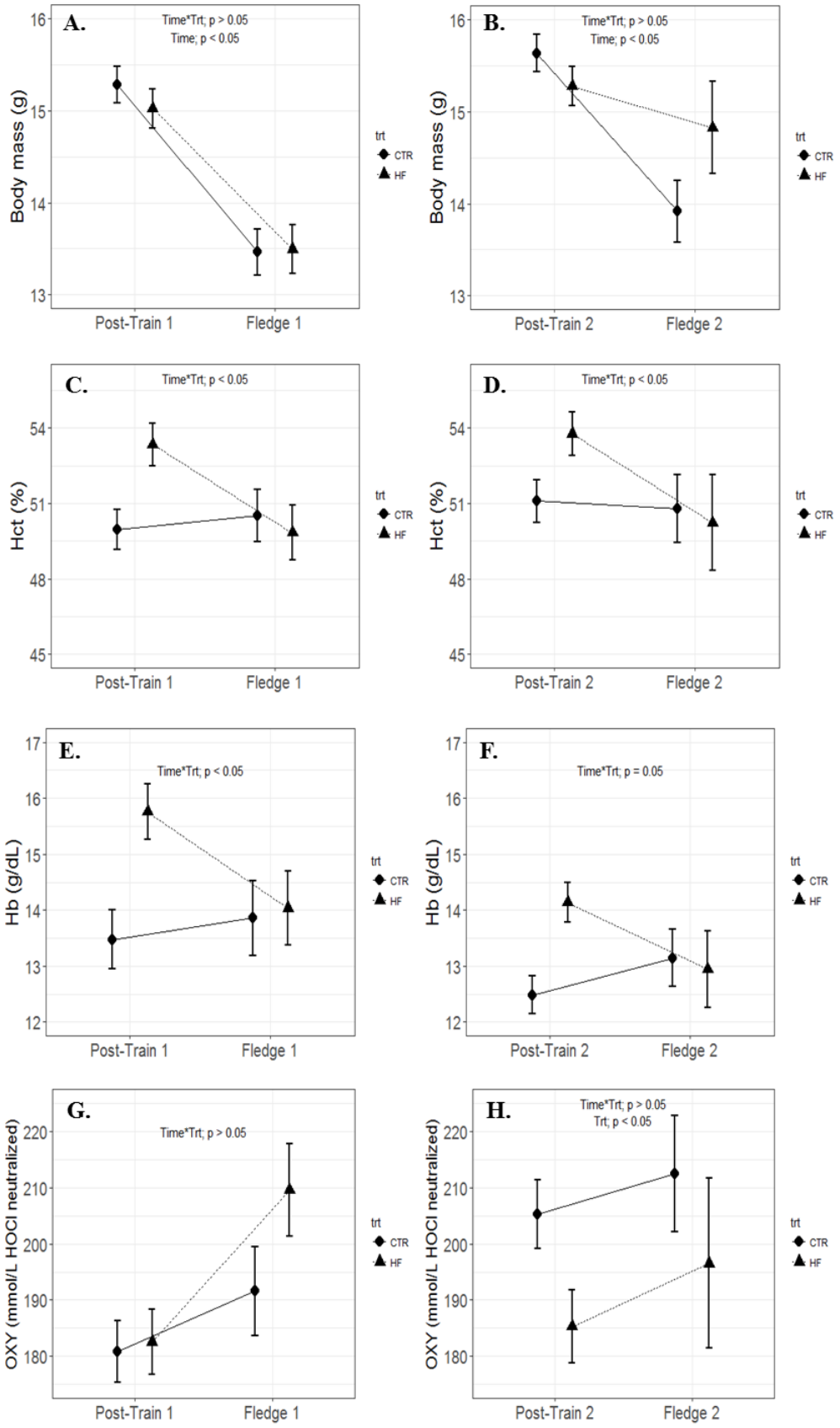

E.
**
Hb (g/dL)
17
16
15
14
13
12
CTR
HF
F.
**
Hb (g/dL)
17
16
15
14
13
12
CTR
HF
G.
NS
OXY (mmol/L HOCl neutralized)
210
200
190
180
CTR
HF
H.
*
OXY (mmol/L HOCl neutralized)
210
200
190
180
CTR
HF
I.
**
ROMs (mmol/L H2O2 equivalent)
5
4
3
2
CTR
HF
J.
NS
ROMs (mmol/L H2O2 equivalent)
5
4
3
2
CTR
HF
K.
**
OS (ROMs x 1000/OXY)
25
20
15
10
5
CTR
HF
L.
*
OS (ROMs x 1000/OXY)
25
20
15
10
5
CTR
HF

Figure 6-3 **Effects of increased foraging effort and reproduction on A) body mass in the first breeding attempt, B) body mass in the second breeding attempt, C) Hct in the first breeding attempt, D) Hct in the second breeding attempt, E) Hb in the first breeding attempt, F) Hb in the second breeding attempt, G) OXY in the first breeding attempt, H) OXY in the second breeding attempt, I) ROMs in the first breeding attempt, J) ROMs in the second breeding attempt, K) OS in the first breeding attempt, and L) OS in the second breeding attempt. Data shown are least-squared means ± s.e.**


A.
Time*Trt; p > 0.05
Time; p < 0.05
Body mass (g)
Post-Train 1
Fledge 1
B.
Time*Trt; p > 0.05
Time; p < 0.05
Body mass (g)
Post-Train 2
Fledge 2
C.
Time*Trt; p < 0.05
Hct (%)
Post-Train 1
Fledge 1
D.
Time*Trt; p < 0.05
Hct (%)
Post-Train 2
Fledge 2
E.
Time*Trt; p < 0.05
Hb (g/dL)
Post-Train 1
Fledge 1
F.
Time*Trt; p = 0.05
Hb (g/dL)
Post-Train 2
Fledge 2
G.
Time*Trt; p > 0.05
OXY (mmol/L HOCl neutralized)
Post-Train 1
Fledge 1
H.
Time*Trt; p > 0.05
Trt; p < 0.05
OXY (mmol/L HOCl neutralized)
Post-Train 2
Fledge 2
trt
CTR
HF

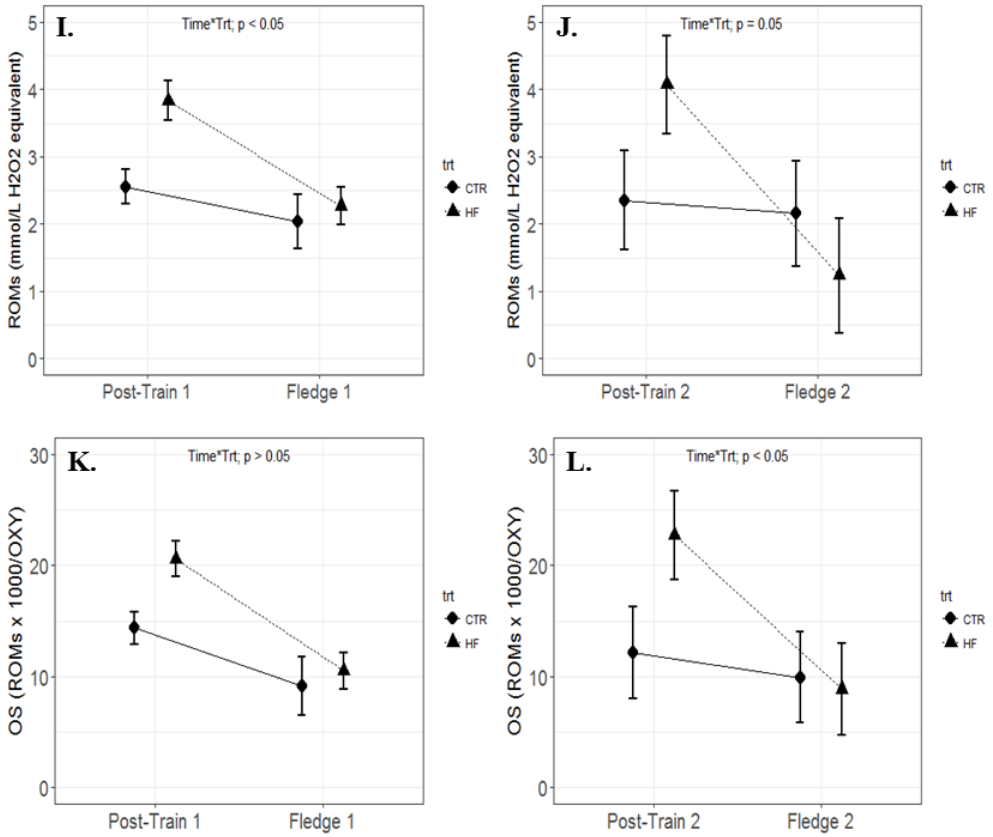

I.
Time*Trt; p < 0.05
ROMs (mmol/L H2O2 equivalent)
Post-Train 1
Fledge 1
trt
CTR
HF
J.
Time*Trt; p = 0.05
ROMs (mmol/L H2O2 equivalent)
Post-Train 2
Fledge 2
trt
CTR
HF
K.
Time*Trt; p > 0.05
OS (ROMs x 1000/OXY)
Post-Train 1
Fledge 1
trt
CTR
HF
L.
Time*Trt; p < 0.05
OS (ROMs x 1000/OXY)
Post-Train 2
Fledge 2
trt
CTR
HF

Figure 6-4 **Effects of increased foraging effort on A) hatching success, B) fledging success, C) laying interval, D) egg mass, E) clutch size, F) brood size at hatch, and G) brood size at fledge. Data shown in Fig. 4A-B are raw mean percentages. Data shown in Fig. 4C-G are least-squared means ± s.e.**

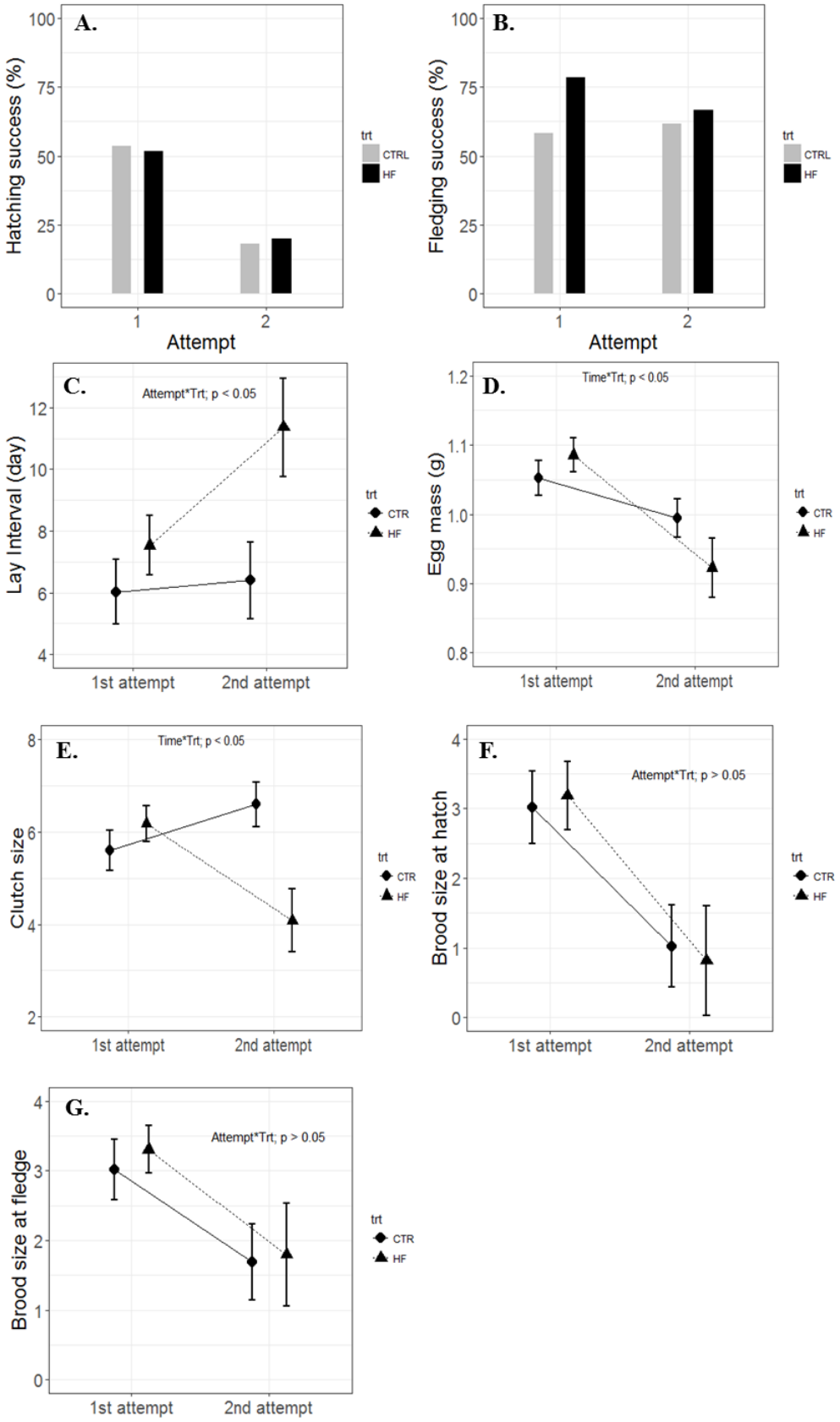
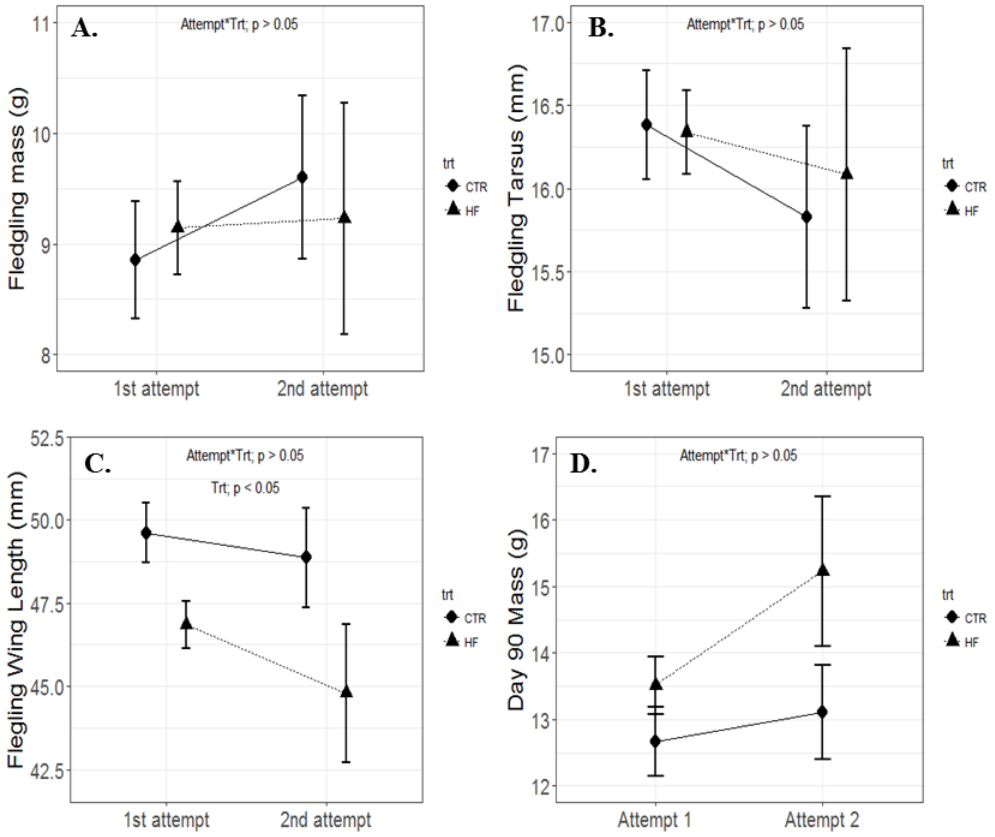

A.
Hatching success (%)
100
75
50
25
0
1
2
Attempt
trt
CTRL
HF
B.
Fledging success (%)
100
75
50
25
0
1
2
Attempt
trt
CTRL
HF
C.
Attempt*Trt; p < 0.05
Lay Interval (day)
12
10
8
6
4
1st attempt
2nd attempt
trt
CTR
HF
D.
Time*Trt; p < 0.05
Egg mass (g)
1.2
1.1
1.0
0.9
0.8
1st attempt
2nd attempt
trt
CTR
HF
E.
Time*Trt; p < 0.05
Clutch size
8
6
4
2
1st attempt
2nd attempt
trt
CTR
HF
F.
Attempt*Trt; p > 0.05
Brood size at hatch
4
3
2
1
0
1st attempt
2nd attempt
trt
CTR
HF
G.
Attempt*Trt; p > 0.05
Brood size at fledge
4
3
2
1
0
1st attempt
2nd attempt
trt
CTR
HF

Figure 6-5 **Effects of increased foraging effort on offspring A) mass at 21 d of age, B) tarsus at 21 d of age, C) wing length at 21 d of age, D) mass at 90 d of age, E) tarsus at 90 d of age, F) wing length at 90 d of age, G) Hct at 90 d of age, H) Hb at 90 d of age, I) OXY at 90 d of age, and J) ROMs at 90 d of age. Data shown in are least-squared means ± s.e.**



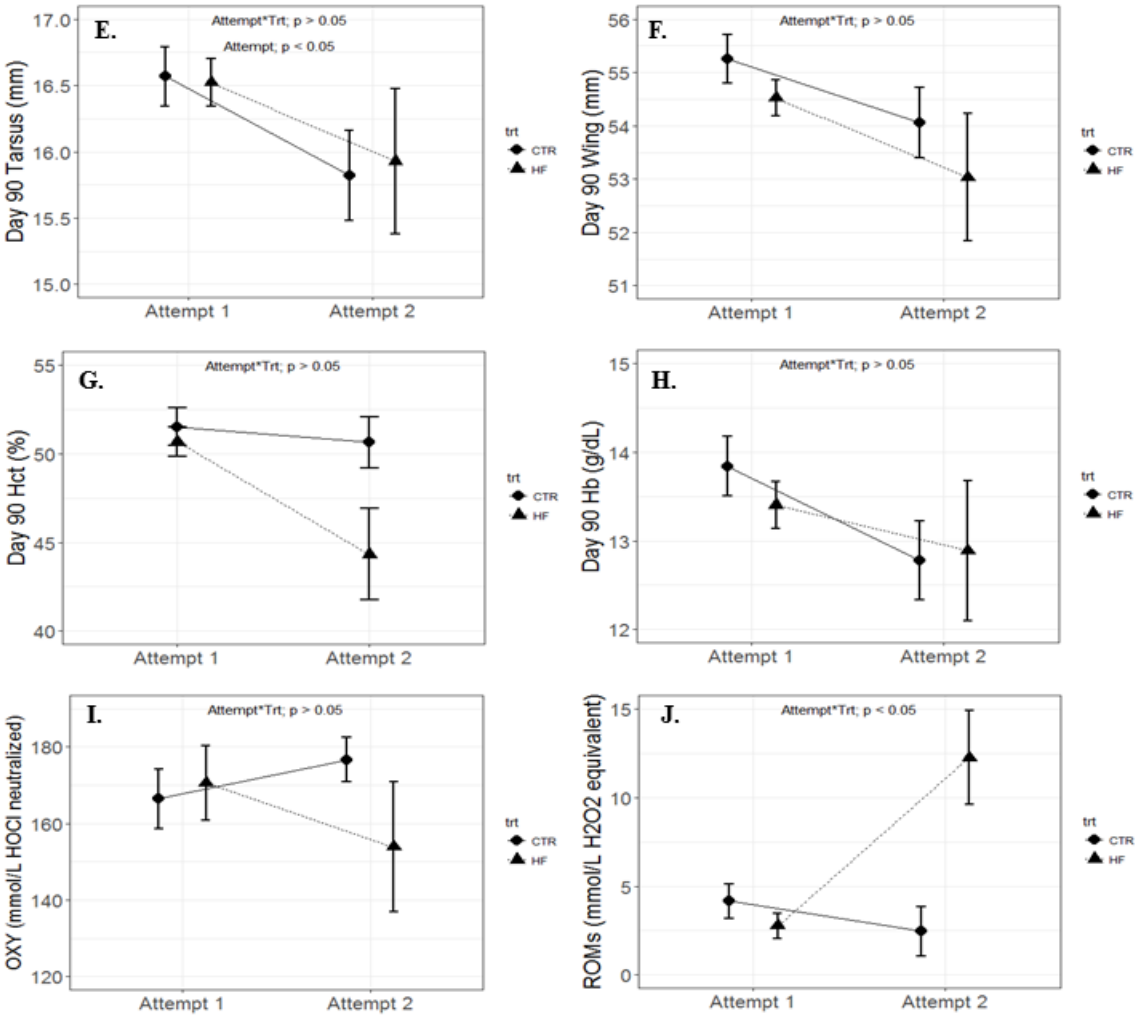

E.
Attempt*Trt; p > 0.05
Attempt; p < 0.05
Day 90 Tarsus (mm)
17.0
16.5
16.0
15.5
15.0
Attempt 1
Attempt 2
trt
CTR
HF
F.
Attempt*Trt; p > 0.05
Day 90 Wing (mm)
56
55
54
53
52
51
Attempt 1
Attempt 2
trt
CTR
HF
G.
Attempt*Trt; p > 0.05
Day 90 Hct (%)
55
50
45
40
Attempt 1
Attempt 2
trt
CTR
HF
H.
Attempt*Trt; p > 0.05
Day 90 Hb (g/dL)
15
14
13
12
Attempt 1
Attempt 2
trt
CTR
HF
I.
Attempt*Trt; p > 0.05
OXY (mmol/L HOCl neutralized)
180
160
140
120
Attempt 1
Attempt 2
trt
CTR
HF
J.
Attempt*Trt; p < 0.05
ROMs (mmol/L H2O2 equivalent)
15
10
5
0
Attempt 1
Attempt 2
trt
CTR
HF

Table 6-1 Body mass, Hct, Hb, OXY, ROMs, and OS values for all 6 timepoints. Data shown are least-squared means ± s.e.

	Attempt 1						Attempt 2					
	Pre-train 1		Post-train 1		Fledge 1		Pre-train 2		Post-train 2		Fledge 2	
	CTR	HF	CTR	HF	CTR	HF	CTR	HF	CTR	HF	CTR	HF
Mass (g)	15.56 ± 0.28	15.69 ± 0.28	15.29 ± 0.26	15.01 ± 0.23	13.28 ± 0.37	13.23 ± 0.29	15.54 ± 0.27	14.62 ± 0.26	15.91 ± 0.27	14.81 ± 0.26	14.28 ± 0.47	14.72 ± 0.48
Hct (%)	50.55 ± 1.19	50.47 ± 1.13	50.09 ± 1.13	52.06 ± 0.94	49.01 ± 1.47	50.81 ± 1.18	51.76 ± 1.18	52.64 ± 1.01	50.76 ± 1.19	54.89 ± 1.04	48.33 ± 1.80	48.47 ± 1.85
Hb (g/dL)	14.81 ± 0.57	14.72 ± 0.56	14.10 ± 0.44	15.28 ± 0.37	14.14 ± 0.57	14.58 ± 0.46	12.90 ± 0.47	11.72 ± 0.42	12.76 ± 0.48	13.79 ± 0.43	13.10 ± 0.70	13.56 ± 0.88
OXY (mmol/L HOCl neutralized)	203.88 ± 5.66	195.88 ± 6.02	184.15 ± 4.84	184.57 ± 4.26	193.51 ± 6.87	207.97 ± 5.46	182.89 ± 5.17	196.69 ± 5.12	203.62 ± 5.27	186.29 ± 5.02	218.57 ± 8.16	194.92 ± 10.39
log ROMs (mmol/L H_2O_2 equivalent)	0.42 ± 0.05	0.45 ± 0.05	0.43 ± 0.04	0.50 ± 0.04	0.26 ± 0.06	0.28 ± 0.05	0.51 ± 0.05	0.56 ± 0.04	0.40 ± 0.05	0.49 ± 0.04	0.30 ± 0.07	0.27 ± 0.08
log OS (ROMs × 1000/OXY)	1.10 ± 0.05	1.16 ± 0.05	1.16 ± 0.04	1.23 ± 0.04	0.96 ± 0.06	0.95 ± 0.05	1.24 ± 0.05	1.28 ± 0.04	1.10 ± 0.05	1.23 ± 0.04	0.98 ± 0.07	0.99 ± 0.08

Chapter 7.
Conclusions and Future Directions

General Conclusions

This thesis took an exercise perspective and investigated the physiological basis of aerobic capacity and workload ability in birds, using both a comparative, phylogenetic approach, as well as various laboratory-based experimental approaches. In surveying the literature and synthesizing findings from experimental studies in different taxa, we identified several potential common physiological markers underlying individual variation in exercise performance and costs of exercise (Chapter 2). Using a comparative and phylogenetic approach, we also found that hematological traits co-vary with life-history variables, and to a certain extent, energy metabolism in birds (Chapter 3). In chapter 4, we provided experimental evidence for physiological responses to flight at high attitude and showed that the relationship between hematocrit and flight performance is dependent on altitude. Using a previously established technique to manipulate foraging effort in captive zebra finches, we provided experimental evidence for behavioural and physiological adjustments to high workload (Chapter 5). In a follow-up experiment, we showed that high workload can negatively impact reproduction (Chapter 6).

Despite finding several potential common physiological markers underlying individual variation in exercise performance and costs of exercise after surveying the literature, few studies have actually investigated how physiological adjustments to exercise can directly affect survival and reproduction. Furthermore, it is difficult to extrapolate the findings to free-living animals because most laboratory-based studies were conducted in conditions that are dissimilar to what animals experience in nature (Fonseca et al., 2014; Killen et al., 2017). We argue that there is a need to develop more ecologically relevant laboratory systems (Briga et al., 2017; Fonseca et al., 2014; Simons et al., 2014), incorporating physiological systems at multiple levels, as well as fitness endpoints such as survival and reproduction (Lailvaux, S. P., & Husak, 2014; Zera and Harshman, 2001).

Our work suggests that at the interspecific level, hematocrit and hemoglobin co-vary with life-history traits like migratory status and altitude, and that hematocrit seems

to be a predictor of energetic constraints in birds. Given the potential link between energetics and fitness measures such as survival and reproduction (Crossin et al., 2014; Lemon, 1993; Williams, 2012), it is still unclear whether interspecific variation in reproductive effort and output such as clutch size and egg size can be explained by variation in hematocrit. In addition, despite showing evidence for covariation of hematological traits with life-history variables, our study is correlational in nature and thus, it remains to be determined if experimental manipulation of hematocrit can affect energy allocation and functional traits such as migratory performance in animals.

Having established the link between hematological traits and life-history variables such as migratory status and altitude at the interspecific level, experimental manipulation of hematocrit at the intraspecific level reveals that the relationship between hematocrit and flight performance is dependent on altitude. More specifically, birds with decreased hematocrit had lower flight performance at low altitude but had higher flight performance at high altitude. We argue that the lower flight performance observed at low altitude is due to low blood oxygen carrying capacity (Hammond et al., 2000; Petit and Vezina, 2014), whereas the higher flight performance observed at high altitude is due to low blood viscosity (Jenni et al., 2006; Schuler et al., 2010). However, a limitation of this study is that altitude was simulated to increase at a relatively high rate, while free-living birds may climb at slower speeds and thus adjust their physiology better to the changing altitude. Thus, it remains to be determined whether birds adaptively modulate their hematocrit level based on the altitude they fly at during migration.

Echoing the call to develop more ecologically relevant laboratory studies to examine the physiology of workload (Briga et al., 2017; Fonseca et al., 2014; Simons et al., 2014), we experimentally manipulated foraging effort in captive zebra finches using a previously described technique (Briga et al., 2017; Koetsier and Verhulst, 2011; Simons et al., 2014) and investigated behavioural and physiological responses to increased foraging effort. In a follow-up study, we also looked at how physiological adjustments to increased foraging effort affect subsequent reproduction. Our studies have shown that birds made drastic behavioural adjustments in response to increased foraging effort, although physiological adjustments appeared to be time-dependent. Increased foraging effort during reproduction also negatively impacted fecundity and offspring quality. We argue that the effect of increased foraging effort on reproduction was mediated by oxidative stress. A study in mice found improved reproductive performance with

increased activity (Zhang et al., 2018a), suggesting that the relationship of activity level and reproductive performance is likely 'dose-dependent'. It is unclear whether lower intensity and/or shorter duration of exercise training would affect reproduction differently in birds.

Future Directions

One important facet of physiology that we did not explore in this thesis is the role of the brain (motivation) on exercise performance and workload ability in animals (Kolb et al., 2010; Marcora et al., 2009; McCormick et al., 2015; Pollak et al., 2014). Whole organism performance and fitness are determined by a complex suite of physiological systems, in addition to morphological and behavioural traits (Irschick, D. J., & Higham, 2016; Lailvaux, S. P., & Husak, 2014; Williams and Fowler, 2015; Yap et al., 2017a). Moving forward, future studies should capitalize on established paradigms such as operant conditioning (Fonseca et al., 2014; Mazur, 2016) to simulate different levels of activity experimentally and look at how it would affect subsequent reproduction, as well as whether reproduction can in turn modulate any subsequent physiological responses to exercise training. Future studies should also consider and integrate multiple physiological systems, such as the nervous system (Rhodes et al., 2003; Rhodes et al., 2005), hormones (Williams, 2008; Zera and Harshman, 2001) and immune functions (Harshman and Zera, 2007; Pedersen and Hoffman-Goetz, 2000) when studying the physiology underlying workload ability, and more generally, life-history trade-offs in animals.

Lastly, although we have made an effort to incorporate the element of resource acquisition into our experimental studies, it is still unclear whether findings from our studies can be extrapolated to free-living animals. Future studies should aim to experimentally manipulate animals in semi-artificial enclosure or even in the field, either by increasing workload (e.g. wing clipping, cold exposure) or through pharmacological means (Costantini et al., 2016; Fronstin et al., 2016) and look at how physiology is affected, as well as how the altered physiological state affects survival and reproduction.

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Appendix A.
Supplemental Information for Chapter 3

Figure S3-1 Consensus tree for analyses of the relationship between life-history traits and haematology.

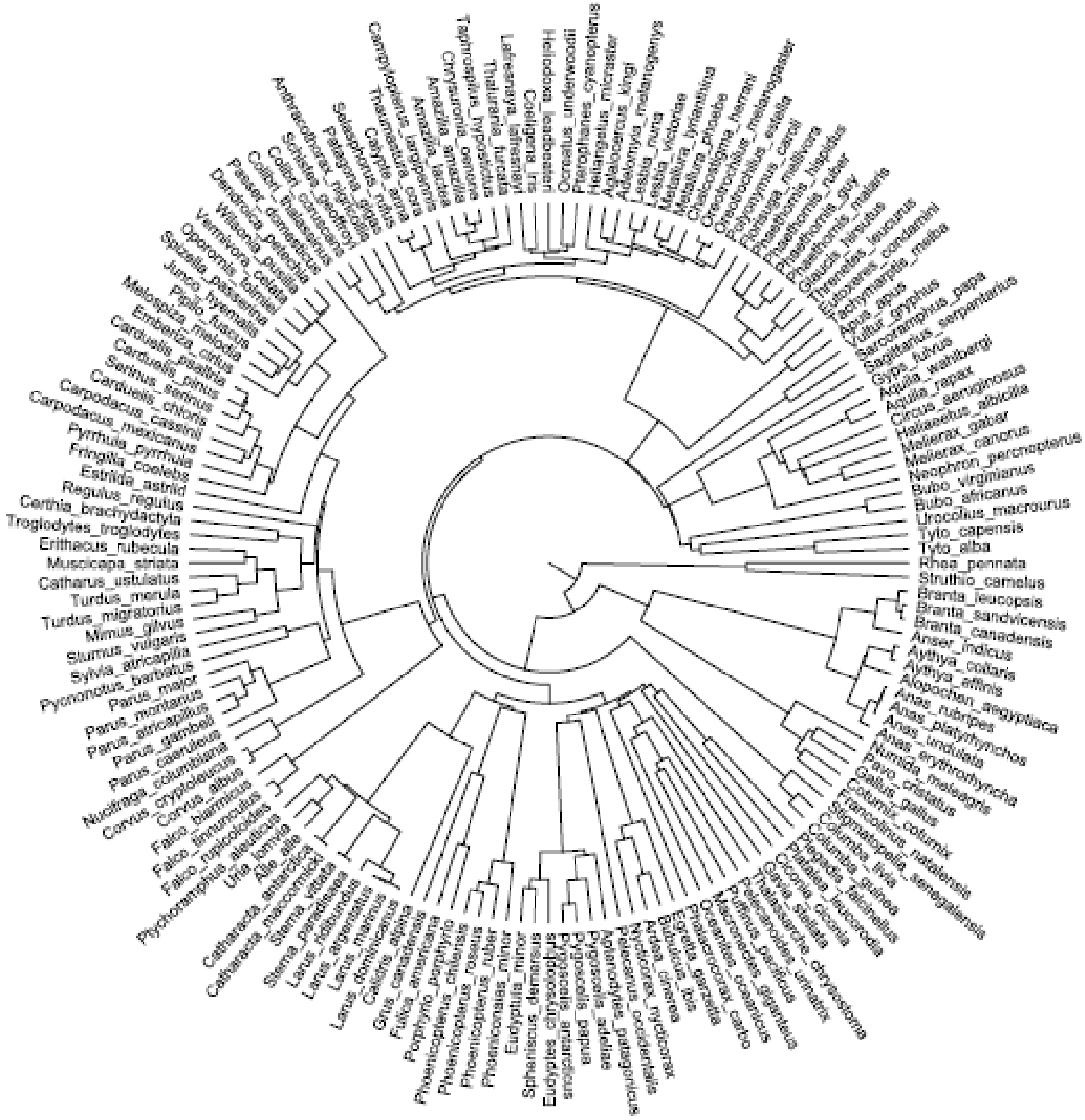
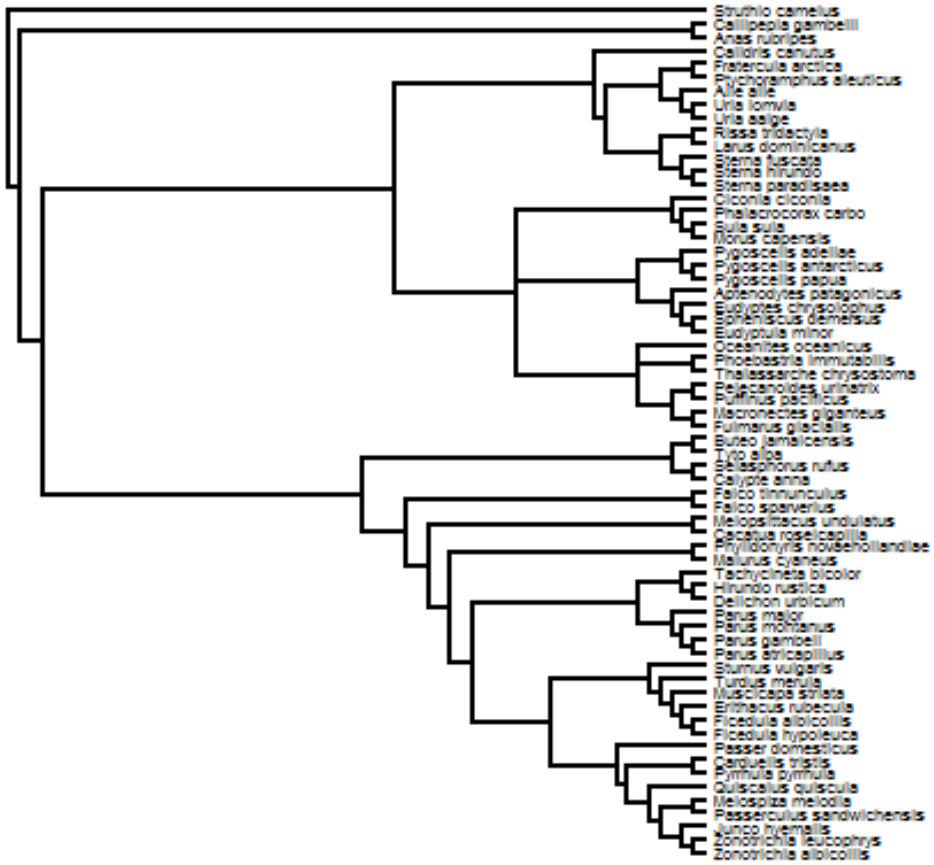


Figure S3-2 Consensus tree for analyses of the relationship between haematocrit and measures of energy expenditure.



Appendix B.
Supplemental Information for Chapter 4

Table S3-3 Statistical output showing all variables and PGLS models. Response variables are highlighted in bold.

PGLS model	num*DF*	Residual *DF*	*F*-value	Slope	Intercept	R^2	*P*-value
log Hb ~ Hct	.	158	.	0.007	0.85	0.07	< 0.01
Hct ~ migration + √ altitude + log mass	2	155	4.95	.	.	.	< 0.01
log Hb ~ migration + √ altitude + log mass	2	155	4.31	.	.	.	0.015
log Hb ~ migration + √ altitude + Hct + log mass	2	154	12.80	.	.	.	< 0.01
Hct ~ √ altitude + log mass	.	157	.	0.03	49.35	< 0.01	0.22
log Hb ~ √ altitude + log mass	.	157	.	0.001	1.21	0.09	< 0.01
Hct ~ log mass	.	158	.	-1.57	49.59	< 0.01	0.22
log Hb ~ log mass	.	158	.	-0.02	1.20	0.02	0.24
log FMR ~ log mass	.	56	.	0.62	-1.93	0.64	< 0.01
log BMR ~ log mass	.	56	.	0.67	-3.33	0.73	< 0.01
log AEE ~ log mass	.	56	.	0.56	-2.11	0.37	< 0.01
BMR ~ AEE	.	56	.	0.04	-2.36	0.37	0.85
BMR ~ FMR	.	56	.	0.40	-0.81	0.69	< 0.01
log BMR ~ Hct + log mass	.	54	.	-0.02	-2.40	0.09	0.06
log FMR ~ Hct + log mass	.	54	.	0.02	-3.21	< 0.01	0.05
log AEE ~ Hct + log mass	.	54	.	0.06	-5.28	0.05	< 0.01

S4 1 Sample sizes for each behavioural and physiological measurements (organized by timepoint, treatment, and altitude exposure).

	Pre-treatment			Post-treatment (High Alt. only)			Post-treatment (Low Alt. only)		
	Anti-EPO	EPO	Veh	Anti-EPO	EPO	Veh	Anti-EPO	EPO	Veh
Strikes	8	7	7	6	6	6	2	1	1
30-Strikes	8	7	7	6	6	6	2	1	1
COT	8	7	7	6	6	6	2	1	1
Power	8	7	7	6	6	6	2	1	1
Duration	8	7	7	6	6	6	2	1	1
Altitude	-	-	-	6	6	6	-	-	-
Alt. Duration	-	-	-	6	1	4	-	-	-
Hct	8	7	7	6	6	6	2	1	1
Hb	7	7	7	6	6	6	2	1	1
Glucose	7	7	7	6	6	6	0	0	1
OXY	8	7	7	6	6	6	0	0	1
dROMs	8	7	7	6	6	6	0	0	1

Appendix C.
Supplementary Information for Chapter 5

Fig. S5-1 Picture of high foraging cost treatment (HF) cage setup.

Table S5-2. Sample sizes for each physiological measurement (organized by sex and treatment)

	HF Male	HF Female	CTR Male	CTR Female
BMR	18	9	18	11
Hct	18	9	18	11
Hb	18	9	18	11
Body composition	18	9	16	11
Glucose	10	6	8	5
Triglyceride and glycerol	15	7	7	8
OXY	16	8	13	11
dROMs	15	8	9	8

Table S5-3 **Statistical model showing Time by Treatment interaction in body mass, BMR, hematocrit, hemoglobin, and glucose, and treatment effect on behavior, immediate food consumption, tissue masses, glycerol, triglyceride, OXY and dROMs. Data shown are least-squared means ± s.e. with both sexes pooled.**

Trait	Pre-trt		Day 3		Day 60		Day 90		Random factor	Estimated Variance	Residual Variance	numDF	denDF	*W*-value	*Z*-value	*F*-value	*P*-value
	CTR	HF	CTR	HF	CTR	HF	CTR	HF									
Body mass (g)	14.43 ± 0.29	14.37 ± 0.30	14.69 ± 0.29	13.85 ± 0.30	14.60 ± 0.29	14.15 ± 0.30	NA	NA	Bird ID	1.930	0.468	2	108	.	.	4.499	0.01
BMR (mL O2/h)	44.72 ± 1.32	48.42 ± 1.37	47.08 ± 1.32	49.47 ± 1.38	44.39 ± 1.32	47.35 ± 1.37	NA	NA	Bird ID	7.491	42.834	2	107	.	.	0.137	0.87
Hematocrit (%)	52.69 ± 0.72	51.30 ± 0.74	52.67 ± 0.72	53.62 ± 0.75	52.05 ± 0.72	51.96 ± 0.75	NA	NA	Bird ID	9.479	5.500	2	107	.	.	1.163	0.32
Hemoglobin (g/dL)	14.91 ± 0.26	14.35 ± 0.27	14.82 ± 0.27	14.23 ± 0.28	14.92 ± 0.26	14.86 ± 0.27	NA	NA	Bird ID	0.937	1.079	2	107	.	.	1.095	0.34
Glucose (mmol/L)	15.23 ± 0.62	16.39 ±	15.27 ±	14.33 ±	14.37 ±	14.32 ±	NA	NA	Bird ID	1.393	3.578	2	53	.	.	2.225	0.12

		0.56	0.58	0.56	0.59	0.56											
Trips to feeder	NA	NA	NA	NA	NA	NA	1.921 ± 2.130	15.843 ± 2.209	.	.	.	1	54	215	.	.	< 0.01
Time spent resting (s)	NA	NA	NA	NA	NA	NA	1106.66 ± 61.831	1011.625 ± 59.382	.	.	.	1	54	.	1.10	.	0.27
Immediate food consumption (g)	NA	NA	NA	NA	NA	NA	0.177 ± 0.029	0.275 ± 0.029	.	.	.	1	54	215	.	.	0.009
Leg muscle mass (g)	NA	NA	NA	NA	NA	NA	0.077 ± 0.003	0.080 ± 0.003	.	.	.	1	52	.	-0.79	.	0.43
Flight muscle mass (g)	NA	NA	NA	NA	NA	NA	0.719 ± 0.014	0.750 ± 0.014	.	.	.	1	52	.	-1.59	.	0.11
Heart	NA	NA	NA	NA	NA	NA	0.509 ±	0.500 ±	.	.	.	1	52	.	0.41	.	0.68

mass (g)							0.001	0.001									
Lung mass (g)	NA	NA	NA	NA	NA	NA	0.055 ± 0.018	0.061 ± 0.019	.	.	.	1	52	.	-1.59	.	0.11
Crop mass (g)	NA	NA	NA	NA	NA	NA	0.012 ± 0.00008	0.011 ± 0.00008	.	.	.	1	52	.	0.54	.	0.59
S. intestine mass (g)	NA	NA	NA	NA	NA	NA	0.023 ± 0.001	0.021 ± 0.001	.	.	.	1	52	.	1.09	.	0.27
L. intestine mass (g)	NA	NA	NA	NA	NA	NA	0.138 ± 0.007	0.132 ± 0.007	.	.	.	1	52	.	0.59	.	0.56
Gizzard mass (g)	NA	NA	NA	NA	NA	NA	0.097 ± 0.003	0.096 ± 0.003	.	.	.	1	52	.	-0.10	.	0.92
Liver mass (g)	NA	NA	NA	NA	NA	NA	0.171 ± 0.01	0.157 ± 0.01	.	.	.	1	52	.	1.01	.	0.31
Kidney mass (g)	NA	NA	NA	NA	NA	NA	0.033 ± 0.002	0.035 ± 0.002	.	.	.	1	52	.	-0.73	.	0.47
Glycerol	NA	NA	NA	NA	NA	NA	0.950 ±	1.067 ±	.	.	.	1	35	.	-0.	.	0.57

(mmol/L)							0.155	0.127							57		
Triglyceride (mmol/L)	NA	NA	NA	NA	NA	NA	4.611 ± 0.362	4.527 ± 0.296	.	.	.	1	35	.	0.18	.	0.86
OXY (mmol/L HOCl neutralized)	NA	NA	NA	NA	NA	NA	243.06 ± 9.803	233.28 ± 9.803	.	.	.	1	46	.	0.70	.	0.48
dROMs (mmol/L H2O2 equivalent)	NA	NA	NA	NA	NA	NA	4.919 ± 0.318	5.769 ± 0.253	.	.	.	1	38	.	-2.11	.	0.03

Fig S5-4 **Day 3 and Day 60 data for (A) body mass, (B) basal metabolic rate (BMR), (C) hematocrit (Hct) and (D) hemoglobin (Hb), using pre-treatment (Day 0) values as covariate. Filled circles and solid lines represent CTR birds; Filled triangles and dashed lines represent HF birds. Data shown are least-squared means ± s.e.**