ENERGY BALANCE IN MIGRATORY BIRDS: INSIGHTS FROM MITOCHONDRIAL GENOMES AND LEPTIN RECEPTOR BIOLOGY

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Energy balance in migratory birds: Insights from mitochondrial genomes and leptin biology

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Migratory birds undergo seasonal migratory journeys that are energetically challenging and require appropriate regulation of energy intake and expenditure. We are beginning to understand the physiological changes that migration entails, but how energy balance is regulated in avian migrants remains poorly understood. The hormone leptin is a key modulator of food intake and energy expenditure in vertebrates. Leptin is produced in proportion to body adiposity and reduces food intake and body fat through leptin receptors in the brain. Leptin could be involved in the physiological changes that migratory birds undergo. However, while leptin receptors and physiological responses to leptin are seen in birds, leptin has yet to be found in the avian genome. Chapter One describes the history of leptin research in birds and the controversy that currently surrounds the topic. In seasonally breeding and migratory mammals, leptin's role in energy regulation changes seasonally. The brain's sensitivity towards leptin is reduced during periods of high energy demand, thus muting the anorexic effects of leptin to facilitate increases in food intake and body fat that are needed for energetically intensive periods. Chapter Two documents similar changes in migratory birds; migratory white-throated sparrows (*Zonotrichia albicollis*) are not responsive to exogenous leptin administration, while leptin reduces food intake and fat mass in wintering birds. Chapter Three documents the expression of leptin receptor and suppressor of cytokine signaling 3 (SOCS3), an inhibitor of leptin signaling, in several sparrow tissues. Changes in leptin receptor and SOCS3 are thought to be partly responsible for the reduction in leptin sensitivity seen in migratory and seasonally breeding

mammals. The expression of these genes was not different between sparrows in migratory and wintering state, suggesting that other mechanisms are responsible for the changes in responsiveness to leptin seen in Chapter 2. Chapter 4 provides DNA sequences of the complete mitochondrial genome of *Tachycineta* swallows to 1) construct the mitochondrial gene tree and 2) test for signatures of positive directional selection across the mitochondrial genome that would indicate correlations with organismal ecology, such as migration. However, we found no evidence of positive selection in the mitochondrial genomes of *Tachycineta*.

Biological Sketch

David Cerasale attended the College of William and Mary in Williamsburg, Virginia, USA between 1996 and 2000. He graduated with a Bachelor's of Science degree, majoring in both biology and history. Following graduation David worked for the United States Geological Survey, Biological Resources Division, supervising field-work on endangered Hawaiian birds. In 2002, he matriculated at the University of Montana studying the physiology of migratory birds. David graduated with a Master's of Science degree from the University of Montana in 2004. Since 2005, David has attended Cornell University as a PhD student in the field of Ecology and Evolutionary Biology. He has studied energy balance in migratory birds both in the field and in the laboratory. The following dissertation is the culmination of his PhD work at Cornell.

Acknowledgements

I preface this section by admitting that I am not one for acknowledgements. I would first like to thank my PhD supervisor, David W. Winkler, for his patience over the past six years. He allowed me to explore my own interests while succeeding and failing on my own. This has taught me valuable lessons not only in how to navigate science as an institution, but also in all aspects of my life. I could say much more about his willingness to discuss research ideas, argue about the best approaches to ask questions and how to juggle multiple projects and deadlines. However, there is one simple characteristic that helped keep my head straight during my tenure here: Wink is a nice guy. I can only hope that he realizes how important that is to his students.

The other members of my committee were always patient with me, while supporting my interests. Irby Lovette's lab adopted me from the word "go", and he was always there for support and a laugh, or some ribbing, whichever was necessary. Yves Boisclair agreed to be on the committee as a member outside of the field. I know this was at times difficult for him, as ecologists and particularly evolutionary biologists have a much different view than do physiologists, both on what constitutes an important biological question and how to go about answering it. The most excited I ever saw him was when he discovered my wife was a Canadian as well; told me that his heart was in the right place.

A number of others had a giant hand in my finishing. The Guglielmo lab at the University of Western Ontario not only were wonderful collaborators, but finished my experiments there when I was indisposed. Theunis Piersma, Joop Jukema and the Castricum Ringing group were amazing hosts in The Netherlands, and Sjoerd and Jacintha will never be forgotten. The labs of Ned Place, Rick Harrison and Esther Angert also helped see me through. Jamie, Paulo, Vale, Anna and Amos were constant lab companions, although they did not always

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The history of avian leptin: From promise to controversy

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The discovery and characterization of the "obese" gene in mice and humans, which codes for the hormone leptin, was thought to provide a key to elucidating the mechanisms of vertebrate energy balance to a host of awaiting molecular physiologists (Zhang et al., 1994). Excitement surrounding leptin centers on its role as a peripheral signal to the brain regarding the extent of energy stores. Leptin, produced by adipose tissue in proportion to total body adiposity, links peripheral fat storage to the central nervous system by influencing both food intake and energy expenditure through leptin receptors expressed by hypothalamic neurons (reviewed in Friedman and Halaas, 1998). Under normal conditions, elevated body fat levels produce high leptin concentrations in the circulation that induce a reduction in food intake and an increase in energy expenditure (see Ahima et al., 2000), while signaling adequate energy for other physiological processes, such as the growth and maintenance of reproductive tissues (e.g., Cheung et al., 1997). Thus, leptin is considered a key modulator of energy balance, and interest in understanding leptin's action upon energy balance has exploded (over 25,000 hits in a recent Web of Science search of "leptin"), particularly within the biomedical field that is motivated to discover a potential treatment for obesity (e.g., Campfield et al., 1998).

The interest in leptin as an important mechanism in the regulation of energy balance in vertebrates has spread to scientific fields outside of biomedical research. Zhang et al.'s (1994) original characterization of mouse and human leptin suggested that leptin-like sequences could be found in a wide array of vertebrates, including the chicken (*Gallus gallus*) and an eel (Anguilliformes). In the 16 years since its publication, sequences of both leptin-like compounds and leptin receptors have now been identified in the genomes of several non-avian vertebrate groups, including: scores of mammalian species (see Doyon et al., 2001), teleost fish (Froiland et al., 2010; Gorissen et al., 2009; Kurokawa and Murashita, 2009; Kurokawa et al., 2005; Li et al.,

2010; Murashita et al., 2008; Yacobovitz et al., 2008), anurans (Crespi and Denver, 2006) and Ambystomid salamanders (Boswell et al., 2006). Despite low amino acid and sequence similarity among vertebrate leptins (e.g., amino acid sequence of frog leptin is only 35% similar to human and 13% similar to fish), its tertiary peptide structure is remarkably conserved (see Crespi and Denver, 2006).

This conserved structure is responsible for the fact that recombinant leptins produced from frog and fish sequences can bind and activate mammalian leptin receptors (Crespi and Denver, 2006, Yacobovitz e al,. 2008), and *Xenopus* leptin receptor can bind both frog and human leptin (Crespi and Denver, 2006). These leptins induce strong anorexic effects *in vivo,* consistent with findings in mammals (Crespi and Denver, 2006; Li et al., 2010; Murashita et al., 2011; Murashita et al., 2008; Yacobovitz et al., 2008). Although a leptin gene has yet to be identified in lepidosaurs, injections of mammalian leptins also reduce food intake, increase metabolic rate, raise body temperature and delay regression of testes, again consistent with findings in mammals (Niewiarowski et al., 2000; Putti et al., 2009). Leptin-like compounds, as measured by radioactive-immuno assays and immuno-histochemistry, are also found in tissues of some members of the Squamata (Muruzabal et al., 2002; Paolucci et al., 2001; Spanovich et al., 2006) and Chondrichthyes (Gambardella et al., 2010).

Two independent reports of the characterization of a chicken "leptin" gene appeared soon after the discovery of the mouse leptin gene (Ashwell et al., 1999; Taouis et al., 1998), and the purported "leptin" gene was mapped to the chicken genome (Pitel et al., 1999). The expression of chicken "leptin" mRNA was also reported in the chicken using a variety of techniques (Richards et al., 1999, 2000). Chicken "leptin" was subsequently purified via recombinant technology using the reported sequences, and its biological activity confirmed by the ability to

activate cells expressing the human leptin receptor (Dridi et al., 2000a; Raver et al., 1998). Two radio-immuno assays, using antibodies raised against the amino acid sequence of the reported chicken ”leptin” were developed to provide a tool to measure leptin protein concentrations in the plasma of birds (Dridi et al., 2000b; Evock-Clover et al., 2002). These apparent discoveries seemed to place the field of avian energetics on the brink of a major advance in the understanding of the control of energy balance.

This excitement, however, was soon replaced by controversy. Several prominent poultry endocrinologists could not reproduce the findings of Taouis et al. (1998) and Ashwell et al. (1999). Multiple efforts to amplify the chicken leptin gene from both genomic DNA and EST libraries failed (see Amills et al., 2003; Friedman-Einat et al., 1999). Results of the initial mapping study were later retracted after sequencing revealed that the primers used to map the purported chicken leptin gene did not amplify products that matched the reported chicken “leptin” sequences (Pitel et al., 2000). Moreover, mRNA with high sequence similarity to mouse leptin was not found in the liver or adipose tissue of several avian species (Friedman-Einat et al., 1999). Phylogenetic evidence also suggested that the reported leptin sequence did not follow the expected model of vertebrate evolution; the chicken “leptin” sequence was far more closely related to murine leptin than the latter is to other mammalian leptins, including sequences from rats and other rodents (Doyon et al., 2001). Thus, some researchers suggested that the reported chicken “leptin” sequences were actually the results of contamination of murine origin (see Sharp et al., 2008).

Following the initial controversy created by the publication of the reported chicken “leptin”, new genetic information supporting or denying the existence of an avian leptin was sparse (but see Dai et al., 2007), and the discussion of the controversy in the literature fell silent.

However, efforts to document and measure leptin-like compounds in avian tissues continued. Leptin-like compounds have now been found in the plasma of members of several avian orders: Galliformes, Passeriformes and Procellariiformes (Dridi et al., 2000b; Evock-Clover et al., 2002; Kordonowy et al., 2010; Quillfeldt et al., 2009). Leptin-like immunoreactivity has also been reported in tissues of chicken (Neglia et al., 2008), geese (*Anser anser*) (Sar et al., 2009) and in the liver of migrating dunlin (*Calidris alpina*) (Kochan et al., 2006). These studies assay leptin-like compounds in birds using antibodies raised against chicken "leptin" sequences, which is very similar to mouse leptin. However, nothing is known about either the identity of the compounds to which these antibodies bind or the cross-reactivity these antibodies exhibit with non-leptin compounds. Thus, the importance of these findings remains severely limited by the controversy over whether leptin actually occurs naturally in birds.

However, two lines of functional evidence have emerged that provide support for the existence of an avian leptin-like system. The first is the uncontroversial discovery of leptin receptor genes in the chicken and turkey (*Meleagris gallopavo*) that are approximately 60% and 50% similar to the human leptin receptor at the nucleotide and amino acid level, respectively (Horev et al., 2000; Richards and Poch, 2003). Avian leptin receptors are expressed in the hypothalamus, the center of feeding regulation, as well as in peripheral tissues, including liver, adipose tissue and ovary (Horev et al., 2000; Richards and Poch, 2003). The chicken leptin receptor or its leptin-binding domains can bind numerous mammalian leptins and frog leptin (Adachi et al., 2008; Dridi et al., 2000a; Hen et al., 2008), but not fish leptins (Yacobovitz et al., 2008). Moreover, the chicken leptin receptor is fully functional: experimental studies have shown that when it is exposed to ovine, mouse or frog leptins, the receptor activates downstream signal transduction pathways (Adachi et al., 2008; Hen et al., 2008).

The existence in birds of a functional avian leptin receptor that can bind leptins from evolutionary distant vertebrate groups (i.e., mammals and amphibians) not only lends support to the existence of a leptin-like system in birds, but also provides a compelling underpinning for a second line of evidence. Since the first reports of the discovery of chicken "leptin," studies of the effects of exogenous leptin injections on avian physiology have become increasingly common in the literature. In several species, injections of leptin *in vivo* influence numerous physiological processes, usually inducing similar effects to those seen in mammals. For example, the injection of leptin decreases food intake, body weight and foraging behavior in numerous avian species (Cassy et al., 2004; Denbow et al., 2000; Dridi et al., 2000a; Kuo et al., 2005; Lohmus and Sundstrom, 2004; Lohmus et al., 2003; Lohmus et al., 2006). Moreover, immunization against the chicken "leptin" protein increases food intake and fat mass in chickens, effectively mimicking a loss of leptin activity (Shi et al., 2006). Leptin injections increase both fatty acid synthase (Dridi et al., 2005) and stearoyl-CoA desaturase (Dridi et al., 2007) in the liver of chickens, although these effects are opposite to those in mammals. Leptin also increases the proliferation of T-cells (Lohmus et al., 2004) and improves immune function in general (Alonso-Alvarez et al., 2007; Lohmus et al., 2011). In addition, administration of leptin attenuates reproductive physiology and behavior in birds. Leptin hastens sexual maturation in chickens (Paczoska-Eliasiewicz et al., 2006) and Japanese quail (*Coturnix japonica*; Macajova et al., 2002), delays the cessation of laying caused by fasting in chickens (Paczoska-Eliasiewicz et al., 2003) and facilitates the growth of regressed ovaries in ducks (Song et al., 2009). The influence of leptin also influences life history decisions: female great tits (*Parus major*) that receive leptin are more likely to lay second clutches (Lohmus and Bjorklund, 2009). However, the physiological effects of leptin in birds are not ubiquitous, particularly on food intake. Leptin

does not reduce food intake in some strains and ages of chickens and quail, particularly young chicks (Bungo et al., 1999; Cassy et al., 2004; Kuo et al., 2005; Macajova et al., 2003). Such exceptions, however, are not inconsistent with the action of leptin in mammals: leptin administration does not reduce food intake in newborn mice and rats (Mistry et al., 1999, Proulx et al., 2002), and in seasonally breeding mammals, changes in leptin sensitivity interfere with its ability to reduce feeding and fat mass (Klingenspor et al., 2000). We have documented similar findings in migratory birds (Chapter 2). Thus, the fact that leptin injections do not influence food intake under all conditions does not provide strong support for arguments against the existence of an avian leptin.

The presence of a functional avian leptin receptor that can be activated by leptins from divergent vertebrate groups and the fairly consistent physiological and behavioral responses to leptin administration provide evidence for the existence of a leptin-like system in birds. However, these lines of evidence are far from conclusive, and indeed they have not convinced many of the leaders in the field of avian endocrinology. In 2008, the debate surrounding avian leptin was reignited when Peter Sharp, a member of the editorial board of the journal *General and Comparative Endocrinology*, authored an opinion piece that appeared in the same journal. He provided an elegant argument that concluded that the published nucleotide sequences of chicken leptin were erroneous. The article also called for a blanket editorial policy to reject ***all*** submissions related to avian leptin or the biological effects of leptin in birds until the genetic sequence was determined (Sharp et al., 2008). The responses to this editorial were twofold. First, the commentary prompted a discussion concerning the suggestion that journals should cease publishing on topics related to avian leptin. The following adequately and succinctly sums the responses: “Such [a] suggestion is unacceptable from a scientific point of view…” (Simon et

al., 2009), and "…would have the potential of a chilling effect on science and discussion" (Scanes, 2008). However, Sharp et al. (2008) did convince the field that the chicken "leptin" gene had not been described, and shifted the discussion from the validity of the reported chicken "leptin" sequence to the very existence of an avian leptin.

The reliance on genetic and protein sequence similarity as a means to discover avian leptin is potentially responsible for both our inability to find leptin in birds, as well as the controversy that has ensued surrounding reports of its discovery. Most efforts to find an avian leptin gene have used the chicken genome to search for genetic sequences similar to mammalian leptins (e.g., Friedman-Einat et al., 1999; Pitel et al., 2010; Taouis et al., 1998). However, the utility of the chicken genome for this purpose is questionable, as 5-10% of *known* chicken genes are missing from recent assemblies (ICGSC, 2004), while the percentage and number of currently unidentified chicken genes that are also missing are necessarily unknown. Moreover, vertebrate leptins do not share a high degree of similarity at either the nucleotide or amino acid level. For example, as mentioned above, frog leptin is only 35% and 13% similar to human and fish leptin, respectively (Crespi and Denver, 2006). Even within mammals, leptin coding sequence is surprisingly divergent (see Pitel et al., 2010), highlighting the fact that amongst vertebrate cytokines, leptin has one of the lowest sequence conservation levels (Huising et al., 2006). Thus, concentrating on sequence similarity is likely not the most effective means to discover an avian leptin, although some current efforts continue to search using these genomic approaches (e.g., Pitel et al., 2010).

The cloning of the chicken leptin receptor (Horev et al., 2000) has provided an alternative strategy for discovering avian leptin. Two laboratories have successfully created stable cell lines that express chicken leptin receptor linked to a reporter gene involved in its signal transduction

pathway (Adachi et al., 2008; Hen et al., 2008). These cell lines can be used as a bioassay by determining if leptin receptor is bound by compounds in avian plasma. Any compound that binds to the chicken leptin receptor would constitute a strong candidate for avian leptin. Optimism that this approach would be successful in finding avian leptin was initially high, because the method is based on receptor binding instead of genetic or protein sequence (Yosefi et al., 2010). Initial findings did demonstrate that 1) the chicken leptin receptor was fully functional and 2) the cell lines were sensitive enough to detect leptin in mammalian plasma at concentrations that were physiologically relevant (Hen et al., 2008; Yosefi et al., 2010). However, these cell lines have failed to detect leptin from plasma of several strains of chicken and turkey (Adachi et al., 2008; Hen et al., 2008). Samples from wild-caught Adelie penguins (*Pygoscelis adeliae*) and bar-tailed godwits *(Limosa lapponica*), representing substantial natural variation in body fat composition also did not contain a compound that bound to chicken leptin receptor (Yosefi et al., 2010). Although these studies are based on a few species, they do represent a serious challenge to the view that an avian leptin exists, but has yet to be found.

These various lines of evidence leave the field with two possibilities about the existence of avian leptin: either it does not exist, or it has yet to be discovered. The fact that leptin is evolutionarily ancient (as evidenced by its presence in bony fish, amphibians and mammals) and has similar physiological effects across these groups suggests that the regulatory control of food intake is rather conserved. Yet, while birds do possess and express a functional leptin receptor, the ligand has yet to be found. Recent commentary correctly indicates that we cannot conclude that a leptin-like system exists in birds solely from the presence of a functional receptor (Pitel et al., 2010); other types of orphan receptors are now known to occur in numerous taxonomic groups and gene families (Markov et al., 2008). Whether the avian leptin receptor is an orphan

receptor because its ligand has been lost through evolutionary time or has yet to be discovered remains to be answered. Arguments for a broader comparative approach to fully evaluate data supporting the existence of avian leptin are welcome (Pitel et al., 2010). However, we also cannot conclude that avian leptin does not exist based largely on genomic data from a single species, the chicken, as these same authors have done (Pitel et al., 2010). While it is possible that the leptin gene was lost in birds, this outcome would require that the receptor present today has remained functional after ~250 million years of evolution without its ligand. Surely the issue of the presence or absence of avian leptin, described as the most important question in avian endocrinology over the last decade (Ohkubo and Adachi, 2008), deserves a broader inquiry than that employed under the current paradigm, which consists mostly of work on a single domesticated species that has been subject to intense artificial selection for traits directly related to fat metabolism.

The following chapters and appendices present evidence that supports both sides of the current leptin controversy. As such, it does not bring this issue closer to resolution. However, the research reported here underscores the utility of using wild avian species, which have evolved mechanisms to cope with large fluctuations in energy demand, in studies of the mechanisms of avian energy balance. Future study will no doubt determine whether the management of avian energy stores relies on a leptin-like system or on some entirely different pathway. Yet, only the successful completion of such research will be able to determine whether the existence of a leptin receptor and its mediation of the physiological effects of exogenous leptin are part of the control of energy balance in birds or merely remnants of an ancient system of energy regulation.

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Cerasale Dissertation: Chapter 2

Behavioral and physiological effects of photoperiod-induced migratory state and leptin on a migratory bird, *Zonotrichia albicollis*: I. Anorectic effects of leptin administration

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Abstract

The hormone leptin is involved in the regulation of energy balance in mammals, mainly by reducing food intake and body adiposity and increasing energy expenditure. During energetically demanding periods, leptin's action is often altered to facilitate fat deposition and maintain high rates of food intake. Despite the present controversy over the existence of an avian leptin, there is evidence that a leptin receptor exists in birds and that its activation influences energy intake and metabolism. However, it is unknown whether the effects of the activation of leptin receptor on energy balance are modulated during avian migration. We manipulated photoperiod to induce migratory behavior in captive white-throated sparrows (*Zonotrichia albicollis*) and injected migratory and wintering sparrows with either murine leptin or PBS for seven days. We measured food intake, changes in body composition and foraging behavior to test if leptin's effects are altered during migratory state. Leptin decreased foraging behavior, food intake and fat mass in wintering sparrows, but had no effect on foraging behavior or food intake in migratory sparrows. Migratory sparrows injected with leptin lost less fat than sparrows injected with PBS. Our results provide further evidence that a leptin receptor exists in birds and regulates energy intake. The response to leptin changes with migratory state, possibly to aid in the increase and maintenance of rates of food intake and fat deposition. Further study of leptin-like compounds and other regulators of energy balance in avian migrants will increase our understanding of the physiological mechanisms that are responsible for their ability to undergo energetically demanding journeys.

Introduction

Migratory birds are able to exercise at very high aerobic intensities for extended periods (Guglielmo, 2010; McWilliams et al., 2004); some species undergo nonstop flights of over 11,000 km lasting several days (Gill et al., 2009). Accumulated fat stores constitute most of the energy used to fuel these journeys (Jenni and Jenni-Eiermann, 1998; McWilliams et al., 2004). Consequently, some species double in mass and deposit upwards of 50% of body mass as fat in preparation for migratory periods (e.g., Battley and Piersma, 2005; Piersma and Gill Jr, 1998). Most species periodically interrupt migratory flights to replenish energy stores, and many of the behavioral decisions during stopover are affected by the amount of lipid and other energy stores (Alerstam and Hedenstrom, 1998; Carpenter et al., 1993; Cimprich and Moore, 2006; Weber et al., 1998; Ydenberg et al., 2002). The substantial changes that avian migrants undergo in preparation for, and during, migratory journeys require a change in the regulated levels of body mass and fatness. Thus, the ability to monitor and regulate energy stores appropriately clearly provides major benefits for wild birds, and yet we understand little about the physiological mechanisms involved.

The discovery of the hormone leptin was a major advance towards understanding the regulation of energy stores in vertebrates because it represents a direct link between adipose tissue and the central nervous system (Zhang et al., 1994). In mammals, leptin is synthesized primarily by adipose tissue, and its circulating concentration is proportional to total body adiposity (Ostlund et al., 1996). Leptin provides a signal indicating the magnitude of lipid storage to the brain through hypothalamic receptors (Ahima et al., 1996). Reports of direct effects of leptin and the presence of leptin receptors in peripheral tissues also suggest that some of leptin's control over energy balance occurs locally, but most regulation appears to be centrally

mediated (see Bjorbaek and Kahn, 2004). The major effect of leptin on behavior is a reduction in food intake, but it can also increase metabolic rate and fat oxidation to reduce fat mass (see Woods et al., 2000). Thus, elevated leptin levels signal an abundance of energy stores and influence behavior and physiology to increase energy expenditure and decrease energy intake.

The presence and expression of leptin homologues have been described in several non-mammalian vertebrates, including fish (Froiland et al., 2010; Gorissen et al., 2009; Johnson et al., 2000; Kurokawa et al., 2005) and amphibians (Boswell et al., 2006; Crespi and Denver, 2006), and leptin-like compounds have been detected by immunological assays in lepidosaurs (e.g., Paolucci et al., 2001). As in mammals, leptin acts to regulate lipid metabolism, reduce food intake and increase energy expenditure in frogs (Crespi and Denver, 2006), fish (Londraville and Duvall, 2002; Murashita et al., 2008) and lizards (Niewiarowski et al., 2000), although not all of these effects are always observed. For example, leptin does not reduce food intake (Londraville and Duvall, 2002) and may not be involved in long-term regulation of feeding (Huising et al., 2006) in some fish species.

For birds, leptin could be integral to the control and success of migration by regulating the availability and amount of fuels that are used to power migratory journeys, thus influencing decisions involved in stopover timing and behavior. However, despite several independent reports of the expression of leptin in the chicken (*Gallus gallus*; Ashwell et al., 1999a; Ashwell et al., 1999b; Taouis et al., 1998) and mallard (*Anas platyrynchos*; Dai et al., 2007), and the presence of leptin-like immuno-reactivity in a variety of avian species (e.g., Dridi et al., 2000b; Kochan et al., 2006; Kordonowy et al., 2010; Quillfeldt et al., 2009), the validity of these findings is highly debated (e.g., Friedman-Einat et al., 1999; Pitel et al., 2010; Scanes, 2008; Sharp et al., 2008; Simon et al., 2009). Despite multiple efforts, the reported avian leptin gene

has not been found in either the genome or EST libraries of any bird species, (see Friedman-Einat et al., 1999; Sharp et al., 2008). Thus, avian leptin has yet to be discovered. However, there is an abundance of evidence that: 1) a leptin receptor does exist in birds (e.g.,(Horev et al., 2000), 2) the receptor is functional (Adachi et al., 2008; Hen et al., 2008) and 3) its activation reduces food intake (Cassy et al., 2004; Denbow et al., 2000; Dridi et al., 2000a; Kuo et al., 2005; Lohmus and Sundstrom, 2004; Lohmus et al., 2003; Raver et al., 1998) and influences fatty acid metabolism (Dridi et al., 2005; Dridi et al., 2007).

While much of the research on leptin in mammals and birds has centered on determining its existence (in birds) and its action at the molecular level (e.g., Dridi et al., 2005; Porte et al., 2002), studies of how the functional effects of leptin are influenced by organismal ecology, especially during periods of high energy demand, have produced intriguing results. For example, during energetically demanding periods of the annual cycle, some seasonally breeding and migratory mammals increase fat storage in the face of increased circulating leptin levels; in this context, their elevated leptin does not reduce food intake or increase energy expenditure (Clarke et al., 2003; Klingenspor et al., 2000; Krol et al., 2006; Krol and Speakman, 2007; Nieminen et al., 2001; Nieminen et al., 2002; Rousseau et al., 2002; Tups et al., 2004). This change in response to leptin and the disruption of its role in the control of food intake has been interpreted as a possible adaptive mechanism that allows for the maintenance of high rates of energy intake and storage in spite of high body fatness (i.e., a means to regulate energy storage at a different set point during energy-intensive periods (Townsend et al., 2008)). For avian migrants, seasonal changes in the leptin receptor system might provide similar advantages, allowing for hyperphagia, high rates of fat deposition and high fat loads in preparation for migratory journeys. Whereas leptin administration has been shown to decrease food intake and influence fatty acid

metabolism in birds, it is unknown whether or not these effects of leptin change seasonally in migratory birds.

We induced migratory condition in white-throated sparrows (*Zonotrichia albicollis*) by photoperiod manipulation and administered murine leptin to activate the leptin receptor system and test whether its response is altered during migratory periods. We expected migratory sparrows to increase food intake, body fat content and body mass in preparation for migration. If the avian leptin receptor system is modulated to facilitate the maintenance of both high body fat and rates of energy intake in response to the energetic requirements of migration, then we expected leptin administration to be less effective in decreasing body mass, fat mass, food intake and foraging behavior in migratory than in non-migratory sparrows.

Methods

Birds

The white-throated sparrow is a small (25 g) passerine that migrates between wintering areas in central and southern North America and breeding sites throughout sub-arctic Canada (Falls and Kopachena, 2010). We captured 48 sparrows with mist nets during fall migration (06 - 17 October, 2008) on private land (42°38'20.14"N, 80°34'21.49"W) approximately 10 km north of Long Point Provincial Park, Ontario, Canada. Sparrows were immediately transported to captive facilities at the University of Western Ontario, and singly housed in cages (40 x 45 x 45 cm) at 21 ºC. We acclimated birds for 14 days to captive conditions on short-days (Light:Dark (L:D): 8:16) with *ad libitum* access to water, mixed wild bird seed (Wild Bird ChowTM, Purina Mills®, Gray Summit, MO) and millet sprays (Nutriphase®, Petsmart Inc., Phoenix, AZ, USA). Sparrows were then acclimated over 21 days to bird feed (Mazuri® Small Bird Breeder, PMI

Nutrition International, Brentwood, MO), which was ground to a powder to facilitate consumption, and were maintained on *ad libitum* access to the ground feed for the duration of the study. All animal procedures were approved by The University of Western Ontario Institutional Animal Care and Use Sub-Committee (protocol # 2005 -060). Birds were captured under a scientific collection permit from the Canadian Wildlife Service (CA 0170).

Photoperiod conditions

All birds were initially held on short days (8:16 L:D) to mimic wintering light conditions for 60 days to break photorefractoriness. We then switched the photoperiod for half of the sparrows to long days (16:8 L:D) to induce migratory restlessness (Miller and Weise, 1978) and changes in metabolic physiology (see Falls and Kopachena, 2010; McFarlan et al., 2009), while the remaining half were held on the short-day light regime. We commenced experiments after birds had experienced the long-day photoperiod for 21 days. Mini-infrared cameras (Advanced Security Products, Bellville, IL, USA) and an infrared light source (Sony Digital 8 camcorder with night vision) were used to monitor nighttime activity following the change in light cycle and throughout the experimental period. All sparrows held on the long-day light regime ("migratory sparrows," hereafter) exhibited migratory restlessness throughout the experimental period, while sparrows kept on short days ("wintering sparrows," hereafter) displayed little to no nocturnal activity (D.J. Cerasale, unpublished data).

Experimental Protocol

Following the 21 days of long-day light conditions, we randomly split migrating sparrows into three groups—leptin-injected, PBS-injected and non-injected—consisting of nine, nine and six birds, respectively. Leptin and PBS-injected sparrows were injected intra-muscularly in the *pectoralis major* twice daily for seven days with either murine leptin (Shenandoah

Biotechnology Inc. Warwick, PA, USA, purity > 95% as measured by RP-HPLC) dissolved in PBS (1 mM Phosphate Buffer Solution, Ph ~ 7.4; Bio Basic Inc. Markham, ON, Canada) or PBS only. We injected sparrows each day at lights on (9 AM EST: long-days, 10 AM EST: short-days) and six hours after lights on. Sparrows received an injection dosage (1 µg/g body mass) that reduces food intake and foraging behavior in passerines (Lohmus et al., 2003) and other avian species (Lohmus and Sundstrom, 2004). In other passerines, exogenous leptin injected at this dosage is observed at high concentrations (75 ng/ml) in the circulation 20-30 minutes post-injection (see Appendix A). The use of injections to induce transient increases in plasma leptin mimics studies that have investigated changes in leptin sensitivity in seasonally breeding mammals (Klingenspor et al., 2000; Tups et al., 2004). Non-injected birds were handled daily, but never injected during this period. We weighed birds each morning at lights on and measured their daily food intake by weighing food dishes every morning and capturing spilled food on plastic tray liners.

Each day, we monitored foraging behavior in 16 of the 24 sparrows, eight of which were injected with leptin and eight with PBS, for one hour immediately following injections with digital cameras (Panasonic 300x camcorders). Videos were subsequently transferred to digital format by ADStech DVD Xpress software (Adesso, Inc., Walnut, CA, USA). Body mass was measured throughout the experiment on a digital balance (Ohaus ± 0.1g). We measured body composition changes in all groups of sparrows using a quantitative magnetic resonance body composition analyzer (QMR) customized for small birds (Echo-MIR-B, Echo-Medical Systems, Houston, TX; Taicher et al., 2003). The QMR measures fat and wet lean masses of sparrows with accuracies of ± 11%, and ± 1%, respectively, but wet lean mass and fat mass do not sum to total body mass because QMR does not detect some body components such as feather and the

skeleton (Guglielmo et al., unpublished data). QMR fat was corrected to predicted gravimetric fat using the equation Fat = QMR fat*0.943, and QMR wet lean was corrected to predicted gravimetric wet lean using the equation Wet Lean = QMR lean*1.021 + 1.347 (Guglielmo et al., unpublished data). All birds were scanned before the change in light cycle, and prior to and following the seven-day injection regime.

After the one-hour observation period on the morning of the seventh day of the injection regime, we euthanized sparrows by isoflurane overdose and decapitation. We collected the heart, liver and pectoralis muscle and flash froze them in liquid nitrogen dry shippers (Taylor-Wharton Cx-100) for subsequent analyses of the proteins involved in fatty acid transport and metabolism (Zajac, Cerasale, et al., unpublished data). Wintering sparrows were subject to the same injection regime following the completion of injections in migratory sparrows. We weighed and measured body composition on sparrows before the commencement of euthanasia procedures.

Foraging videos

All foraging videos were observed and transcribed by a single observer (DJC) to reduce variability. The treatment group to which individual sparrows belonged was unknown at the time of observation. We excluded one wintering sparrow injected with leptin from analyses because we could not accurately observe its feeding behavior. We divided the one hour observation into 12 five-minute periods. For each sparrow at each time period, we calculated the cumulative: 1) number pecks at the food inside the food dish ("foraging rate," hereafter), 2) number of feeding trips to the food dish and 3) amount of time spent on the food dish since the injection of either leptin or PBS.

Statistical Analyses

We used general linear models to test for a difference in body mass and body composition (lean and fat mass) due to both photoperiod and leptin administration. The dependent variables were the change in fat mass (ΔFM^p), lean mass (ΔLM^p) or body mass (ΔBM^p) due to the photoperiod manipulation, and the change in fat mass (ΔFM^L), lean mass (ΔLM^L) or body mass (ΔBM^L) due to leptin injections. We calculated the photoperiod change in masses as the difference between the masses at the change in light cycle ("initial fat mass, lean mass and body mass", hereafter, Fig. 1) and the beginning of the injection regime ("injection fat mass, lean mass and body mass", hereafter, Fig. 1). We calculated the injection change in masses as the difference between the injection mass and the mass at end of the injection regime ("final fat mass, lean mass and body mass," hereafter, Fig. 1). If there was a significant correlation between ΔFM^p, ΔLM^p and ΔBM^p and their respective initial masses or between ΔFM^L, ΔLM^L and ΔBM^L and their respective injection masses, we used initial masses or injection mass as covariates in models testing for differences in ΔFM^p, ΔLM^p and ΔBM^p between photoperiods and ΔFM^L, ΔLM^L and ΔBM^L between injection treatments, respectively. We combined non-injected and PBS-injected groups ("control sparrows", hereafter) for analyses of body composition changes due to injection treatments because ΔFM^L, ΔLM^L and ΔBM^L did not differ between non-injected sparrows and PBS-injected birds within a photoperiod (all $P > 0.19$).

We modeled the response of daily food intake to the injection regime using a repeated measures design in a linear mixed model. Wintering sparrows injected with PBS differed from non-injected wintering sparrows, thus we did not combine these treatment groups for analysis of food intake. The day since the beginning of the injection regime, leptin treatment, photoperiod treatment and their interactions were considered explanatory variables explaining daily food intake. We included the day of the injection regime as a repeated effect for each individual

sparrow with an autoregressive covariance structure to account for repeated measurements on the same individual.

We used linear mixed models to test for differences between injection treatments in cumulative measure of: 1) foraging rate, 2) number of feeding trips to the food dish and 3) amount of time spent on the food dish since the injection of either leptin or PBS. The cumulative number of pecks at the food dish and number of trips to the food dish were square root transformed, and time spent on the food dish was $\log_{10}(x+1)$ transformed to satisfy model assumptions. We included the day of the injection regime, the 5 min time period post-injection, leptin treatment and their interactions as explanatory variables. Time period was considered a repeated effect for each individual bird within a day with an autoregressive covariance matrix to account for the correlation among observations in time. We also included individual bird as a random effect to account for multiple days of observation on the same individual. We modeled each 1 hr observation period (i.e., following morning or afternoon injections) separately to more easily account for time-correlated data. We used linear contrasts to test for differences between leptin treatment at a given time period and used a sequential Bonferonni procedure to adjust for multiple comparisons. The cumulative number of trips to the food dish and time spent on the food dish were modeled similarly.

All covariates were retained in models at $P < 0.10$. We considered differences between photoperiod and injection treatments to be significant at $P < 0.05$. All statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC, USA).

Results

Photoperiod and body composition – The correlation between ΔFM^p, ΔLM^p and ΔBM^p and their respective initial masses differed among body composition measurements (Table 1). We found

no statistically significant difference in ΔFM^p between migrating and wintering sparrows, although migratory sparrows tended to gain more fat ($P < 0.11$). Wintering sparrows gained significantly more lean mass than did migrating sparrows ($F = 10.42$, df = 1 and 45, $P < 0.003$). There was no difference in ΔBM^p between migratory and wintering sparrows ($P > 0.82$).

Daily food intake – Migratory sparrows had higher daily food intake than wintering sparrows ($F = 62.74$, df =1 and 42, $P < 0.0001$). Overall, food intake did not differ amongst treatment groups (non-injected, leptin-injected, PBS-injected) in migratory sparrows (all $P > 0.47$). When compared on a day-by-day basis, food intake in migratory sparrows injected with leptin never differed from PBS-injected sparrows (all $P > 0.28$). In wintering sparrows, non-injected birds had higher daily food intake that did either leptin-injected ($F = 6.00$, df = 1 and 42, $P = 0.018$) and PBS-injected sparrows ($F = 4.88$, df = 1 and 42, $P = 0.032$), but food intake did not differ overall between leptin and PBS-injected sparrows ($P > 0.79$). Wintering sparrows injected with leptin had significantly lower food intake than PBS-injected sparrows on the first day of the injection regime ($F = 4.00$, df = 1 and 210, $P = 0.049$), but food intake did not differ between these groups on any other day (all $P > 0.29$).

Injection treatment and body composition – The relationships between ΔFM^L and ΔBM^L and injection fat mass and body mass, respectively, were influenced by photoperiod (ΔFM^L: photoperiod*injection fat mass: $F = 4.06$, df = 1 and 44, $P = 0.05$; ΔBM^L: photoperiod*injection body mass: $F = 10.17$, df = 1 and 44, $P < 0.01$; also see Table 1). Thus, we analyzed changes in body composition due to injection treatment separately by photoperiod.

Wintering sparrows injected with leptin lost significantly more fat mass ($F = 7.39$, df = 1 and 21, $P = 0.013$, Fig. 2). There was no difference in ΔLM^L between control and leptin-injected

sparrows ($P > 0.56$, Fig. 2). Wintering sparrows injected with leptin tended to lose more body mass than control birds, but this effect was not statistically significant ($P < 0.15$, Fig. 2).

Migratory sparrows injected with PBS lost more fat than those injected with leptin ($F = 6.92$, df = 1 and 21, $P = 0.016$, Fig. 2). There was no difference in ΔLM^L between leptin-injected and control birds ($P > 0.75$, Fig. 2). Control birds lost significantly more body mass than leptin-injected sparrows ($F = 2.33$, df = 1 and 22, $P = 0.03$, Fig. 2).

Foraging behavior – Following the morning injection, wintering sparrows injected with leptin significantly reduced their number of pecks at the food dish overall ($F = 7.13$, df = 1 and 1027, $P < 0.01$). After sequential Bonferonni correction, this reduction was statistically significant beginning at 35 min. post-injection ($P < 0.01$, Fig. 2A), and remained significantly lower for all remaining time periods (all $P < 0.01$). Leptin had similar effects following the afternoon injections; sparrows injected with leptin significantly reduced their number of pecks at the food dish overall ($F = 7.06$, df = 1 and 1019, $P < 0.01$). After sequential Bonferonni correction, cumulative pecks were significantly lower in sparrows injected with leptin beginning at 30 min. post-injection ($P < 0.01$, Fig. 2B) and remained significantly lower for the remaining time periods (all $P < 0.01$). In migratory sparrows, leptin did not decrease foraging rate after either the morning injection ($P > 0.59$, Fig. 2C) or the afternoon injection ($P > 0.76$, Fig. 2D), and there was no difference in foraging rate at any time period for all migratory birds (all $P > 0.32$).

In wintering sparrows, the number of visits to the food dish did not differ between sparrows injected with leptin or PBS after either the morning ($P > 0.63$) or afternoon injections ($P > 0.66$). In migrating sparrows, there was no difference in number of trips to the food dish between birds injected with leptin or PBS after the morning injection ($P > 0.26$), but sparrows

injected with leptin tended to visit the food dish less often following the afternoon injection ($F = 3.65$, $df = 1$ and 922, $P = 0.056$).

Wintering sparrows injected with leptin tended to spend less time on the food dish following both morning ($F = 3.17$, $df = 1$ and 1027, $P = 0.075$) and afternoon injections ($F = 12.60$, $df = 1$ and 1019, $P < 0.001$). In migratory sparrows, there was no effect of leptin administration on the time spent on the food dish following either morning ($P > 0.17$) or afternoon ($P > 0.52$) injections.

Discussion

This study demonstrates that leptin administration influences both body composition and foraging rate in a migratory passerine, but the effects of leptin are dependent on migratory state. In accordance with our hypotheses, birds in wintering state decreased foraging rate and fat mass in response to leptin, while migrating birds did not reduce foraging rate, and actually lost less fat mass following leptin injections. The ability to perceive and control the amount of stored energy is imperative for migratory birds, and this study illustrates that avian migrants can alter their response to leptin during migratory periods. This modulation of the response to leptin is beneficial for the maintenance of both high food intake rates and body fat levels and could be involved in the regulation of seasonal changes in body mass that migratory birds regularly undergo.

Foraging behavior

Video monitoring of foraging activity following injections provided an informative measure of sparrows' response to leptin because it allowed for the measurement of foraging over the period of time when leptin was likely to be increased in the plasma (see Appendix A) and

acting to reduce energy intake (Lohmus and Sundstrom, 2004; Lohmus et al., 2003). The response of sparrows to leptin differed with migratory state; wintering sparrows that were injected with leptin reduced foraging rates while migratory sparrows injected with leptin did not. Avian migrants increase food intake substantially during migratory periods to achieve the high body fat levels necessary for migratory flights. However, leptin's action to reduce food intake would directly impede this outcome. These data show that the ability of avian migrants to modulate the response to leptin's signal of energy availability is similar to how mammals prepare for energetically intensive periods (e.g., Kronfeld-Schor et al., 2000; Townsend et al., 2008) and provides insight into how their physiology might change to obtain nutrients and regulate energy storage at varying set-points during different life history stages.

We found no difference in the number of trips to the food dish or the time spent feeding over the one-hour period following injections between sparrows injected with either leptin or PBS. However, wintering sparrows injected with leptin did tend to spend less time feeding than birds injected with PBS. This trend is similar to those in chicken and quail in which birds injected with leptin did not reduce the number of approaches to food sources, but rather decreased the amount of time spent foraging (Dridi et al., 2000a; Lohmus and Sundstrom, 2004). Thus, it appears that in birds, the reduction of food intake in response to leptin may be driven mainly by a decrease in time spent feeding, as in mammals (i.e., leptin decreases meal size; see (Schwartz, 2004).

Food Intake

Wintering sparrows that were not injected had higher daily food intake than either leptin or PBS-injected sparrows, suggesting that the twice-daily injection regime itself had some effect on feeding behavior. This effect was not seen in migratory sparrows, perhaps because injected

birds had a longer day-length to recover from injections. Following the first day of injections, leptin significantly decreased food intake in wintering sparrows, in parallel with decreased foraging rates immediately following injections (see Fig. 2A, 2B). However, there was no effect of leptin on food intake on any other day of the experiment. The lack of an effect of leptin on food intake on a daily scale may be explained by compensatory feeding following leptin injections (see (Lohmus et al., 2003). Our leptin administration regime entailed injections that increase leptin-like compound in the plasma of other migratory passerines (Cerasale et al., unpublished data) and reduce food intake (Lohmus and Sundstrom, 2004; Lohmus et al., 2003). But these increases are temporary, as exogenous leptin likely clears the plasma of birds fairly quickly (i.e., within 30 min.; see McMurtry et al., 2004), and its anorectic effects on food intake and foraging behavior disappear within 1 - 2 hours (Lohmus and Sundstrom, 2004; Lohmus et al., 2003). Thus, sparrows injected with leptin could have reduced their food intake immediately following injections, but eaten more food during the rest of the daylight hours to compensate for this reduction. Given our experimental protocol, daily food intake may not be the most representative measurement of the feeding response to leptin injections.

Body composition

Another fundamental effect of leptin on energy balance in mammals is a reduction in body adiposity. Leptin significantly reduced fat mass and body mass in wintering sparrows, but not in migratory sparrows, similar to findings from studies of seasonal mammals (e.g., Rousseau et al., 2003; Tups et al., 2004). In contrast, migratory sparrows that were injected with leptin actually lost less fat and body mass under the injection regime than did migratory control sparrows. This effect was unexpected and has not been reported in other birds or migratory mammals. Nonetheless, because many birds require massive increases in fat stores for migratory

journeys (up to 50% of body mass; Battley and Piersma, 2005), our data show that an altered response to leptin that reduces its anorectic effects on body composition could be advantageous in order to build and maintain fat stores during migratory periods.

Leptin Controversy

Despite reports of the presence of leptin-like compounds (Ashwell et al., 1999a; Ashwell et al., 1999b; Dai et al., 2007; Dridi et al., 2000b; Sato et al., 2003; Taouis et al., 1998), the existence of avian leptin remains unproven (Pitel et al., 2010; Sharp et al., 2008). Multiple efforts have failed to: 1) amplify the published leptin sequences (e.g., Amills et al., 2003; Friedman-Einat et al., 1999), 2) identify a leptin sequence in the chicken genome or EST libraries (van Hemert et al., 2003) or 3) find leptin in microarray or proteomic analyses of chicken tissues (Cogburn et al., 2007; Cogburn et al., 2004; Wang et al., 2009; Wang et al., 2007). Moreover, an assay based on the binding of chicken leptin receptor rather than sequence similarity has also failed to detect leptin in several avian species (Yosefi et al., 2010).

However, there is an abundance of functional studies that support the existence of an avian leptin receptor that, when activated, influences many behavioral and physiological processes in birds. Avian leptin receptors that can bind vertebrate leptins and activate signal transduction pathways are not controversial and have been described in multiple species (Adachi et al., 2008; Hen et al., 2008; Horev et al., 2000; Ohkubo et al., 2007; Ohkubo et al., 2000). In several species, injections of leptin *in vivo* influence numerous physiological processes, including immune function (Alonso-Alvarez et al., 2007; Figueiredo et al., 2007; Lohmus et al., 2004), reproductive condition (Paczoska-Eliasiewicz et al., 2006), reproductive effort (Lohmus and Bjorklund, 2009) and fatty acid metabolism (Dridi et al., 2005; Dridi et al., 2007). Leptin injections also decrease food intake and foraging behavior in numerous avian species (Cassy et

al., 2004; Denbow et al., 2000; Dridi et al., 2000a; Kuo et al., 2005; Lohmus and Sundstrom, 2004; Lohmus et al., 2003). However, the ubiquity of these functional effects is uncertain; leptin fails to reduce food intake in several strains of chicken (Bungo et al., 1999; Cassy et al., 2004; Kuo et al., 2005), and leptin injections also did not affect fat metabolism in peripheral tissues of wild birds (Zajac, Cerasale, et al., unpublished data). Our data add to the growing functional evidence that a leptin receptor system exists in birds by demonstrating that leptin injections reduce food intake and body fat levels in white-throated sparrows. This study also illustrates that the response to leptin in birds can change seasonally, which could help explain the observed lack of consistency of the effects of exogenous leptin on food intake in birds.

Leptin administration

The implications of the failure to find a leptin gene in birds limit our ability to observe the effects of migratory state on the leptin receptor system in sparrows. Currently there is no accepted assay to detect endogenous leptin in birds (Sharp et al., 2008), thus we could not determine how circulating leptin changes in response to our manipulations. However, there is direct evidence that injections of murine leptin induce physiological responses through avian leptin receptors. Mouse leptin binds to chicken leptin receptor and activates leptin signaling transduction pathways (Adachi et al., 2008; Hen et al., 2008). Moreover, the tertiary structure of vertebrate leptins is very conserved (Crespi and Denver, 2006), such that the chicken leptin receptor can bind to bovine, ovine, mouse and frog leptins (Adachi et al., 2008; Hen et al., 2008; Yosefi et al., 2010). Thus, it is highly likely that administration of murine leptin influences food intake and body composition through the avian leptin receptor (see (Ohkubo and Adachi, 2008).

Injections of leptin at the dosage used in this experiment (1 μg/g body mass) were effective at influencing immune function (Alonso-Alvarez et al., 2007; Lohmus et al., 2004),

foraging behavior (Lohmus and Sundstrom, 2004) and food intake (Lohmus et al., 2003) in other bird species. The measurement of the degree to which leptin injections increased plasma leptin concentrations in our sparrows, however, was hampered by our inability to measure endogenous leptin in avian plasma (see above). Our findings from other passerines indicate that 20-30 min. following injections, exogenous leptin is found in the circulation at high concentrations (75 ng/ml; see Appendix A). These concentrations are close to the upper range of plasma leptin observed in obese mammals (75-80 ng/ml; Friedman-Einat et al., 2003), suggesting that the doses sparrows received in the study could be physiologically relevant to avian migrants, particularly those that deposit substantial fat loads (e.g., Battley and Piersma, 2005). Future studies are required to determine how variation in both the dosage and method of delivery (i.e. injections vs. constant infusion) influences the responses of avian migrants to leptin.

Photoperiod

Contrary to effects seen in other studies (e.g., Landys-Ciannelli et al., 2002), our photoperiod manipulation did not result in significantly higher body mass in migratory sparrows, although fat mass did trend higher in migratory sparrows. However, in free-living sparrows, we have found that wintering birds have higher body mass than those captured during migratory periods (McFarlan et al., 2009). Also, the migratory sparrows in our study likely had higher energy expenditure because of nocturnal restlessness. Thus, we are confident that our sparrows kept on the long-day light cycle were in migratory condition. As expected, daily food intake and nocturnal activity increased substantially in migratory sparrows. Moreover, our data on muscle fatty acid metabolism (Zajac, Cerasale et al., unpublished data) are similar to data collected from free-living birds captured during migratory periods (Guglielmo et al., 2002; McFarlan et al., 2009).

Putative mechanisms for seasonal changes in the response to leptin

Seasonal changes in the relationships between plasma leptin and food intake and body fat also occur in several species of seasonal mammals, presumably in response to the energetic challenges associated with periods of their annual cycle. Dissociation of plasma leptin from its expected relationships with body fat (i.e., high body fat associated with high leptin levels) and other physiological processes (e.g., increased metabolic rate) is found in pre-hibernatory and pre-migratory bats (Kronfeld-Schor et al., 2000; Townsend et al., 2008). Extreme leptin insensitivity, termed resistance, is also related to photoperiod and seasonal conditions in a number of mammalian species (e.g., Klingenspor et al., 2000; Rousseau et al., 2002; Rousseau et al., 2003; Tups et al., 2004). In these studies, leptin levels increased in concert with higher fat loads but did not reduce food intake or increase energy expenditure and fat oxidation. Several putative mechanisms have been identified that could be involved in leptin resistance. Increases in both mRNA of SOCS3 and protein inhibitor of activated STAT, cytokine signaling inhibitors, that prevent the transduction of leptin's signal are associated with changes in leptin sensitivity in seasonal mammals (Tups et al., 2006; Tups et al., 2004), including pre-migratory bats (Townsend et al., 2008). Additionally, the relative expression of different leptin receptor isoforms is modulated during pre-migratory fattening in bats, such that those with reduced signaling capacity are overly expressed relative to isoforms with higher signaling capacity (Townsend et al., 2008). The fact that leptin supplementation did not elicit a reduction in food intake or fat mass in migrants suggests that leptin resistance is most likely the means by which migratory birds change their response to leptin's effects.

Conclusions

Our data add to the growing functional evidence for the presence of a leptin receptor system in birds, and they demonstrate that migratory birds alter their response to leptin administration, thus facilitating the deposition of fuel stores required for migratory periods. Our work also highlights the importance and utility of examining mechanisms of energy balance in wild birds in addition to domesticated species. The selective forces acting upon the regulatory mechanisms of energy balance in wild species are much different than those imposed on domesticated species that are most often studied but are the result of intense artificial selection for specific traits, such as high growth rates. However, while the existence and functionality of avian leptin receptor are well known, the controversy over its biological significance will continue until the existence of its ligand is either confirmed or refuted. Yet, if leptin is found not to exist in birds, why a functional leptin receptor in birds would subsist after millions of years of evolution without its ligand will constitute an intriguing question for future inquiry. Only continued research on the avian leptin receptor system and its role in energy balance will answer this question.

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Table 1.

Average ± standard deviation of fat mass (g), lean mass (g) and body mass (g) in white-throated sparrows (*Zonotrichia albicollis*) kept on either a short-day light cycle to simulated wintering conditions ("wintering sparrows"; see methods) or switched to a long-day light cycle to induce migratory behavior ("migratory sparrows"; see methods). Initial, injection and final time periods refer to values at the change in light cycle, and the start and end of the leptin injection regime, respectively (see methods and Fig. 1 for details).

	Initial	Injection	Final	r^P	r^L
"Wintering sparrows"					
Body Mass (g)	24.28 ± 1.86	27.28 ± 2.78	24.80 ± 1.95	-0.18	-0.77*
Fat Mass (g)	3.70 ± 1.64	4.80 ± 2.18	2.83 ± 1.41	-0.39*	-0.82*
Lean Mass (g)	17.65 ± 1.01	18.33 ± 1.05	18.05 ± 0.93	-0.29*	-0.46*
"Migratory sparrows"					
Body Mass (g)	24.68 ± 2.05	27.94 ± 3.17	27.45 ± 3.30	-0.18	-0.08
Fat Mass (g)	3.78 ± 1.56	6.04 ± 2.95	5.48 ± 2.83	-0.39*	-0.33
Lean Mass (g)	17.89 ± 0.86	17.83 ± 1.03	18.04 ± 1.00	-0.29*	-0.35

r^P = Pearson's r correlation between (Injection mass –Initial mass) and Initial mass with data from "wintering" and "migratory" sparrows pooled. (see methods for details)
r^L = Pearson's r correlation between (Final mass-Injection mass) and Injection mass; data from "wintering" and "migratory" sparrows are reported separately (see methods for details)
* = statistically significant correlation at $P < 0.05$ (see methods for details)

Figure Legends

Figure 1. Body mass (g) ± standard deviation of "migratory" white-throated sparrows (*Zonotrichia albicollis*) kept on a short-day photoperiod, then switched to a long-day photoperiod for 21 days to induce migratory behavior. Initial, injection and final mass refer to measurements at the start of the change in photoperiod and the beginning and end of the injection regime, respectively (see methods for details). The leptin injection experiment began on experiment day 0 and lasted 7 days. The experimental protocol for "wintering" sparrows was similar with the exception of the lack of a photoperiod manipulation (see methods for details).

Figure 2. Least squared means ± standard error of the change in fat mass (ΔFM), lean mass (ΔLM) and body mass (ΔBM) in white-throated sparrows (*Zonotrichia albicollis*) kept on a short-day ("wintering") or long-day photoperiod ("migrating"; see methods for details) and either injected with leptin (open bars) or considered control birds (closed bars; see methods). Significant differences between control sparrows and those injected with leptin are denoted by *** ($P < 0.05$). Only differences within photoperiods were tested (see methods).

Figure 3. Back-transformed least squared means ± standard error of cumulative foraging rate in white-throated sparrows (*Zonotrichia albicollis*) injected with either PBS (closed circles) or leptin (open circles). Sparrows were kept on either a short-day ("wintering"; Fig. 2A, 2B) or long-day ("migrating"; Fig. 2C, 2D) photoperiod and observed after injections at lights on (Fig. 2A, 2C) and six hours after lights on (Fig. 2B, 2D). The first time period in which there was a significant difference between PBS and leptin-injected sparrows is denoted by ***.

Figure 1.

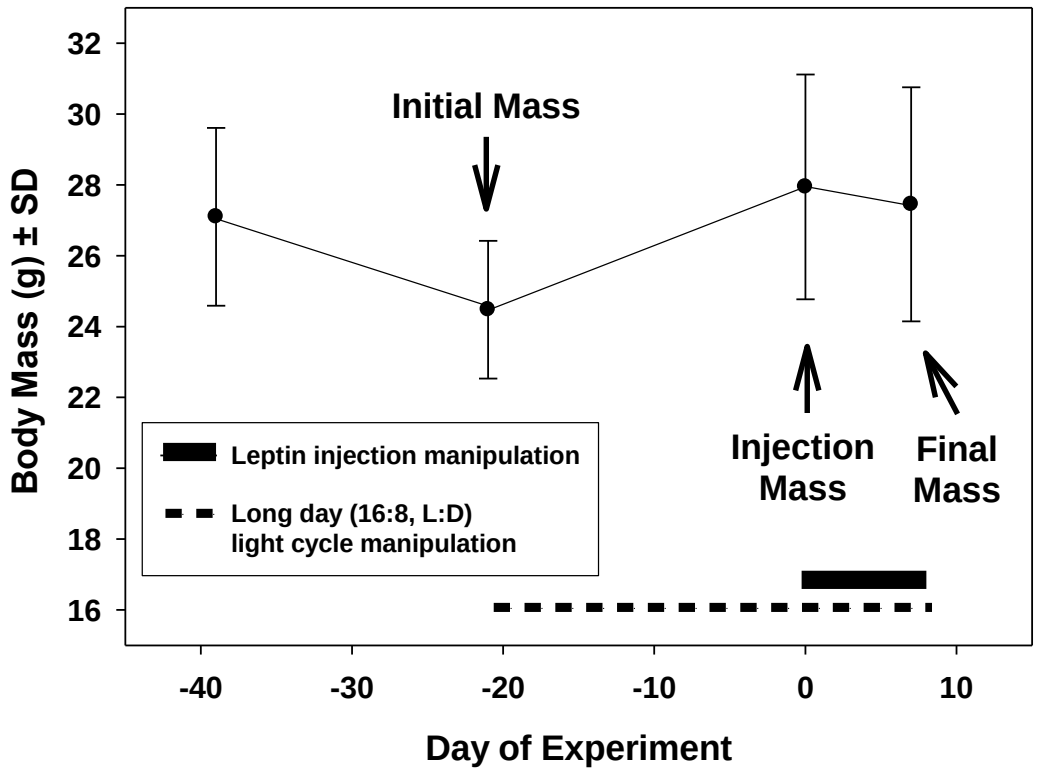

Body Mass (g) ± SD
32
30
28
26
24
22
20
18
16
Initial Mass
Injection Mass
Final Mass
Leptin injection manipulation
Long day (16:8, L:D) light cycle manipulation
-40
-30
-20
-10
0
10
Day of Experiment

Figure 2.

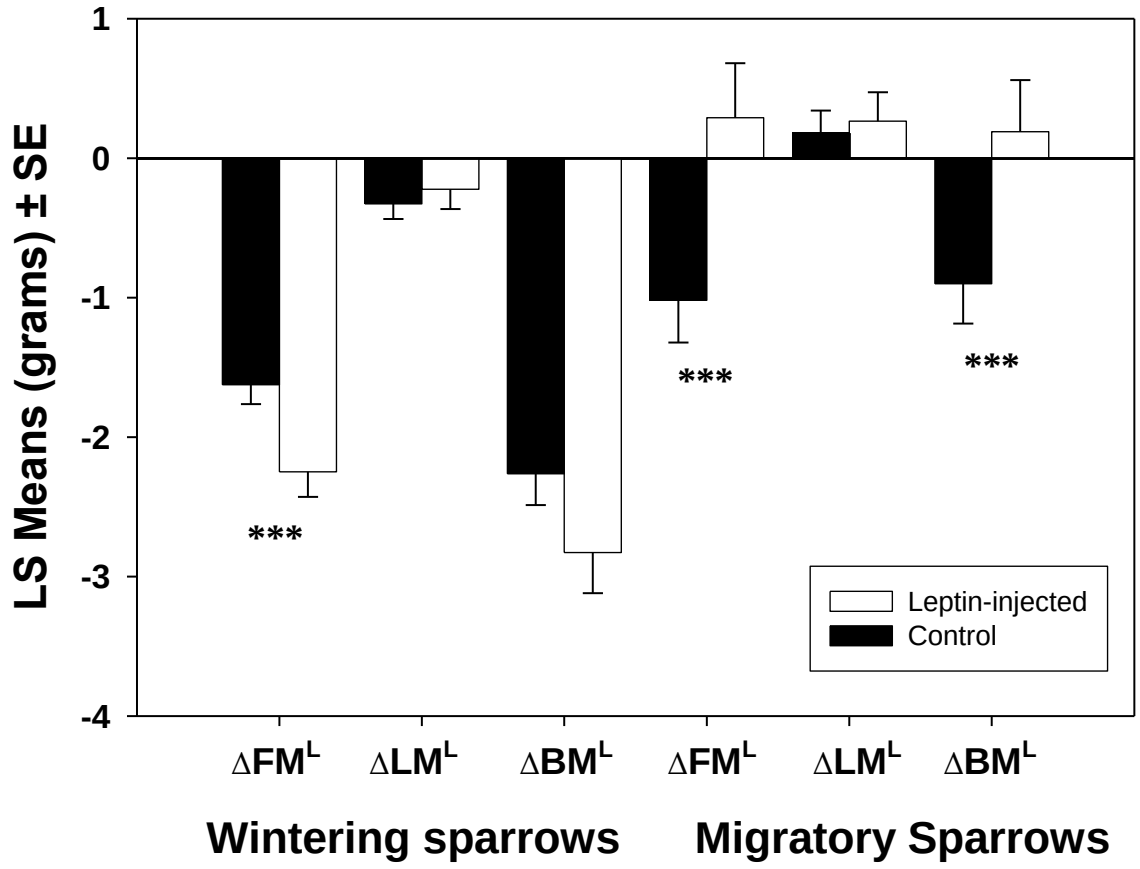
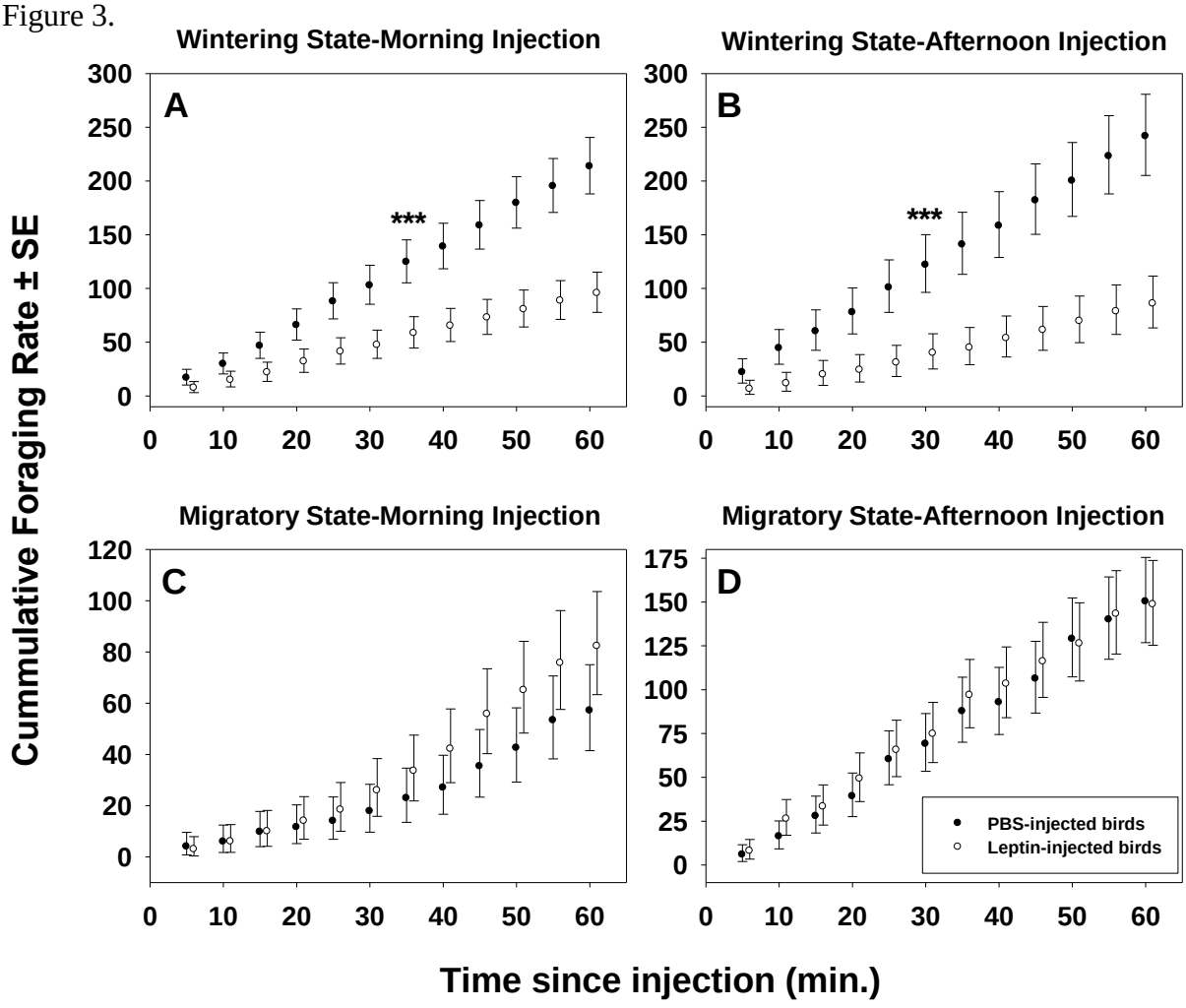

LS Means (grams) ± SE
1
0
-1
-2
-3
-4
***
***
***
Leptin-injected
Control
ΔFM^L
ΔLM^L
ΔBM^L
ΔFM^L
ΔLM^L
ΔBM^L
Wintering sparrows
Migratory Sparrows

Figure 3.


Wintering State-Morning Injection
Wintering State-Afternoon Injection
Migratory State-Morning Injection
Migratory State-Afternoon Injection
A
B
C
D
***
***
Cummulative Foraging Rate ± SE
Time since injection (min.)
PBS-injected birds
Leptin-injected birds

Expression of leptin receptor and SOCS3 in a migratory passerine, *Zonotrichia albicollis*

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Abstract

Migratory birds must cope with extreme energetic challenges during migratory periods, yet the physiological mechanisms involved in the regulation of energy balance in avian migrants are not fully known. The hormone leptin provides a signal of energy availability and serves as a direct link between adipose tissue and the brain centers that regulate feeding. While birds possess functional leptin receptors, the existence of avian leptin remains unresolved. However, consistent with findings in mammals, exogenous leptin administration reduces food intake and fat mass in birds. Our previous work suggests that, as in seasonally breeding mammals, changes in leptin receptor sensitivity could promote high rates of food intake and fat deposition during avian migration. In mammals, changes in the expression of leptin receptor isoforms and inhibitors of leptin signaling are thought to be involved in regulating seasonal modulations in leptin sensitivity. We identified partial mRNA sequences of leptin receptor and the suppressor of cytokine signaling 3 (SOCS3) in white-throated sparrows (*Zonotrichia albicollis*) and documented their expression in the hypothalamus, liver and adipose tissue. There was no evidence that expression of SOCS3, long-form leptin receptor or the relative expression of leptin receptor isoforms differed between sparrows held on short-day or long-day light regimes. This study represents the first description of leptin receptor and SOCS3 expression in migratory birds, but suggests that changes in their expression are not responsible for the changes in leptin receptor sensitivity seen in these sparrows.

Introduction

The effective control of both body weight and composition is critical to the appropriate regulation of energy balance in vertebrates. The negative effects of excessive fat storage (see Witter and Cuthill, 1993) must be balanced with the necessity of sustaining adequate energy stores to avoid starvation and ensure sufficient energy availability for other activities, such as reproduction. However, our understanding of the mechanisms that control vertebrate energy balance is incomplete, especially in free-living species. The discovery of the "obesity" gene in mice and humans caused great excitement in the field of energetic physiology (Zhang et al., 1994). This gene encodes for leptin, a peptide hormone produced by adipose tissue. Leptin constituted a particularly important discovery because it is a peripheral signal to the brain conveying the extent of somatic energy availability (see Ahima et al., 2000). Adipose tissue produces leptin in proportion to total body adiposity (Klein et al., 1996), and leptin action is mediated through leptin receptors in the hypothalamus and potentially through receptors in peripheral tissues (Ahima et al., 1996). Under normal conditions, the main consequence of leptin action is a reduction in food intake and a decrease in body fat. However, leptin also signals an abundance of energy storage that can be reallocated to other physiological processes, such as increased immune function and the maintenance of reproductive tissues. Indeed, in addition to mediating food intake, leptin influences numerous physiological states, processes and behaviors including reproductive condition (e.g., Schneider et al., 2000), fatty acid metabolism (e.g., Wang et al., 1999), immune function (Lord et al., 1998) and energy expenditure (Halaas et al., 1995).

Leptin's role as a key mediator in the control of food intake is strongly conserved; all vertebrate leptins that have been purified induce a reduction in food intake (Crespi and Denver, 2006; Halaas et al., 1995; Murashita et al., 2008; Yacobovitz et al., 2008). These anorectic

effects, however, are not always beneficial. During seasonal periods of high energy demand, many vertebrates must regulate body weight and fat around a higher set point than during other times in their annual cycle. The increase and maintenance of high levels of both food intake and fat deposition are required to achieve this change in set point. The anorectic effects of leptin would operate to limit both the means to approach (i.e., high food intake rates) and the ability to maintain this higher set point. Seasonally breeding mammals address this challenge by altering their sensitivity towards leptin. For example, leptin reduces food intake in Siberian hamsters (*Phodopus sungorus*) kept under short-day photoperiods, but has no effect on hamsters kept on long days (Rousseau et al., 2002). This phenomenon, termed leptin resistance, occurs under natural conditions in several seasonally breeding mammals, presumably as an adaptive response to seasonally high energetic requirements (e.g. Krol et al., 2006; Krol et al., 2007; Rousseau et al., 2002).

Migratory birds face similar energetic challenges to those faced by breeding mammals, and proper control of energy balance is crucial for their successful migration. Many migrants increase body weight and fat deposition in preparation for long-distance flights. Some species deposit approximately 100% of lean body weight as fat in preparation for nonstop flights of over 11,000 km (Battley and Piersma, 2005; Gill et al., 2009). Moreover, decisions concerning the timing and length of migratory flights are dependent on the amount of energy stored (Alerstam and Hedenstrom, 1998; Weber et al., 1998).

Given the presence of leptin in several major vertebrate groups (fish, amphibians and mammals), leptin constitutes a putative modulator and signal of energy status that could be central to the ability of migratory birds to handle the physiological challenges that migration entails. Indeed, functional leptin receptors are found in birds, and are expressed in the

hypothalamus, as well as numerous peripheral tissues (Adachi et al., 2008; Hen et al., 2008; Horev et al., 2000; Liu et al., 2007; Ohkubo et al., 2007; Ohkubo et al., 2000). Yet, leptin remains undiscovered in the chicken genome, and the likelihood of its existence is a topic of considerable debate (Pitel et al., 2010; Scanes, 2008; Sharp et al., 2008; Simon et al., 2009). Nevertheless, there is substantial evidence that a leptin receptor system exists in birds and is involved in the regulation of energy balance. As in mammals, exogenous leptin administration reduces food intake, lowers fat mass and body weight, and influences numerous other physiological processes and behaviors in diverse species of birds (Alonso-Alvarez et al., 2007; Cassy et al., 2004; Denbow et al., 2000; Dridi et al., 2005; Dridi et al., 2000; Dridi et al., 2007; Kuo et al., 2005; Lohmus and Bjorklund, 2009; Lohmus et al., 2004; Lohmus et al., 2011; Lohmus and Sundstrom, 2004; Lohmus et al., 2003; Paczoska-Eliasiewicz et al., 2003; Paczoska-Eliasiewicz et al., 2006; Song et al., 2009). Moreover, we have shown that white-throated sparrows (*Zonotrichia albicollis*) modulate their response to leptin during migration; leptin reduces food intake and body composition in sparrows under winter photoperiods, but has no effect when sparrows are in migratory state (Chapter 2).

Changes in leptin sensitivity in mammals have received substantial attention, as complete lack of leptin action, termed leptin resistance, is associated with human obesity and other metabolic diseases (Myers et al., 2008). In seasonally breeding mammals, the current model of adaptive changes in leptin sensitivity is focused upon a reduction in signaling capacity downstream of the leptin receptor, mainly through the suppressor of cytokine signaling 3 (SOCS3; Tups, 2009). SOCS3 expression is induced by leptin signaling, and acts to inhibit the signal transducer and activator of transcription 3 (STAT3), a primary signaling pathway for leptin action (Bjorbaek et al., 1999). An alternate mechanism to achieve seasonal changes in

leptin sensitivity is a modulation in the amount of leptin receptors or in the relative expression of different receptor isoforms. Because short isoforms of the leptin receptor lack full signaling capacity, a relative increase in this isoform could reduce leptin sensitivity, as is suggested from findings in migratory bats (Townsend et al., 2008).

While at least two isoforms, including the long-form receptor, are found in poultry (Horev et al., 2000; Liu et al., 2007; Ohkubo et al., 2000), the presence and relative expression of leptin receptor isoforms has not been reported in migratory birds. Neither the existence nor expression of SOCS3 has been reported in any species other than the chicken. Here, we report partial sequences and the expression of leptin receptor and SOCS3 in tissues of a migratory passerine, the white-crowned sparrow. We also test the hypothesis that the modulation in leptin responsiveness seen in migratory sparrows (Chapter 2) could be mediated by changes in the expression of leptin receptor isoforms and SOCS3.

Methods

Study system.

The white-throated sparrow is a small (25 g) passerine that migrates between breeding grounds throughout sub-arctic Canada to wintering areas in central and southern North America (see Falls and Kapochena, 2010). We captured 11 sparrows during fall migration (18 October-21 October) with mist nets on private land approximately 10 km north of Long Point Provincial Park, Ontario, Canada. Birds were immediately transported to captive facilities at the University of Western Ontario where they were initially held in outdoor aviaries. We provided sparrows with mixed wild bird seed (Wild Bird Chow TM, Purina Mills®, Gray Summit, MO) and water *ad libitum* throughout the experiment. All animal procedures were approved by The University of Western Ontario Institutional Animal Care and Use Sub-Committee (protocol #2005-060).

Sparrows were captured under a scientific collection permit from the Canadian Wildlife Service (CA 0170).

Experimental conditions

After being held outdoors for three weeks at an approximate photoperiod of (11:13 Light:Dark (L:D)), sparrows were brought indoors and singly housed in wire cages (40 x 45 x 45 cm) at 20 C° and on a light cycle regime of 9:15 (L:D) for six weeks. Six sparrows were then switched to a photoperiod mimicking wintering conditions (8:16 L:D) and five to a long-day photoperiod (16:8 L:D) to induce migratory restlessness. Nocturnal migratory behavior was monitored by video cameras equipped with an infrared light source. Forty days after the switch in photoperiod, we euthanized all birds via a brief (< 1 min.) isoflurane overdose followed by decapitation. Carcasses were immediately dissected and hypothalami, livers and adipose tissues flash-frozen in liquid nitrogen for further analyses.

We measured body mass (± 0.1g) and body composition of sparrows immediately prior to euthanasia to assess the effect of photoperiod changes. Body composition was assessed using a quantitative magnetic resonance body composition analyzer (QMR) that was customized for small birds (Echo-MIR-B, Echo-Medical Systems, Houston TX; Taicher et al., 2003). The QMR measures fat and wet lean masses of sparrows with accuracies of ± 11%, and ± 1%, respectively, but wet lean mass and fat mass do not sum to total body mass because QMR does not detect some body components such as feather and the skeleton (Guglielmo et al., unpublished data). QMR fat was corrected to predicted gravimetric fat using the equation Fat = QMR fat*0.943, and QMR wet lean was corrected to predicted gravimetric wet lean using the equation Wet Lean = QMR lean*1.021 + 1.347 (Guglielmo et al., unpublished data).

RNA extraction and reverse transcription

Portions of sparrow tissues, hypothalamus (40-60mg), adipose (60-90 mg) and liver (15-25 mg) were homogenized using a hand-held electronic Kontes pellet pestle (VWR). RNA was subsequently isolated using a RNeasy lipid tissue mini kit (Qiagen) according to the manufacture's directions. RNA was eluted in nuclease-free water and was subject to two DNAase treatments. First, we used an on-column RNase-free DNAase kit (Qiagen) during the RNA extraction protocol. After final elution, RNA was also treated with Turbo DNAase (Ambion) according to manufacturer's instructions. Following DNAase treatment, RNA was quantified on a nanospectrophotometer. All samples had Å260/Å280 values greater than 1.8, indicating high quality RNA. We reverse-transcribed 1 µg of RNA per sample using a Superscript III first-strand synthesis for qRT-PCR kit (Invitrogen) following the manufacturer's instructions. The resulting cDNA was stored at -20 C° until analysis.

Quantitative PCR

We aligned chicken (GenBank Accession No. AB033383), mouse (NM_146146) and predicted zebra finch (XM_002195330) leptin receptor sequences to develop degenerate primers to amplify a 683 bp portion of sparrow leptin receptor from sparrow liver cDNA. This amplicon included the following exons as described for chicken leptin receptor (Horev et al., 2000): part of exon 15, all of exons 16, 17, 18 and 19, and part of exon 20 (table 1). We aligned mouse SOCS3 (NM_007707), human SOCS3 (NM_003955) and chicken SOCS3 (AF424806) to develop primers to amplify a 236bp amplicon of sparrow SOCS3 from sparrow liver cDNA. The resulting PCR products were sequenced via Sanger (3730XL) DNA sequencing on an Applied Biosystems Automated 3730 DNA Analyzer (Cornell University Life Science Core Laboratories Center).

Quantitative PCR (qPCR) primers and custom Taqman-MGB probes for leptin receptor and SOCS3 were developed from these sequences using Primer Express 3 (Applied Biosystems) software (Table 1). Primers and probes for β-actin (which served as the housekeeping gene for analyses) were developed from known sequences from white-throated sparrow (EU556706). Two different primer/probe sets were used to amplify sparrow leptin receptor. One pair amplified an amplicon that included sequence from exon 20 of the chicken leptin receptor (Fig. 1). Exon 20 is only present in the long-form leptin receptor of birds (Liu et al., 2007). The second primer/probe set amplified a region that included the transmembrane domain (Fig. 1) but no portions of exon 20. In this way we could separately measure the expression of all isoforms of leptin receptor that are membrane-bound and the long-form leptin receptor. We tested the specificity of qPCR primers by amplifying pooled cDNA from sparrows and sequencing the resulting products.

We performed qPCR reaction on an Applied Biosystems 7300 Real Time system in 25 µl reactions using 12.5 µl Taqman gene expression master mix (Applied Biosystems), 900 nM of each primer, 250 nM probe and 100 ng cDNA template diluted in nuclease free water. Cycling conditions were 50 C° for two minutes, 95 C° for ten minutes and 40 cycles at 58 C° for one minute. We used serial dilutions of PCR products, amplified using primers for PCR described above (see Table 1), to create a standard curve and calculate amplification efficiencies for each gene. Pooled cDNA was used as a calibrator and its expression measured for each gene on every sample plate. Inter-assay coefficients of variance were < 5% for each gene. Our assays were not sensitive enough to detect leptin receptor in all samples, particularly adipose tissue, and these were excluded from analyses. Thus, sample sizes across tissues differed slightly (see Fig. 2). Amplification efficiencies were similar among genes: 1.91 for long-form leptin receptor, 1.92 for

all membrane-bound leptin receptor isoforms, 1.93 for SOCS3 and 1.87 for β-actin. To allow for the comparison of expression between long-form and all membrane-bound leptin receptor isoforms, we constrained the level at which critical threshold (CT) was calculated to be equal for these genes.

Data analysis

β-actin was considered an appropriate housekeeping gene for comparing relative expression of each gene because our prior findings indicate that its expression does not differ between sexes, ages and across seasons (Zajac, 2010). In this study, β-actin also did not differ between photoperiods or across tissues ($P > 0.21$). We calculated the expression ratio (see McFarlan et al., 2009) of each gene using the following equation:

Relative Expression = $(E_{\text{target}})^{\Delta Ct_T} / (E_{\beta\text{-actin}})^{\Delta Ct_H}$

where E is amplification efficiency and ΔCt is the difference between the calibrator and the target gene (ΔCt_T) or the house-keeping gene, beta-actin (ΔCt_H).

The differences in body mass and body composition between birds held on long and short days were tested using two-sample t-tests, assuming unequal variance. We used linear mixed models to test if the expression of each gene differed between photoperiods and among tissues. Photoperiod and tissue type were included as fixed effects, and individual included as a random effect to account for different tissues originating from the same bird. Differences were considered statistically significant at $P < 0.05$ and contrasts among tissues were adjusted by Tukey's post-hoc tests to account for multiple comparisons. We used a paired t-test to determine if CTs differed between long-form and all membrane-bound leptin receptor isoforms within an individual. We also performed statistical power analyses post-hoc for comparisons of each gene

between photoperiod treatments. All analyses were performed using SAS 9.2 (SAS Institute, Cary, NC).

Results

Body mass and body composition.

Body mass in migratory sparrows was higher than in wintering sparrows, although only this effect was only marginally significant ($t = 2.20$, $df = 9$, $P = 0.055$, Table 2). Similarly, fat mass was higher in migratory than in wintering sparrows, but was not quite significantly different ($t = 2.17$, $df = 9$, $P = 0.058$, Table 2). Lean mass did not differ between wintering and migratory sparrows ($P > 0.61$).

Description of leptin receptor and SOC3

Aligned sequences of chicken and sparrow leptin receptor indicated several conserved regions of nucleotide sequence (Figure 1). These regions included the transmembrane and signaling box motif 1. The partial sparrow leptin receptor sequence shares 77 - 78% identity with both chicken and turkey (*Meleagris gallopavo*) leptin receptor and 66 - 67% identity with both human and mouse leptin receptor. The partial nucleotide sequence of sparrow SOCS3 shares 94% identity with chicken and 88 - 90% identity with human and mouse SOCS3.

Relative gene expression

All three genes of interest were expressed in the tissues examined, however, their pattern of expression among these tissues varied. The expression of the long form of leptin receptor differed among tissues ($F = 52.18$, $df = 2$ and 12, $P < 0.0001$, Fig. 2A). The hypothalamus had significantly higher expression of long-form leptin receptor than did either adipose tissue ($t = 3.60$, $df = 12$, $P < 0.0001$) or liver ($t = 4.04$, $df = 12$, $P < 0.0001$), but expression did not differ between adipose and liver tissue ($P > 0.63$). The expression of all membrane-bound leptin

receptor isoforms differed similarly among tissues ($F = 26.16$, df = 2 and 11, $P < 0.0001$, Fig. 2B), with significantly higher expression in the hypothalamus than in either adipose tissue ($t = 3.66$, df = 11, $P < 0.001$) or liver ($t = 3.76$, df = 11, $P < 0.001$), and no detectable differences in expression between the liver and adipose tissue ($P > 0.98$). The expression of SOCS3 also differed among tissues ($F = 12.09$, df = 2 and 17, $P < 0.001$, Fig. 2C); however, in contrast to leptin receptor, SOCS3 expression was significantly lower in the hypothalamus than in either adipose ($t = 3.83$, df = 17, $P < 0.004$) or liver ($t = 4.56$, df = 17, $P < 0.001$), and there was no difference in expression between adipose tissue and liver ($P > 0.69$). Within tissue types, none of the three genes differed in expression levels between photoperiod treatments (all $P > 0.20$). The mean difference between CT values of long-form and all membrane-bound leptin receptor isoforms within a sample was small (0.20 cycles) and not statistically significant (long-form mean CT = 33.25; all membrane-bound mean CT = 33.45, $t = -1.45$, df = 25, $P > 0.08$), indicating little if any expression of any isoform other than the long form.

Our power to detect a difference in gene expression between photoperiod treatments was low for all genes. Statistical power in all tissues for both long form and all membrane-bound leptin receptor isoforms was less than 0.08, and less than 0.18 for SOCS3.

Discussion

We demonstrate both the existence and expression of leptin receptor in a migratory bird, the white-throated sparrow. This represents the first description of leptin receptor in a non-domesticated avian species. Several regions of the sparrow leptin receptor show considerable conservation with homologous sections of chicken and mammalian leptin receptors. These areas correspond to the transmembrane domain and signaling box 1, which is involved in STAT signaling. Leptin receptor is widely expressed but concentrated mostly in the hypothalamus, the

site of feeding regulation (see Ahima et al., 1996). We also identify coding sequence for and describe tissue expression of sparrow SOCS3. Neither the expression of leptin receptor nor SOCS3 differed between photoperiods, suggesting that transcriptional changes in neither leptin receptor nor SOCS3 are responsible for changes in the response to leptin seen in sparrows in photoperiod-induced migratory state (Chapter 2).

Leptin resistance is characterized by a decrease in sensitivity towards leptin, which results in a lack of anorectic effects of leptin on food intake, body weight and fat mass (Myers et al., 2008). Research on the neuroendocrine mechanisms involved in leptin resistance has largely been undertaken from the perspective of pathological diagnosis (Tups, 2009). However, leptin resistance is also an adaptive response that allows mammals to cope with seasonal changes in energy demand. The classic example of this type of leptin resistance is in the Siberian hamster, as it undergoes dramatic seasonal changes in body weight and food intake that are modulated, in part, by leptin sensitivity (Klingenspor et al., 2000; Rousseau et al., 2002). Our prior work suggests that, despite the controversy over the existence of avian leptin (e.g., Pitel et al., 2010), a similar pattern of altered physiological and behavioral responses to leptin is seen in migratory birds. Birds in non-migratory state respond to leptin by reducing foraging and body fat, while those in migratory condition do not reduce food intake or fat mass (Chapter 2).

Leptin Receptors

Several mechanisms have been proposed to explain leptin resistance in mammals. Changes in leptin receptor expression or abundance in the hypothalamus could modulate the ability of leptin to reach its target neurons, thus reducing sensitivity towards leptin. Evidence of a saturatable leptin transport system lends support to this possibility (Adam and Findlay, 2010; Adam et al., 2006; Banks et al., 1999). However, a reduction in leptin sensitivity is unlikely to

be a result of a decrease in the total expression of leptin receptors in the hypothalamus, as total leptin receptor expression actually increases during periods of leptin insensitivity (Mercer et al., 2000a; Mercer et al., 2000b; Townsend et al., 2008). Changes in the relative expression of different leptin receptor isoforms, however, may reduce leptin sensitivity. In migratory bats, total leptin receptor expression increases in the hypothalami of bats fattening for migration, but short isoforms are over-expressed relative to the long-form of leptin receptor (Townsend et al., 2008). Because the short-form has reduced signaling capacity, these data can be interpreted as an adaptive response to dampen the effects of leptin on energy acquisition during periods when high rates of food intake and fat deposition are required.

Our data do not support this mechanism as a means of changing leptin responsiveness in avian migrants. We found no evidence that relative changes in either leptin receptor isoforms or long-form receptor alone were associated with photoperiod. In fact, there was little indication of any expression of membrane-bound isoforms of leptin other than the long isoform. This is consistent with findings from Liu et al. (2007) who identified short-form receptor in chicken, but found very low expression in most tissues, and little to no expression in the hypothalamus, liver and adipose. Thus, it does not appear that changes in the transcription of leptin receptor isoforms are responsible for the modulated responses to leptin seen in migratory sparrows (Chapter 2).

SOCS3

Evidence from seasonally breeding mammals that exhibit leptin resistance strongly suggests that mechanisms downstream of the leptin receptor are responsible for changes in leptin sensitivity (Tups, 2009; Tups et al., 2006; Tups et al., 2004). SOCS3 has emerged as the central actor in regulating changes in leptin sensitivity in several seasonally breeding mammals and perhaps in pregnant rats (see Tups, 2009). Under normal conditions, leptin binding to its

receptor activates STAT3 signaling which subsequently increases SOCS3 expression. SOCS3 then operates as a negative feedback signal for STAT signaling (Bjorbaek et al., 1998). However, SOCS3 expression is also up-regulated under long-day photoperiods, independent of leptin binding. This increase causes blunted physiological responses to leptin administration and immediately precedes an increase in body weight and fat stores (see Krol and Speakman, 2007; Tups 2009; Tups et al., 2006).

Our study identified part of the mRNA sequence for sparrow SOCS3 and demonstrates its expression in several tissues, including the hypothalamus. However, our data are not consistent with an increase in SOCS3 as the primary mechanism responsible for changes in the response to leptin in avian migrants; SOCS3 expression did not differ between sparrows kept on short- and long-day photoperiods. It is possible, however, that most of the changes in SOCS3 expression occur only transiently after a change in photoperiod. For example, in field voles (*Microtus agrestis*) switched to long days, SOCS3 temporarily increased 2-4 weeks following the shift in photoperiod, but then decreased to levels similar to voles kept on short days (Krol et al., 2006; Krol and Speakman, 2007; Krol et al., 2007). This transient increase is sufficient to develop leptin insensitivity and is involved in the regulation of body weight at a higher set point. In birds, if changes in SOCS3 are seen only temporarily after a change in photoperiod, our experimental design, which measured expression 40 days after birds were switched to long-days, could have missed detecting such an increase. Future studies in migratory birds should incorporate a time course of measurements of SOCS3 and leptin receptors to address this possibility.

Limitations

Whereas our results suggest that leptin receptor changes and SOCS3 expression are not associated with photoperiod in migratory birds, these conclusions are limited by several caveats. First, we could not distinguish changes in gene expression amongst different regions of the hypothalamus. Most research on changes in hypothalamic leptin signaling in seasonal mammals has focused on signaling in the arcuate nucleus (ARC) (e.g., (Krol et al., 2006; Krol and Speakman, 2007; Krol et al., 2007; Rousseau et al., 2002; Tups et al., 2006). While long-form leptin receptor and SOCS3 are heavily expressed in the ARC and other areas that control feeding and satiety (e.g., Bjorbaek et al., 1998; Mitchell et al., 2009), leptin receptor is expressed in other areas of the hypothalamus (e.g., Leinninger et al., 2009). Thus, it is possible that either leptin receptor or SOCS3 expression did differ between photoperiods in the ARC and that we could not detect this because a lack of changes in other areas of the hypothalamus masked any effect in the ARC.

Also, we only measured gene expression and did not quantify the amount or activity of leptin receptor and SOCS3 proteins. While changes in gene expression might be involved in the modulation of leptin's signal, it is likely that multiple steps share control over the leptin-signaling pathway. Thus, post-transcriptional regulation of leptin receptors or SOCS3 could be involved in the seasonal changes in leptin sensitivity seen in avian migrants. In fact, evidence from leptin signaling in the mouse brain illustrates this point well; changes in leptin receptor number do occur before any changes in gene expression (Mitchell et al., 2009). We also did not measure all compounds involved in the control of leptin signaling that could account for seasonal changes in the response to leptin in birds. For example, expression of PIAS-3, a constitutively expressed inhibitor of cytokine signaling, increases in the hypothalamus of little brown bats (*Myotis lucifugus*) during pre-migratory fattening (Townsend et al., 2008) and could be involved

in the dissociation of leptin with food intake and body fat in this species (Kronfeld-Schor et al., 2000). Future efforts to measure changes at multiple steps within the leptin signaling pathway, including the abundance and activity of leptin receptors and SOCS3 proteins, will prove valuable in understanding seasonal changes in leptin sensitivity in migratory birds.

Finally, our ability to detect differences in expression of leptin receptor isoforms and SOCS3 between photoperiod treatments was particularly low due to small sample sizes and large variances. Thus, further study is needed to conclusively determine whether or not changes in the expression of both SOCS3 and leptin receptor isoforms are associated with migratory state in sparrows.

Conclusions

This study demonstrates that leptin receptor is expressed in several tissues of an avian migrant. This not only represents the first report of its existence outside of a domesticated bird species, but its expression in the hypothalamus is also consistent with the existence of an avian leptin-like system that is involved in the regulation of energy balance. These data also provide evidence that the responses to exogenous leptin injections observed in our previous experiments with white-throated sparrows are mediated through a leptin receptor. Although an avian leptin has yet to be discovered, further investigation of both leptin receptor and its signaling pathway in migratory birds could shed light on the mechanisms involved in avian energy balance. If avian leptin does not exist, as is suggested by some findings (see Pitel et al., 2010), why a functional leptin receptor in birds would subsist after millions of years of evolution without its ligand will remain an intriguing question for future inquiry. Continued research on avian leptin receptor and its role in the control of food intake and energy balance seems most likely to answer this question. Migratory birds provide an excellent model for these research efforts, as they express

leptin receptor, exhibit seasonal changes in response to leptin and must cope with extreme energetic challenges during migratory periods.

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Table 1. Primers (5’ to 3’) used in standard PCR and sequence primers (5’ to 3’) and taqman-MGB™ probes (5’ to 3’) used in quantitative PCR of leptin receptor, SOCS3 and β-actin in white-throated sparrows (*Zonotrichia albicollis*).

Gene	**Forward Sequence Primer**	**Reverse Sequence Primer**	**Taqman MGB probe**	**Amplicon Size (bp)**
for PCR:				
Leptin Receptor	CCCTGAGTGCTTACCTGGTGAAC	TCCARRAGAAGWGGCTCAMATGAC		683 bp
SOCS3	ATGGTCACCCACAGCAAGTTYCC	GCGYAGGTTCTTGGTSCCCGACT		236 bp
for qPCR:				
Leptin Receptor (long form)	AAGAAGCTGCTCTGGGAGGAT	TGGCAAAGAGATGCTCCAGAGT	CCAACCCCAAGAAC	100 bp
Leptin Receptor (membrane-bound)	CCACGTGGTTCTGTCCATTG	AACATCCTCCCAGAGCAGCTT	TTTCTCCACCTCAGTTCT	100 bp
SOCS3	TGCGCCTCAAGACGTTCAG	CCTGTCACCGTGCTCCAATA	TCCAAGAGCGAGTACCAG	100 bp
β-actin	TCCCCTGAACCCCAAAGC	GGTAACACCATCACCAGAGTCCAT	AACAGAGAGAAGATGACACAGA	157 bp

Table 2. Mean ± standard deviation of body mass, fat mass and lean mass of white-throated sparrows kept on either a long day (16:8 Light:Dark (L:D)) or short day (8:16 L:D) photoperiod. The P-value corresponds to a test of a difference between photoperiods using a t-test assuming unequal variances (see methods).

	Long day (16:8 L:D)	**Short day (8:16 L:D)**	**P-value**
Body mass (g) ± SD	29.61 ± 1.75	26.46 ± 2.76	0.055
Fat mass (g) ± SD	6.53 ± 1.99	4.21 ± 1.56	0.058
Lean mass (g) ± SD	18.97 ± 1.51	18.56 ± 1.13	0.615

Figure Legends

Figure 1. Comparison of partial mRNA sequence of white-throated sparrow (WTSP; *Zonotrichia albicollis*) and chicken (*Gallus gallus*; Genbank Accession No. AB033383) leptin receptor. Exons are identified according to Horev et al. (2000). Transmembrane (bold) and cytokine signaling box 1 (bold and underlined) regions are identified according to Richards and Poch (2003).

Figure 2. The relative mRNA expression + standard error of A) long-form leptin receptor, B) all leptin receptor isoforms with a transmembrane domain and C) SOCS3 in the hypothalamus, liver and adipose tissue of white-throated sparrows (*Zonotrichia albicollis*). β-actin was used as a housekeeping gene for the calculation of relative expression for all target genes (see 2.7, 2.8).Sparrows were held on a short-day photoperiod and either switched to a long-day photoperiod to induce migratory restlessness (closed bars), or kept on short-days (open bars). Expression is shown relative to liver expression in birds held on short-days. Within each gene, bars with the same letter were not significantly different ($P < 0.05$) following Tukey's adjustment for multiple comparisons. Numbers above and in bars denote sample sizes.

Figure 1.

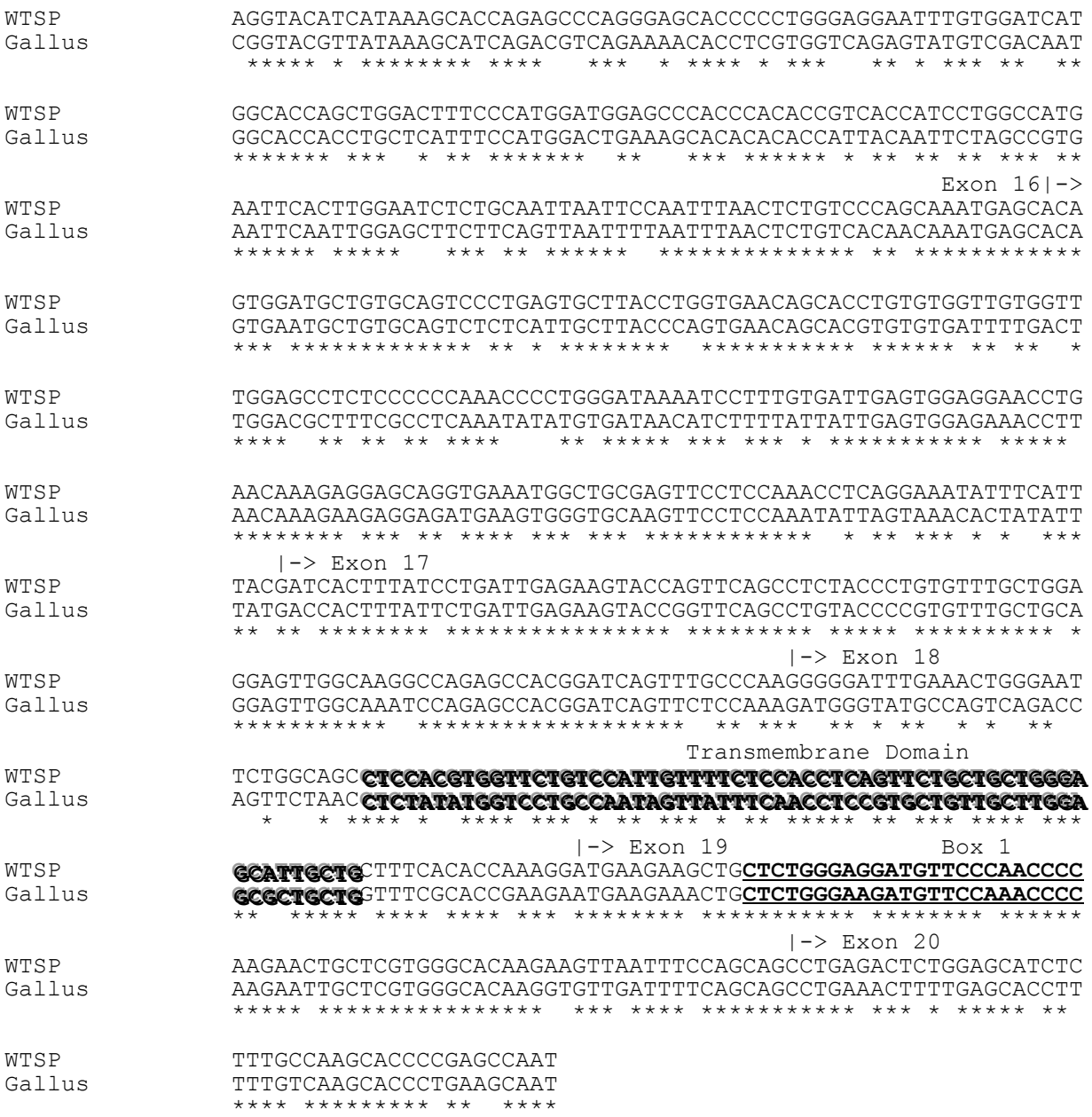
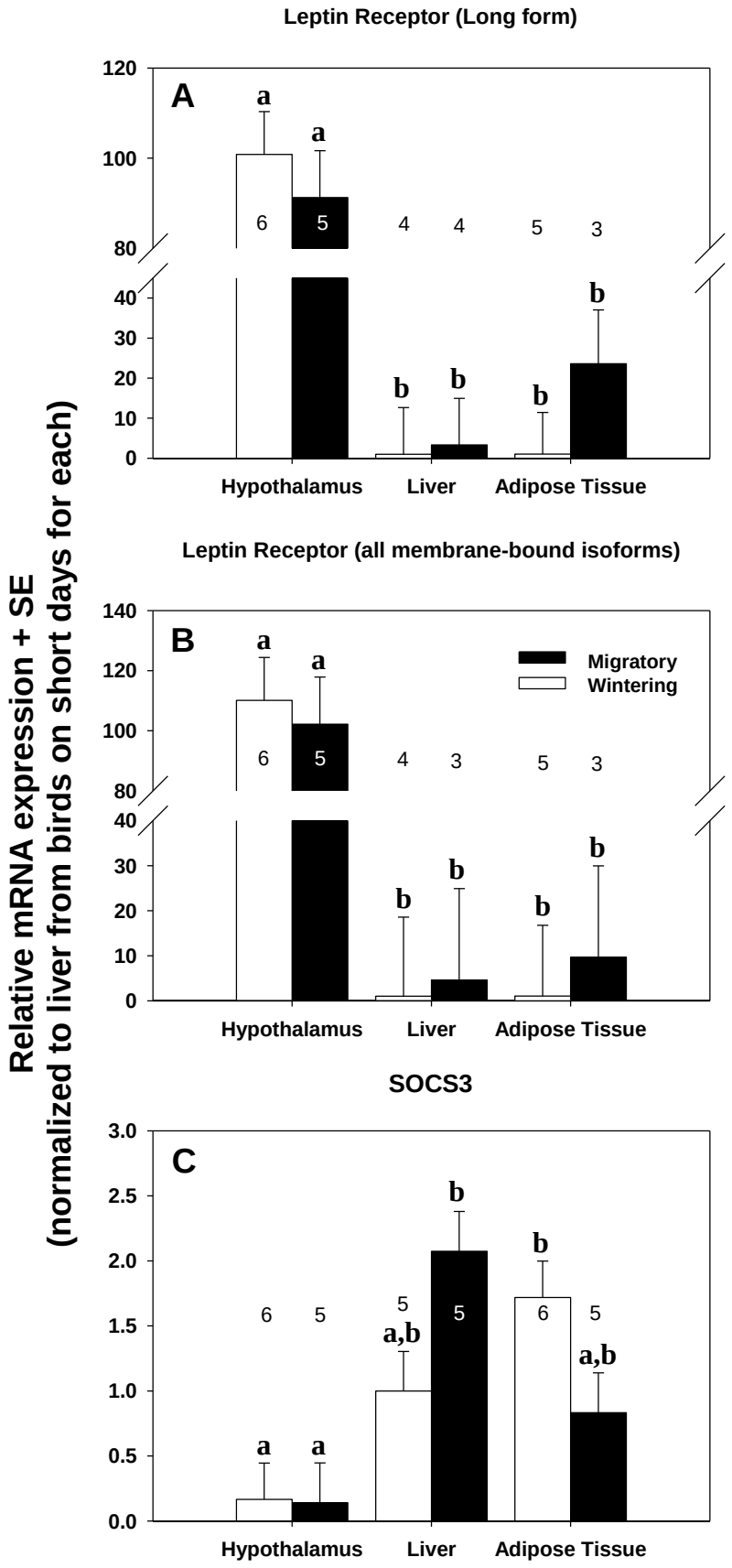

WTSP AGGTACATCATAAAGCACCAGAGCCCAGGGAGCACCCCCTGGGAGGAATTTGTGGATCAT
Gallus CGGTACGTTATAAAGCATCAGACGTCAGAAAACACCTCGTGGTCAGAGTATGTCGACAAT
WTSP GGCACCAGCTGGACTTTCCCATGGATGGAGCCCACCCACACCGTCACCATCCTGGCCATG
Gallus GGCACCACCTGCTCATTTCCATGGACTGAAAGCACACACACCATTACAATTCTAGCCGTG
Exon 16|->
WTSP AATTCACTTGGAATCTCTGCAATTAATTCCAATTTAACTCTGTCCCAGCAAATGAGCACA
Gallus AATTCAATTGGAGCTTCTTCAGTTAATTTTAATTTAACTCTGTCACAACAAATGAGCACA
WTSP GTGGATGCTGTGCAGTCCCTGAGTGCTTACCTGGTGAACAGCACCTGTGTGGTTGTGGTT
Gallus GTGAATGCTGTGCAGTCTCTCATTGCTTACCCAGTGAACAGCACGTGTGTGATTTTGACT
WTSP TGGAGCCTCTCCCCCCAAACCCCTGGGATAAAATCCTTTGTGATTGAGTGGAGGAACCTG
Gallus TGGACGCTTTCGCCTCAAATATATGTGATAACATCTTTTATTATTGAGTGGAGAAACCTT
|-> Exon 17
WTSP TACGATCACTTTATCCTGATTGAGAAGTACCAGTTCAGCCTCTACCCTGTGTTTGCTGGA
Gallus TATGACCACTTTATTCTGATTGAGAAGTACCGGTTCAGCCTGTACCCCGTGTTTGCTGCA
|-> Exon 18
WTSP GGAGTTGGCAAGGCCAGAGCCACGGATCAGTTTGCCCAAGGGGGATTTGAAACTGGGAAT
Gallus GGAGTTGGCAAATCCAGAGCCACGGATCAGTTCTCCAAAGATGGGTATGCCAGTCAGACC
Transmembrane Domain
WTSP TCTGGCAGCCTCCACGTGGTTCTGTCCATTGTTTTCTCCACCTCAGTTCTGCTGCTGGGA
Gallus AGTTCTAACCTCTATATGGTCCTGCCAATAGTTATTTCAACCTCCGTGCTGTTGCTTGGA
|-> Exon 19
Box 1
WTSP GCATTGCTGCTTTCACACCAAAGGATGAAGAAGCTGCTCTGGGAGGATGTTCCCAACCCC
Gallus GCGCTGCTGGTTTCGCACCGAAGAATGAAGAAACTGCTCTGGGAAGATGTTCCAAACCCC
|-> Exon 20
WTSP AAGAACTGCTCGTGGGCACAAGAAGTTAATTTCCAGCAGCCTGAGACTCTGGAGCATCTC
Gallus AAGAATTGCTCGTGGGCACAAGGTGTTGATTTTCAGCAGCCTGAAACTTTTGAGCACCTT
WTSP TTTGCCAAGCACCCCGAGCCAAT
Gallus TTTGTCAAGCACCCTGAAGCAAT

Figure 2.


Leptin Receptor (Long form)
A
Leptin Receptor (all membrane-bound isoforms)
B
SOCS3
C
Migratory
Wintering
Relative mRNA expression + SE
(normalized to liver from birds on short days for each)
Hypothalamus
Liver
Adipose Tissue

Cerasale Dissertation: Chapter 4

Phylogeny of the Tachycineta genus of New World swallows: Insights from complete mitochondrial genomes

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Abstract

The *Tachycineta* genus of swallows is comprised of nine species that range from Alaska to southern Chile. We sequenced the entire mitochondrial genome of each member of *Tachycineta* and generated a completely resolved phylogenetic hypothesis for the corresponding mitochondrial gene tree. Our analyses confirm the presence of two sub-clades within *Tachycineta* that are associated with geography: a North American/Caribbean clade and a South/Central American clade. We found considerable variation among regions of the mitochondrial genome in both substitution rates and the level of information that each region supplied for phylogenetic reconstruction. We found no evidence of positive directional selection within mitochondrial coding regions, but we identified numerous sites under negative stabilizing selection. This finding suggests that, despite differences in life history traits and distributions, mitochondrial genes in *Tachycineta* are predominantly under purifying selection for conserved function.

Introduction

Tachycineta swallows comprise nine species that are distributed throughout the Western Hemisphere, ranging from Alaska to southern Chile (see Whittingham et al., 2002). Although *Tachycineta* species are ecologically similar, they exhibit substantial interspecific variation in life history traits, including variation in clutch size (Dyrcz, 1984; Massoni et al., 2007), seasonal migration (e.g., Liljesthrom et al., 2009) and the level of extra-pair paternity (Ferretti and Winkler, 2009; Moore et al., 1999). Moreover, of the members of *Tachycineta*, the Tree Swallow (*Tachycineta bicolor*) is notable for being one of the most comprehensively studied birds in North America, providing copious life history data for comparative analyses (Jones, 2003). Efforts to collect comparable data from other *Tachycineta* species have recently increased (e.g., Bulit and Massoni, 2011), and the genus is now considered a model avian group for comparative research (Jones, 2003; also see golondrinas.cornell.edu).

Despite the promise that *Tachycineta* swallows offer for comparative studies of physiological, ecological and life history traits, the evolutionary relationships amongst its members remain only partly resolved. The first molecular phylogeny of *Tachycineta*, based on mitochondrial sequences, identified two sister clades delineated largely by geography, with one clade including the North American and Caribbean species (*T. bicolor*, *T. thalassina*, *T. euchrysea* and *T. cyaneoviridis*), and the other clade the South and Central American species (*T. stolzmanni*, *T. albilinea*, *T. albiventer*, *T. leucorrhoa* and *T. meyeni*; Whittingham et al., 2002). Subsequent analyses that included a nuclear marker supported these same clades, although species sampling was not complete (Sheldon et al., 2005). However, support for some of the relationships within each of

these clades was poor, particularly for the relative placement of *T. bicolor* and *T. thalassina* within the North American clade (Whittingham et al., 2002). The relationships of these two species are important because they represent the members of the genus with the most extensive geographic ranges, and the only species to breed in North America; data from both *T. bicolor* and *T. thalassina* are therefore integral to comparative research with *Tachycineta*, including the association of life history traits with latitude (Dunn et al., 2000) and the response of birds to climate change (see Winkler et al., 2002).

Here, we provide the complete mitochondrial genomes of all members of the *Tachycineta* genus and use them to generate phylogenetic reconstructions of the mitochondrial gene tree. We describe which portions of the genome provide most phylogenetic information, document variation in substitution rates among regions of the genome, and test for regions of positive selection within mitochondrial coding sequences. Beyond resolving the phylogenetic relationships within *Tachycineta*, the availability of entire mitochondrial genomes can provide information on the evolution and timescale of changes in mitochondrial gene order (e.g., Gibb et al., 2007; Singh et al., 2008), and on how molecular divergence metrics vary across regions of the mitochondrial genome. They also foster comprehensive tests for correlations of both ecological and physiological traits with genetic changes in mitochondria. Recent efforts have drawn attention to the potential fitness effects of non-neutral variation in mitochondrial genes (Dowling et al., 2008). For example, studies in both mammals and birds indicate that variation in mtDNA is associated with both altitude and latitude (Cheviron and Brumfield, 2009; Ehinger et al., 2002; Ruiz-Pesini et al., 2004) and is correlated with thermogenic capacity

(Fontanillas et al., 2005). The *Tachycineta* genus represents a collection of ecologically similar species that occupy quite different thermal environments and that vary in several traits that have potential metabolic side effects, including migration, clutch size and breeding latitude (e.g., Dunn et al., 2000; Massoni et al., 2007). Analyses of the mitochondrial genomes of *Tachycineta* therefore provide opportunities to explore their potential functional variation.

Methods

Samples and Laboratory techniques

We obtained blood and tissues samples from all members of *Tachycineta* and from *Progne chalybea*, which we employed as the outgroup for phylogenetic reconstruction (see Table 1). Genomic DNA was extracted using a DNAeasy blood and tissue kit (Qiagen). Primers described by Kessing et al. (1989), Hunt et al. (2001), Eberhard and Bermingham (2004) and Lovette (2004) were used to amplify portions of ND2, ND3, COI, ATPase 6 and cytochrome b oxidase (see Table 2). Sequences generated from these products were aligned with the *Smithornis sharpei* (GenBank accession no. NC_000879), *Vidua chalybeata* (NC_000880) and *Corvus frugilegus* (NC_002069) complete mitochondrial sequences in Sequencer 4.5 (Gene Codes) to generate specific and degenerate primers (Table 2) for the amplification of overlapping PCR products 500-3000 bp long for the remaining regions of the genome. Primers were constructed using Primer Select 5.07 (DNASTAR). PCR amplification reactions had a 10 µl total volume using 1 µl Jumpstart Taq polymerase (2.5 units/µl; Sigma), 1 µl genomic DNA (concentration 10-50 ng/µl), 0.25mM of each nucleotide, 1 µl 10x PCR buffer w/out $MgCl_2$ (Sigma), variable $MgCl_2$ (range 2-3 mM), and 0.25mM of each

primer. PCR amplification was performed in a PTC-220 Dyad Thermal Cycler (MJ Research) with an initial denaturing incubation (95 C°, 4.5 min), followed by 30-35 cycles of 95 C° (1 min.), variable primer annealing temperature (see Table 2; 45 sec), and extension at 72 C° (1 min./1000 bp of amplified product), terminated by a final extension incubation (72 C°, 4.5 min).

PCR products were visualized on agarose gels, purified, and sequenced using both amplification and additional internal sequencing primers following the general protocols of Lovette and Rubenstein (2007) and Dor et al. (2010). Sequences were read using an Applied Biosystems Automated 3730 DNA Analyzer. Overlapping contiguous sequences were aligned in Sequencher 4.7; >99% of each genome was confirmed by sequencing both complementary DNA strands, often with 3x or greater replication.

Data analysis

We used both maximum likelihood (ML) implemented in RAxML v7.0.3 (Stamatakis, 2006) and Bayesian methods implemented in MrBayes v3.12 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) to infer phylogenies. We used DT-ModSel (Minin et al., 2003) to select the best evolutionary model for each analysis performed in MrBayes. Each MrBayes analysis consisted of two independent runs of four chains for 10 million generations (sampling every 1000 generations). We assessed convergence using AWTY (Nylander et al., 2008) and discarded the first 100 trees (100,000 generations) as burn-in. For analyses using RAxML, we applied the General Time Reversible + GAMMA + I (GTR + G + I) evolution model for all analyses and each partition, and resampled using 1000 bootstraps. We analyzed complete genome sequences using two partitioning schemes: 1) no partitions and 2) six partitions, coding

regions by codon position, tRNA, rRNA and control regions. The effect of partitioning was assessed using likelihood ratio tests (LRT). We excluded a 10 bp overlapping region that consisted of a frame-shift between ATPase 6 and ATPase 8, and combined the ATPases as one locus in all analyses. We did not specify an outgroup for analyses in either MrBayes or RAxML, but used *P. chalybea* as the outgroup for visualization of tree topology. *P. chalybea* is a member of *Progne*, a sister genus to *Tachycineta* (Sheldon et al., 2005), and thus is a suitable taxon to root the *Tachycineta* mitochondrial tree.

We assumed that the tree topology derived from analyses of the entire mitochondrial sequences was the true mitochondrial gene tree. To determine the relative support provided by each gene or region to this topology, we ran both ML and Bayesian analyses on each gene or region separately. To obtain the estimated model parameters for each mitochondrial region, we performed analyses of each specific region separately as described above, using the GTR + G + I model of evolution. We calculated both uncorrected genetic distances and genetic distances corrected by GTR + G + I for the complete genome and for each mitochondrial gene separately, using PAUP v.4.0 (Swofford, 2003). Because most analyses of genetic differences for calibration of molecular clocks use cytb sequences (Lovette, 2004; Weir and Schluter, 2008), we then compared differences among genes by subtracting the genetic differences calculated from cytb sequences from the estimated genetic differences for each locus using independent contrasts of four species pairs: *T. euchrysea* and *T. cyaneoviridis*, *T. albilinea* and *T. albiventer*, *T. meyeni* and *T. leucorrhoa*, *T. stolzmanni* and *T. bicolor*.

To determine if any areas of the coding regions of the mitochondrial genome were under positive selection in *Tachycineta*, we implemented tests of dN/dS by codon in the

HYPHY software package (Pond et al., 2005). We analyzed the entire coding region by estimating synonymous and non-synonymous substitution rates independently by codon using a two-rate fixed effects likelihood model. Nucleotide substitution rates were estimated with a M694 x GTR + G + I model. The significance of signatures of selection on each codon was analyzed by likelihood ratio tests using the neutral model as a null hypothesis (i.e., synonymous and non-synonymous rates are equal). The significance value from these tests takes into account error of estimation of synonymous and non-synonymous rates (Pond et al., 2009). Codons were considered to be under positive or purifying selection at $P < 0.05$.

Results

Sequence characteristics and partitions

Total mitochondrial genome lengths differ among *Tachycineta* species (Table 1), due largely to differences in the length of their control regions (CR). All genomes have a gene arrangement indicating that ND6 is transcribed from the light strand. The mtDNA of all species (including *P. chalybea*) also have a duplicate CR with both copies having near identical sequences in each individual. The region including ND6, tPro and tGlu occurs in between these two sets of CRs. This gene order is similar to the order found in other passerines (Singh et al., 2008) and in *Amazona* parrots (Eberhard et al., 2001), and is termed the duplicate CR gene order by Gibb et al. (2007). In all species, a variable microsatellite consisting of a series of complex cytosine-adenine repeats is present at the 3' end of the CR copy directly adjacent to the 12S ribosomal coding region. Preliminary analyses indicate that the length of this microsatellite is variable both within species and potentially within individuals (Lovette et al., unpublished data). The estimated model

parameters varied among loci (Table 3). As expected for mtDNA loci, transitional substitution rates are all substantially greater than transversional rates, and second codon positions and the 12S gene have a high proportion of invariant sites. All sequences obtained during this study are available in GenBank (see Table 1).

Phylogenetic reconstructions

The gene tree topology and node support calculated from the entire mitochondrial sequences is shown in Figure 1. Model likelihood of the analysis of complete mtDNA sequences was greater when partitioned (ML = -51967.07) than with no partitions (ML = -55989.39), and the overall fits of the trees under partitioned and non-partitioned sets differed significantly (LRT: $P < 0.0001$). However, the topology for the and between partitioned and un-partitioned analyses was identical and nodal support was similar, thus we report results from the un-partitioned analysis only.

Separate analyses of each gene or region, treated as a subset of the mitochondrial gene, indicate variability in the amount of support each region provides to the topology of the overall mitochondrial gene tree (Table 4). Analysis of the coding sequence alone yielded the same topology, although with lower support for the sister relationship between *T. euchrysea* and *T. cyaneoviridis*. Most regions provided support for the relationships within the South American clade, but the level of support for relationships in the North American clade was poor for most individual loci. In fact, only analyses of CO1 produced the same topology as the overall mitochondrial gene tree, although the sister relationship between *T. euchrysea* and *T. cyaneoviridis* was not well supported (Table 4).

Genetic distances

Uncorrected genetic distances among species within the South American clade were smaller (range 2%-6%) than distances within the North American clade (range 6%-7%). Distances were larger between the two different clades (range 7%-8%) and between all *Tachycineta* species and *P. chalybea* (range 10%-11%) (Table 5). Genetic distances that were corrected with the GTR + G + I evolution model yielded similar patterns (Table 5): distances among species within the South American clade (range 3%-8%) were smaller than distances between species within the North American clade (range 9%-11%). Distances between members of different clades (range 11%-13%) and between all *Tachycineta* species and *P. chalybea* (range 17%-20%) were larger.

The difference in substitution rates between cytb and each locus varied among mitochondrial regions (Fig. 2). As expected, 12S and 16S ribosomal coding regions had relatively lower substitution rates. All of the NADH complexes, except ND3, had higher genetic differences than cytb, while all cytochrome oxidase complexes had lower genetic differences than cytb (Fig. 2).

Positive selection in the genome

We found no evidence of positive selection in any area of the mitochondrial coding region. No codons showed evidence of positive selection, but 321 codons showed evidence of purifying selection. These sites were dispersed throughout the coding sequences of the mitochondrial genome, suggesting that it is subject to purifying selection throughout. This outcome is consistent with the known high level of amino acid conservation across the mitochondrial genome in many taxa.

Discussion

Phylogenetic relationships

We provide here a phylogenetic hypothesis for a fully resolved mitochondrial gene tree for *Tachycineta* swallows based on analyses of the entire mtDNA genome from each species. While this phylogenetic reconstruction does not constitute a species tree, it provides useful information concerning the evolutionary history of this genus. Prior analyses have provided substantial evidence for two clades within *Tachycineta* that are largely geographically distinct (Whittingham et al., 2002). Our analyses offer additional support that these are monophyletic groupings as reflected in their mitochondrial gene tree. The Northern American clade consists of two widespread continental species (*T. thalassina* and *T. bicolor*) and two range-restricted Caribbean species (*T. euchrysea* and *T. cyaneoviridis*). In the mtDNA tree, *T. thalassina*, a species of western North America, is sister to the Caribbean species *T. euchrysea* and *T. cyaneoviridis.* Prior phylogenetic reconstructions have instead placed *T. thalassina* as sister only to *T. euchrysea*, albeit with minimal support (Whittingham et al., 2002). The topology derived by Sheldon et al. (2005) was similar to the topology based on complete genomes presented here, but had weak support for the relationships among *T. thalassina*, *T. euchrysea* and *T. cyaneoviridis*. The relationships among species within the Southern Hemisphere clade are completely resolved in our mtDNA genome tree, and are consistent with prior phylogenetic reconstructions (Sheldon et al., 2005; Whittingham et al., 2002).

Relative support provided by mitochondrial regions

The sequencing of entire mitochondrial genomes allows for an analysis of the relative amount of phylogenetic information and support that each region of the genome provides towards the topology of the full-genome mitochondrial tree. This information can aid in the selection of the mitochondrial regions that are best suited for future studies

of evolutionary relationships or population structure in this genus. Previous analyses have used ND2 and cytb sequences (Sheldon et al., 2005) or sequences from cytb, ND2, COII, ATPase 8 and two tRNA regions (Whittingham et al., 2002) to infer evolutionary relationships within *Tachycineta*. Our results suggest that even analysis of the complete protein-coding sequence does not provide full support for the relationships within the North American clade (Table 5), and the inclusion of non-coding regions helped to further resolve the mitochondrial gene tree (Fig. 1). Of the mitochondrial genes analyzed individually, only COI produced the same topology as the full mitochondrial gene tree, although support for the relationship between *T. euchysea* and *T. cyaneoviridis* was weak (Table 5).

Mitochondrial gene order

While the combination of mitochondrial genes is conserved in vertebrates, the order of these genes within the mitochondrial genome is variable, particularly within birds (Gibb et al., 2007). Within passerines, two gene orders have been reported, both of which include a duplicated CR (Singh et al., 2008). The remnant CR 2 gene order has both a functional and a degenerate CR copy, while the duplicate CR gene order has two nearly identical copies of the CR that both appear to be functional (e.g., Abbott et al., 2005; Eberhard et al., 2001). The duplicate CR gene order also occurs in other avian orders where analyses indicate that the CR copies within an individual are more similar to each other than to CRs of other individuals or species (Sammler et al., 2011; Morris-Pocock et al., 2010). These findings suggest that the CR copies within individuals are evolving in concert.

All *Tachycineta* species and *P. chalybea* have duplicate CR copies that are nearly identical (i.e., duplicated CR gene order) adding to the growing literature suggesting that the duplicated CR gene order occurs in many passerines (Singh et al., 2008). Given that all *Tachycineta* as well as *P. chalybea* possess this gene order, the duplication of the CR likely arose before the divergence of *Tachycineta*. This suggests that the CR copies within an individual are maintained by concerted evolution, but future studies using CR sequences of multiple individuals are needed to provide further evidence for this possibility.

Positive selection

Mitochondria are integral to many aspects of cellular and tissue function in multicellular organisms, including programmed cell death (e.g., Blackstone and Green, 1999), endothermy (e.g., Duong et al., 2006) and metabolic control (see Race et al., 1999). Several studies have correlated differences in mitochondrial sequences with organismal ecology and thermoregulatory performance (Cheviron and Brumfield, 2009; Ehinger et al., 2002; Fontanillas et al., 2005), mainly within mammals and birds. Avian mitochondria are of particular interest as they must meet the substantial energetic requirements necessary for flight (McWilliams et al., 2004) while producing low levels of damaging free radicals (Barja et al., 1994). As a result, birds have higher metabolic rates than similarly-sized mammals, yet they live longer (Speakman, 2005). The *Tachycineta* genus includes species with different movement patterns, life history traits and annual cycles (see golondrinas.cornell.edu), representing an opportunity to test for associations between areas of positive selection and species traits, such as migration and breeding latitude.

Despite the differences among *Tachycineta* species that might lead to directional selection on their mitochondrial function, we found no evidence for positive selection in their mitochondrial coding sequences. Our analyses did identify numerous sites under negative selection, indicating that mitochondria in *Tachycineta* are under selection for conserved function. This result is not surprising, as most of the mtDNAs of animals are known to be under purifying selection, although periods of positive selection within mitochondria can drive mitochondrial evolution (Bazin et al, 2006). However, our dataset is not the most suitable for tests of positive selection: we sequenced mitochondrial genomes from a single individual of each species, which preempted intraspecific comparisons that might have detected correlations between mitochondrial haplotypes and either mitochondrial function or ecological traits. Intraspecific comparisons in both mammals and birds have provided much of the data that correlate mitochondrial genetic changes to organismal ecology, metabolic performance and distribution (e.g., Ehinger et al., 2002 Cheviron and Brumfield, 2009). Future research efforts with *Tachycineta* should focus on comparing mitochondrial genetic variation among conspecific populations that differ in ecological traits or are subject to different environmental selective pressures (e.g., thermoregulatory costs).

Conclusions

This study reports the nucleotide sequence for the complete mitochondrial genomes of *Tachycineta* swallows and *P. chalybea*. These sequences provide a fully resolved phylogenetic hypothesis of the mitochondrial gene tree for the *Tachycineta* genus. The complete mitochondrial tree and documentation of the variation in

substitution rates that occurs across the genome will prove valuable in future comparative research in this widely studied group.

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Table 1. Specimens and their mtDNA length (bp) included in this study. Abbreviations: T=tissue, B=blood, LSUMNS= Louisiana State Museum of Natural Science, CUMV= Cornell University Museum of Vertebrates.

Species	Common Name	Tissue Type	Museum	Sample #	Collection Locality	mtDNA length (bp)
Tachycineta cyaneoviridis	Bahama Swallow	T	LSUMNS	B-48916	Bahamas	18,154
Tachycineta albiventer	White-winged Swallow	T	LSUMNS	B-12853	Bolivia	17,916
Tachycineta stolzmanni	Tumbes Swallow	T	LSUMNS	B-25372	Ecuador	17,932
Progne chalybea	Gray-breasted Martin	T	CUMV	50672	Uruguay	18,030
Tachycineta albilinea	Mangrove Swallow	T	CUMV	50162	Belize	17,929
Tachycineta bicolor	Tree Swallow	T	CUMV	50502	USA	17,947
Tachycineta thalassina	Violet-green Swallow	T	CUMV	50184	USA	18,118
Tachycineta meyeni[a]	Chilean Swallow	T			Argentina	18,012
Tachycienta leucorrhoa[a]	White-rumped Swallow	T			Argentina	17,965
Tachycineta euchrysea[a]	Golden Swallow	B			Dominican Republic	17,930

[a] Samples collected from individuals in the field and are not part of museum collections (see acknowledgements).

Table 2. List of PCR primers used for amplification of *Tachycineta sp.* and *P. chalybea* mitochondrial genomes. Unless specified, numbers in primer names refer to location on *Vidua chalybeata* mitochondrial genome (AF090341). Abbreviations: H=heavy strand, L=light strand, CR=control region copy.

Primer Name	Sequence 5' to 3'	Gene	Annealing Temp.
Tachy1383L	CCCAAAGACAAAAGACTTAGTCCTAACC	12S	61 C°
Tachy1544L	CACCCCCACGGGTATTCAGC	12S	62 C°
Tachy1875H	TAAGCGTTTGTGCTCGTAGTTCTC	12S, CR2	64 C°
Tachy2768H	CAGGCAACCAGCTATCACCCAG	16S	61 C°
Tachy2953H	CGCACTCTTTGTTGATGGCTGC	16S	62 C°
Tachy3873L	CGGAGCAATCCAGGTCGGTT	16S	62 C°
Tachy2384L	GGTGATAGCTGGTTGCCTGTG	16S	63 C°
Tachy4016H	CCGAGCTCTGCCACGCTA	16S	63 C°
GQL[2]	GACAATGCTCAGAAATCTGCGG	ATP6ase	62 C°
HMH[2]	CATGGGCTGGGGTCRACTATGTG	ATP6ase	62 C°
Tachy9817L	CGACTAACAGCAAACCTCACAGC	ATPase 6	61 C°
Tachy9147H	TTAGTTGTGGCATGTCATTAAGGAGG	ATPase 8	61 C°
CO1a[1]	AGTATAAGCGTCTGGGTAGTC	CO1	54 C°
CO1f[1]	CCTGCAGGAGGAGGAGAYCC	CO1	54 C°
IL6591L[a, 5]	GCCGATAAGAAGAGGAATTG	CO1	54 C°
IL7389H[a, 5]	GAGATGATTCCAAATCCTGG	CO1	54 C°
IL7925L[a, 5]	KGTAAACYTAACCTTCTTCCCC	CO1	54 C°

IL8760H[a, 5]	GACTCGGATAGTRGAGTTTATGGG	CO1	54 C°
Tachy6545L	CARCAGGCTTCTGCCTAAGACC	CO1	57 C°
Tachy7076H	GAGTCAGAAGCTTATGTTGTTTATTCG	CO1	57 C°
Tachy6698H	ATAATCATCGGTTGATGAATGTCA	CO1	59 C°
Tachy7855L	CCACTACGTACTATCCATAGGAGCAG	CO1	57 C°
Tachy8297H	AAGTGGTTTATATGCGGTTGGC	CO1	57 C°
Tachy8679L	CGAAGTCAATGAACCAGACATGACC	CO2	61 C°
Tachy10968H	TCAAAGCCGCATTCGTATGG	CO3	61 C°
H16065HBC[a, 3]	GGAGTCTTCAGTCTCTGGTTTACAAGAC	Cytb	54 C°
IL15311HBC[a, 3]	CTACCATGAGGACAAATATC	Cytb	54 C°
IL14828L[a, 3]	CCACCCTCCACTCAGGCCTAATCAA	Cytb	54 C°
H15710[a, 3]	GTAGGCGAATAGGAAGTATC	Cytb	54 C°
Tachy15058H	GTTTCATCAGGTTGAGATGTTTGA	Cytb	56 C°
Metb[4]	CGAAAATGATGGTTTAACCCCTTCC	ND2	54 C°
TRPc[4]	CGGACTTTAGCAGAAACTAAGAG	ND2	54 C°
Tachy5450H	TGGTTGCGGCTTCAATGGC	ND2	62 C°
Tachy5851L	CCTAGCATTCTCCTCCATCTCC	ND2	59 C°
L10755[a, 3]	GACTTCCAATCTTTAAAATCGG	ND3	50 C°
H11151[a, 3]	GATTTGTTGAGCCGAAATCAAC	ND3	50 C°
Tachy11071L	CTCCTATTCGACCTAGAAATCGC	ND3	57 C°
Tachy12209H	GTGTGCTTTAGGGAGTCATAGGTG	ND4	57 C°
Tachy12012L	CCGAACGACTAAACGCAGGCAT	ND4	64 C°
Tachy13420L	CGCAACATGGTACATAGCCTCAGAC	ND5	64 C°
Tachy14322H	CAGGTGGGAGGTGCTTAGGC	ND5	64 C°

Tachy13903L	CTCCAGCACAATAGTAGTAGCCG	ND5	56 C°
Tachy14062H	CTGGAGGTGGAGAAGGCAATG	ND5	64 C°
Tachy13497L	CAACATGGTACATAGCCTCAGACC	ND5	57 C°
Tachy14278H	GAGGCAGGAGGTAGTTGTTGGTA	ND5	57 C°
TachymiddleND6L	AACARAGCAGCMCCACTAGAATCCA	ND6	54 C°
TachymiddleND6H	CGGTTGCRTCTAATCCTTCTCC	ND6	54 C°
TachyND6L	CCGCCAACGAAACAGAATAAAC	ND6, CR2	54 C°
TachyND6H	AATCAGTTTCGTTGGCGGC	ND6, CR1	54 C°
Tachy16046L	TCATCATCATCGGCCAACTAGC	ND6, CR1	57 C°

[a] Numbers in reference to primer position in the chicken mitochondrial genome (X52392)
[1] From Kessing et al. (1989)
[2] From Hunt et al. (2001)
[3] From Lovette and Rubenstien (2007)
[4] From Eberhard and Bermingham (2004)
[5] From Lovette (2004)

Table 3. Comparison among mitochondrial regions of the model of evolution used in Bayesian analyses and estimated model parameters using the GTR + I + G model of evolution.

Locus	Model of evolution	Relative substitution rate (GTR + I + G)						Base Frequencies				ά	P(I)
		A-C	A-G	A-T	C-G	C-T	G-T	A	C	G	T		
Entire Mitochondrion	TrN+I+G	2.50	21.00	1.50	0.50	22.50	1.00	0.30	0.32	0.15	0.23	1.17	0.63
All coding	GTR+I+G	2.50	23.50	1.50	0.50	21.00	1.00	0.29	0.35	0.14	0.23	2.23	0.64
Codon position 1	TIM+I+G	1.33	11.67	1.00	0.00	18.33	1.00	0.28	0.29	0.23	0.20	1.85	0.70
Codon position 2	TVM+I	3.50	23.00	1.00	6.00	15.50	1.00	0.18	0.29	0.13	0.40	60.49	0.92
Codon position 3	TIM+I+G	0.14	7.14	0.29	0.14	5.57	1.00	0.40	0.43	0.06	0.12	1.29	0.50
Non-coding	GTR+I+G	2.33	10.67	1.67	0.67	17.33	1.00	0.33	0.28	0.16	0.23	0.66	0.66
Control region	HKY+I+G	1.33	6.50	0.67	0.83	6.33	1.00	0.34	0.29	0.11	0.26	2.45	0.64
12S	HKY+I	7.00	42.00	2.00	2.00	47.00	1.00	0.31	0.27	0.22	0.20	86.25	0.87
16S	TrN+I+G	2.00	9.50	2.50	0.00	35.00	1.00	0.34	0.27	0.20	0.20	7.28	0.70
ND1	TrN+G	3.00	60.00	2.00	1.00	33.00	1.00	0.29	0.37	0.13	0.22	85.84	0.68
ND2	TrN+I	3.00	59.00	3.00	2.00	32.00	1.00	0.31	0.37	0.09	0.22	95.50	0.60
ND3	HKY+I	0.67	9.17	0.67	0.33	4.83	1.00	0.30	0.37	0.11	0.22	89.16	0.65
ND4	HKY+G	3.00	48.00	3.00	0.00	43.00	1.00	0.61	0.38	0.11	0.20	61.81	0.67

ND5	HKY+G	4.00	45.00	3.00	2.00	45.00	1.00	0.32	0.37	0.10	0.20	1.18	0.55
ND6	K81uf+I+G	12.50	102.50	5.00	5.00	125.00	1.00	0.10	0.07	0.41	0.42	3.44	0.54
cytb	HKY+G	1.00	11.50	1.00	0.25	10.75	1.00	0.29	0.38	0.12	0.21	82.46	0.72
COI	HKY+I	4.00	42.00	2.00	1.00	50.00	1.00	0.28	0.34	0.15	0.22	25.75	0.72
COII	HKY+G	1.00	11.25	0.50	0.25	11.00	1.00	0.32	0.33	0.14	0.21	47.77	0.65
COIII	HLY+I	3.00	18.50	2.00	0.50	25.50	1.00	0.27	0.34	0.16	0.21	90.49	0.72
ATPases	TrN+I	0.40	13.20	0.60	0.20	4.60	1.00	0.32	0.37	0.08	0.23	80.48	0.63

Table 4. Comparison of the support of different regions of the mitochondrial genome for each node of the mitochondrial tree derived from complete mitochondrial genomes of *Tachycineta* swallows and *P. chalybea* (see Fig. 1). Node support is indicated as Bayesian posterior probabilities/bootstrap values from maximum likelihood analysis. The dashes (-) indicate instances where analyses of the mitochondrial region did not produce the relationships indicated by the true mitochondrial gene tree (see Fig. 1).

	Node (sensu Fig.1)						
Region	1	2	3	4	5	6	7
Entire Mitochondrion	1.0/99	1.0/100	1.0/93	1.0/100	1.0/100	1.0/100	1.0/100
All coding sequence	1.0/100	1.0/100	0.77/48	1.0/100	1.0/100	1.0/100	1.0/100
All non-coding sequence	-	1.0/100	0.91/88	1.0/100	-	-	1.0/100
Control region	-	-	-	1.0/100	-	-	1.0/100
12S	-	0.81/38	0.58/55	0.82/81	0.74/67	-/44	1.0/99
16S	-	-	-	1.0/100	-	-	1.0/99
ND1	-/43	-/27	-/21	1.0/100	0.99/99	1.0/99	1.0/100
ND2	-	-	0.69/51	1.0/100	1.0/99	0.88/99	1.0/100
ND3	-	0.63/63	0.68/78	1.0/99	1.0/98	0.84/82	0.87/70
ND4	-	-	-/49	1.0/100	1.0/98	0.98/84	1.0/100
ND5	-	-	0.83/66	1.0/100	0.98/98	1.0/99	1.0/100
ND6	0.96/11	-	-	-	-	-/75	0.99/100
cytb	-	-	-	1.0/100	1.0/96	0.95/89	1.0/98
COI	0.98/99	0.99/66	0.61/37	1.0/100	0.98/72	0.99/86	1.0/100
COII	-	-	-	1.0/100	-	-	1.0/100
COIII	-/4	-	-	0.61/71	0.99/84	1.0/96	0.89/91
ATPases	0.91/33	-	-	1.0/100	1.0/99	0.70/49	1.0/100

Table 5. Uncorrected genetic distances (above the diagonal) and genetic distance corrected by the GTR +G + I evolution model (below the diagonal) for the complete mitochondrial genomes of *Tachycineta* swallows and *P. chalybea.*

Species	*P. chalybea*	*T. albilinea*	*T. albiventer*	*T. bicolor*	*T. cyan.*	*T. euchrysea*	*T. leucorrhoa*	*T. meyeni*	*T. stolzmanni*	*T. thalassina*
P. chalybea	0	0.10	0.10	0.10	0.10	0.10	0.10	0.11	0.11	0.11
T. albilinea	0.18	0	0.03	0.07	0.08	0.08	0.05	0.05	0.05	0.08
T. albiventer	0.18	0.03	0	0.08	0.08	0.08	0.06	0.06	0.05	0.08
T. bicolor	0.18	0.11	0.11	0	0.07	0.07	0.07	0.07	0.08	0.07
T. cyaneoviridis	0.18	0.12	0.12	0.11	0	0.06	0.07	0.07	0.08	0.07
T. euchrysea	0.18	0.11	0.11	0.11	0.09	0	0.07	0.07	0.08	0.07
T. leucorrhoa	0.17	0.07	0.08	0.11	0.11	0.11	0	0.02	0.06	0.07
T. meyeni	0.17	0.07	0.08	0.11	0.11	0.11	0.03	0	0.06	0.07
T. stolzmanni	0.19	0.07	0.07	0.12	0.12	0.11	0.08	0.08	0	0.08
T. thalassina	0.20	0.12	0.12	0.11	0.10	0.10	0.11	0.11	0.13	0

Figure Legends

Figure 1. Consensus mitochondrial gene tree for *Tachycineta* swallows. Node support is indicated as Bayesian posterior probabilities/bootstrap values from maximum likelihood analysis. The nodes are labeled one through seven for reference with Table 6.

Figure 2. Average ± SE of the difference between the genetic difference calculated for each locus and the genetic difference calculated from cytochrome b oxidase. Open bars and closed bars represent uncorrected genetic differences and differences corrected by the GTR + G + I evolution model, respectively. Four independent contrasts were used to calculate means and SE: *Tachycineta euchrysea* and *T. cyaneociridis*, *T. albilinea* and *T. albiventer*, *T. meyeni* and *T. leucorrhoa*, *T. stolzmanni* and *T. bicolor*.

Fig. 1

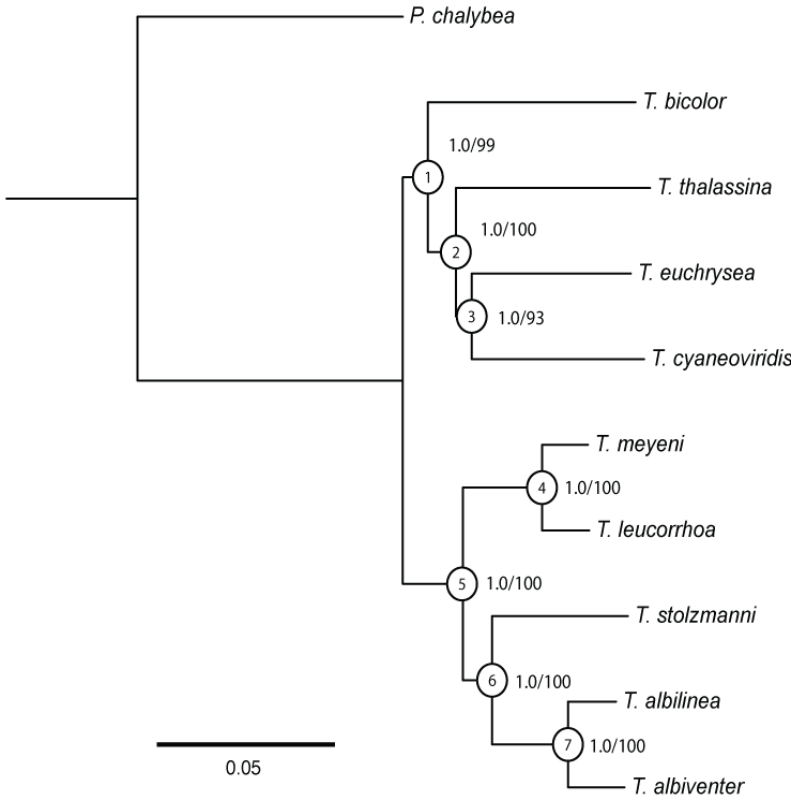
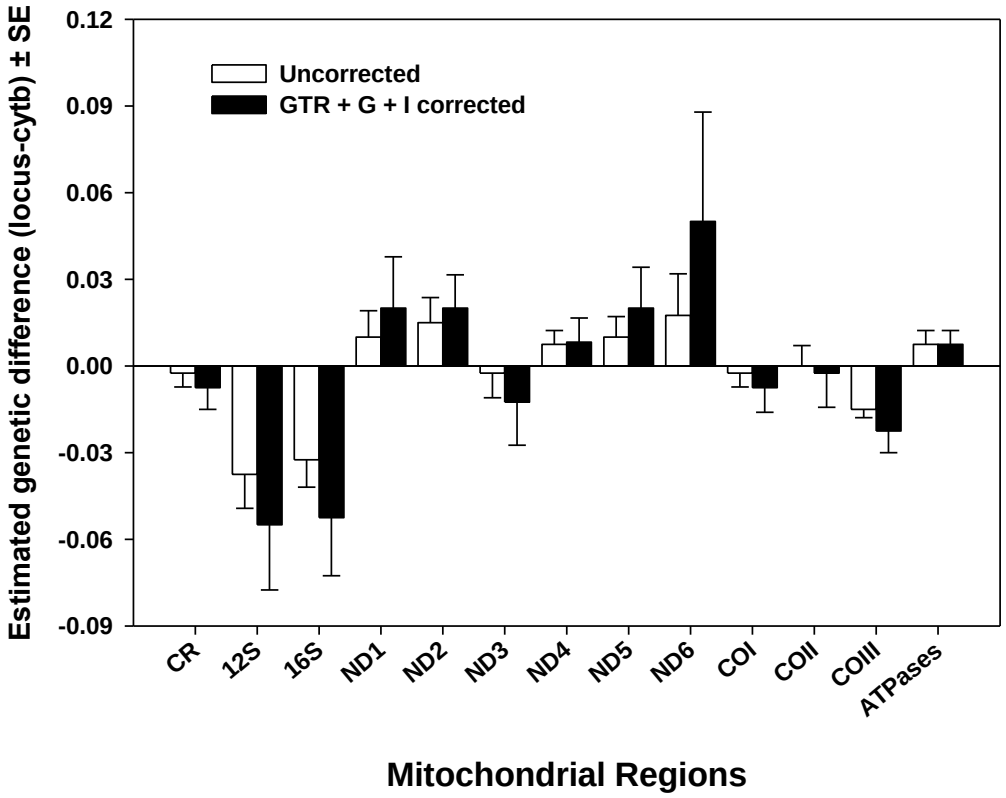

P. chalybea
T. bicolor
1.0/99
1
T. thalassina
1.0/100
2
T. euchrysea
3
1.0/93
T. cyaneoviridis
T. meyeni
4
1.0/100
T. leucorrhoa
5
1.0/100
T. stolzmanni
6
1.0/100
T. albilinea
7
1.0/100
T. albiventer
0.05

Fig. 2


Estimated genetic difference (locus-cytb) ± SE
0.12
0.09
0.06
0.03
0.00
-0.03
-0.06
-0.09
Uncorrected
GTR + G + I corrected
CR
12S
16S
ND1
ND2
ND3
ND4
ND5
ND6
COI
COII
COIII
ATPases
Mitochondrial Regions

Leptin-like compound is associated with metabolic state in a migratory passerine, *Dolichonyx oryzivorus*

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Avian leptin could be an integral hormone in the control and success of migration. Leptin compound might not only be a signal of the availability of energy stores that could influence behavioral decisions during migration (see Chapter 2), but might also directly influence the availability of oxidizable fuels that could be used to power migratory journeys. Higher leptin levels in mammals increase the release of free fatty acids from adipose tissue (William et al., 2002) perhaps to provide an abundance of metabolic fuel during periods of positive energy balance. For long-distance avian migrants, increased availability of energy stores, particularly fatty acids, may be crucial in the preparation for impending endurance flights (e.g., Landys-Ciannelli et al., 2002). There are several independent reports of the characterization of the leptin gene in the chicken (*Gallus gallus*; Ashwell et al., 1999a; Ashwell et al., 1999b; Taouis et al., 1998) and mallard duck (*Anas platyrynchos*; Dai et al., 2007), and there is an abundance of functional evidence that a leptin-like compound exists in birds and acts to influence energy balance and behavior (e.g., Dridi et al., 2000a; Dridi et al., 2000b; Lohmus and Bjorklund, 2009; Lohmus et al., 2003). Moreover, leptin-like compounds have been found in the tissues of several bird species (Dridi et al., 2000b; Kochan et al., 2006; Kordonowy et al., 2010; Quillfeldt et al., 2009). However, a debate over the validity of these findings and the existence of an avian leptin remains (see Pitel et al., 2010; Scanes, 2008; Sharp et al., 2008; Simon et al., 2009), thus, following the recommendation of Scanes (2008), we refer to leptin in birds as a leptin-like compound. Here we measure a leptin-like compound in a long-distance passerine migrant, the bobolink (*Dolichonyx oryzivorus*), to determine its relationship with metabolic state (i.e. feeding vs. fasting). We also test if injections of exogenous leptin are observed in the plasma following injections.

Methods

We captured twenty-six adult male bobolinks (*Dolichonyx oryzivorus*) with mist nets in Tompkins County near Ithaca, NY, between 12 May and 2 June 2006. We immediately transferred all birds to indoor holding facilities at Cornell University, Ithaca, NY. We singly housed individuals in wire cages (75cm x 45cm x 45cm) at 21 (± 2) C°, fed birds a seed mix supplemented with waxworms (*Galleria mellonella*) and mealworms (*Tenebrio molitor*), and provided water *ad libitum*. Initially, we held all birds on a light cycle similar to ambient conditions at capture (15 hours light (L):9 hours dark (D)) for 3 months. We then switched the light cycle to 12L:12D to induce migratory fattening, and kept birds on this light cycle for 3 months before performing experiments and euthanizing all birds.

Experiment 1

We randomly split 22 birds into two groups and removed food dishes from the cages of one group at lights on (0800 EST) on the day prior to euthanasia. We provided the remaining group *ad libitum* food and water during this period. Before euthanizing birds we weighed them (±0.1g) and placed them under anesthesia with isofluorane. While the birds were anesthetized, we sampled whole blood (500-1000 µl) via cardiac puncture with an EDTA washed syringe and a 22 gauge needle. We immediately centrifuged the blood at 10 x g for 5 minutes, and removed and stored the plasma at -80° C until analysis. We measured plasma leptin-like compound using a radioimmunoassay as described by (Kordonowy et al., 2010).

Experiment 2

We randomly chose four additional birds, fed them *ad libitum* and gave them intramuscular injections of chicken leptin-like compound (National Hormone and Peptide Program) 20 minutes before euthanasia. We used a dosage for injections, 1 µg/g of body mass dissolved in 100 µl of phosphate buffer (Lohmus et al., 2003), known to affect feeding rates in other passerines (Lohmus et al., 2003) and galliformes (Lohmus and Sundstrom, 2004). We euthanized birds, collected plasma and assayed plasma leptin-like compound as described above. The objective of this study was not to provide a calibration for the effect of exogenous injections of leptin on plasma levels, but rather to demonstrate that plasma leptin can be manipulated in migratory birds. Thus, we did not include a control group that received vehicle injections.

Statistical analysis

To test if birds gained mass in preparation for migratory activity we compared body mass within each experimental group between the date we switched the light cycle from 15L:9D to 12L:12D (initial mass; Table 1) and the day of blood sampling (final mass; Table 1) by paired t-tests. To determine if mass was needed as a covariate in future analyses, we tested the relationship between mass and plasma leptin-like compound in birds pooled across experiments by simple regression. We compared body mass between fasted and *ad libitum* fed birds by one-way ANOVA. We tested if plasma leptin differed between fasting and *ad libitum* fed birds (exp. 1 above) by one-way ANOVA. To determine if leptin levels differed between *ad libitum* fed birds and birds injected with endogenous leptin (exp. 2 above) we used Welch's one-way ANOVA to account for unequal variances between treatments. We performed all statistical tests in SAS 9.2 (SAS Institute Inc.,Cary, NC) and deemed models significant at $P < 0.05$.

Results

Birds in all groups substantially gained body mass between the change in light cycle and blood sampling (Table 1), indicating fattening in preparation for migration. Body mass at the time of blood sampling did not differ between fasted and fed birds ($F_{1, 20} = 2.54$, $P > 0.12$, Table 1), and was not related to plasma leptin in birds from both experimental groups pooled or each experimental group alone (all $P > 0.11$). Thus, body mass was not included as a covariate in subsequent models. Fasted birds had significantly lower plasma leptin-like compound (mean: 8.32 ng/ml ± 1.28 SD) than *ad libitum* fed birds (mean: 10.09 ng/ml ± 1.66 SD; $F_{1, 20} = 7.61$, $P < 0.012$, Fig. 1). Experimentally injected birds had significantly higher plasma leptin-like compound (mean: 75.98 ng/ml ± 10.69 SD) than birds fed *ad libitum* ($F_{1, 3.05} = 150.85$, $P < 0.002$, Fig. 1). These results indicate that 1) plasma leptin-like compound is associated with metabolic state and 2) injections of exogenous leptin increase the leptin-like compound in the plasma of avian migrants.

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Table 1. Comparison of mass changes for each experimental group between the change in light cycle (15L:9D to 12L:12D see methods; initial mass) and blood sampling (final mass) by paired t-tests.

Treatment	Initial Mass	Final Mass	T-statistic	P-value	Sample Size
ad libitum	34.85 ± 2.04	56.38 ± 3.91	17.781	<0.001	12
fasted	34.30 ± 2.72	53.57 ± 4.37	13.138	<0.001	10
injected	36.08 ± 5.30	51.75 ± 1.88	7.514	<0.005	4

Figure 1. Comparison of plasma leptin levels between bobolinks (*Dolichonyx oryzivorus*) fasted for 24 hrs. and those fed *ad libitum* (one-way ANOVA, see methods), and between *ad libitum* fed birds and *ad libitum* fed birds injected with chicken leptin-like compound (Welch's one-way ANOVA, see methods). * and *** indicate P <0.012 and <0.002, respectively. Numbers above each bar represents sample size.