MOLECULAR MECHANISMS UNDERLYING AVIAN METABOLIC VARIATION: A GENOMIC APPROACH

BY

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THESIS

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ABSTRACT

Physiological adaptive acclimatization allows an organism to balance the competing demands of its life cycle and environment. This thesis seeks to further our understanding of the processes underlying physiological acclimation by characterizing two molecular mechanisms that enable organisms to mount physiological responses to changing environmental conditions. Comprised of two investigations, it focuses on the molecular mechanisms underlying variation in avian aerobic performance. In the first, I explore regulatory changes that act on relatively short time periods, enabling organisms to respond to rapid environmental changes within an individual’s lifetime. I show that thermogenic flexibility manifested from simultaneous changes in the expression of genes within hierarchical regulatory networks in response to temperature in the Dark-eyed Junco (Junco hyemalis). This transcriptomic variation resulted in rapid physiological modifications within individuals. In the second, I instead characterize coding mutations that occur on the timescale of generations. I show that differences in the mitochondrial sequences of the genes encoding the machinery of cellular metabolism could result in canalized differences in whole-organism aerobic performance among Tachycineta swallow species. Sites of positive selection were concentrated in species with ‘slow’ life histories that may be associated with low metabolic rates. Together these investigations provide a concise survey of the molecular tools birds are equipped with to metabolically respond and adapt to environmental variation.
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CHAPTER 1

INTRODUCTION

Physiological adaptive acclimatization allows an organism to balance the competing demands of its life cycle and environment. Variation in physiological responses can therefore have dramatic fitness consequences and understanding physiological adaptation is thus critical for understanding large-scale evolutionary processes. This thesis seeks to further our understanding of the processes underlying physiological adaptation by characterizing the molecular mechanisms that enable organisms to mount physiological responses to changing environmental conditions. Specifically, I focus here on a physiological activity fundamental to all eukaryotic life: aerobic metabolism.

Rates of energy expenditure vary tremendously among organisms — up to 200-fold within a single class (e.g., Cephalopods; Seibel, 2007) — yet the underlying metabolic pathways are highly conserved. The metabolic network that enables a sessile coral reef sponge to consume as little as 0.02 μmol O₂ g⁻¹ min⁻¹ (Hadas et al., 2008) is essentially the same as that which enables a hummingbird to sustain hovering flight at a rate of 82 μmol O₂ g⁻¹ min⁻¹ (Suarez, 1992). These pathways are well characterized and, while we often think of metabolism in terms of whole-organism performance, at lower levels, cellular respiration can be decomposed into a series of enzyme-catalyzed reactions. Each component part feeds one into the next in a step-like fashion that enables the conversion of biological molecules (carbohydrates, fats, and proteins) into usable energy (ATP). Together, these vital reactions sum to form an organism’s metabolic rate, a universal measure of energy exchange.

Metabolic performance varies both within and among individuals and broad-scale patterns in metabolic performance exist (e.g., across seasons, elevations, and latitudes). Variation within a single individual might represent an adaptive acclimatization response to short-term environmental pressures. For instance, small endotherms exhibit seasonal changes in aerobic capacity to meet the thermogenic demands of winter (e.g., Swanson, 2010). Variation among populations may arise from differences in the abiotic selective pressures of their respective environments, and could be indicative of adaptive evolution. For instance, basal (or resting) metabolic rate has been shown to increase with increasing latitude for many groups of
vertebrates (e.g., birds, Wiersma et al., 2007a,b; fish, Naya & Bozinovic, 2012; mammals, MacMillen & Garland, 1989; Lovegrove, 2000) and has been correlated with rates of reproduction and survival (Wiersma et al., 2007a,b). Thus investigations of the molecular basis of metabolic variation both within and among individuals can allow us to explore the underlying mechanisms that enable organisms to respond differentially to changes in their environments.

At the biochemical level, metabolic variation can manifest from differences in the regulatory mechanisms that govern aerobic respiration (e.g., Cheviron et al., 2012; 2014). Chapter 2 investigates the molecular basis of metabolic flexibility in response to seasonally changing environmental stimuli. We exposed Dark-eyed Juncos (Junco hyemalis) to two, experimentally manipulated seasonal cues: photoperiod and temperature. We then tested for associations between changes in gene expression profiles of skeletal muscle and the activities of key enzymes, as well as whole-organism thermogenic performance. This integrative, manipulative experiment allowed us to make explicit connections from each environmental stimulus, to the transcriptomic and proteomic responses, to the resulting changes in organismal aerobic capacity.

In contrast, coding mutations in the genes encoding the machinery of cellular metabolism can alter the functional properties of metabolic enzymes and result in canalized differences in whole-organism metabolic performance. Genomic comparisons among closely related populations or species are particularly useful for characterizing these genetic differences. To this end, Chapter 3 explores the genetic basis of metabolic variation among Tachycineta swallows within a phylogenetic framework. Avian life histories consistently vary with latitude, and many of these differences can be attributed to underlying differences in rates of metabolic energy expenditure. We therefore inferred differences in metabolic demands among Tachycineta species using an index of life-history variation, and then tested for evidence of adaptive mitochondrial divergence using complete mitochondrial genome sequences and suites of comparative genomic analyses. Using this approach, we uncovered a number of amino acid substitutions that bear the signature of positive selection across the mitogenome, and we make inferences regarding the functional significance of this genetic variation on whole-organism metabolic performance.

These investigations serve to illuminate the molecular mechanisms underlying physiological variation in non-model organisms. Separately, these chapters describe two distinct processes underlying different levels of metabolic variation acting at disparate scales.
Collectively, they provide a concise survey of the molecular tools organisms are equipped with to metabolically respond and adapt to environmental variation.

**LITERATURE CITED**


CHAPTER 2

REGULATORY MECHANISMS OF METABOLIC FLEXIBILITY IN THE DARK-EYED JUNCO (JUNCO HYEMALIS)

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ABSTRACT

Small temperate birds can reversibly modify their aerobic performance to maintain thermoregulatory homeostasis amidst winter conditions, and these physiological adjustments may be attributable to changes in the expression of genes in the underlying regulatory networks. Here we report the results of an experimental procedure designed to gain insight into the fundamental mechanisms of metabolic flexibility in the Dark-eyed Junco (Junco hyemalis). We combined genomic transcriptional profiles with measures of metabolic enzyme activities and whole-animal thermogenic performance from juncos exposed to four experimental treatments varying in exposure to temperature (cold=3°C; warm=24°C) and photoperiod (SD=8h light:16h dark; LD=16h light:8h dark). Cold-acclimated birds increased thermogenic capacity compared to warm-acclimated birds, and this enhanced performance was associated with upregulation of genes involved in lipid transport and oxidation, and with catabolic enzyme activities. These physiological changes occurred over ecologically relevant timescales, suggesting that birds make regulatory adjustments to interacting, hierarchical networks in order to seasonally enhance thermogenic capacity.

INTRODUCTION

Phenotypic flexibility is an organism’s ability to reversibly modify its phenotype with respect to changes in its environment. Because it allows an organism to optimally match its phenotype to prevailing biotic and abiotic conditions, phenotypic flexibility has profound consequences for organismal fitness (Piersma & Drent, 2003; DeWitt & Scheiner, 2004).
Elucidating the mechanisms that underlie phenotypic flexibility therefore has important implications for understanding how organisms cope with rapid environmental change.

Temporal variation in organismal bioenergetics is a prominent example of phenotypic flexibility. Endotherms reversibly alter their metabolic rates across the annual cycle to meet seasonal demands associated with migration, reproduction, and thermoregulation. Such metabolic flexibility is especially important for small endotherms that are resident in seasonally variable environments, as metabolic thermoregulatory performance has acute consequences for survival during prolonged cold stress (Hayes & O’Connor, 1999). Birds that winter at high latitudes can provide a valuable model for understanding the physiological mechanisms underlying metabolic flexibility because they do not hibernate, and therefore must rely largely on physiological adjustments that enhance thermogenic performance for survival. For instance, in winter, temperate-zone birds elevate their capacities for shivering thermogenesis to maintain thermoregulatory homeostasis in the face of falling temperatures, and this increased thermogenic performance is associated with elevated basal and summit metabolic rates (e.g., Arens & Cooper, 2005). However, winter is also accompanied by reductions in productivity, decreased foraging time, and increased fasting periods overnight (Marsh & Dawson, 1989), so birds must potentially meet these increased thermoregulatory demands in the face of reduced energetic input (Swanson, 2010). Thus, short-term physiological adjustments that optimally balance competing energetic demands are crucial for survival during the harsh winters in high-latitude environments.

At the biochemical level, variation in aerobic thermogenic performance is manifest through differences in the expression and functional properties of the genes encoding the machinery of cellular metabolism. For instance, high-elevation deer mice (Peromyscus maniculatus) exhibit greater thermogenic capacities compared to their lowland counterparts, and this enhanced performance has been linked to regulatory changes in the expression of genes that influence metabolic fuel use and oxygen utilization (Cheviron et al., 2012; 2014). Because changes in gene regulation allow the genome to rapidly and reversibly respond to environmental variation, transcriptomic studies can provide key insights into the mechanistic underpinnings of phenotypic flexibility and the adaptive modification of complex physiological traits, both of which require concerted changes in hierarchical and interacting regulatory networks (Cheviron & Brumfield, 2012). The hierarchical nature of complex physiological traits is well illustrated by
physiological adjustments that enhance aerobic thermogenic performance, which comprise three broad levels of physiological organization (Figure 2.1):

(1) Enhancing the size or structure of thermogenic organs. Small birds rely on flight muscles for facultative thermogenesis (Hohtola, 1982; Hohtola et al., 1998; Dawson & O’Connor, 1996; Yacoe & Dawson, 1983), and several studies have demonstrated a prominent role for avian pectoralis size on thermogenic capacity (e.g., Vézina et al., 2006; Vézina et al., 2007; Swanson et al., 2013). Muscle hypertrophy can be achieved by altering the expression of genes in several key growth signaling pathways. Specifically, the rapamycin (mTOR) signaling pathway is recognized as the major player in protein synthesis regulation for adult mammalian skeletal muscle (McCarthy & Esser, 2010). The mTOR pathway integrates signals from a number of upstream pathways, including the insulin-like growth factor 1 (IGF1)-Akt signaling pathway (also called PIK3-Akt signaling pathway), which plays a principal role in stimulating cell growth and proliferation. Increased expression of the first component in this pathway (IGF1) has been linked to muscle hypertrophy in birds and mammals (e.g., Rennie et al., 2004; Heinemeier et al., 2007; Price et al., 2011). Inhibitory growth factors that interact with the IGF1-Akt3 and mTOR pathways (e.g., myostatin) impede protein synthesis and induce muscle atrophy (Welle et al., 2009). Blocking myostatin activity can therefore result in subsequent growth to myocytes (e.g., McPherron et al., 1997). In principle, modifications could also be made to the relative proportion of fiber types within the muscle. This has been shown in mammals, where endurance training is associated with the alteration of relative proportions of fast glycolytic fibers and fast oxidative-glycolytic fibers in skeletal muscle (Holloszy & Coyle, 1984; Hudlicka, 1985); however, this has not been documented in small birds. Instead, numerous studies have shown that the pectoralis of small birds is composed almost exclusively of fast oxidative-glycolytic fibers, providing both an intermediate contraction velocity and resistance to fatigue that allows for sustained flapping flight (e.g., Lundgren & Kiessling, 1988; Welch & Altshuler, 2009). Therefore, while it is possible that the avian pectoralis does grow and atrophy seasonally to meet changing thermogenic demands, the fiber composition does not seem to be altered to any appreciable degree.
(2) **Enhancing oxygen delivery.** Increased oxygen delivery can be accomplished by a number of cardiovascular changes throughout the body. Within the pectoralis, oxygen diffusion distance can be reduced through increased capillary density in the muscle, as has been shown in altitude-acclimated pigeons (Mathieu-Costello & Agey, 1997). Angiogenesis is primarily stimulated by the vascular endothelial growth factor (VEGF) signaling pathway, which mediates endothelial cell proliferation and migration (Klagsbrun & D’Amore, 1996; Ferrara, 1997). The VEGF pathway in turn can be activated by cellular hypoxia via the hypoxia-inducible factor 1 (HIF-1) signaling cascade (Bruick & McKnight, 2001). In addition to initiating angiogenesis, the HIF-1 cascade regulates oxygen homeostasis in a number of other ways, including eliciting erythropoiesis via hormonal control (Rankin et al., 2007) and inducing vasodilation via nitric oxide production (Beall et al., 2012). These many interacting mechanisms can serve to boost oxygen delivery to the muscle, enabling individuals to sustain increased aerobic performance.

(3) **Enhancing the cellular aerobic capacity of thermogenic organs.** Increases in fuel supplies and oxygen utilization can increase the cellular aerobic capacity of thermogenic organs like the pectoralis. Although mammals shift between carbohydrate and lipid fuel supplies according to exercise intensity (McClelland, 2004), energy-rich lipids are the primary fuel source for avian skeletal muscle for all intensity levels — from rest to the prolonged exercise associated with shivering and flight (Rothe et al., 1987; Suarez et al., 1990; Vaillancourt et al., 2005; Guglielmo, 2010; Kuzmiak-Glancy & Willis, 2014). Adipose stores have been shown to contribute 80% of the energy required for avian shivering (Vaillancourt et al., 2005). Fuel availability is not only dependent upon the size of adipose stores, but also upon an individual’s ability to rapidly mobilize, catabolize, and oxidize these fuels. The first step, mobilization, occurs by modifying expression to the many pathways that comprise the greater fatty acid supply pathway. Fats are not water-soluble; therefore, carrier proteins are required to move fatty acids from the adipocyte to the muscle and, ultimately, across the cellular membrane to the mitochondria. To that effect, birds meet the heightened demands of migration by seasonally increasing fatty acid transporter proteins in the flight muscle (Guglielmo et al., 1998, Pelsers et al., 1999, Guglielmo et al., 2002, McFarlan et al., 2009). Moreover, increased metabolic intensity of the pectoralis can be achieved by increasing mitochondrial density, which has been documented in premigratory birds (Gaunt et al., 1990; Evans et al, 1992), or by elevating activities of catabolic enzymes involved in fatty
acid beta-oxidation, which has been documented in both wintering and migratory birds (e.g., Lundgren & Kiessling, 1985; Carey, 1989; O'Connor, 1995; Guglielmo et al., 2002). Birds also exhibit corresponding seasonal changes in activities of oxidative enzymes that are involved in the final steps of aerobic metabolism — the citric acid cycle and oxidative phosphorylation pathways (e.g., Liknes, 2005; Liknes & Swanson, 2011; Zheng et al., 2008). Thus adjustments can be made to fine-tune fuel supplies and oxidative utilization at many points along this extensive cascade.

Despite this range of possibilities, it is still unclear how organisms effectively modify and combine these modular and hierarchical trait components in order to rapidly respond to exogenous factors, such as extreme weather events. Although regulatory adjustments could occur at any step in a given pathway, theory predicts that flux control should be unevenly distributed among pathway components (Wright & Rausher, 2010). Specifically, it is the upstream steps of linear pathways that are expected to be most rate limiting (Wright & Rausher, 2010; Eanes, 2011). Indeed in *Drosophila* species, genes at the top of the glycolytic pathway exhibit signatures of selection in the form of latitudinal allele clines and elevated rates of amino acid substitution, suggesting that these positions have been targeted for increased pathway flux (Sezgin et al., 2004; Flowers et al., 2007; Eanes, 2011). If flux control is also concentrated in the upstream portions of pathways influencing thermogenic performance, we would expect to see regulatory changes in the upstream components (e.g., the upper tier of the fatty acid supply pathway). This hypothesis can be tested using transcriptomic pathway-level approaches that allow for simultaneous analyses of many interacting regulatory networks to identify regional patterns in regulatory change.

Here we report the results of an experimental procedure designed to gain insight into the fundamental mechanisms of avian metabolic flexibility. We combine new data on genomic transcriptional profiles with previously published data on thermogenic performance and enzyme activities for the same individuals (Swanson et al., 2014). This work focuses on Dark-eyed Juncos (*Junco hyemalis*), a small passerine that winters from Alaska to Arizona (Nolan et al., 2002). Wintering juncos exhibit increases in thermogenic performance and cold tolerance compared to summer-acclimatized birds (Swanson, 1991; 2001). To induce changes in junco thermogenic performance, we exposed wild-caught juncos to synthetic photoperiod and
temperature regimes, two seasonal cues that have been previously implicated in altering avian metabolic rate and thermogenic performance (i.e. Cooper & Swanson, 1994; Liknes & Swanson, 1996; Zajac et al., 2011). We then related variation in thermogenic performance to differences in underlying gene expression profiles of a plastic, thermogenically-active tissue, the pectoralis. We explicitly surveyed the genes associated with the cell-, tissue-, and organ-level responses outlined above to test a priori hypotheses regarding the changes likely to occur at each of the three levels of biological organization. By concurrently examining the effects of photoperiod and temperature, we address how each environmental stimulus induces changes in metabolic gene expression and how these transcriptomic responses, in turn, affect whole-organism thermogenic performance.

METHODS

Bird capture and acclimation treatments

We subjected juncos to a two-by-two experimental design using two relevant environmental cues. Details of the sampling methods have been previously described by Swanson et al. (2014). Briefly, we captured juncos overwintering near Vermilion, South Dakota (~42°47’ N, 97°W) in mid-December. We housed birds individually and, after a two-week adjustment period, subjected individuals to one of four experimental temperature-photoperiod regimes, each lasting six weeks. The treatments were 16 h light:8 h dark at 24°C (warm LD, n = 10), 8 h light:16 h dark at 24°C (warm SD, n = 10), 16 h light:8 h dark at 3°C (cold LD, n = 10), or 8 h light:16 h dark at 3°C (cold SD, n = 9). To obtain appropriate sample sizes, procedures were performed over the course of two consecutive winter seasons (2011-2012 and 2012-2013).

Measures of metabolic performance

We measured individual thermogenic performance before and after the treatments (at 6 weeks). We quantified summit metabolic rate (M_{sum}), which is the maximum metabolic rate attained by birds under cold exposure (Swanson, 2010) and is a proxy for thermogenic capacity, using open-flow respirometry in a heliox atmosphere (21% oxygen/79% helium) with sliding cold exposure (as per Swanson et al., 1996). Following the M_{sum} trial, we measured cloacal temperature to verify that individuals were hypothermic (body temperature ≤ 37°C). We identified M_{sum} as the period of peak oxygen consumption over a 5-minute window and report it
To control for existing differences in individual $M_{sum}$ before acclimation treatments, we calculated the change in individual $M_{sum}$ before and after treatments ($\Delta M_{sum}$) for use in subsequent analyses. We tested for differences among treatment groups after the acclimation period using a one-way ANOVA on $\Delta M_{sum}$.

At the end of the acclimation treatment, immediately following the final $M_{sum}$ measurement, we euthanized juncos via cervical dislocation, measured body mass, and rapidly excised pectoralis tissue on ice. We weighed pectoralis tissue to the nearest 0.1 mg, then flash froze it in liquid N$_2$ for use in enzyme activity assays or stored it in RNAlater and froze it at -60°C for transcriptomic analyses. We collected birds under appropriate state (11-7, 12-2) and federal (MB758442) scientific collecting permits and all procedures were approved by the University of South Dakota Institutional Animal Care and Use Committee (Protocol 79-01-11-14C).

**Functional genomic analyses**

*Generation and sequencing of RNA-seq libraries*

We used massively parallel sequencing of pectoralis muscle transcriptomes (RNA-seq; Wang *et al.*, 2009) to quantify genome-wide patterns of gene expression for each of the 39 individuals. We isolated mRNA from pectoralis using Trizol reagent and prepared cDNA libraries for sequencing using the Illumina TruSeq RNA Sample Preparation Kit. Libraries were sequenced as 100nt single-end reads on the Illumina HiSeq2000 platform. We multiplexed 10 individuals in a single flow cell lane using Illumina index primers. Image analysis and base calling were performed using Illumina pipeline software.

*Read mapping, normalization and differential expression*

We performed a series of sequential filtering steps to remove low quality reads and index primer sequences. First, we removed all reads with mean Solexa quality scores less than 30. Second, we trimmed low-quality bases (Solexa score < 30) from these remaining high-quality sequences. Finally, we scanned reads for adaptor sequences, and if detected, they were trimmed from the sequence read. The filtering steps resulted in a final dataset of nearly 743 million sequence reads, with an average of 19 million reads per individual (range = 3.5 – 63.0 million reads), and the trimming steps resulted in an average read length of 97.7 nt.
We estimated transcript abundance by mapping sequence reads to the Zebra Finch (*Taeniopygia guttata*) genome (build taeGut3.2.4) using CLC Genomics workbench (v. 6.0.4). In total, sequence reads mapped to 15,362 unique genes. However, we excluded genes with less than an average of 5 reads per individual because genes with low count values are typically subject to increased measurement error (Robinson & Smyth, 2007). This filtering step resulted in a final dataset of 10,868 detected genes.

We performed normalization and differential expression analyses using the package edgeR (Robinson *et al*., 2010) in Program R (ver. 3.0–R Development Core Team, 2013). We first normalized read counts and controlled for differences in total library size (number of total reads) among individuals using the function calcNormFactors (Robinson & Oshlack, 2010). Following normalization, we tested for differences in transcript abundance among treatment groups using a generalized linear model approach. We estimated model dispersion for each gene separately using the function estimateTagwiseDisp (McCarthy *et al*., 2012). We tested for genes that exhibited significant expression differences across treatments using a GLM likelihood ratio test and controlled for multiple tests by enforcing a genome-wide false discovery rate (FDR) of 0.05 (Benjamini & Hochberg, 1995).

Connecting gene expression and phenotypic variation

By detecting suites of genes that exhibit correlated transcriptional patterns, previously undetected modules of ostensibly co-regulated genes can be defined. These modules may expose biologically meaningful associations between surprising assemblages of genes, and thus their use in subsequent functional analyses can provide insight into the mechanistic underpinnings of complex traits (Ayroles *et al*., 2009; Stone & Ayroles, 2009). To identify coordinated expression changes among interacting genes, we assessed the degree of correlation in transcript abundance among genes with significant treatment effects (FDR < 0.05) using Modulated Modularity Clustering (MMC; Stone & Ayroles, 2009). This method calculates Pearson correlation coefficients among transcript abundances for all pair-wise combinations then identifies suites (modules) of highly intercorrelated genes (Stone & Ayroles, 2009).

For each of the modules, we then summarized overall module expression using a principal component analysis and retained the first principal component axis (PC1), which represented on average 84% of within module variation (49% - 100%). To determine if suites of
differentially expressed (DE) genes were related to whole-organism thermogenic performance, we performed linear regressions of individual $\Delta M_{\text{sum}}$ on module expression (PC1) and identified correlations at a significance level of $P \leq 0.05$. We repeated this procedure to identify correlations between pectoralis size ($M_p$; expressed as a proportion of body mass) and module expression. For modules that exhibited significant associations with either phenotype, we performed functional enrichment analyses using the Zebra Finch GO Analysis (Wu & Watson, 2009) to identify functional categories that were over-represented among the genes that comprise a given trait-associated module.

To isolate the independent contributions of photoperiod and temperature on gene expression, we followed a procedure identical to that described above for the overall treatment effects: we tested for genes that exhibited significant expression differences between the two photoperiod treatments, identified suites of genes with correlated expression patterns, summarized module expression, tested for associations between module expression (PC1) and $\Delta M_{\text{sum}}$ or $M_p$, and performed enrichment analyses. We repeated this procedure for the temperature treatments. Overall-treatment-sensitive modules are denoted by A, photoperiod-sensitive modules by P, and temperature-sensitive modules by T.

**Targeted pathway and gene analyses**

We performed analyses on targeted genes and pathways to test *a priori* hypotheses about their influences on metabolic flexibility. These included all genes in the four major metabolic pathways (glycolysis, fatty acid oxidation, citric acid cycle, oxidative phosphorylation), and in muscle growth (mTOR signaling), angiogenesis (vascular endothelial growth factor), and fuel supply (glycerolipid metabolism, glycerophospholipid metabolism, peroxisome proliferator-activated receptor (PPAR) signaling, pyruvate metabolism) pathways. We identified these genes using *Taeniopygia guttata* KEGG Database pathway maps (Kanehisa & Goto, 2000; Kanehisa et al., 2014), downloaded from CytoKEGG (ver. 0.0.5). We also included select genes in the insulin-like growth factor (IGF1) and hypoxia inducible factor 1 (HIF-1) signaling pathways for which complete *T. guttata* maps were not available. We identified the presence/absence of these genes among those differentially expressed (either among photoperiod or temperature treatments) and noted fold changes with respect to the environmental stimuli where applicable.
Because phenotypic responses could manifest from large-scale changes in expression across the entire pathway, we tested for concerted expression across these same pathways in response to both photoperiod and temperature treatments. To do this we mapped expression values onto *T. guttata* pathway maps and counted the number of genes exhibiting positive and negative log fold-change values. We performed binomial tests to test for concerted expression across each pathway with the null expectation that an equal number of genes would be up- and down-regulated in given pathway simply by chance. We identified concerted expression at a significance level of $P \leq 0.05$.

*Enzyme quantification and transcript abundance*

Variation in transcript abundance does not consistently produce an equivalent or proportional response in corresponding protein abundance (Abreu et al., 2009). To investigate the relationship between the junco transcriptome and its corresponding gene products, we quantified the activities of three metabolic enzymes that serve as biomarkers for the flux capacity of important metabolic pathways. Enzyme activity is a product of both enzyme concentration and kinetic efficiency, and consequently is not strictly equivalent to protein abundance. Nonetheless, because we expect any potential variation in kinetic efficiency to be randomly distributed among individuals that were assigned to acclimation treatments, we assumed that differences in enzyme activities largely reflected differences in enzyme concentration, which in turn should be related to differences in the transcript abundance of enzyme-encoding genes. The enzymes we surveyed included: carnitine palmitoyl transferase (CPT), an indicator of fatty acid transport capacity across the mitochondrial membrane; beta-hydroxyacyl Coenzyme-A dehydrogenase (HOAD), an indicator of fatty acid oxidation capacity; and citrate synthase (CS), an indicator of mitochondrial abundance and cellular metabolic intensity. We utilized pectoralis tissue to quantify enzymes using standard assays (*e.g.*, CPT as in Guglielmo et al., 2002; CS and HOAD as in Liknes & Swanson, 2011) conducted at $39 \pm 2°C$ with a Beckman DU 7400 spectrophotometer. Further details have been previously described in Swanson et al. (2014). We report all enzyme activities as mean mass-specific activity ($\mu$moles $\cdot$ min$^{-1} \cdot$ g$^{-1}$). To test whether differences in transcript abundance translated into variation in enzyme activity, we performed linear regressions of individual metabolic enzyme activity on the corresponding transcript abundances for each of the
enzyme-encoding genes. We identified associations between enzyme activity and transcript abundance at a significance level of $P \leq 0.05$.

**RESULTS**

*Phenotypic flexibility in thermogenic performance, not pectoralis size*

We exposed juncos to two synthetic cues (temperature and photoperiod) using a two-by-two experimental design in order to induce changes in thermogenic performance. As previously reported (Swanson et al., 2014), these six-week acclimation treatments effectively altered junco summit metabolic rate ($\Delta M_{\text{sum}}; P = 1.43 \times 10^{-5}$; Figure 2.2). Cold-acclimated birds increased $M_{\text{sum}}$ by 1.19 ml O$_2$ min$^{-1}$ compared to warm-acclimated birds ($P = 8.63 \times 10^{-7}$), but the photoperiod treatments did not result in changes to $M_{\text{sum}}$ ($\Delta M_{\text{sum}} = 0.05$ ml O$_2$ min$^{-1}$, $P = 0.684$). Mass adjusted pectoralis size ($M_p$), however, did not vary among any of the acclimation treatments (one-way ANOVA, $P_{\text{total}} = 0.263$; $P_{\text{temp}} = 0.133$; $P_{\text{photo}} = 0.234$; Appendix A).

*Transcriptomic responses to overall treatment*

We used a general linear model approach to identify genes that exhibited a significant difference in transcript abundance due to acclimation treatment. A total of 1882 genes (17.3% of the total number of transcripts after filtering) exhibited differential expression (DE) among acclimation treatments (FDR < 0.05). Within this subset of DE genes, we identified modules of coregulated genes using MMC (Stones and Ayroles, 2009). This analysis revealed 47 transcriptional modules composed of highly intercorrelated genes (Figure 2.3A). We then employed principal component and regression analyses to determine which of these transcriptional modules were associated with thermogenic performance ($\Delta M_{\text{sum}}$). Overall module expression (summarized as PC1 scores) correlated with individual $\Delta M_{\text{sum}}$ for 13 modules (Table 2.1). Gene enrichment analyses revealed that these modules were significantly enriched for terms related to lipid metabolism (A18, A26, A27), fatty acid oxidation (A26), oxygen transport (A26), angiogenesis (A23, A27, A28), and muscle growth (A28, A43) (see Appendix B for full list of terms enriched in each module). Additionally, module expression (PC1) correlated with mass-adjusted pectoralis size ($M_p$) for five modules. Gene enrichment analyses revealed that these modules were enriched for terms largely related to ion transport (A7, A19).
Isolating transcriptomic responses to each cue

We used a similar approach to isolate the effects of photoperiod and temperature on junco transcriptomic responses. A total of 1325 genes (12.2% of total transcripts) were differentially expressed among the photoperiod treatments and 756 genes (7.0% of total transcripts) were differentially expressed among the temperature treatments. Of these, 196 DE genes were shared among the two treatment classes, and these were also detected in the overall treatment analyses (Figure 2.4). Likewise, many of the DE genes particular to a single treatment class were also detected in the overall treatment analysis. As a result, 252 novel DE genes were detected among the photoperiod treatments alone and 92 novel DE genes were detected among the temperature treatments. We again employed the combination of MMC, principal component analysis, and regression analysis to determine which photoperiod-specific (or temperature-specific) transcriptional modules were associated with $\Delta M_{\text{sum}}$ and $M_p$.

Photoperiod-sensitive DE genes (those with significant photoperiod effects) clustered into 51 transcriptional modules (Figure 2.3B). In regression analyses, three transcriptional modules exhibited strong associations with $\Delta M_{\text{sum}}$ (Table 2.1). These modules were enriched for terms related to glycolysis (P36) and angiogenesis (P45; Appendix B). Similarly, five transcriptional modules showed strong associations with $M_p$. These modules were enriched for terms involved in ion transport (P8, P21) and lipid metabolism (P32, P51).

Temperature-sensitive DE genes (those with significant temperature effects) clustered into 38 transcriptional modules (Figure 2.3C). In regression analyses, 23 transcriptional modules exhibited strong associations with $\Delta M_{\text{sum}}$. These modules were enriched for terms related to lipid metabolism ($n = 5$ modules), oxidative phosphorylation ($n = 5$), glycolysis ($n = 2$), angiogenesis and oxygen transport ($n = 4$), and growth ($n = 7$; Table 2.2). Additionally, three transcriptional modules showed strong associations with $M_p$. These modules was enriched for terms involved in muscle contraction (T5) and growth and angiogenesis (T38).

Pathway-level expression patterns

Our dataset included the majority of unique genes (84-100%) from each of the ten pathways that we examined in entirety. A high proportion of the genes involved in fatty acid metabolism (27%), glycerolipid metabolism (27%), glycolysis (33%), and pyruvate metabolism (29%) pathways exhibited significant differential expression among temperature treatments.
Photoperiod also had a strong effect on the expression of genes involved in fatty acid metabolism; 27% of the genes in this pathway differed in expression between photoperiod treatments. We used a binominal test to examine large-scale changes in expression across each pathway. This analysis revealed evidence of concerted expression changes in response to photoperiod across three integrated pathways (fatty acid metabolism, the citric acid cycle, and oxidative phosphorylation; Table 2.3). A disproportionate number of genes exhibited trends towards upregulation in SD across both the citric acid cycle and oxidative phosphorylation, while the reverse pattern (upregulation in LD) was exhibited for the fatty acid oxidation pathways. Additionally, the fatty acid metabolism pathway exhibited concerted expression in response to temperature (upregulation in cold), and the citric acid cycle also exhibited a non-significant trend in this same direction ($P = 0.057$).

*Transcript abundance correlates to enzyme activity*

Expression patterns were largely paralleled by changes in the activities of key enzymes in relevant metabolic pathways (Figure 2.5). We quantified enzyme activities for enzymes involved in fatty acid transport capacity (CPT), fatty-acid oxidation capacity (HOAD), and cellular metabolic intensity (CS). Of these, only HOAD activity exhibited an overall effect of treatment (one-way ANOVA, $P = 0.002$). Enhanced capacity for aerobic thermogenesis was associated with elevated activities of CPT alone ($P = 0.044$), and CPT was also the only enzyme to reveal temperature-sensitive activity patterns ($P = 0.039$). In contrast, CS and HOAD enzyme activities exhibited photoperiod effects, such that LD individuals demonstrated increased activity of both enzymes ($P_{\text{CS}} = 0.039, P_{\text{HOAD}} = 0.001$) but neither was associated with $\Delta M_{\text{sum}}$. We then verified that differences in transcript abundance translated into variation at the protein level by examining the relationship between transcript abundance and enzyme activity using linear regression.

Transcript abundance for the gene encoding CS correlated with CS activity (Figure 2.5). Likewise, each of the three genes encoding HOAD subunits correlated with HOAD activity. However, CPT activity was not correlated with CPT transcript abundance. The effect of treatment on HOAD activity was mirrored by overall treatment effects on transcript abundance for all three HOAD subunit genes, as well as photoperiod-sensitive expression patterns in $HADHA$ and $HADHB$, and temperature-sensitive expression patterns in $HADHB$. CS and CPT transcript abundances did not exhibit effects of acclimation treatment.
DISCUSSION

Phenotypic flexibility allows an organism to optimally match its phenotype to prevailing environmental conditions. This reversible modification of complex physiological traits can be driven by changes in the regulation of genes at several levels of hierarchical organization (Cheviron & Brumfield, 2012). Such regulatory adjustments could occur at any step in the underlying linear pathways, but theory predicts that flux control is disproportionately allocated among the upstream steps (Wright & Rausher, 2010; Eanes, 2011). Our acclimation treatments successfully altered junco thermogenic performance, and we found that these changes in performance were associated with underlying transcriptomic changes in genetically independent but interacting hierarchal pathways. Although juncos in our acclimation treatments only utilized a subset of the potential physiological strategies for elevating aerobic thermogenic performance, regulatory modifications were concentrated in upstream portions of integrated pathways. Additionally, seasonal cues associated with the onset of winter (changes in temperature and photoperiod) induced distinct transcriptomic responses, highlighting the importance of temperature and photoperiod cues in mediating distinct, seasonal responses in avian physiology. Cold-acclimated individuals exhibited elevated thermogenic capacities and transcriptomic signatures indicative of enhanced O\(_2\) delivery as well as enhanced transport and beta-oxidation of fatty acids, suggesting that enhancement of thermogenic capacity is mediated through the changes to upstream, fuel supply portions of the metabolic pathways. These differences in transcript abundances were largely associated with the activities of enzymes that influence flux through the fatty-acid oxidation and citric acid cycle pathways, suggesting that changes in gene regulation in these pathways are translated to functional variation at the proteomic level. Conversely, changes in photoperiod were associated with differential regulation of downstream pathways that influence cellular oxidative capacities. However, photoperiod manipulation was not associated with significant changes in thermogenic performance. Taken together, these results provide important clues into the differential regulation of hierarchal, multistep step pathways that influence complex physiological adjustments to abiotic stressors.

Transcriptomic signatures of muscle growth
As in other bird species (e.g., Swanson, 2010), winter increases in junco thermogenic performance and metabolic rate are often accompanied by pectoralis muscle hypertrophy (Swanson, 1991). However, we found that pectoralis size was not affected by acclimation treatment in this study, thus increases in thermogenic performance were not accompanied by corresponding increases in pectoralis size (Appendix A). Seasonal increases in \( M_{\text{sum}} \) without corresponding increases in pectoralis size have also been documented in populations of American Goldfinches (Spinus tristis; Carey et al. 1978; Liknes et al. 2002). The lack of pectoralis size changes as a driver of improved thermogenic performance further emphasizes the importance of cellular adjustments that enhance lipid oxidation.

Despite the lack of changes in pectoralis mass across the acclimation treatments, we still found transcriptomic responses suggesting the induction of muscle growth. Both metabolic performance and pectoralis size were correlated with the expression of transcriptomic modules containing genes related to muscle development. Temperature-sensitive modules associated with thermogenic capacity included collagen (two in T28), myoblast proliferation (T22), skeletal muscle cell differentiation (T23), transforming growth factor (T29 & T33), and regulation of growth (T38) terms. Considering that pectoralis sizes were similar among treatments, differential expression among these genes could reflect a potential lag between gene expression signaling and ensuing changes in muscle growth.

Very few genes (6%) in the mTOR signaling pathway were differentially regulated among temperature treatments, and those that were differentially expressed were counterintuitively upregulated in warm-acclimated birds. Upstream regulators of the mTOR pathway, myostatin (MSTN) and insulin-like growth factor 1 (IGF1), are major components of muscle growth regulation in mammals (Rennie et al., 2004). These same proteins are central in regulating avian embryonic muscle development (Guernec et al., 2003; Duclos, 2005; Sato et al., 2006; Kim et al., 2007; McFarland et al., 2007), but their role in seasonal regulation of avian adult muscle size is unclear. Swanson et al. (2009) found that increases in pectoralis size of wintering House Sparrows (Passer domesticus) were associated with decreased MSTN expression, but increases in flight muscle size of photo-stimulated White-throated Sparrows were conflictingly accompanied by increased expression of both IGF1 and MSTN (Price et al., 2011). Although IGF1 expression was not detected in our dataset, we found that MSTN expression differed significantly among the acclimation treatments. Contrary to our expectations however,
individuals in the winter-like treatment (cold SD) upregulated MSTN expression relative to other treatment groups (FDR = 0.016), though MSTN was not associated with pectoralis size. Taken as a whole, this indicates that cold-acclimated individuals were not employing any of the major muscle growth elements to enhance thermogenic performance.

Transcriptomic signatures of angiogenesis

We found some evidence to indicate that juncos may be enhancing oxygen delivery to the muscle via angiogenesis. Although the temperature treatments were not associated with differential expression of either hypoxia inducible factors (HIF), and were only associated with differential expression of a single gene (paxillin, PXN) across the vascular endothelial growth factor (VEFG) pathway, VEGFC was upregulated in cold-acclimated individuals and included in Msum-associated module T28. VEGFC is a VEGF family member largely associated with lymphangiogenesis (Joukov et al., 1996). Although it is not clear how increased lymphatic vessels might specifically aid cold individuals, VEGFC has also been shown to increase angiogenesis under certain experimental conditions (Cao et al., 1998; Witzenbichler et al., 1998), including in avian embryonic development (Eichmann et al., 1998). Similar function in cold-acclimated individuals could lead to decreased oxygen diffusion distances at the muscles, enabling increased oxygen delivery for use in oxidative phosphorylation, and ultimately aerobic thermogenesis.

Modifications to fuel supply pathways

In addition to enhancements of O₂ delivery, increased thermogenic performance could also result from regulatory changes that enhance the supplies of metabolic fuels to thermogenic organs. Indeed, enhancements to lipid mobilization have been documented in both migrating and winter-acclimatized birds (Swanson, 2010). In particular, the processes of circulatory transport and muscular uptake have been previously implicated as limiting steps in the avian fatty acid supply pathway (Guglielmo et al., 2002; Jenni-Eiermann & Jenni, 1992; McFarlan et al., 2009; Vock et al., 1996; Weber, 1992). The fatty acid supply pathway has been well characterized for birds (Figure 2.1C; for a thorough review see Price, 2010). To briefly summarize the main steps in this pathway: Before transport, lipids — largely stored as triacylglycerols in both muscle and adipose tissues, but also as phospholipids in the cell membrane — are hydrolyzed by hormone-
sensitive lipases (Ramenofsky, 1990). The resulting fatty acids then bind to albumin in the blood for transport to the muscle or, alternatively, to the liver where they are repackaged as very low density lipoproteins (VLDL) before continuing on to the muscle (Jenni-Eiermann & Jenni, 1992). Muscle lipoprotein lipases can then hydrolyze VLDL in the capillary endothelium to enable uptake of fatty acids by the tissue (Nilson-Ehle et al., 1980; Oscai et al., 1990). Conversion to VLDL is optimal because it allows improved fatty acid transport rate (via directed uptake at the tissues) without altering blood viscosity and subsequently affecting blood oxygen transport (Robinson, 1970; Jenni-Eiermann & Jenni, 1992). Once at the myocyte, transport proteins are similarly required to cross the cellular membrane including plasma membrane-bound fatty acid binding protein (FABPpm) and fatty acyl translocase (FAT/CD36; Luiken et al., 1999; Bonen et al. 2004; McFarlan et al., 2009). Within the cell, transport to the mitochondria occurs with the aid of cytosolic fatty acid binding proteins (FABP; Guglielmo et al., 1998). Here fatty acids are converted to acyl-CoA by acyl-CoA synthetase, and then to acyl-carnitine via carnitine palmitoyl tranferase I (Price, 2010). In the final step of fat transport, translocase (or carnitine acyl transferase) moves acyl-carnitine across the mitochondrial membrane and carnitine palmitoyl tranferase II reforms acyl-CoA for beta-oxidation, ultimately resulting in ATP production (Price, 2010). For these reasons, we targeted the genes in the glycerolipid, glycerophospholipid, and fatty acid metabolism pathways, as well as the many carrier protein genes in the peroxisome proliferator-activated receptor (PPAR) signaling pathway in order to determine if seasonal increases in thermogenic capacity could in part be driven by increased fuel supplies to the pectoralis mitochondria.

Juncos have been shown to seasonally increase muscle lipoprotein lipase during migration (Ramenofsky, 1990; but see Savard et al., 1991), but its role in enhancing circulatory lipid transport capacity during winter remains inconclusive (Swanson, 2010). We did not find evidence of differential regulation in hepatic, endothelial or lipoprotein lipases in response to our acclimation treatments, suggesting that these lipases did not contribute to elevated thermogenic performance. Similarly, increases in the expression of sarcolemmal fatty acid transporters in migrating White-throated Sparrows (Zonotrichia albicollis) suggest that these could also mediate seasonal variation in fatty acid transport capacities (McFarlan, 2007), but fatty acyl translocase (CD36) was not differentially expressed in juncos across the acclimation treatments. Seasonal increases in the abundance of heart-type FABP, which correlates with flight muscle fatty acid
oxidation capacity (Haunerland, 1994), have been documented in the pectoralis of a number of avian species during winter [e.g., Black-capped Chickadees, *Poecile atricapillus*; White-breasted Nuthatches, *Sitta carolinensis* (Liknes et al., 2014)]. This points to the central role of H-FABP in accelerating fatty acid uptake from the circulation (Guglielmo et al., 2002). Although we did not detect H-FABP expression in our dataset, brain-type FABP was upregulated in the pectoralis of cold-acclimated individuals, suggesting that it may also play a critical role in the uptake of fats by skeletal muscle myocytes in juncos. Finally, seasonal increases in the enzymes facilitating fat transport across the mitochondrial membrane (carnitine acyl CoA transferases) have also been shown in migrating Semipalmated Sandpipers (*Calidris pusilla*; Driedzic et al., 1993) and Western Sandpipers (Guglielmo et al., 2002) but their role in avian metabolic performance outside of migration (i.e. for winter birds) is unknown (Swanson, 2010). Though we did not find differential expression among any of the genes encoding the three enzymes that facilitate transport across the mitochondrial membrane (CPT1, CPT2, and carnitine-acylcarnitine translocase), we did find a trend towards increased CPT enzyme activity in cold-acclimated individuals ($P = 0.039$). Taken together, these results indicate that increased junco thermogenic performance was not enabled by wholesale changes in gene expression across the lipid fuel supply pathway. Rather, while upstream components of the supply pathway did not exhibit differential regulation (i.e. mobilization of fatty acids and circulatory transport), downstream components were apparently modified to enhance lipid uptake in response to temperature (i.e. myocyte and mitochondrial uptake).

Apart from limitations to the machinery for fatty acid transport and oxidation, there is also evidence that rates of mobilization and utilization are strongly influenced by fatty acid type. Differential mobilization of fatty acids from the adipocyte occurs due to differences in the molecular structure of fatty acid species (i.e. length, saturation, and relative location of double bonds), and is thought to be a conserved feature of adipose tissue (Raclot et al., 1995; Raclot, 2003). Accordingly, selective mobilization has been shown *in vitro* from the adipocytes of Ruff (*Philomachus pugnax*) and White-crowned Sparrows, regardless of migratory or exercise state (Price et al., 2008; Price, 2009). Fatty acid type also influences lipid oxidation rates in avian muscle (Price, 2009). Deposition of fatty acids in the adipose tissues is largely dependent on the types of fats consumed in the diet (Pierce & McWilliams, 2005), and experimental manipulation of dietary fats has been shown to influence whole-animal aerobic performance in birds (Pierce et
al., 2005; Price & Guglielmo, 2009; McWilliams & Pierce, 2006). While it is possible that selective mobilization and/or utilization of fatty acids contributed to this variation in performance, the mechanisms underlying this effect are yet unclear (Price, 2010). Because juncos were fed a uniform diet for the 8-week duration of the experiment, we do not expect that adipose stores differed greatly in fatty acid content among treatment groups. Therefore selective mobilization and/or utilization of fatty acids are not likely to explain the differences in thermoregulatory performance shown here.

**Enhanced cellular aerobic capacity**

Regulatory changes to genes involved in lipid oxidation and oxidative phosphorylation are known to be important for thermogenic capacity in mammals (Cheviron et al. 2012, 2014). We found similar patterns in juncos, although our results emphasize the importance of the former, suggesting that regulatory changes that enhance thermogenic performance in juncos are made to upstream portions of these integrated pathways. Module T32 (negatively correlated with $\Delta M_{\text{sum}}$, Table 2.1) included 1 subunit of NADH dehydrogenase (Complex I – NDUFB9), which was downregulated in cold-acclimated individuals, but this is the only member of the oxidative phosphorylation pathway to exhibit temperature-sensitive differential expression. However, it is unclear how differential regulation of a single component of NADH dehydrogenase — a large complex composed of 41 avian subunits — could influence the function of Complex I and subsequently the electron transport chain as a whole. Increased metabolic performance in cold-acclimated juncos was instead associated with heightened expression of genes related to the beta-oxidation of fatty acids, the major source of fuel for sustained avian thermogenesis. For instance, temperature-sensitive transcriptional module T31 was enriched for fatty-acyl-CoA biosynthesis and HOAD activity GO terms, while the expression of T33 was negatively correlated with thermogenic capacity and included terms related to fatty acid elongation. Roughly one third of the genes involved in the fatty acid metabolism pathway were differentially expressed among temperature treatments. In combination with trends in CPT activity, temperature-sensitive expression patterns in the genes involved in fatty acid oxidation are largely indicative of enhanced lipid oxidative capacity in cold-acclimated individuals.

Cold-acclimated birds also downregulated 25% of the genes involved in glycolysis, indicating a preference for lipid over carbohydrate fuels to enhance thermogenic capacity. This
preference has similarly been shown in Ruff and in rodents under conditions of prolonged shivering (Vaillancourt et al., 2005; 2009; Vaillancourt & Weber, 2007). Recent in vitro studies of House Sparrow flight muscle mitochondria suggest that selection of fatty acid over glycolytic fuels is likely due to suppression of pyruvate oxidation rather than relative lipid catalytic potentials (Kuzmiak-Glancy & Willis, 2014). Although we found temperature-sensitive expression patterns in 29% of the genes in the pyruvate metabolism pathway, cold-acclimated birds downregulated the expression of four of these genes while upregulating the expression of the remaining two genes. Thus these regulatory changes were not wholly indicative of pyruvate oxidation suppression in cold-acclimated individuals.

**Enhanced catabolic enzyme activity**

For a number of birds, winter increases in $M_{\text{sum}}$ are associated with increased cellular metabolic and lipid oxidation capacities of the pectoralis, as inferred from citrate synthase (CS), cytochrome c oxidase (COX), and beta-hydroxyacyl CoenzymeA dehydrogenase (HOAD) enzyme activities (e.g., Guglielmo et al., 2002; Vézina & Williams, 2005; Zheng et al., 2008; Liknes & Swanson, 2011). Of the three muscle metabolic enzyme activities that we measured, enhanced capacity for aerobic thermogenesis was only associated with elevated activities of CPT. CPT was also the only enzyme to reveal temperature-sensitive activity patterns, while CS and HOAD enzyme activities exhibited photoperiod-sensitive patterns. Transcript abundances of the enzyme-encoding genes were correlated with enzyme activities for CS and HOAD but not for CPT.

Enzyme activity patterns largely paralleled concomitant changes in expression across the corresponding metabolic pathways. Increased thermogenic capacity among populations of deer mice is associated with increased HOAD and COX activities and concerted expression patterns across both the fatty acid beta-oxidation and oxidative phosphorylation pathways (Cheviron et al., 2012; 2014). Similarly, CPT is considered to be the rate-limiting step in fatty acid oxidation, and we found that increased CPT activity in cold-acclimated individuals was accompanied by concerted expression across the fatty acid oxidation pathway in response to temperature. Likewise, in response to photoperiod we found concomitant changes across fatty acid oxidation, the citric acid cycle, and oxidative phosphorylation pathways, and these patterns corresponded with increased CS and HOAD activities among the same treatments. Together, these results
suggest differential effects of temperature and photoperiod on the two ends of the pathway: supply is manipulated in response to temperature with seemingly little effect further downstream, while photoperiod has less of an effect to upstream supply steps and largely modifies downstream oxidative capacity.

CONCLUSIONS

The mechanisms underlying seasonal phenotypic flexibility remain uncertain, but recent advances in genomic technologies have enabled high-throughput characterization of the distinct and concurrent physiological adjustments an organism concurrently employs when responding to changes in abiotic stressors. Previous studies that surveyed a limited number of parameters suggest that individuals can increase aerobic performance by making modifications to three broad levels of physiological organization – enhancements to thermogenic organ size, oxygen delivery, and cellular aerobic capacity (Figure 2.1). We found evidence that juncos are using only a small subset of these potential mechanisms to enhance thermogenic performance in response to experimental manipulations of their abiotic environment. This limited response could be due, in part, to the strength of the stressor that we employed and the subsequent strength of the physiological response that was required to offset it. The magnitude of increase by cold-acclimated individuals is representative of seasonal changes to $M_{\text{sum}}$ documented in free-living passerines (usually 10-50% from summer to winter; Swanson, 2010). However, changes in thermogenic performance shown here are likely less dramatic than those juncos sustain in the wild. $M_{\text{sum}}$ varies with environmental temperature (Swanson, 2010) and the temperature employed for cold-acclimation (3°C) was milder than what juncos likely experience throughout much of their winter range. We suggest that, in the face of maintaining energetically expensive metabolic machinery, juncos may only engage the minimum degree of change that is necessary to meet their specific aerobic demands. Given the modular structure of trait components that influence aerobic performance, efficient regulation of metabolic flexibility may involve differential regulation of trait components according to the relative costs and benefits of particular adjustments. Although we successfully increased thermogenic performance through exposure to relatively mild temperatures, it seems likely that lower temperatures would stimulate an even more pronounced metabolic response, perhaps uncovering additional transcriptomic signatures as well. Subsequent investigations to tease apart the relationship between stimulus
strength and pathway-level responses will help to elucidate which physiological changes might be more or less costly to induce, and how more severe stresses are mediated.

Changes in thermogenic performance were elicited within a relatively short time period, with cold-acclimated individuals already showing incremental increases in thermogenic capacity when measured midway through the acclimation period (at 3 weeks; Swanson et al., 2014), indicating that temperature is a proximal cue that can be integrated to make rapid physiological adjustments. This is not surprising as temperatures can be highly variable in the temperate zone—in contrast to daylength—and birds must quickly respond to precipitous drops in temperature that could serve as selective events (e.g., polar vortices; Bumpus, 1898). It therefore follows that these changes would occur at the level of fuel mobilization and oxygen utilization, representing a rapid vehicle for response. In comparison, relatively immediate (and temporary) responses in the oxygen cascade come in the form of accelerated breath rate and enhanced cardiac output, which require no physiological reorganization, while changes that increase vasculature and mitochondrial abundance represent more persistent changes that are achieved over prolonged time periods. We found that temperature-sensitive transcriptional modules associated with increased thermogenic performance showed upregulation of genes related to beta-oxidation and oxygen utilization. These modules also contain genes related to muscle development, possibly reflecting longer-term changes in muscle size and structure; however, individuals did not exhibit corresponding pectoralis hypertrophy. Taken together these results suggest differential regulation of pathway components that are associated with short-term and long-term phenotypic adjustments. It remains to be seen how quickly birds can mount responses to temperature and photoperiod manipulations, and which pathway components respond most rapidly to these changes. The unpredictable nature of adverse weather events suggests that temperate endotherms can achieve metabolic flexibility quite rapidly, but further work is needed to characterize the metabolic — and corresponding transcriptomic — responses associated with such rapid physiological adjustments in order to determine whether the same physiological mechanisms are employed.

Finally, many previous investigations of avian seasonal metabolic flexibility have focused on the migratory period. Avian migration is an extreme athletic feat associated with dramatic reorganization of multiple physiological systems (van Gils & Piersma, 2011), yet it represents an acute stressor that lasts for a relatively short period of time (on the order of days).
This is in stark contrast to the chronic stress of winter, which lasts six months or more in the temperate north. Therefore, while it may be possible for birds to concurrently maintain many physiological responses for the duration of a migratory event, it could prove too costly to sustain these same responses for an entire winter season. Further investigations are thus needed to determine how free-living birds employ these mechanisms of metabolic flexibility amidst winter conditions.

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CHAPTER 2 TABLES & FIGURES

TABLE 2.1. Associations between transcriptional modules and thermogenic performance ($\Delta M_{\text{sum}}$) or pectoralis size ($M_p$). Number of genes and proportion of variance explained by PC1 are shown for each module. Modules are illustrated in Figure 2.3. Direction of correlation (positive or negative), r-squared, and p-values from linear regressions. NS = uncorrected $P > 0.05$. Values in bold are significant after Bonferroni correction from multiple tests. Modules A19 and P21 are comprised of the same genes.

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<th>Module</th>
<th>No. genes</th>
<th>Prop. variance</th>
<th>Thermo. performance</th>
<th>Pectoralis size</th>
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<td></td>
<td></td>
<td></td>
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<td>$R^2$</td>
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<td>NS</td>
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TABLE 2.1 (continued).

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<th>Module</th>
<th>No. genes</th>
<th>Prop. variance</th>
<th>Thermo. performance +/-</th>
<th>$R^2$</th>
<th>$P$</th>
<th>Pectoralis size +/-</th>
<th>$R^2$</th>
<th>$P$</th>
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<td>12</td>
<td>0.82</td>
<td>-</td>
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<td>T25</td>
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<td>-</td>
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<td>$&lt;0.001$</td>
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<tr>
<td>T27</td>
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<td>+</td>
<td>0.16</td>
<td>0.007</td>
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</tr>
<tr>
<td>T28</td>
<td>41</td>
<td>0.50</td>
<td>+</td>
<td>0.24</td>
<td>$&lt;0.001$</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>T29</td>
<td>17</td>
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<td>-</td>
<td>0.15</td>
<td>0.008</td>
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<td>-</td>
<td>0.18</td>
<td>0.004</td>
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<td>T37</td>
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<td>0.82</td>
<td>+</td>
<td>0.14</td>
<td>0.010</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>T38</td>
<td>15</td>
<td>0.91</td>
<td>-</td>
<td>0.16</td>
<td>0.007</td>
<td>+</td>
<td>0.10</td>
<td>0.026</td>
</tr>
</tbody>
</table>
**TABLE 2.2.** Summarized enrichment results for temperature-sensitive modules of differentially expressed genes associated with thermogenic capacity. Individual modules were significantly enriched for terms related to categories indicated in black.

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</tbody>
</table>
TABLE 2.3. Targeted pathway analyses. Number of unique genes in each pathway, number detected within our dataset, and number significantly differentially expressed (DE) across temperature and across photoperiod treatments. *P*-values shown from binomial tests performed to evaluate the presence of concerted expression across the pathway (significant values in bold).

<table>
<thead>
<tr>
<th>Pathway</th>
<th>No. unique genes</th>
<th>No. genes in dataset</th>
<th>Temperature DE</th>
<th>Temperature <em>P</em></th>
<th>Photoperiod DE</th>
<th>Photoperiod <em>P</em></th>
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<tr>
<td>Citric acid cycle</td>
<td>14</td>
<td>14</td>
<td>1</td>
<td>0.057</td>
<td>0</td>
<td>0.002</td>
</tr>
<tr>
<td>Fatty acid metabolism</td>
<td>15</td>
<td>15</td>
<td>4</td>
<td>0.035</td>
<td>4</td>
<td>0.035</td>
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<tr>
<td>Glycerolipid metabolism</td>
<td>16</td>
<td>15</td>
<td>4</td>
<td>0.302</td>
<td>6</td>
<td>0.119</td>
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<tr>
<td>Glycerophospholipid metabolism</td>
<td>28</td>
<td>25</td>
<td>4</td>
<td>1.000</td>
<td>5</td>
<td>0.690</td>
</tr>
<tr>
<td>Glycolysis</td>
<td>26</td>
<td>24</td>
<td>8</td>
<td>0.152</td>
<td>4</td>
<td>0.152</td>
</tr>
<tr>
<td>mTOR signaling</td>
<td>33</td>
<td>31</td>
<td>2</td>
<td>0.281</td>
<td>4</td>
<td>0.720</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
<td>97</td>
<td>84</td>
<td>1</td>
<td>1.000</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PPAR signaling</td>
<td>38</td>
<td>32</td>
<td>4</td>
<td>0.215</td>
<td>5</td>
<td>0.377</td>
</tr>
<tr>
<td>Pyruvate metabolism</td>
<td>21</td>
<td>21</td>
<td>6</td>
<td>1.000</td>
<td>5</td>
<td>0.189</td>
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<td>VEGF signaling</td>
<td>24</td>
<td>24</td>
<td>1</td>
<td>0.839</td>
<td>1</td>
<td>0.152</td>
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</table>
FIGURE 2.1. (A) Modifications to enhance avian metabolic performance can occur via increases to the size of thermogenic organs (hypertrophy of pectoralis), to oxygen delivery (angiogenesis), or to cellular aerobic capacity. These modifications are achieved by altering the expression of genes in associated pathways, bulleted below. (B) Each pathway is a network of many interacting genes; those comprising the citric acid cycle are shown in the inset. (C) The trajectory of lipids (gray circles) from adipocyte to myocyte and into the mitochondria with the aid of enzymes (in italics) and transporter proteins (white boxes). Solid lines represent enzymatic conversion of lipid forms; dotted lines represent transport. Acyl carnitine (AC); carnitine-acylcarnitine translocase (CACT); fatty acid translocase (CD36); carnitine palmitoyl transferase (CPT); fatty acid (FA); fatty acid binding protein (FABP); plasma-membrane bound fatty acid binding protein (FABPpm); hormone-sensitive lipase (HSL); triacylglyceride (TAG); very low density lipid (VLDL).

A

1. **Thermogenic organ size**
   - mTOR signaling
   - PI3K-Akt signaling

2. **Oxygen delivery**
   - HIF-1 signaling
   - VEGF signaling

3. **Cellular aerobic capacity**
   - Citric acid cycle
   - Fatty acid β-oxidation
   - Glycerolipid metabolism
   - Glycerophospholipid metabolism
   - Glycolysis
   - Oxidative phosphorylation
   - PPAR signaling
   - Pyruvate metabolism
FIGURE 2.2. Change in thermogenic performance ($\Delta M_{\text{sum}}$) from before and after six-week acclimation treatments.
FIGURE 2.3. Correlated transcriptional modules. (A) Clustering of 1882 transcripts with significant treatment effects into 47 modules. (B) Clustering of 1325 transcripts with significant photoperiod treatment effects into 51 modules. (C) Clustering of 756 transcripts with significant temperature treatment effects into 38 modules. For each, ordered genes listed along x- and y-axes with pair-wise Pearson correlation coefficients shown in color (red = highly correlated; blue = highly uncorrelated). Red blocks along diagonal indicate clusters of genes that are highly co-regulated in expression pattern.
FIGURE 2.4. Number of genes differentially expressed among all treatments, temperature treatments, and photoperiod treatments.
FIGURE 2.5. Metabolic enzyme activity and corresponding transcript abundance for the enzyme-encoding genes. (A) CPT activity and $CPT1\alpha$ abundance. (B) CS activity and $ACLY$ abundance. (C) HOAD activity and $HADH$ (blue), $HADHA$ (black), and $HADHB$ (gray) abundance. Lines fit with linear regression for significant associations, $P$-values shown.
CHAPTER 3

SIGNATURES OF NATURAL SELECTION IN THE MITOCHONDRIAL GENOMES OF TACHYCINETA SWALLOWS AND THEIR IMPLICATIONS FOR LATITUDINAL PATTERNS OF THE ‘PACE OF LIFE’

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ABSTRACT

Latitudinal variation in avian life histories can be summarized as a slow-fast continuum, termed the ‘pace of life’, that encompasses patterns in life span, reproduction, and rates of development among tropical and temperate species. Much of the variation in avian pace of life is tied to differences in rates of long-term metabolic energy expenditure. Given the vital role of the mitochondrion in metabolic processes, studies of variation in the mitochondrial genome may offer opportunities to establish mechanistic links between genetic variation and latitudinal ‘pace of life’ patterns. Using comparative genomic analyses, we examined complete mitochondrial genome sequences obtained from nine, broadly distributed Tachycineta swallow species to test for signatures of natural selection across the mitogenome within a phylogenetic framework. Our results show that although purifying selection is the dominant selective force acting on the mitochondrial genome in Tachycineta, three mitochondrial genes (ND2, ND5, and CYTB) contain regions that exhibit signatures of diversifying selection. Two of these genes (ND2 and ND5) encode interacting subunits of NADH dehydrogenase, and amino residues that were

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inferred to be targets of positive selection were disproportionately concentrated in these genes. Moreover, the positively selected sites exhibited a phylogenetic pattern that could be indicative of adaptive divergence between “fast” and “slow” lineages. These results suggest that functional variation in cytochrome b and NADH dehydrogenase could mechanistically contribute to latitudinal ‘pace of life’ patterns in *Tachycineta*.

**INTRODUCTION**

Over the past century, biologists have accumulated a multitude of comparative data on latitudinal patterns in avian life histories. One of the strongest generalizations emerging from this large body of work is that tropical birds tend to exhibit long life spans and low reproductive and developmental rates compared to their temperate counterparts (Lack, 1947; Ricklefs, 1969; James, 1970; Ricklefs, 1976; Skutch, 1976). More recently, these general patterns have been summarized as a “slow-fast” continuum in the ‘Pace of Life’ (POL) hypothesis of latitudinal variation in avian life histories (Ricklefs & Wikelski, 2002; Wiersma *et al.*, 2007b). The mechanistic underpinnings of latitudinal pace of life patterns are poorly understood, but recent comparative studies suggest that much of this variation may be linked to the rate of long-term metabolic energy expenditure (Wiersma *et al.*, 2007a). In general, temperate-zone species tend to have higher basal metabolic rates, and higher aerobic capacities than closely related tropical species (Wiersma *et al.*, 2007a,b), both of which may be necessary to support an increased pace of life in the temperate zone. For example, increased energetic investment may be necessary to support faster developmental rates or greater provisioning rates for larger clutches in temperate zone birds. Increased rates of aerobic metabolism can also increase rates of cellular oxidative damage, which may contribute to the reduced life span of temperate zone species (Vleck *et al.*, 2007; Monaghan *et al.*, 2008). Given the central role of the mitochondrion in cellular metabolic processes, mitochondrial variants that are segregating within and among closely related species may affect both cellular and whole-organism metabolic performance (Ballard & Melvin, 2010; Toews *et al.*, 2013), and therefore, studies of mitochondrial variation may offer opportunities to begin to link genetic variation to latitudinal ‘pace of life’ patterns.

The vertebrate mitochondrial genome contains 13 protein-coding genes, all of which are central to the process of oxidative phosphorylation and aerobic metabolism. Moreover, in animals, the mitochondrial genome exhibits an elevated substitution rate, smaller effective
population sizes, and reduced recombination rates compared with the nuclear genome, all of which facilitate rapid evolution and selective pressures that favor compensatory evolutionary changes in interacting nuclear genes (Rand *et al*., 2004; Burton & Barreto, 2012; Osada & Akashi, 2012). As a result, the mitochondrial genome represents an important potential target of natural selection in taxa that are distributed across environmental gradients. Indeed, recent empirical studies have documented a number of putative examples of local adaptation in mitochondrial variants of species that are distributed along latitudinal and elevational gradients (Fontanillas *et al*., 2005; Cheviron & Brumfield, 2009; Hassanin *et al*., 2009; Ribeiro *et al*., 2010; Scott *et al*., 2011; Toews *et al*., 2013). In birds specifically, mitochondrial variants have been linked to differences in kinetic properties of cytochrome c oxidase (Scott *et al*., 2011), and the degree of coupling efficiency between substrate oxidation and ADP phosphorylation (Toews *et al*., 2013), both of which can influence overall cellular aerobic capacity. Similarly, a wide variety of mitochondrial variants have been linked to metabolic disorders and the rate of production of reactive oxygen species in humans (Wallace, 2005).

Lineages with broad latitudinal distributions are well suited for examining the mechanistic underpinnings of latitudinal POL patterns. *Tachycineta* is a monophyletic genus of nine swallow species (Dor *et al*., 2012) that are continuously distributed from Alaska to Cape Horn (Figure 3.1A). This exceptionally broad latitudinal distribution makes it possible to make multiple comparisons of temperate and tropical species (Figure 3.1B). Multiple aspects of latitudinal variation in the life histories of *Tachycineta* species conform to POL predictions: species that occur nearer to the tropics tend to have smaller clutches, higher investment per offspring, slower developmental rates, and longer nestling periods (Appendix C). Additionally, preliminary evidence suggests that tropical *Tachycineta* species also have lower adult provisioning rates (Ardia *et al*., unpub. data) and generally higher adult survival rates (Winkler *et al*., unpub. data).

Here, we utilized a suite of comparative genomic approaches to analyze complete mitochondrial genome sequences from each of the nine *Tachycineta* species. Using a phylogenetic framework, we specifically tested for evidence of adaptive mitochondrial divergence among lineages along a “slow-fast” POL continuum. We summarized interspecific life-history variation as a multivariate POL score to test the prediction that mitochondrial variation is associated with latitudinal patterns of POL variation in *Tachycineta*. First, we
predicted that substitutions at codon positions that are under positive diversifying selection would disproportionately occur along branches that separate taxa that exhibit the greatest differences in POL scores (i.e., adaptive divergence at mitochondrial genes should be associated with divergence between sister taxa that differ strongly in their life histories). Second, we predicted that the “slowest”, most derived species would also exhibit the largest accumulation of derived, positively selected substitutions. We show that although purifying selection is the dominant selective force acting on the mitochondrial genome in *Tachycineta*, 3 of 13 mitochondrial genes contain regions that exhibit signatures of diversifying selection. These three genes (*ND2*, *ND5* and *CYTB*) encode two enzymes (NADH dehydrogenase and cytochrome b) that are central to cellular metabolic processes. Moreover, the number of positively selected sites was positively correlated with POL score, demonstrating that derived species with slow life histories (high POL scores) are characterized by relatively high levels of adaptive variation at these mitochondrial genes. Taken together, these results suggest that mitochondrial variation could potentially contribute to metabolic differences that are related to latitudinal pace of life patterns in *Tachycineta*.

**METHODS**

*Taxon sampling and mitogenomic data*

We downloaded complete mitochondrial genome sequences for each of the nine *Tachycineta* species and one outgroup, *Progne chalybea*, from GenBank (Table 3.1). From these mitogenomic sequences, we extracted protein-coding regions, and constructed gene-specific alignments using Sequencher (ver. 5.0).

*Summarizing life-history variation*

To determine where each *Tachycineta* species fell on the POL continuum, we gathered life-history data for traits that were measured in all nine species from the literature (clutch size, egg size, growth rate, nestling period, adult body mass, and absolute latitude; Appendix C). Because the POL hypothesis assumes a continuous axis from “slow” to “fast” life histories, we chose to summarize variation among traits using a principal component analysis, rather than classifying each species as either temperate or tropical. We performed a principal component analysis on the z-normalized values for each trait and retained PC components with eigenvalue
>1 (Kaiser, 1960), which proved true of PC1 alone. We used the resulting PC1 scores, which we refer to as the POL score, as a continuous variable with which to perform ancestral state reconstruction. To do this, we reconstructed ancestral states using R (ver. 2.15.0–R Development Core Team, 2012) with ancestral character estimation (*ace*) based on restricted maximum likelihood and a model of Brownian motion (Paradis, 2006). We calculated absolute changes in POL score between the terminal branches and their ancestral nodes for use in determining whether substitutions occur disproportionately along branches with greater POL differences. We then used the POL scores to predict which species are the most derived from the temperate, basal lineage (Appendix D). Species with the highest POL score are considered “slow” and derived, and thus are likely to exhibit the highest rate of positive selection; while species with the lowest POL scores are considered to be “fast” and are likely to exhibit low rates of positive selection.

To test this prediction, we used a generalized linear model with Poisson distribution for count data (*i.e.* number of substitutions) and verified that overdispersion was not present. We chose not to test for phylogenetic signal among these variables as estimates of phylogenetic signal among datasets of such a limited size (*e.g.*, 9 species) lack power and can therefore be inaccurate (Boettinger *et al.*, 2012). We therefore did not employ phylogenetically independent contrasts (PICs), as use of PICs without proper assessment of phylogenetic signal can be inappropriate or misleading (Revell, 2010).

*Detecting selection*

We examined each of the thirteen protein-coding genes for evidence of negative selection using the single likelihood ancestor counting (SLAC) method performed with the HyPhy software package (Kosakovsky Pond *et al.*, 2005). We estimated non-synonymous to synonymous substitution (dN/dS) ratios conditioned using neighbor-joining trees, and we identified gene-specific nucleotide substitution models using a model selection procedure performed in HyPhy that tests 203 hierarchical time-reversible substitution models. The most appropriate rate matrix was selected using both likelihood ratio tests (LRTs) and AIC selection. We identified sites under purifying selection at a significance level of \( P \leq 0.05 \). Although SLAC can also be used to detect signatures of positive selection, it does not allow for variation in dN/dS ratios across branches and individual codons. We therefore employed two additional methods with greater sensitivity for detecting positive selection.
First, we implemented branch-site models, which allowed us to test for positive selection at individual codons using a modified branch-site model A test 2 (Zhang et al., 2005) in the program CODEML (PAML 4.6; Yang, 2007). For each of the thirteen genes, we used LRTs to compare the null neutral model (model = 2, NSsites = 2, fix_omega = 1, omega = 1) against alternate models of branch-specific positive selection (model = 2, NSsites = 2, fix_omega = 0, omega = 1.5). Each branch (terminal and internal) served independently as the foreground lineage and we tested sites within the foreground lineage for selection. To control for phylogenetic uncertainty, we conditioned substitution models using four different trees for each of the models. Input trees were modified from the consensus mitochondrial tree (Cerasale et al., 2012), the consensus nuclear tree comprised of 16 nuclear loci (Dor et al., 2012), and two nuclear trees for which we coded polytomies at each of two nodes with posterior probability less than 0.85 (Figure 3.1B). The mitochondrial tree differs from the nuclear tree in that it is composed of two distinct clades—a North American/Caribbean clade and a Central/South American clade—whereas the nuclear tree does not have this distinction, and thus some of the sister relationships, such as that of *T. euchrysea*, vary between the nuclear and mitochondrial phylogenies. Branch lengths were estimated for each gene in CODEML (Yang, 1996). Positive selection was inferred if the *P*-value derived from the LRT between the neutral and branch-specific models was significant (at *P* ≤ 0.05) based on the chi-squared asymptotic distribution. Bayesian empirical Bayes (BEB; Yang et al., 2005) procedures were performed for branches exhibiting dN/dS >1 to identify individual sites subject to diversifying selection.

Branch-site models of this kind can be limiting due to the necessary specification of foreground lineages and the assumption that dN/dS = 1 for all background lineages (Kosakovosky Pond et al., 2011). We therefore also examined each of the thirteen genes for signatures of episodic diversifying selection using a mixed effects model of evolution (MEME; Murrell et al., 2012) performed with HyPhy. This method allows the distribution of the estimated dN/dS ratio to vary from site to site and from branch to branch and, in so doing, identifies individual episodes of positive selection that affect only a subset of lineages, which are often overlooked using traditional methods that assume dN/dS is shared by all sites in the alignment or that selective pressures are constant throughout time (Murrell et al., 2012). We identified positively selected sites at a significance level of *P* ≤ 0.05.
Ancestral reconstruction

Following detection of selection, we identified amino acid substitutions for each of the ten species at all sites exhibiting signatures of positive selection and mapped substitutions to the gene tree (the consensus mitochondrial tree; Cerasale et al., 2012). To do this, we reconstructed ancestral states with ancestral character estimation (ace) using R (ver. 2.15.0–R Development Core Team 2012) with three standard models: equal rates, symmetric, and all rates different (Paradis, 2006). We compared alternative models using LRTs at a significance level of \( P \leq 0.05 \). We calculated the degree of physicochemical similarity of each substitution using Grantham’s difference (\( D \)), a measure of amino acid exchangeability based on volume, weight, polarity, and carbon composition (Grantham, 1974). Using \( D \), we then classified amino acid substitutions as conservative (0-50), moderately conservative (50-100), moderately radical (100-150), or radical (>150) according to the criteria proposed by Li et al. (1984). Due to our specific interest in sites that were inferred to be under positive selection and the prohibitively large number of negatively selected sites, we did not repeat this procedure for sites exhibiting signatures of negative selection.

Mapping selected residues

To provide insight regarding the physical location of sites under selection within the protein, we mapped all sites to topologies of the transmembrane segments of electron transport proteins. We predicted topologies based on the consensus of multiple models (Granseth et al., 2006; Hessa et al., 2007; Bernsel et al., 2009) using the software TOPCONS (http://topcons.ebr.su.se/). Topology of these proteins is largely conserved across eukaryotes (Degli Eposti et al., 1993; Brandt 2006) and so we anticipated that the overall architecture of the proteins would be conserved at the species level. We therefore randomly chose T. leucorrhoa as the query sequence. We then mapped selected sites to predicted topologies for genes exhibiting signatures of both positive and negative selection.

RESULTS

Pace of life (POL) scores, representing life-history variation among Tachycineta species, show a continuous distribution from “slow” (high POL values) to “fast” (low POL values) with T. stolzmanni and T. bicolor at each end of the spectrum, respectively (Table 3.1). As predicted,
we found a significant correlation between the POL score and the number of amino acid substitutions for a given taxon (Poisson regression, $P = 0.05$), such that “slow”, derived species with high POL scores ($T. albilinea$, $T. albiventer$, and $T. stolzmanni$) exhibited the highest degree of positive selection (Figure 3.2). However, we found equivocal evidence that positively selected substitutions occur disproportionately along branches separating taxa with the greatest differences in POL score (Appendix D).

Signatures of negative selection were pervasive across the mitogenomes of *Tachycineta* species. We identified 320 negatively selected sites across all 13 protein-coding genes. The percentage of codons under negative purifying selection within a single mitochondrial gene averaged 7.5% with *COX1* exhibiting the highest (11%) and *ATP8* the lowest (3%) rates of purifying selection (Table 3.2).

Signatures of positive diversifying selection were much less prevalent across the mitogenome than those of purifying selection and were concentrated in three genes: *ND2*, *ND5* and *CYTB*. Using branch-specific models, we detected signatures of positive selection in 2 genes, both of which are subunits of NADH dehydrogenase (Appendix E). Two codons in *ND5* were inferred to be under positive selection within the *T. stolzmanni* lineage, site 16 (92.5% probability) and site 545 (55.2%), and this result was robust across tree topologies (Table 3.3). *ND5* also exhibited signatures of positive selection within branch B at site 27 (86.8-88.7%), and this result was also robust across the three tree topologies to which branch B is common (Phylogenies 2-4). Additionally, two codons in *ND2* exhibited signatures of positive selection along branch A: sites 222 (99.4%) and 327 (79.5%). This model could only be tested with Phylogeny 1 as this branch is not common to the other three trees.

Using MEME, we identified episodic positive selection in 2 of 13 genes: *CYTB* (site 161) and *ND5* (sites 27, 67, 190, and 519; Table 3.2). Thus, 1 of the 10 sites (*ND5* 27) was identified by both methods. Additionally, one site each in *ND2* and *ND4* exhibited signatures of positive selection that were marginally significant (sites *ND2* 222, $P = 0.07$; *ND4* 192, $P = 0.06$).

The equal rates model proved best for all instances of ancestral state reconstruction (Appendix F). Positively selected substitutions produce polarity changes in the resulting amino acids for 3 of the 5 sites identified by branch-site models and in 2 additional sites of episodic selection; the remaining 3 substitutions do not result in polarity changes (Figure 3.3). The physicochemical dissimilarity of the amino acid change was categorized as conservative for 4
substitutions, moderately conservative for 3 substitutions, and moderately radical for 1 substitution (ND5 519, D = 103; Figure 3.3). When mapped to the phylogeny, the outgroup alone exhibited differences for sites ND4 192 and ND5 67 (Appendices G & H), and they are therefore not considered for the remainder of this work.

Consistent with other vertebrate studies (e.g., Whitehead, 2009; Birrell & Hirst 2010), predicted protein topologies contained 8, 11, and 14 transmembrane regions in CYTB, ND2, and ND5, respectively. However, mammalian ND5 has been shown to contain an additional one to two transmembrane helices (da Fonseca et al., 2008; Bridges et al., 2011; Lemay et al., 2013). All of the sites that exhibited signatures of episodic positive selection in the MEME analyses were restricted to the predicted loop regions of their respective proteins (Figure 3.4). However, 2 of the sites identified by branch-site models were located wholly within transmembrane regions (ND2 327 and ND5 16). Sites of negative selection occurred in both loop and transmembrane regions for all three genes that also exhibited positive selection (Figure 3.4).

**DISCUSSION**

*Mitochondrial variation and latitudinal patterns in the ‘pace of life’*

Latitudinal variation in ‘pace of life’ (POL) has been well documented in a wide variety of taxa (Ricklefs & Wikelski, 2002; Wiersma et al., 2007b). However, little is known about the mechanistic underpinnings of POL variation within and among species. Several lines of evidence suggest that much of this variation may be functionally linked to differences in rates of metabolic energy expenditure. Given the vital role of the mitochondrion in cellular metabolism, the mitochondrial genome therefore represents a suitable target to begin investigations of the genetic basis underlying life-history variation. Using a genus of broadly distributed passerines, we identified mitogenomic regions that may contribute to a mechanistic understanding of this macroecological pattern. Although purifying selection is the dominant selective force acting on the mitochondrial genome in *Tachycineta*, we found evidence of diversifying selection in three mitochondrial genes.

The positively selected sites exhibited a phylogenetic pattern that is consistent with adaptive divergence between “fast” temperate and “slow” tropical lineages. Derived substitutions at both positively selected sites in ND2 were shared by the three equatorial species whose life-history variation is at the “slow” end of the spectrum (*T. albilinea*, *T. albiventer*, and *T.*
Moreover, substitutions at two positively selected sites within ND5 were unique to *T. stolzmanni* alone, which is likely to be the “slowest” of the *Tachycineta* species (Table 3.1), exhibiting both the lowest fecundity and lowest growth rate of the genus (Stager *et al.*, 2012). The remaining sites under positive selection were distributed across the phylogeny resulting in an overall pattern consistent with the POL hypothesis, (*i.e*., “slow” lineages were characterized by more positively selected substitutions than “fast” lineages; Figure 3.2).

The high rate of positively selected substitutions within *T. stolzmanni* could alternatively be explained by the narrow distribution and relatively small effective population size of this lineage. However, this pattern does not hold across the genus: the two island species, *T. euchrysea* and *T. cyaneoviridis*, are characterized by even more restricted range sizes and smaller populations — listed as vulnerable and endangered, respectively (IUCN, 2013) — yet they do not show the same elevation in positively selected sites. In fact, *T. cyaneoviridis* does not exhibit a single positively selected substitution, yielding this an unlikely mechanism for the interspecific patterns we have shown here. Likewise, interspecific divergence levels across the mitochondrial genome are similar among *Tachycineta* species with both small and large distributions (Cerasale *et al.*, 2012). This finding is supported by the work of Bazin and colleagues (2006) who have demonstrated across ~3000 animal species that mitochondrial DNA diversity cannot be predicted by effective population size, but rather is better explained by adaptive evolution.

The three positively selected genes encode subunits of two separate complexes of the electron transport chain. Seven of the eight positively selected sites occurred within genes (*ND2* and *ND5*) that encode subunits of NADH dehydrogenase, a large, multimeric enzyme comprising respiratory chain complex I. This complex has been previously implicated in the adaptive evolution of the mitogenome in other taxa (41 placental mammals, da Fonseca *et al.*, 2008; salmon, Garvin *et al.*, 2011; monkeys, Yu *et al.*, 2011; wild donkeys, Luo *et al.*, 2012; herring, Teacher *et al.*, 2012; hares, Melo-Ferreira *et al.*, 2014). Located in the arm of L-shaped complex I, subunits *ND2*, *ND4*, and *ND5* collectively serve as a proton pump for H⁺ ions across the inner membrane (Brandt, 2006; Radermacher *et al.*, 2006). These three subunits are linked by the arm of *ND5*, allowing for coordinated shifts in proton pumping that in turn drive ATP production (Efremov *et al.*, 2010; Hunte *et al.*, 2010).

Within NADH subunits, sites exhibiting positive selection largely occurred in the predicted loop regions of the protein. Regions lacking sites of positive selection may reflect the
presence of conserved functional domains that are subject to purifying selection (Whitehead, 2009), and we do indeed find evidence of purifying selection in many of the regions that are devoid of codons under positive selection (Figure 3.4). The low incidence of positively selected sites in these particular regions of complex I is consistent with previous studies of fish (Whitehead, 2009) and mammals (da Fonseca et al., 2008). Although one positively selected site in each of ND2 (site 327) and ND5 (site 27) occurred within the transmembrane region, these substitutions are physiochemically conservative. The most radical substitutions were restricted to loop regions (Figures 2.3 & 2.4).

Cytochrome b is an integral component of respiratory chain Complex III (cytochrome bc₁ complex), which converts ubiquinol to cytochrome c, thereby generating an electrochemical gradient across the inner mitochondrial membrane that is utilized for ATP production (Mitchell, 1976; Trumpower, 1990; Hunte et al., 2003). In this process, ubiquinol is first converted to ubiquinone via oxidation at the Qₒ binding site and ubiquinone is then reduced to cytochrome c at the Qᵢ binding site (Gao et al., 2003; Kolling et al., 2003; Crofts, 2004). Both of these sites are located within the cytochrome b subunit. This process ultimately relies on the capacity of the Qᵢ site to bind water molecules for the reduction of ubiquinone to ubiquinol (Crofts, 2004). Because amino acid replacements can result in regional changes to hydrophobicity and structure within the protein, amino acid replacements within CYTB have the potential to alter the efficiency of this binding site. Replacements at cytbI7T characteristic of human mitochondrial haplogroup H likely enhance the binding of water at Qᵢ and have been linked to patterns of increased longevity for this group (Beckstead et al., 2009). Similarly, mutations to key residues at Qₒ in yeast alter this binding site and have resulted in decreased catalysis efficiency, as well as increased damaging, oxygen radical-producing bypass reactions (Wenz et al., 2007). However, the isoleucine to leucine substitution at CYTB 161 is among the most conservative amino replacements that occur at positively selected residues (D = 5), making the functional significance of this substitution in Tachycineta unclear.

Model assumptions and consistency of inferences across methods

Our results differ from a previous investigation of mitogenomic variation in Tachycineta in which positive selection was not detected (Cerasale et al., 2012). This disparity is likely due to our use of methods that are more sensitive to detecting lineage-specific selection. Cerasale et al.
used a two-rate fixed effects likelihood (FEL) approach, whereas we have employed a mixed effects model of evolution (MEME). The two methods differ in that MEME allows dN/dS to vary across both sites and branches (such that some branches may exhibit positive selection at the same time that others exhibit negative selection). Conversely, FEL assumes constant selection across time (prohibiting variation across branches), thereby limiting its power to detect selection occurring in only a single or few lineages (Murrell et al., 2012). We also implemented a branch-specific model of positive selection in CODEML to control for phylogenetic uncertainty. Cerasale et al. did not employ branch-site models.

CODEML inferences were largely robust to tree topology but only a subset of the sites inferred to be under selection by CODEML were also identified by MEME: one site was detected by both methods (ND5 27) and another (ND2 222) was found to be marginally significant. The differences can likely be attributed to differences in the underlying assumptions of the two methods (namely the use of one vs. multiple dN/dS ratios across branches), and also to the constraint of testing one focal branch at a time using branch-site tests. The latter issue prevented detection of sites under positive selection at CYTB 161, which exhibits a parallel amino acid change in two non-sister taxa (Figure 3.3).

CONCLUSIONS & FUTURE DIRECTIONS

Taken together, the results presented here suggest that the mitochondrial genome may be a promising candidate locus to begin to tie molecular genetic variation to latitudinal patterns in the pace of life. We have found evidence of positive selection acting on DNA sequence variation in the mitogenome of a widespread avian genus that exhibits latitudinal patterns of life-history variation that are consistent with POL expectations. While we found weak support for our prediction that branches separating taxa with greater POL variation also exhibit higher rates of positive diversifying selection, “slow” species did harbor the largest accumulation of derived, positively selected substitutions. We speculate that these substitutions are associated with functional metabolic differences among species and hope that our work will motivate future studies to characterize whole-organism metabolic rates, energy expenditure, and cellular metabolic processes within Tachycineta. Because the metabolic machinery is also encoded by the nuclear genome, similar investigations should be performed as sequences become available for the adjacent nuclear subunits: these could likely be targets of intergenomic coevolution.
(Burton & Barreto, 2012) and responsible for some of the remaining POL variation among species.

At the biochemical level, variation in metabolic rates is manifest through differences in the expression of genes that encode the machinery of cellular metabolism and the kinetic properties of these metabolic enzymes. Thus, although the positively selected amino acid substitutions we have identified here may contribute to functional variation in NADH dehydrogenase and cytochrome b, variation in whole-organism metabolic rate can also be driven through regulatory mechanisms. Indeed, recent studies of metabolic performance in deer mice (Peromyscus maniculatus) have demonstrated that elevated thermogenic performance is associated with large-scale upregulation of genes that participate in the beta-oxidation of lipids and oxidative phosphorylation, and these transcriptomic changes paralleled differences in the activities of metabolic enzymes that influence flux through these same pathways (Cheviron et al., 2012; 2014). Therefore, studies of regulatory variation in relation to pace of life expectations are likely to be particularly fruitful, and would complement our analyses of DNA sequence variation. Similarly, detailed studies of mitochondrial respiration rates coupled with measurements of whole-organism metabolic rates could provide insights into the functional consequences of putatively adaptive amino acid substitutions (Storz & Wheat, 2010; Toews et al., 2013). Collectively, these studies may begin to elucidate the genetic and physiological mechanisms that underlie an important broad-scale macrophysiological pattern – latitudinal variation in the ‘pace of life’ (Gaston et al., 2009).

ACKNOWLEDGEMENTS

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CHAPTER 3 TABLES & FIGURES

TABLE 3.1. Pace of life variation among the nine *Tachycineta* species. Variation in life history traits was summarized using a principal component analysis on the z-normalized values. The resulting PC1 scores (POL score) represent the degree of variation among species; positive scores represent “slow” species and negative scores represent “fast”. GenBank accession numbers of mitochondrial sequences for each species and one outgroup used in this study are also indicated (these data were originally reported by Cerasale *et al.*, 2012). Life history data are presented in Appendix C.

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession #</th>
<th>POL score</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. bicolor</em></td>
<td>JQ071614</td>
<td>-2.81</td>
</tr>
<tr>
<td><em>T. leucorrhoa</em></td>
<td>JQ071621</td>
<td>-1.95</td>
</tr>
<tr>
<td><em>T. meyeni</em></td>
<td>JQ071622</td>
<td>-0.76</td>
</tr>
<tr>
<td><em>T. thalassina</em></td>
<td>JQ071615</td>
<td>-0.69</td>
</tr>
<tr>
<td><em>T. cyaneoviridis</em></td>
<td>JQ071617</td>
<td>0.38</td>
</tr>
<tr>
<td><em>T. albilinea</em></td>
<td>JQ071619</td>
<td>0.82</td>
</tr>
<tr>
<td><em>T. euchrysea</em></td>
<td>JQ071616</td>
<td>0.90</td>
</tr>
<tr>
<td><em>T. albiventer</em></td>
<td>JQ071620</td>
<td>1.21</td>
</tr>
<tr>
<td><em>T. stolzmanni</em></td>
<td>JQ071618</td>
<td>2.90</td>
</tr>
<tr>
<td><em>Progne chalybea</em></td>
<td>JQ071623</td>
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</tr>
</tbody>
</table>
TABLE 3.2. Proportion of sites undergoing positive episodic (Pos) or negative (Neg) selection (identified with MEME and SLAC, respectively) for each of thirteen *Tachycineta* mitochondrial genes. Sites identified at $P \leq 0.05$.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Pos.</th>
<th>Neg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP6</td>
<td>0</td>
<td>0.044</td>
</tr>
<tr>
<td>ATP8</td>
<td>0</td>
<td>0.036</td>
</tr>
<tr>
<td>COX1</td>
<td>0</td>
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</tr>
<tr>
<td>COX2</td>
<td>0</td>
<td>0.070</td>
</tr>
<tr>
<td>COX3</td>
<td>0</td>
<td>0.080</td>
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<tr>
<td>CYTB</td>
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<td>0.097</td>
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<tr>
<td>ND1</td>
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<tr>
<td>ND2</td>
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</tr>
<tr>
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<tr>
<td>ND4</td>
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<tr>
<td>ND4L</td>
<td>0</td>
<td>0.071</td>
</tr>
<tr>
<td>ND5</td>
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<td>0.091</td>
</tr>
<tr>
<td>ND6</td>
<td>0</td>
<td>0.076</td>
</tr>
</tbody>
</table>
TABLE 3.3. Genes inferred to be under positive selection using branch-site models with CODEML. Trees and branches correspond to Figure 3.1B. Degrees of freedom = 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Branch</th>
<th>Tree</th>
<th>lnL(_{\text{Neutral}})</th>
<th>lnL(_{\text{Selection}})</th>
<th>LRT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND2</td>
<td>A</td>
<td>1</td>
<td>-3488.26</td>
<td>-3486.06</td>
<td>4.41</td>
<td>0.0357</td>
</tr>
<tr>
<td>ND5</td>
<td>B</td>
<td>2</td>
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<td>-5787.08</td>
<td>8.15</td>
<td>0.0043</td>
</tr>
<tr>
<td>ND5</td>
<td>B</td>
<td>3</td>
<td>-5849.67</td>
<td>-5845.53</td>
<td>8.27</td>
<td>0.0040</td>
</tr>
<tr>
<td>ND5</td>
<td>B</td>
<td>4</td>
<td>-6005.78</td>
<td>-6000.92</td>
<td>9.72</td>
<td>0.0018</td>
</tr>
<tr>
<td>ND5</td>
<td>T. stolzmanni</td>
<td>1</td>
<td>-5767.91</td>
<td>-5770.08</td>
<td>4.33</td>
<td>0.0374</td>
</tr>
<tr>
<td>ND5</td>
<td>T. stolzmanni</td>
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<td>-5787.52</td>
<td>4.25</td>
<td>0.0392</td>
</tr>
<tr>
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<td>T. stolzmanni</td>
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<td>-5847.01</td>
<td>4.56</td>
<td>0.0327</td>
</tr>
<tr>
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<td>T. stolzmanni</td>
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<td>-6004.54</td>
<td>-6003.22</td>
<td>2.64</td>
<td>0.1040</td>
</tr>
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</table>
FIGURE 3.1. (A) Color-coded distributions of the nine Tachycineta species; colors correspond to those used in phylogenies. Dots indicate approximate localities of specimen origin for each sequence. Gray line indicates the equator. (B) The four phylogenies employed in CODEML analyses are shown: the consensus mitochondrial (Phylogeny 1; Cerasale et al., 2012), consensus nuclear (Phylogeny 2; Dor et al., 2012), and nuclear trees with polytomy at node 17 (Phylogeny 3) and at node 14 (Phylogeny 4). Nodes 14 and 17 indicated by black dots in Phylogeny 2. Branches A and B are highlighted in red. Branch lengths not to scale.
**FIGURE 3.2.** Number of substitutions exhibited by each species as a function of their respective pace of life (POL) scores. Line fit by Poisson regression, p-value shown. Colors correspond to Figure 3.1.
FIGURE 3.3. Ancestral state reconstruction of polarity changes resulting from amino acid substitutions for 8 residues exhibiting positive selection: sites (A) CYTB 161; (B) ND2 222; (C) ND2 327; (D) ND5 16; (E) ND5 27; (F) ND5 190; (G) ND5 519; (H) and ND5 545. Amino acids are indicated by color and their polarity specified: nonpolar (np) or polar (p). Pie charts represent the proportional likelihood of particular state changes at each of the ancestral nodes. Grantham’s difference ($D$) shown for each amino acid pair within Tachycineta (Grantham, 1974).
FIGURE 3.4. Predicted topologies of transmembrane domains for genes exhibiting signatures of selection. The x-axis represents amino acid sites for each protein, and transmembrane regions are shown in gray while loop regions are shown in white. Sites under negative selection identified with SLAC are indicated with triangles. Sites that are inferred to be targets of positive selection identified with MEME are indicated with circles, and closed circles represent amino acid substitutions resulting in a change in polarity, whereas open circles represent substitutions that do not change polarity. Sites inferred to be under positive selection in the CODEML analysis are indicated with squares (closed = polarity change; open = no change in polarity). Colors correspond to the different branches on which they were identified: red = *T. stolzmannii*; green = branch A; blue = branch B (branches correspond to Figure 3.1).
CHAPTER 4

CONCLUSION

Together, these investigations elucidate two distinct molecular mechanisms underlying variation in avian metabolic performance. Regulatory changes can act on relatively short time periods, enabling organisms to respond to rapid environmental changes within the lifetime of an individual. Junco thermogenic flexibility manifested from simultaneous changes in the expression of genes within hierarchical regulatory networks in response to temperature. This transcriptomic variation resulted in rapid physiological modifications within individuals. In comparison, amino acid substitutions occur over the course of generations. Differences in the mitochondrial sequences of the genes encoding the machinery of cellular metabolism could result in canalized differences in whole-organism aerobic performance among *Tachycineta* swallow species. Here sites of positive selection were concentrated in species with ‘slow’ life histories that may be associated with low metabolic rates.

While our investigations focused on each mechanism in isolation, these two molecular mechanisms could in fact act in concert to generate phenotypic variation among individuals. An individual possesses a single genome; therefore phenotypic flexibility cannot be driven by sequence variation. In contrast, it is likely that regulatory changes also contribute to interspecific differences in aerobic performance, though we did not examine the presence of transcriptomic variation among *Tachycineta* species here. Future studies investigating the mechanisms underlying physiological adaptation among individuals should therefore integrate methodologies to simultaneously characterize both regulatory and sequence variation. Quantifying transcriptomic variation among *Tachycineta* species, for instance, would allow us to determine the degree to which metabolic variation results from standing sequence variation in mitochondrial protein-coding genes vs. differential regulation of metabolic genes. Likewise, further studies among junco populations could illuminate whether these regulatory mechanisms are conserved across evolutionary lineages, and genomic sequencing could help to uncover mechanisms of potential phenotypic canalization.

Given that we find evidence for each of these mechanisms here, it begs the question: Which is a stronger driver of interindividual differences in avian aerobic performance? This
question is particularly interesting in light of the diverse habitats that avian lineages inhabit around the globe, encompassing a wide range of environmental conditions that can exert an equally wide breadth of selective pressures on metabolic performance. With the aid of recent advances in genomic technology, the answer is within our reach.
APPENDIX A. Effect of acclimation treatment on pectoralis size ($M_p$).
APPENDIX B. Significant enrichment results for all transcriptional modules significantly associated with thermogenic performance or pectoralis size ($P \leq 0.05$). See supplemental file “Appendix_B.xls”. 
APPENDIX C. Life-history traits for each of the nine *Tachycineta* species. Data for clutch size (*Clutch*), ratio of egg volume to adult body mass (*V_{egg:M_{ad}}*), growth rate (*Growth*), and nestling period (*Nest Per.*) were gathered from the literature with corresponding absolute latitudes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Latitude</th>
<th>Clutch</th>
<th><em>V_{egg:M_{ad}}</em></th>
<th>Growth</th>
<th>Nest Per.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. bicolor</em></td>
<td>42.44</td>
<td>5.4</td>
<td>0.09</td>
<td>0.50</td>
<td>21.0</td>
</tr>
<tr>
<td><em>T. leucorrhoa</em></td>
<td>35.57</td>
<td>4.9</td>
<td>0.08</td>
<td>0.46</td>
<td>23.3</td>
</tr>
<tr>
<td><em>T. meyeni</em></td>
<td>54.80</td>
<td>3.8</td>
<td>0.09</td>
<td>0.43</td>
<td>26.0</td>
</tr>
<tr>
<td><em>T. thalassina</em></td>
<td>48.70</td>
<td>4.3</td>
<td>0.11</td>
<td>0.41</td>
<td>24.5</td>
</tr>
<tr>
<td><em>T. cyaneoviridis</em></td>
<td>26.67</td>
<td>3.0</td>
<td>0.10</td>
<td>0.36</td>
<td>22.8</td>
</tr>
<tr>
<td><em>T. euchrysea</em></td>
<td>18.85</td>
<td>3.0</td>
<td>0.12</td>
<td>0.51</td>
<td>25.6</td>
</tr>
<tr>
<td><em>T. albilinea</em></td>
<td>9.16</td>
<td>4.0</td>
<td>0.11</td>
<td>0.41</td>
<td>25.0</td>
</tr>
<tr>
<td><em>T. albiventer</em></td>
<td>7.45</td>
<td>3.0</td>
<td>0.10</td>
<td>0.37</td>
<td>24.7</td>
</tr>
<tr>
<td><em>T. stolzmanni</em></td>
<td>6.48</td>
<td>2.7</td>
<td>0.12</td>
<td>0.36</td>
<td>28.3</td>
</tr>
</tbody>
</table>

APPENDIX D. Pace of life (POL) score ancestral state reconstruction using each the consensus mitochondrial tree (A) and the consensus nuclear tree (B). Numbers indicate the POL score for the associated node. Taxa names are color-coded in accordance with Figure 3.1.

A

B
APPENDIX E. Branch-specific models of positive selection for each of the 4 trees. All branches examined for positive selection using branch-site model A test 2 are shown by gene with log-likelihoods for both neutral (ln\textsubscript{neutral}) and branch-specific models (ln\textsubscript{selection}), likelihood ratio test (LRT) values, and \(P\)-values (\(P\)). Terminal branches denoted by species name; internal branches denoted by letter, corresponding to Appendix I. Degrees of freedom = 1. See supplemental file “Appendix_E.xls”.
**APPENDIX F.** Ancestral reconstruction methods results. Log-likelihood values for each of the three models used: equal rates (ER), symmetric (SYM), and all rates different (ARD). The $P$-value represents the chi-square distribution for the LRT between the ER and ARD models.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Site</th>
<th>ER</th>
<th>SYM</th>
<th>ARD</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYTB</td>
<td>161</td>
<td>-4.808</td>
<td>-4.808</td>
<td>-4.143</td>
<td>0.249</td>
</tr>
<tr>
<td>ND2</td>
<td>222</td>
<td>-4.737</td>
<td>-4.737</td>
<td>-4.746</td>
<td>0.892</td>
</tr>
<tr>
<td>ND2</td>
<td>327</td>
<td>-4.737</td>
<td>-4.737</td>
<td>-4.746</td>
<td>0.892</td>
</tr>
<tr>
<td>ND4</td>
<td>192</td>
<td>-2.284</td>
<td>-2.284</td>
<td>-1.364</td>
<td>0.175</td>
</tr>
<tr>
<td>ND5</td>
<td>16</td>
<td>-5.651</td>
<td>-5.395</td>
<td>-4.663</td>
<td>0.160</td>
</tr>
<tr>
<td>ND5</td>
<td>27</td>
<td>-3.961</td>
<td>-3.961</td>
<td>-3.765</td>
<td>0.532</td>
</tr>
<tr>
<td>ND5</td>
<td>67</td>
<td>-2.284</td>
<td>-2.284</td>
<td>-1.364</td>
<td>0.175</td>
</tr>
<tr>
<td>ND5</td>
<td>190</td>
<td>-4.190</td>
<td>-4.190</td>
<td>-4.044</td>
<td>0.589</td>
</tr>
<tr>
<td>ND5</td>
<td>519</td>
<td>-5.302</td>
<td>-5.302</td>
<td>-4.311</td>
<td>0.159</td>
</tr>
<tr>
<td>ND5</td>
<td>545</td>
<td>-3.539</td>
<td>-3.539</td>
<td>-2.558</td>
<td>0.161</td>
</tr>
</tbody>
</table>
APPENDIX G. Ancestral state reconstruction of polarity changes resulting from amino acid substitutions for ND4 site 192. Amino acids are indicated by color and their polarity specified: nonpolar (np) or polar (p). Pie charts represent the proportional likelihood of particular state changes at each of the ancestral nodes. Grantham’s difference ($D$) shown for each amino acid pair within Tachycineta (Grantham, 1974).

**ND4 192**

- Threonine (p)
- Glycine (np)

$D = 59$
APPENDIX H. Ancestral state reconstruction of polarity changes resulting from amino acid substitutions for ND5 site 67. Amino acids are indicated by color and their polarity specified: nonpolar (np) or polar (p). Pie charts represent the proportional likelihood of particular state changes at each of the ancestral nodes. Grantham’s difference ($D$) shown for each amino acid pair within Tachycineta (Grantham, 1974).

**ND5 67**

![Amino acid substitution diagram](image-url)
**APPENDIX I.** Branch labels corresponding to Appendix E. (A) Nuclear phylogeny with nodes and branches labeled. Branch labels identical for shared branches in Phylogeny 3 and 4. (B) Mitochondrial phylogeny with branches labeled.

**A**

![Nuclear phylogeny diagram](image)

**B**

![Mitochondrial phylogeny diagram](image)