MIGRATION, MITOCHONDRIA, AND THE YELLOW-RUMPED WARBLER

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Discordance between mitochondrial and nuclear DNA has been noted in many systems. Asymmetric introgression of mitochondria is a common cause of such discordances, although in most cases the drivers of introgression are unknown. In the yellow-rumped warbler, evidence suggests that mtDNA from the eastern, myrtle warbler, has introgressed across much of the range of the western form, the Audubon's warbler. Within the southwestern United States myrtle mtDNA comes into contact with another clade that occurs in the Mexican black-fronted warbler. Both northern forms exhibit seasonal migration, whereas black-fronted warblers are nonmigratory. We investigated the link between mitochondrial introgression, mitochondrial function, and migration using novel genetic, isotopic, biochemical, and phenotypic data obtained from populations in the transition zone. Isotopes suggest the zone is coincident with a shift in migration, with individuals in the south being resident and populations further north becoming increasingly more migratory. Mitochondrial respiration in flight muscles demonstrates that myrtle-type individuals have a significantly greater acceptor control ratio of mitochondria, suggesting it may be more metabolically efficient. To our knowledge this is the first time this type of intraspecific variation in mitochondrial respiration has been measured in wild birds and we discuss how such mitochondrial adaptations may have facilitated introgression.

KEY WORDS: Adaptation, biogeography, hybridization, introgression, mitochondrial DNA, physiology.

Most well-studied hybrid zones between divergent taxa are characterized by steep, coincident clines at various genetic loci and phenotypic traits. These patterns are consistent with limited introgression across a hybrid zone and strong selection against hybrids (Barton and Hewitt 1985). In some instances, however, there is geographic discordance among genes or traits, where clines are strongly displaced in their spatial distribution compared to the rest of the genome (Barton 1993). Often this pattern is consistent with introgression following secondary contact, which can be promoted by various demographic and/or selective factors (Toews and Brelsford 2012). Hence the biogeographic patterns associated with these types of discordant clines can reveal novel insights into evolutionary processes (e.g., Brumfield et al. 2001).

One linked suite of genes that commonly shows biogeographic discordance with other genetic markers or phenotypic traits are the genes encoded in the mitochondrial genome (mtDNA; Takahata and Slatkin 1984; Barton 1993). Discordant biogeographic patterns in mtDNA and nuclear markers have been identified in numerous animal systems and most cases are likely due to introgression of mtDNA between taxa (approximately 90% of the studies reviewed by Toews and Brelsford 2012). Mitochondrial introgression can be a result of neutral diffusion and genetic drift or be driven by demographic asymmetries such as female-biased dispersal (mtDNA is maternally inherited in most animals) or geographically varied patterns of selection that differentially affect mitochondrial and nuclear genes (Rheindt and Edwards 2011; Irwin 2012). Although many authors have noted these
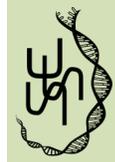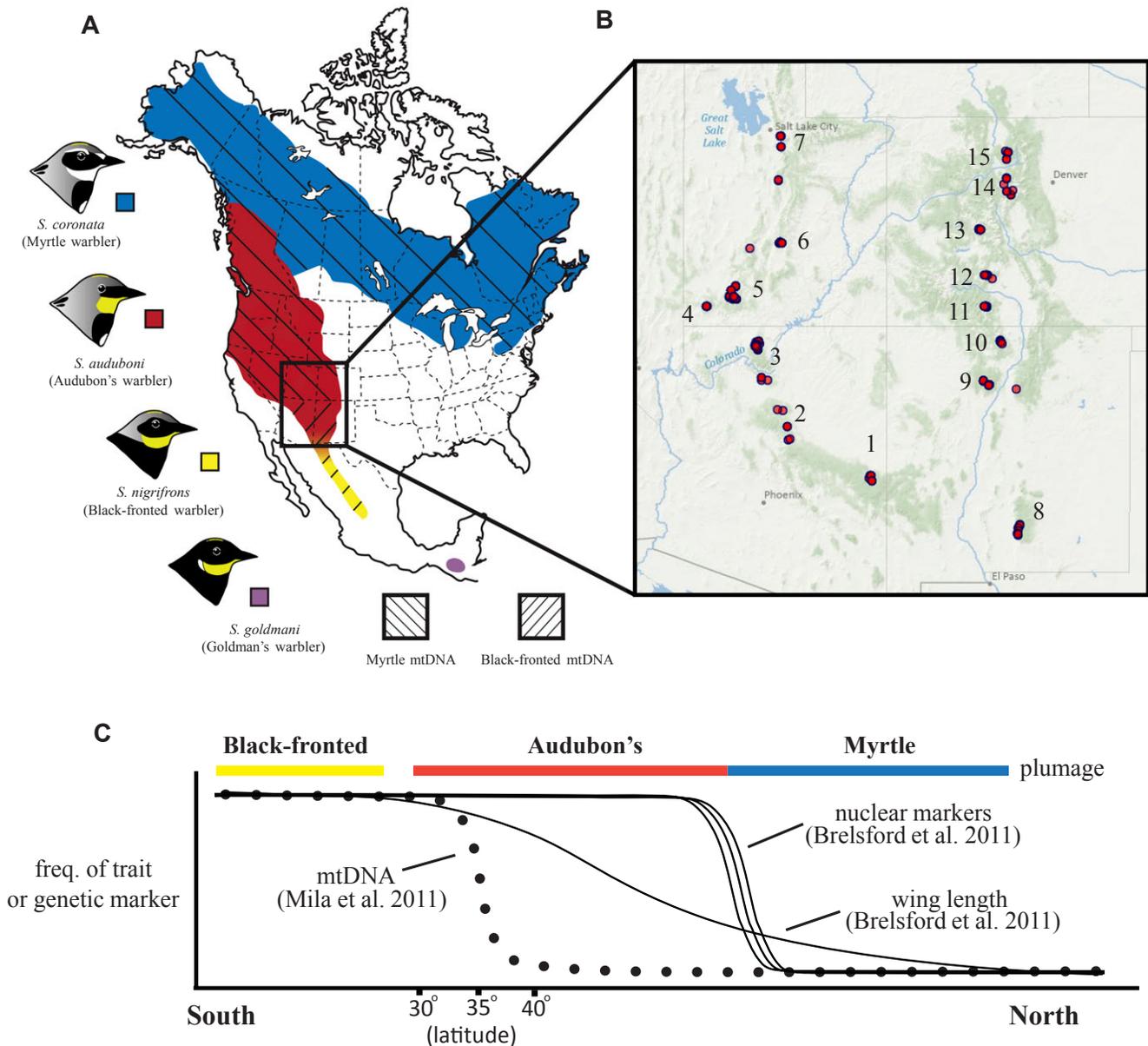
Figure 1. (A) The distribution of the four currently classified species in the yellow-rumped warbler (*Setophaga spp.*) complex. According to Brelsford et al. (2011) there are three distinct nuclear groups that align with the areas shaded blue (myrtle warbler), red/yellow (the Audubon's phenotype and black-fronted warbler phenotype), and violet (Goldman's warbler). The hatched areas distinguish the myrtle-type from black-fronted type mtDNA. (B) Sampling localities in the southwestern United States where we studied the cryptic mtDNA transition zone. Locations 1–7 indicate sites along the western transect, 8–15 in the eastern transect (see Table 1 for location information). (C) A simplified schematic adapted from Brelsford et al. (2011) to illustrate the discordances between plumage, morphometric, nuclear, and mitochondrial patterns in this system.

biogeographic patterns and have speculated as to the potential drivers of mito-nuclear discordance, most have not collected additional evidence to test the various introgression hypotheses (Toews and Brelsford 2012).

Here we investigate the discordance between mtDNA and nuclear DNA in the yellow-rumped warbler (*Setophaga [coronata]* spp.) species complex, one of the most abundant and

widespread warblers in North America. The species complex is composed of four currently recognized taxa (formerly of the genus *Dendroica*; designated as separate species by the International Ornithological Council but as subspecies of a single species by the American Ornithologists' Union; Fig. 1A): *Setophaga coronata*, the myrtle warbler, which breeds in the boreal forest east of the Rocky Mountains and winters in eastern North America, Central

America, and the Caribbean; *Setophaga auduboni*, the Audubon's warbler, which breeds west of the Rocky mountains and winters in the southwestern United States, Mexico, and central America; *Setophaga nigrifrons*, the black-fronted warbler, which is a resident year-round in Mexico; and finally, *Setophaga goldmani*, the Goldman's warbler, which consists of a small population of resident birds confined to Guatemala (Hubbard 1970).

Studies of multilocus nuclear markers (AFLPs) suggest that there are three distinct nuclear clusters in this group of four recognized taxa, one consisting of the myrtle warbler, another that includes Audubon's and black-fronted warblers, and one distinct group corresponding to Goldman's warblers (Brelsford et al. 2011). Within the cluster that includes the Audubon's and black-fronted warbler there is a gradual latitudinal gradient in the nuclear genome that is also mirrored in some morphological traits (e.g., wing length; Brelsford et al. 2011). Darker plumage patterns and migratory behavior of the birds in Mexico (i.e., *S. nigrifrons*) separate these individuals from the rest of the Audubon's group in the United States (i.e., *S. auduboni*), however there is no known reproductive boundary between these taxa (Milá et al. 2011).

These patterns in phenotype and the nuclear genome contrast with the distribution of mtDNA: over much of the range of the Audubon's warbler, myrtle warbler mtDNA is fixed (see Fig. 1C for a simplified schematic of discordant clines). Near the border of Utah and Arizona there is a transition to a second, deeply divergent mitochondrial DNA clade (Brelsford et al. 2011), which was previously assumed to be geographically restricted to Mexico in the black-fronted warbler. This cryptic transition occurs within what are otherwise phenotypically and morphologically Audubon's warblers (Brelsford et al. 2011; Milá et al. 2011) and where there is no observed break in nuclear markers across this zone. Evidence suggests that this mito-nuclear discordance was generated from mitochondrial introgression that is estimated to have occurred relatively recently (divergence between northern Audubon's and myrtle mtDNA is estimated to have occurred roughly 16,000 years ago, although there is very large uncertainty in that estimate; Milá et al. 2011). Past hybridization and introgression seem possible given that myrtle and Audubon's warblers are known to currently interbreed extensively and form viable hybrids in the Rocky Mountains of British Columbia and Alberta, although this is far from the contemporary transition in mtDNA (Hubbard 1969; Brelsford and Irwin 2009).

Although all of the individuals in the Utah/Arizona mtDNA transition zone resemble Audubon's warblers in plumage and morphometric traits (Milá et al. 2011), Brelsford et al. (2011) suggest the possibility that this area may instead align with another important phenotypic trait—a shift in seasonal migratory behavior, from resident birds in the south to migratory birds in the north. Although this suggestion is based on limited observational data (Hunt and Flaspohler 1998), given the important role

that mitochondria play in energy production during the metabolically demanding act of migration (e.g., Scott et al. 2009) there is an intuitive link between this behavior and variation in mitochondrial phenotype. This "migration adapted mitochondrion" hypothesis posits that in the past, natural selection favored myrtle mitochondrial variants in migratory Audubon's warblers compared to those with the ancestral black-fronted mitochondria, which presumably evolved in primarily resident or short-distance migrant populations.

Empirical examinations of whether selection may play a role in facilitating mtDNA introgression have been rare, although simulations have shown that local adaptation of mtDNA can occur with only a small selective advantage (Irwin 2012). This is likely due in part to the widespread assumption of neutrality and in part to the difficulty of detecting selection in the mitochondria, both at the molecular and biochemical level. Despite the long held assumption that variation in mtDNA is primarily neutral, a number of studies have identified intra- and interspecific variation in the proteins encoded by genes in the mitochondrial genome that authors have attributed to natural selection (Blier et al. 2001; Bazin et al. 2006; Meiklejohn et al. 2007; Ballard and Melvin 2010; Scott et al. 2011; Correa et al. 2012; Pichaud et al. 2012). In those studies that have tested for evidence of adaptive introgression (e.g., Blier et al. 2006; Boratynski et al. 2011), few have found evidence for functional differences in introgressed mitochondria. It has been suggested, however, that more sensitive techniques that test mitochondrial function directly, such as measuring potential variation in respiratory capacity in mitochondrial preparations (e.g., Pichaud et al. 2012), may be more appropriate to determine if introgressed mitochondria differ phenotypically from a native type. While collecting such functional data is useful, eventually connecting it with potential fitness consequences can be an important yet challenging additional step (Storz and Wheat 2010).

We investigated the potential link between mitochondrial introgression, mitochondrial function, and migration in yellow-rumped warblers using novel genetic, isotopic, biochemical, and phenotypic data obtained from several natural populations in the mtDNA transition zone. We made two major predictions. First, if there is any link between mitochondrial introgression and migratory behavior, we predicted that the mtDNA transition would also be located at the transition in migratory phenotype. Although stochastic processes alone could generate such a correlation, preliminary studies suggest that no other phenotypic trait (e.g., color), environmental factors (e.g., habitat characteristics), or demographic parameters (e.g., population size) covary with mtDNA. To determine the location and shape of the mtDNA cline, we genotyped individuals across two transects (depicted in Fig. 1B) in the mtDNA transition zone and used a maximum-likelihood cline-fitting procedure to estimate the cline center and width. To estimate the migratory movements of individuals, we

Table 1. Sampling localities, sample sizes, and frequency of black-fronted type mitochondrial DNA (*Setophaga nigrifrons*) in populations along the eastern and western transects. Numbers of sampling locations correspond to those shown in Figure 1.

Site (abbreviation)	Avg. Lat.	Avg. Long.	Transect	*n* for mtDNA genotype (*n* for isotopes)	Frequency *S. nigrifrons* mtDNA
(1) Apache National Forest, AZ (ANF)	33.95	−109.42	Western	11 (8)[N.S.]	0.91
(2) Coconino National Forest, AZ (CNF)	35.00	−111.51	Western	8 (7)[N.S.]	1.00
(3) Kaibab National Forest, AZ (KNF)	36.53	−112.21	Western	36 (9)[1]	0.97
(4) Dixie National Forest – Pine Valley, UT (DNF1)	37.37	−113.46	Western	8 (0)	0.50
(5) Dixie National Forest – Navajo Lake, UT (DNF2)	37.59	−112.81	Western	44 (8)[1]	0.32
(6) Fish Lake National Forest, UT (FLNF)	38.61	−111.70	Western	16 (8)[1]	0.13
(7) Uinta National Forest, UT (UNF)	40.42	−111.64	Western	12 (8)[1]	0.00
(8) Lincoln National Forest, NM (LNF)	32.90	−105.76	Eastern	13 (8)[N.S.]	0.85
(9) Santa Fe National Forest, NM (SFNF)	35.84	−106.50	Eastern	11 (8)[1]	0.55
(10) Carson National Forest, NM (CNF)	36.69	−106.22	Eastern	10 (7)[1]	0.30
(11) Rio Grande National Forest – Stunner, CO (RGNF1)	37.38	−106.59	Eastern	15 (8)[1]	0.27
(12) Rio Grande National Forest – Posos, CO (RGNF2)	37.98	−106.56	Eastern	10 (8)[1]	0.40
(13) Gunnison National Forest, CO (GNF)	38.86	−106.73	Eastern	13 (8)[1]	0.15
(14) White River National Forest, CO (WRNF)	39.68	−106.03	Eastern	11 (8)[1]	0.09
(15) Arapaho National Forest, CO (ANF)	40.29	−106.06	Eastern	7 (7)[1]	0.00

[1]Denotes significance (adjusted $P < 0.004$) testing whether the difference between latitude at capture and estimated winter latitude (as estimated by isotope values) for a given site was significantly different from zero.

analyzed stable hydrogen isotopes in feathers to estimate the distance that each individual travels between its breeding and predicted wintering grounds. We expected that individuals in the south would show little difference in the isotopic composition of feathers grown on the breeding and wintering grounds (i.e., year-round residents), whereas individuals in the north would show a larger difference, consistent with moving between disparate localities (i.e., migrants).

Second, we predicted that variation in mitochondrial genotype explains some variation in mitochondrial phenotype. Such a finding is important in that it suggests that there is functional variation present that selection could act upon, although it is important to note that documenting variation in biochemical phenotype does not necessarily imply any effect on fitness (Storz and Wheat 2010). We first sequenced protein coding mtDNA genes involved in the electron transport complexes (ETC) from multiple Audubon's warblers with either myrtle or black-fronted mitochondrial types, examining whether these proteins differ in amino acid sequence between the two groups. To test if these amino acid differences had functional consequences, we directly assayed maximal enzyme activity in one of the electron transport chain complexes (complex I) that had a number of amino acid substitutions. We predicted we would observe higher maximal activities in this enzyme from the northern, myrtle-type mitochondria. We also assayed the respiratory capacity of mitochondria in permeabilized muscle fibers from wild-caught individuals of both mtDNA types in the contact zone. If individuals in the

north of the transition zone, with myrtle-type mtDNA, have mitochondria better adapted for migratory movements, this might be reflected in the ability of their mitochondria to consume oxygen and produce ATP more efficiently. Specifically, we predicted that a mitochondrion associated with a more migratory lifestyle would (1) have a higher maximum capacity for respiration as measured by a higher state III (ADP-stimulated) oxygen consumption rate and/or (2) have a more efficient mitochondria as a result of increased coupling, which can be estimated by calculating the ratio of state III-to-state II consumption rates, also known as the acceptor control ratio (ACR; Nicholls and Ferguson 2002).

Methods
SAMPLING AND MITOCHONDRIAL GENOTYPING
During the spring of 2010 and 2011, we studied 225 yellow-rumped warblers in a cryptic mitochondrial contact zone in the southwestern United States. We captured territorial individuals along two broad transects (Fig. 1B; Table 1): one that was previously identified by Milá et al. (2011) from northern Utah into Arizona (the "western transect"), and another from northern Colorado into New Mexico (the "eastern transect") that had not been examined before this study. We captured individuals using song playback and mist nets and then took morphometric measurements (i.e., bill, wing, and tail length), photographs, feather samples, and a blood sample (10–40 μL) from each individual. For most birds, we applied unique bands and then released the individual.

A subset of individuals were euthanized using isoflurane and tissues were collected for biochemical and physiological analysis. Because of logistical constraints, some specimens were destroyed following blood and tissue sampling. All animal care and experimentation was conducted according to the University of British Columbia protocol Nos. A10-0058 and A09-0131. We aged and sexed all individuals according to Pyle (1997); most captured individuals were males, likely due to the use of song playback to attract and capture the individuals.

Blood samples, taken using a small needle from the brachial vein, were stored in Queen's lysis buffer (Seutin et al. 1991) and left at ambient temperature until returned to the laboratory for analysis of mtDNA genotypes. DNA was extracted using a phenol–chloroform protocol and resuspended with 50–200 μL of buffer (depending on the size of the pellet) containing 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and stored at 4°C. Our choice of molecular markers for distinguishing the mitochondrial types was based on sequence information from Milá et al. (2007). We amplified a 648 bp fragment that spanned 358 bp of the *ATP-synthase 6* gene and the entire *ATP-synthase 8* gene using primers L8929 (5′-GGACAATGCTCAGAAATCTGCGG-3′) and H9855 (5′-ACGTAGGCTTGGATTATKGCTACWGC-3′; Sorenson et al. 1999). Amplification reactions included $1\times$ PCR buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTP mix (New England Biolabs), 0.5 μM forward and reverse primer, 0.04 units/μL Taq DNA polymerase (New England Biolabs), and 2.5 ng/μL genomic template DNA, in a total volume of 10 μL. The thermal cycling profile was 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, and 30 s at 72°C, ending with 10 min at 72°C. We used a restriction fragment length polymorphism (RFLP) assay to genotype a single nucleotide polymorphism (SNP) at 390 bp, where there is a C-T polymorphism, with C fixed in myrtle-type mtDNA and T fixed in black-fronted-type mtDNA; the restriction enzyme *Xba*I cuts only the C variant. We digested 2 μL of the PCR product with two units of the restriction enzyme *Xba*I in its appropriate buffer (New England Biolabs) in a total volume of 6 μL. Products were digested for 2 h at 37°C and the digested DNA was visualized by electrophoresis on 2% agarose gel stained with SYBRSafe (Invitrogen). This digestion cuts the myrtle-type mtDNA PCR product into two fragments (390 and 258 bp) and does not cut the black-fronted product (one 648 bp fragment).

The transitions of allele frequencies and phenotypic traits across many hybrid zones can be modeled effectively by sigmoidal curves (Syzmura and Barton 1986). The estimated center and width of a particular genotypic transition (e.g., Toews et al. 2011) can then be compared to those of other traits (i.e., migratory behavior). We used the program Cfit6 (Gay et al. 2008) to determine the best fitting sigmoid for *ATPase* frequencies. We used this to estimate the location of mtDNA cline center and width (defined as the inverse of the maximum slope) along each transect. Given the apparent north–south orientation of the transition in mtDNA we used the average latitude for each population to represent its location across the contact zone. We anchored each transect with two populations, far away from the current mtDNA transition zone, that are known to be fixed for the black-fronted (in Mexico; 26.47°N) or myrtle (in Idaho; 43.89°N) mtDNA based on Milá et al. (2011). We ran Cfit unconstrained so that each cline along either transect would have its own center and width.

FEATHER ISOTOPE ANALYSIS

For a subset of birds ($n = 110$), we determined the stable hydrogen value (δD) in two greater covert feathers from each individual. Stable hydrogen (i.e., deuterium) was used because it correlates strongly with precipitation and generally varies latitudinally in the study area (Meehan et al. 2004; Hobson et al. 2012), although there is some local variation that likely relates to elevational differences. We sampled feathers from 7 to 9 randomly chosen individuals from each of 14 sites (Table 1). Analysis was done at Environment Canada's Isotope Laboratory at the National Water Research Institute in Saskatoon, SK. We took advantage of a feather molting pattern in Audubon's warblers where in the fall a bird will molt most of its feathers during a prebasic molt, which takes place on the breeding grounds (Pyle 1997), whereas in the spring these birds also molt 3–4 of their inner-covert feathers on their wintering grounds during a prealternate molt (Gaddis 2011). Therefore, on any single individual caught during the spring in the following breeding season, there are two generations of feathers that can be easily distinguished: one set with the isotopic signature of the previous breeding ground and another group from the most recent wintering area.

We assumed that most of the individuals captured in the spring bred and grew their basic feathers the previous fall in a similar location to the capture site. Many small songbirds have high breeding site fidelity, and previous studies of color-banded yellow-rumped warblers studied over multiple years indicate this is true of them (A. Brelsford, unpubl. data). Using these "breeding" feathers, we generated a linear regression of capture site latitude (the response variable) on deuterium value (the explanatory variable). We did this separately for each of the two transects. This allowed us, within the bounds of our study transects, to coarsely predict the latitude at which a feather was grown based on its deuterium value (see Figs. S1 and S2 for regressions). Using these regressions, we then estimated wintering location latitude from the isotopic composition of alternate covert feathers (which grew in the winter), generating a "predicted wintering latitude."

We assumed individuals that are resident or short-distance migrants would show little-to-no difference between their breeding latitude and their inferred wintering latitude (i.e., residents), whereas individuals that are migrants would show a larger

difference between the two latitudes (i.e., migrants). We used a one-sample *t*-test in R to determine whether the mean latitude that individuals moved at a given site was significantly different from zero (i.e., consistent with migratory behavior). For this test, we used an adjusted critical value of 0.004 (0.05/14 comparisons) to control for multiple comparisons for the various sites. We then used the average values for each site to test whether breeding latitude and distance traveled were correlated, using the Pearson product–moment correlation, also in R.

To explicitly examine whether mtDNA type might explain some of the residual variation in migration distance, after controlling for breeding latitude, we performed an analysis of covariance (ANCOVA) using the "step" function in R. We included migration distance (in degrees latitude), mtDNA type, and breeding latitude. This procedure removes parameters in a stepwise manner and determines, with AIC, which is the best model for the data. This allows us to test for an independent signature of mtDNA type on migration, once latitude is accounted for, and any potential interaction between the two. It should be noted that we assume that annual differences in the isotopic patterns reflect movement between disjunct sites. Other factors, such as seasonal diet changes or shifts in local weather patterns can also change patterns in isotopes over a season, however these factors are not expected to show a latitudinal pattern as is predicted for migratory behavior.

mtDNA SEQUENCING

We sequenced mtDNA from six individuals: three of each mtDNA type, with two individuals <250 km from the center of the mtDNA contact zone and one >400 km. We sequenced all or portions of 11 of the 13 protein coding genes encoded in mtDNA. Primer information, accession numbers, and PCR conditions can be found in Table S1. We assembled the sequences with CLC Main Workbench 6 (CLC Bio) using the full mitochondrial genome of the yellow-browed bunting (*Emberiza chrysophrys*; GenBank accession No. HQ896034) as a reference. We then translated the mtDNA sequences into corresponding amino acid sequences to determine the number of nonsynonymous substitutions between the myrtle-type and black-fronted-type variants for each protein. In addition to identifying whether there were any amino acid differences between the mtDNA types, we were interested in determining the distribution of those amino acid (AA) changes (i.e., are they spread throughout the genes sequenced or are they concentrated to genes coding for certain enzyme complexes?). Finally, we calculated the average pairwise Jukes–Cantor genetic distance for each of the protein coding genes between black-fronted and myrtle mtDNA types using the CLC Main Workbench 6 (CLC Bio).

MAXIMAL ENZYME ACTIVITIES

To test whether there were any functional differences in the enzyme complexes or in the respiratory pathway at the mitochondrial level, we measured both maximal enzyme activity and mitochondrial respiratory capacity. For this we captured, euthanized, and collected *pectoralis major* muscle from 24 wild caught individuals from two sites in the center of the western transect of the mtDNA contact zone ($n = 14$ from the DNF2 site and $n = 10$ from KNF; Table 1). Muscle samples were taken from each individual from approximately halfway down the sternum and about 3-mm deep into the tissue. Half of the tissue collected was flash frozen in liquid N_2 for enzyme assays whereas half was used to immediately assess mitochondrial respiration. These individuals were later genotyped as having either a myrtle ($n = 9$) or a black-fronted ($n = 15$) mito-type using the RFLP method described earlier.

We assayed the maximal enzyme activity for one of the five electron transport chain complexes, complex I (NADH:ubiquinone oxidoreductase) and one of the enzymes involved in the tricarboxylic acid cycle of the mitochondria, citrate synthase (CS). Complex I was chosen because the mtDNA sequence data identified a number of fixed amino acid substitutions between the two mito-types in genes that encode for components of this enzyme (see Results). We also identified a single amino acid substitution in complex V (*ATP synthase*), although we were not able to reliably quantify activities for this complex, a result that other authors have previously reported in other study systems (Kirby et al. 2007; Jonckheere et al. 2012). We also assayed CS activity as this has been shown previously to be a good proxy for overall mitochondrial content (Larsen et al. 2012). We did this to test for potential covariation between mitochondrial content and mitochondrial genotype, where higher respiration rates may be a result of greater mitochondrial content as opposed to greater efficiency. We had insufficient muscle tissue from one myrtle-type individual to determine its maximal enzyme activity.

For complex I and CS assays, frozen muscle was broken into small pieces in an insulated, liquid N_2 cooled mortar and pestle. Approximately 100 mg of tissue was quickly weighed and homogenized on ice in six volumes of ice-cold homogenization buffer (25 mM K_2HPO_4, 5 mM $MgCl_2$, pH 7.2) using four, 7 second bursts set on the medium speed setting of a polytron homogenizer. The homogenates were separated into aliquots and frozen immediately at $-80°C$.

Maximal enzyme activities were determined spectrophotometrically on muscle homogenates using a VersaMax spectrophotometer (Molecular Devices, CA) assayed at $25°C$. Complex I activity was monitored by rotenone-sensitive reduction of 2,6-dichloroindophenol sodium (DCIP) at 600 nm (Janssen et al. 2007) and CS activity was monitored by the appearance of 5-thio-2-nitrobenzoic acid as a result of the reaction of free CoA with 5,5'-dithiobis(2-nitrobenzoic acid) at 412 nm over a 15-min incubation period. The assay conditions necessary to measure maximal enzyme activities were as follows (in mM unless

otherwise indicated): complex I: 25 K_2HPO_4, pH 7.2, 5 $MgCl_2$, 2.5 mg/mL bovine serum albumin (BSA), 0.1 DCIP, 0.2 NADH, 0.065 Coenzyme Q_2, 4 μg/mL antimycin, with or without 2 μg/mL rotenone and CS: 50 Tris, pH 8.0, 0.5 oxaloacetate, 0.3 acetyl-CoA, 0.15 5,5-dithiobis-2-nitrobenzoic acid. Rotenone-sensitive complex I activity was obtained by subtracting complex I activity in the presence of rotenone from total activity. Citrate synthase activity was calculated as the difference between activity measured in the presence of oxaloacetate and activity measured in the absence of oxaloacetate. Empirically determined extinction coefficients were used to convert changes in absorbance to changes in maximal enzyme activity. Total soluble protein was determined in each homogenate using the Bradford protein assay (Bradford 1976) and maximal enzyme activities were normalized to both tissue weight and total soluble protein (we found no difference between these measurement types and therefore only protein results are reported). We used two-sample t-tests in R to test whether activities were significantly different between the mitochondrial types.

MITOCHONDRIAL RESPIRATION

Muscle samples were immediately placed into an ice-cold relaxing solution (in mM): 20 taurine, 0.5 dithiothreitol (DTT), 6.56 $MgCl_2$, 50 potassium methane-sulfonate, 20 imidazole, 5.77 Na_2ATP, 15 creatine phosphate, 2.77 CaK_2EGTA, 7.23 K_2EGTA. Muscle fiber bundles were then mechanically separated using tweezers under a dissecting microscope. Bundles were placed into a 2 mL centrifuge tube filled with the relaxing solution containing saponin (50 μg/mL) and gently mixed on ice using a rocking plate for 30 min. This was followed by three 10-min washes in respiration medium (in mM unless otherwise indicated): 20 Taurine, 0.5 DTT, 1.38 $MgCl_2$, 100 potassium methane-sulfonate, 20 imidazole, 3 KH_2PO_4, 2.77 CaK_2EGTA, 7.23 K_2EGTA, and 1 g of BSA. Following the washes, the saponin-permeabilized bundles were quickly dabbed dry, weighed, and placed into fresh respiration medium in the respiratory chamber.

Mitochondrial respiration was measured in 2 mL respiratory solution under continual stirring using the Ocean Optics FOXY oxygen sensor. Temperatures were maintained at 25°C. This is lower than avian body temperature (40°C for most species; Gill 2007) but allowed us to run the entire experiment without depleting the O_2 levels in the chamber and is not expected to affect the comparisons of oxygen consumption rates. After approximately 3 min we added 2 mM malate followed by 5 mM of pyruvate to estimate state II respiration rate. The state II respiration rate is defined as the oxygen consumption rate obtained when substrates are present but ADP is lacking, and this oxygen consumption rate is considered the background respiration rate. This was followed by the addition of 5 mM ADP to determine state III respiration rate, which is also known as the maximal respiration rate. Preliminary

analysis demonstrated that state III oxygen consumption rate was maximal at 2.5 to 5 mM of ADP in yellow-rumped warbler muscle tissue. Measurements were taken only after consumption rate had stabilized and we took measurements for 1–2 min of the trace. We then added 0.5 mM of rotenone, inhibiting complex I, and reoxygenated the respiration solution by removing the lid of the chamber. The chamber lid was then closed and 10 mM of succinate was added, to maximally stimulate complex II (succinate dehydrogenase). We then added 5 mM of antimyacin-A, an inhibitor of complex III, and again reoxygenated the chamber. Finally, complex IV (cytochrome c oxidase; COX) was maximally stimulated with 0.5 mM of tetramethylphenylenediamine (TMPD).

It is important to note that isoflurane has been shown to affect mitochondrial coupling (Ljubkovic et al. 2007); however, given the short duration of isoflurane exposure (until cessation of the heart, usually less than 1 min) and 1 h sample preparation (tweezing of fibers, washing in relaxing solution, and respiration medium) with frequent solution changes, it is likely that any effects of isoflurane on mitochondrial function would be strongly attenuated. Even if the effects of isoflurane persisted during the sample preparation, all samples were processed identically, such that differences in response variables between samples should reflect biological differences, not differences due to procedure.

We used two-sample t-tests in R to test whether consumption rates were significantly different between the mitochondrial types. In one case, when comparing consumption rates under TMPD stimulation between mito-types, we found that variances were likely not equal between the populations and instead used a Welch's t-test, which does not require this assumption.

Results

SAMPLING AND CLINE ANALYSIS

Yellow-rumped warblers in the southwestern United States are most abundant in an archipelago of high-elevation coniferous forests between 2000 and 3500 m above sea level. We confirmed the observations of Milá et al. (2011) and Brelsford et al. (2011) that the individuals in this area are phenotypically and morphometrically aligned to Audubon's warblers along both transects. The cline analysis of mtDNA allowed us to determine center and width of the mtDNA transition from the myrtle to black-fronted warbler types along both transects. For the western (UT to AZ) transect, the center was at 37.40°N and the width was 213 km (Fig. 2A), which is similar to a previous study of populations along this transect, although slightly narrower (37.17°N and 297-km wide as reported by Milá et al. 2011). In contrast to the western transect, our sampling of the eastern transect (CO to NM) revealed a much wider cline, 743 km, although it had a similar center at 35.94°N (Fig. 2A).
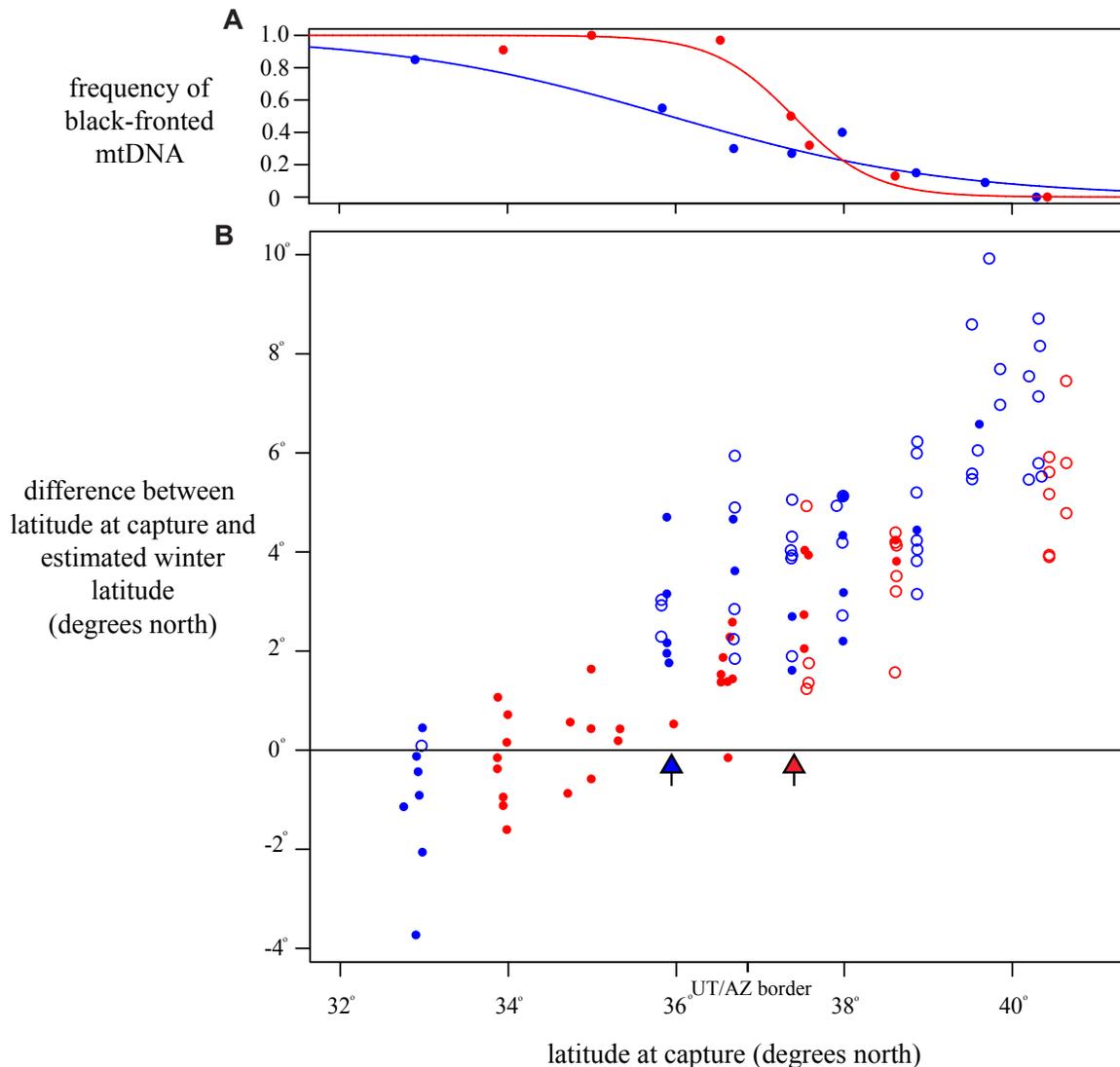
Figure 2. (A) Clines in mtDNA across two transects in a cryptic contact zone in the southwestern United States between myrtle-type and black-fronted type mtDNA. Points are allele frequencies in the mitochondrial *ATPase* genes, locations are averaged latitudes (in degrees), and colors distinguish the western (red) and eastern (blue) transects. The lines represent the best fitting clines as estimated by Cfit6 (Gay et al. 2008). (B) Stable hydrogen isotope data obtained from feather samples across the contact zone. The horizontal axis represents the latitude where an individual was captured (i.e., its breeding latitude). The vertical axis is the difference in degrees between this location and the predicted wintering latitude. Filled circles indicate individuals with black-fronted mtDNA and open circles are individuals with myrtle-type mtDNA. The arrows indicate the centers of the clines for mtDNA for the two clades shown in (A).

FEATHER ISOTOPE ANALYSIS

Our ability to estimate the wintering location for each individual, and subsequently the approximate distance travelled between breeding and wintering grounds, relied on our ability to predict the latitude at which a feather was grown based on its δD content. We found that although there was large variation in δD of feathers grown in summer, it varied predictably with latitude over our study area. For the western transect, we found that the regression equation (latitude) $= 0.09 \times (\permil\, \delta D) + 30.66$ best explained variation in latitude with isotope values ($R^2 = 0.61$; df $= 46$; $P < 0.001$) and along the eastern transect we found that the re-

lationship (latitude) $= 0.12 \times (\permil\, \delta D) + 26.95$ was the best fit ($R^2 = 0.33$; df $= 60$; $P < 0.001$).

Using the regressions based on feathers grown on the breeding grounds, we then used isotopic values of winter-grown feathers to estimate the approximate wintering latitude of each individual and, subsequently, the difference between this value and where we captured the individual (Fig. 2B). For 10 individuals, the deuterium content of the winter feathers fell outside the range of the summer feathers ($-34.7\permil$ to $-119.7\permil\,\delta D$) by $<15\permil\,\delta D$; removing these individuals did not meaningfully affect the results shown and they were therefore retained in further analyses. Both

transects show a similar pattern. We found that the most southerly sites (ANF, CNF, and LNF; Table 1) did not differ significantly from zero degrees of movement, whereas the rest of the sites toward the north were significantly different from zero ($P < 0.004$), evidence of seasonal movements (i.e., migrants). The isotopic data suggest that individuals in the north move between 4° and 10° latitude seasonally. In contrast, the southern sites have movement estimates that are very close to (and not significantly different than) zero, which is consistent with individuals having similar breeding and wintering areas (i.e., residents). This transition from south to north is highly significant (Pearson product–moment correlation = 0.93; $P < 0.0001$; df = 12).

There is an apparently gradual transition between these two migratory patterns (i.e., resident vs. migrant) and this is broadly coincident with the mtDNA cline centers from both transects (the arrows in Fig. 2B). The ANCOVA model-fitting procedure indicates the best model describing migratory movements includes both mtDNA type and breeding latitude, although AIC ranking suggests that the top three models do not differ strongly in their fit to the data (Table 2). However, removing breeding latitude from the model has a large effect on the amount of variation explained, implicating an important relationship between latitude and migratory movements, as illustrated in Figure 2B.

mtDNA SEQUENCING

Accession numbers for sequences can be found in Table S1. Consistent with Milá et al. (2007) we found, on average across all of the mitochondrial genes sequenced, 4.1% sequence divergence between the myrtle and Audubon's/black-fronted mtDNA, although this varied by gene (from 2% in ND2 to 7.2% in ND4; Fig. 3). We found that genes that encode for proteins in complex I of the electron transport chain (NADH dehydrogenase) had far more amino acid (AA) substitutions (16 fixed AA substitutions)

Table 2. Results of an analysis of covariance test for variables that explain variation in the distance an individual moved between its breeding and wintering grounds (i.e., migratory distance), derived from isotopic data. The best model, as chosen by Akaike Information Criteria (AIC; Akaike 1974), is shown in bold, although the small differences between the top three models suggest they are statistically indistinguishable.

Model	AIC
mtDNA type + breeding latitude + mtDNA type × breeding latitude	70.89
Breeding latitude + mtDNA type	**69.42**
Breeding latitude	70.80
mtDNA type	162.61

between the types compared to genes encoding for proteins in other complexes: complex III (cytochrome *b*; 0 fixed AA substitutions), complex IV (cytochrome *c* oxidase; 0 AA substitutions), and complex V (ATP synthase; 1 fixed AA substitution).

MITOCHONDRIAL ENZYME ASSAYS AND RESPIRATION

We found no significant difference in overall maximal enzyme activity (V_{max}) in complex I between individuals with myrtle or black-fronted type mitochondria in the center of the contact zone ($P = 0.179$, df = 21; Fig. 4A). Although complex I had the most fixed AA differences between the mito-types in the mtDNA genes sequenced, these data provide no evidence that these AA changes affect the maximal activity of the enzyme or that there are any differences in expression between the mito-types. We found a similar result for CS activity (Fig. 4B), which also did not differ significantly between the types ($P = 0.559$, df = 21). Citrate synthase, an enzyme involved in the tricarboxylic acid cycle, is correlated with overall mitochondrial content (Larsen
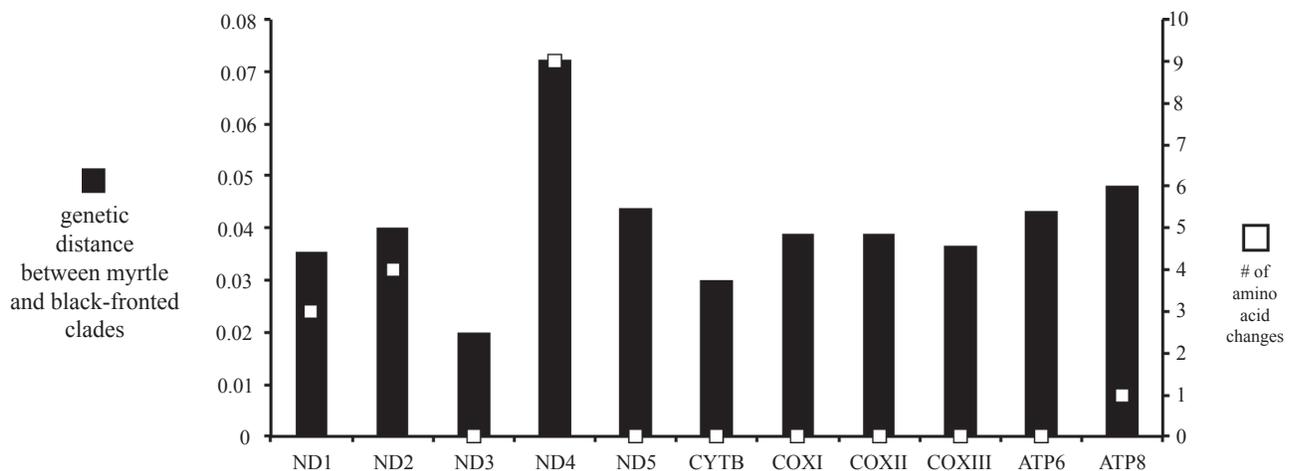

Figure 3. Sequence divergence and the number of fixed amino acid changes between the two mitochondrial clades in the contact zone.
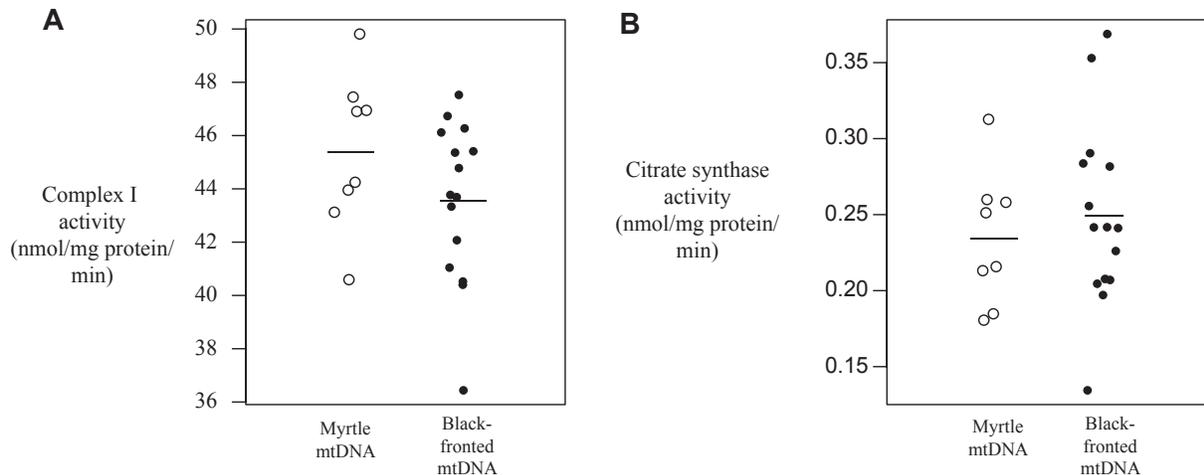
Figure 4. Maximal enzyme activity for **(A)** complex I and **(B)** citrate synthase from frozen *pectoralis* major of yellow-rumped warblers in the mtDNA contact zone distinguished as having a myrtle-type (open; *n* = 8) or black-fronted (filled; *n* = 15) mitochondria. The horizontal line represents the average maximal enzyme activity for each mitochondrial type.

et al. 2012); hence, this result provides no evidence that individuals with differing mtDNA types differ in the amount of mitochondria in their cells.

State II respiration rate obtained in the presence of pyruvate and malate did not differ between the two mito-types ($P = 0.191$, df = 22; Fig. 5A) and state III respiration rate following ADP addition also did not differ ($P = 0.944$, df = 22; Fig. 5A). There was also no difference in the rates of O_2 consumption between the two mtDNA types when mitochondria were respiring on succinate (substrate for complex II; $P = 0.348$, df = 22), or TMPD (a substrate for complex IV; $P = 0.777$, df = 20.8). However, we did find significant differences between the mtDNA types in the ACR, which is the ratio of state III respiration rate to state II, which was higher in birds with the myrtle mtDNA compared with birds with the black-fronted mtDNA ($P = 0.032$, df = 22; Fig. 5B). ACR is commonly used as a proxy measurement of how efficiently substrate oxidation is coupled to ADP phosphorylation (Scott et al. 2009) and in this case, northern and more migratory individuals have a significantly higher ACR than southern, resident individuals. These differences in ACR suggest that mitochondria from the more northern, migratory individuals are more coupled and subsequently more efficient (mean ACR is 2.27 for myrtle mito-types, 1.99 for black-fronted types). The other rate ratios of state III respiration to succinate or TMPD were not significantly different between the two mitochondrial types (succinate/state III: $P = 0.1637$, df = 22; TMPD/state III: $P = 0.908$, df = 20.5).

Discussion

In an effort to understand the drivers of mitochondrial introgression in the yellow-rumped warbler system, we have assembled a diverse data set that includes genetic, biochemical, and phenotypic variation obtained from several natural populations. We sampled individuals in an area in the southwestern United States that is a cryptic transition zone between the myrtle and black-fronted mtDNA (i.e., introgressed vs. ancestral mitochondrial types, respectively). We found that this transition in mtDNA is broadly coincident with a shift in migratory behavior and also with some aspects of mitochondrial phenotype. We discuss and interpret the findings for this novel data set in detail below.

First, our data support and extend previous research (e.g. Milá et al. 2011) suggesting that this mtDNA transition occurs within what are otherwise phenotypically and morphometrically Audubon's warblers in Arizona and Utah and also in previously unsampled sites in Colorado and New Mexico. However, the results of the cline fitting analysis suggest that the evolutionary and/or ecological processes shaping mtDNA variation differ somewhat between eastern and western transects, with the width of the eastern transition being approximately twice that of the west. Although many factors may be involved, including the fact that the intensity of selection between the two transects may vary, one of the most striking differences between the two transects is that the eastern (CO/NM) transect has a more contiguous matrix of suitable forest between sampling locations (green areas in Fig. 1B). More habitat continuity could increase population connectivity and mtDNA gene flow, thereby generating wider clines. In contrast, along the western (UT/AZ) transect, populations are concentrated in high-elevation coniferous forests, such as the Coconino and Kaibab National Forest (AZ) and the Dixie National Forest (UT), that are separated by wider areas of unsuitable habitat, such as the Grand Canyon, potentially inhibiting dispersal and generating narrower clines.
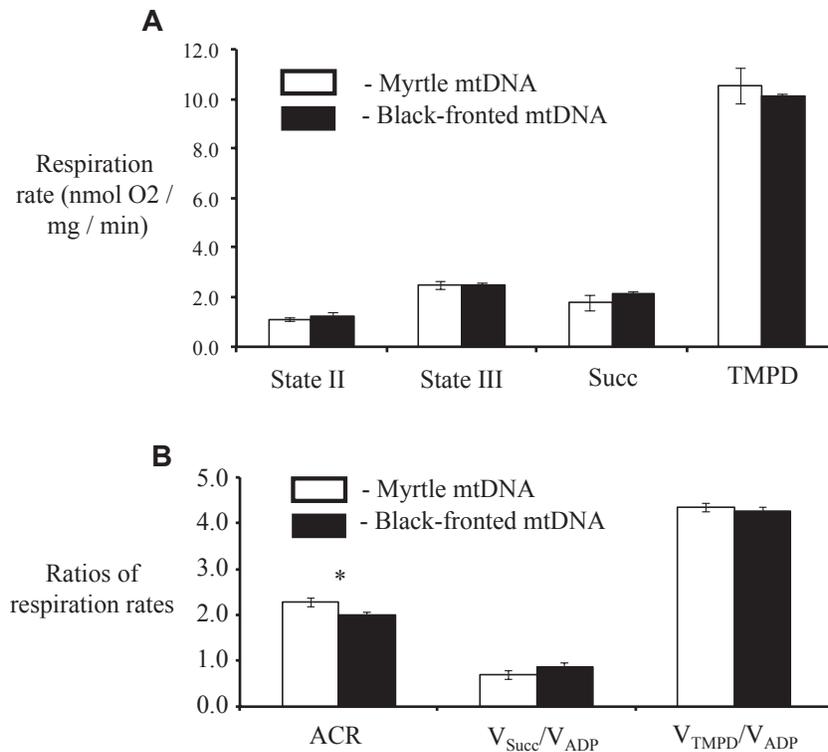
Figure 5. (A) Respiration rates of permeabilized fibers from the *pectoralis major* of yellow-rumped warblers in the mtDNA contact zone distinguished as having a myrtle-type (open; $n = 9$) or black-fronted mitochondria (filled; $n = 15$). Rates are shown for state II (background respiration rate without ADP), state III (maximally stimulated rate with ADP), as well as respiration rates when complex II (succinate dehydrogenase) and complex IV (cytochrome *c* oxidase) are stimulated. (B) rate ratios, illustrating a significant difference ($P < 0.05$) between myrtle-type and black-fronted mitochondria for the acceptor control ratio (ACR), the ratio of state III to state II respiration rate. Error bards indicate standard error.

The mtDNA clines can be compared to a model of neutral diffusion following secondary contact using the equation $w = \sqrt{2\pi\sigma^2 t}$ (Endler 1977; Barton and Gale 1993) where w is cline width, σ is root-mean-squared dispersal, and t is number of generations since contact. Assuming a dispersal distance of 20 km and a generation time of 2 years (Brelsford and Irwin 2009), it would take approximately 400 years following contact for a neutral mtDNA cline to form that is as wide as that observed along the eastern transect (less than half that time is necessary for the narrower cline in the west). This is not long, considering the biogeographic history of western North America, and it seems likely that these populations have been in contact much longer.

Both transitions in the mtDNA contact zone are wider than another well-studied hybrid zone in this species complex, between the myrtle and Audubon's warbler in the northern Rocky Mountains of British Columbia and Alberta, which is estimated to be 132 km wide (Brelsford and Irwin 2009). The narrowness of that hybrid zone in the Rockies suggests that there is moderately strong selection against hybrids (Brelsford and Irwin 2009). In the southern contact zone studied here, however, the observation that no other phenotypic trait studied to date, such as plumage,

morphometrics (Milá et al. 2011), and song (unpubl. data) shows a transition in the same location as mtDNA, combined with the fact these clines in mtDNA are much wider, suggests there is not likely a strong reproductive barrier between individuals with myrtle-type and black-fronted-type mtDNA. This result could be confirmed with additional nuclear markers using next generation technologies. For instance, are there small portions of the nuclear genome that covary with mtDNA, consistent with a pattern of cryptic genomic regions of isolation between individuals with the two mitochondrial types? Such high-resolution genomic data could also be useful in asking whether there is any reproductive isolation between birds with the black-fronted mtDNA in the southern United States and those in Mexico (i.e., across the traditional taxonomic boundary between *S. auduboni* and *S. nigrifons*).

Brelsford et al. (2011) suggests one possible factor driving the introgression of the myrtle-type mitochondria to high frequency in Audubon's warblers: it may be better adapted for the energetic demands of long-distance migration. Supporting this suggestion, our isotopic analysis suggests that there is a broad transition in migratory behavior, from individuals that do not move

large distances between seasons at the southern end of the mtDNA transition zone, to individuals that display behaviors associated with a fully migratory phenotype in the north. The ANCOVA analysis suggests that mtDNA type may explain a small amount of variation in individual movement (Table 2) but that breeding latitude in the contact zone is a much better predictor of migratory distance. Although finer-scale sampling along both transects would have been ideal, our estimates suggest that the shift in migratory behavior occurs between 35°N and 36°N (Table 1), very near the centers of the mtDNA transitions (35.94–37.4°N; Fig. 2). The fact that this transition in migratory behavior is broadly coincident with the shift in mitochondrial DNA is consistent with migration-adapted mtDNA hypothesis suggested by Brelsford et al. (2011). However, another possibility is that the mtDNA contact zone is still moving and it is by chance located in the current location that parallels the shift in migration.

It is interesting to note that inferred wintering latitude did not differ between individuals breeding at different locations. For instance, 95% of the individuals have winter deuterium ratios between −74‰ and −27‰ δD (Fig. S3; deuterium ratios increase toward the south), which would put most of the individuals wintering between approximately 37°N and 33°N latitude (Hobson et al. 2012). This is consistent with field observations of wintering yellow-rumped warblers in the southwestern United States (Hunt and Flaspohler 1998). However, it seems likely that at least some of the southern populations still move a short distance from their breeding locations. For instance, most sites where we captured individuals on their breeding territories across this mtDNA transition are at high elevations and likely still experience harsh winters. It may be that individuals in the south are moving out of the coniferous forests and into low-lying areas, performing a short altitudinal migration. Currently this is speculation, but future studies employing other, more sensitive techniques may be able to address these hypotheses, although it would not alter the conclusions of this study.

The mtDNA sequencing data suggests that there are numerous amino acid differences between the two mitochondrial types (Fig. 3); it is possible that some evolved via selection in myrtle warblers and were subsequently the targets of selective introgression following secondary contact with Audubon's warblers. Consistent with this, data from *ND2*, which has been sequenced in a number of *Setophaga* warblers, suggests that three of the four amino acid substitutions we identified in this gene (Fig. 3) are derived in the myrtle-type mtDNA and are retained in the ancestral-state in the black-fronted mtDNA. However, distinguishing directional selection from genetic drift or purifying selection as causes of mitochondrial DNA sequence differences is difficult (Hudson and Turelli 2003). In addition, the genes in mtDNA are inherited as a linked group, therefore testing the phenotypic effects of certain mutations is impossible without experimental work. Most of the amino acid changes are present in genes that code for protein subunits present in complex I, suggesting it may be a target of directional selection, however there are a number of reasons why this interpretation should be treated with caution. First, complex I is the most poorly understood complex in the electron transport chain, primarily because of its "L-shaped" membrane structure (Efremov and Sazanov 2011), so it is currently not possible to predict if amino acid changes occur in important active sites of the enzyme. Second, the genes coding for proteins involved in complex I have been found to evolve at a high rate in birds and reptiles as compared to other mitochondrial genes, so it is not necessarily surprising that these genes show a number of amino acid differences (Eo and DeWoody 2010). Third, we did not find significant differences in maximal enzyme activity of complex I between individuals with myrtle versus black-fronted type proteins, providing insufficient evidence that these changes affect enzyme function via changes in catalytic efficiency (k_{cat}) or enzyme amount. We note that there are other kinetic properties of complex I that we did not measure which may be influenced by the amino acid differences, such as the binding affinity for its substrates (NADH and ubiquinone) that may vary between the mitochondrial types and could be assayed in future studies. More generally, other nuclear genes involved with mitochondrial products could instead be the target of selective introgression resulting in mitochondrial discordance. Although a previous multilocus nuclear study using AFLPs (Brelsford et al. 2011) did not find sharp changes in the nuclear genome along the mtDNA transition zone, this does not rule out the potential contribution of nuclear-encoded mitochondrial products. We suggest that future studies employing next generation sequencing technologies could address this latter alternative more conclusively.

The data from measures of mitochondrial respiration suggest there is a small but statistically significant difference between how the mitochondrial types consume oxygen. Here we predicted that a mitochondrion associated with a more migratory lifestyle would (1) have a higher maximum capacity for respiration as measured by a higher state III consumption rate and/or (2) be more efficient as a result of increased coupling, measured here as the ratio of state III to state II consumption rates or the ACR. Coupling is defined as the amount of inorganic phosphate that is incorporated into ATP per unit of O_2 consumed by the mitochondria. The uncoupling of oxidative phosphorylation describes any process that decreases this phosphate/oxygen ratio and generally leads to a waste of redox energy (Nicholls and Ferguson 2002). In this case, we find no significant difference in the maximal efficiency of mitochondrial respiration (state III) between myrtle and black-fronted mito-types, but we do find a significant difference in ACR between them. Although this difference is small, it is in the expected direction of more migratory myrtle-type birds exhibiting a higher ACR and a potentially more efficient production of ATP,

due to less proton leak and uncoupling, compared to the southern and sedentary black-fronted type individuals. This ratio in ACR was driven mostly by differences in state II, as it differs most strongly between the types, although the difference is not statistically significant (Fig. 5A).

Previous studies have shown that proton leak decreases with body size in birds and other endotherms (Brand et al. 2003). This suggests that small birds, such as wood warblers studied here, have a high mitochondrial membrane permeability and proton leak and therefore have potentially a greater scope for evolving a more coupled system. Although the molecular basis of the proton leak is currently unclear, we suggest that future studies assessing membrane permeability of the different mitochondrial types could further test our finding of higher ACR and potentially lower state II respiration rate in myrtle-type mitochondria.

To our knowledge this is the first time mitochondrial respiration has been measured in permeabilized muscle fibers in a wild caught bird. Compared to captive geese (Scott et al. 2009), yellow-rumped warblers have higher state III and state II respiration rates and a lower ACR. This is consistent with the allometric relationship of these variables as measured in isolated mitochondria in other birds (Brand et al. 2003). The sensitivity of this method suggests that it could be useful for other applications. For instance, a broader comparative study of migratory versus sedentary species could be used to test whether mitochondrial adaptation to a migratory lifestyle is common. This would be especially useful in cases where past introgression may not have left a distinct biogeographic pattern such as observed in this system. Beyond a migratory phenotype, these types of assays would be ideal for testing hypotheses of mitochondrial adaptation to conditions of hypoxia, such as at high elevation, as highlighted by studies of the rufous-collared sparrow (Cheviron and Brumfield 2009) and deer mice (Cheviron et al. 2012).

Given the current extent of introgression and the functional differences we have identified, it is unclear why the myrtle-type mtDNA has not swept to fixation throughout the entire Audubon's/black-fronted warbler range. One possibility is that it has swept to frequencies at or near fixation in populations for which it is adaptive (i.e., migratory populations) and then, in those populations for which the myrtle mitochondrial-type no longer presents a selective advantage, more local demographic processes such as population size and dispersal may dominate. Although speculative, there may also be a tradeoff between the two mitochondrial types: increased coupling may be important for migration in the north, whereas an increased proton leak may be advantageous in the south due to other environmental factors that we have not measured. There are also scenarios that could generate such discordant mtDNA clines that do not involve selective introgression of mtDNA. For instance, perhaps the original contact between the divergent forms is close to the current tran-

sition zone in the southwestern United States. If so, it is possible that the zone may be trapped in a population sink or in an ecological transition that is not currently obvious. To explore these alternatives, future studies should consider collecting additional environmental and genetic data and also sample other populations further east, west, and along various elevations. This would allow a more robust test of whether the patterns observed here are likely a result of selection, demographics, or simply stochastic variation.

In conclusion, our study adds to the small number of cases where the proposed drivers of mitochondrial and nuclear discordance have been rigorously tested. Indeed, of the 35 studies reviewed by Toews and Brelsford (2012) that invoke adaptive explanations to explain mitochondrial introgression, very few have rigorously tested those explanations (e.g., Aubert and Solignac 1990; Blier et al. 2006; Boratynski et al. 2011). By combining molecular biogeography, stable isotopes, and mitochondrial biochemistry, we have examined the correlation between migratory phenotype and mitochondrial genotype in this system. We suggest that data presented here are consistent with the migration-adapted mitochondrion hypothesis. However, given the correlative nature of this study, other processes, including stochastic shifts in the frequency and distribution of mtDNA that may have produced this mtDNA-nuDNA discordance (and any correlated phenotypic differences), should not be excluded. In the future, a more direct test of the role of selection driving mtDNA introgression in this system will require a combination of the functional data presented here with measures of fitness that these different phenotypes may confer. Although challenging, especially for a complex phenotype such as migration, such cases of mitochondrial introgression present the exciting opportunity to link underlying genetic changes with phenotypic variation at the level of the mitochondria and whole-organism performance (Storz and Wheat 2010). This is a shift from the past, where many previous studies assumed that mtDNA evolves neutrally and where potential differences associated with selection were often disregarded (Ballard and Whitlock 2004; Irwin 2012). Indeed, studies that examine possible adaptive causes of genetic introgression can highlight the role that hybridization plays in providing an important source of adaptive alleles between partially reproductively isolated taxa.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Regression of feather isotopes versus latitude at capture for yellow-rumped warblers along the western (AZ/UT) transect.

Figure S2. Regression of feather isotopes versus latitude at capture for yellow-rumped warblers along the eastern (CO/NM) transect.

Figure S3. Feather isotopes composition of all individuals from both transects against latitude at capture location.

Table S1. Accession numbers, primer information and PCR conditions for mtDNA sequencing.