HYBRIDIZATION, SPECIATION AND THE BIOGEOGRAPHY OF GENETIC AND PHENOTYPIC VARIATION IN SETOPHAGA WARBLERS

by

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Abstract

Contact zones between previously isolated taxa provide natural systems for studying the processes involved in divergence, adaptation and reproductive isolation. In this thesis I use inferences drawn from biogeographic patterns and the characteristics of hybrids to understand the evolutionary processes involved in the production and maintenance of avian contact zones. In chapter two, I use genetic and phenotypic data from two related species of wood warbler to study the hybridization dynamics between them. I found that *Setophaga virens* and *S. townsendi* hybridize extensively where they come into contact but that the hybrid zone between them is narrow, suggesting moderate selection against hybrids. In chapter three and four I examine a possible mechanism of selection within such hybrid zones: differences in seasonal migratory behaviour. I explore this in a hybrid zone between *S. coronata* and *S. auduboni*. In chapter three I use isotopic data from breeding birds to make inferences about the wintering behaviour of hybrids. In chapter four, I use genetic data and video-based orientation assays of birds on fall migration to estimate the migratory tendency of birds in the hybrid zone. I found that (1) isotopes suggest that birds in the hybrid zone mostly wintered in the southeastern U.S. and (2) birds, on average, oriented towards the northeast during fall migration, regardless of their genetic background.

These hybrid zones illustrate patterns of concordance between some characteristics but discordance in others. As I review in chapter five, the biogeographic patterns associated with discordant molecular markers, especially between those in the nuclear versus mitochondrial genome, can reveal novel insights into important evolutionary processes. In chapter six I address an example of such discordance, where previous research has suggested that mitochondria from *S. coronata* have introgressed throughout much of the range of *S. auduboni*. I use genetic, biochemical, and phenotypic variation to show that this shift in mitochondria is correlated with a shift in migratory behaviour and with some aspects of mitochondrial phenotype. In chapter seven, I use a genome-wide assay of tens of thousands of nuclear polymorphisms to test whether mtDNA is truly an outlier as compared to the nuclear genome.
Preface

A version of chapter 2 has been published: Toews D. P. L. Brelsford A and D. E. Irwin. 2011. Hybridization between Townsend’s *Dendroica townsendi* and black-throated green warblers *D. virens* in an avian suture zone. *Journal of Avian Biology* 42: 434-446. I helped plan the study, collected the field samples, conducted the molecular work and statistical analysis, and wrote the manuscript. Darren Irwin initially began studies of this and other systems in the area and helped planned the current study. Alan Brelsford and Darren Irwin contributed to the sampling and the writing of the paper. Ilya Povalyaev assisted in the field. Animal care and experimentation was conducted according to the University of British Columbia protocol No. A09-0131 (Project title: Geographic variation in birds of western Canada). Note the change in genus names, from *Dendroica* to *Setophaga*, between the publication and the current thesis.

A version of chapter 3 has been accepted for publication: Toews, D.P.L. Brelsford A and D. E. Irwin. Isotopic variation across the Audubon’s / myrtle hybrid zone. *Journal of Evolutionary Biology*, in press. I planned the study, conducted the statistical analysis and wrote the paper. Alan Brelsford collected the field samples and, with Darren Irwin, contributed ideas during the writing of this chapter. Mano Young and Keith Hobson conducted feather isotope analysis in Environment Canada’s Isotope Laboratory at the National Water Research Institute in Saskatoon. All animal care and experimentation was conducted according to the University of British Columbia protocol No. A09-0131 (Project title: Geographic variation in birds of western Canada).

Chapter 4 is work conducted at the Kananaskis Biogeoscience Institute. I planned the study, collected and analyzed the data. Phil Taylor and Kira Delmore helped with planning the orientation experiments. Matthew Osmond helped with the statistical analysis of the orientation data. Kira Delmore and Stephanie Cavaghan assisted with the field research. Darren Irwin, Kira Delmore, Phil Taylor and Matthew Osmond contributed ideas during the writing of this chapter. All animal care and experimentation was conducted according to the University of British Columbia protocol Nos. A11-0054 (Project title: Orientation in Migratory Songbirds) and A09-0131 (Project title: Geographic variation in birds of western Canada).
A version of chapter 5 has been published: Toews D. P. L. and A. Brelsford. 2012. The biogeography of mitochondrial and nuclear discordance in animals. *Molecular Ecology* 16: 3907-30. I conceptualized the idea for the review, conducted part of the literature review, analyzed the data and wrote the manuscript. Alan Brelsford also conducted part of the literature review and co-wrote the manuscript.

A version of chapter 6 has been published: Toews D. P. L. Mandic M. Richards J. G. and D. E. Irwin. 2014. Migration, mitochondria and the yellow-rumped warbler. *Evolution* 68: 241-255. I planned the study, collected the field samples, conducted the molecular work and statistical analysis, helped with the biochemical and mitochondrial respiration assays, and wrote the manuscript. Milica Mandic collaborated during the planning and execution of the biochemical and mitochondrial assays. Mano Young and Len Wassenaar conducted feather isotope analysis in Environment Canada’s Isotope Laboratory at the National Water Research Institute in Saskatoon. Milica Mandic, Jeff Richards and Darren Irwin all contributed to the ideas presented in this chapter. All animal care and experimentation was conducted according to the University of British Columbia protocol Nos. A10-0058 (Project title: Mitochondrial function and the Evolution of Long-Distance Migration in a Passerine) and A09-0131 (Project title: Geographic variation in birds of western Canada).

Chapter 7 is based on worked conducted in UBC’s Lab of Molecular Biogeography. I planned the study, collected most of the field samples, prepared the sequencing libraries and conducted the molecular analyses. Some of the samples were also collected by Borja Milá and Alan Brelsford. Darren Irwin, Borja Milá, Alan Brelsford and Christine Grossen contributed ideas during the writing of this chapter. All animal care and experimentation was conducted according to the University of British Columbia protocol No. A09-0131 (Project title: Geographic variation in birds of western Canada).
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CHAPTER 1: General introduction

In writing on evolution and the origin of species, Darwin (1859) proposed natural and sexual selection as the primary processes by which, over many generations, organisms become adapted to their environment and, subsequently, how new species arise. The theory, that acting on heritable variation selection will favor individuals that express traits making them more likely to survive and reproduce, has been immensely powerful in explaining the origin and diversity of biological variation. It seems surprising then that even 150 years after Darwin’s foundational research, there are still a number of important gaps in our understanding of these processes. For instance: how prevalent are non-adaptive or neutral mechanisms in producing patterns of diversity (Schluter 2000; Coyne and Orr 2004; Rundell and Price 2009)? How often does selection act on ‘standing variation’ versus new mutations in populations (Barrett and Schluter 2008)? What is the relative role of hybridization between ‘independent’ evolutionary lineages in generating biological variation (Rieseberg 1997; Mallet 2007)? How are the underlying genetic changes that arise via mutation connected to an organism’s phenotype and subsequent fitness (Orr and Coyne 1992; Hoekstra et al. 2006)? And finally, how does adaptation to different environments produce reproductive isolation if and when individuals from divergent populations interbreed (Schluter 2000; Coyne and Orr 2004; Schluter 2009)?

Addressing diverse questions such as these is challenging. This is especially true among non-model organisms that are not amenable to experimental crosses and laboratory studies, which have proved so successful in a number of systems (e.g. Ting et al. 1998; Colosimo et al. 2005; Hoesktra et al. 2006). Using areas where divergent populations/subspecies/species occur in sympatry and naturally interbreed can be especially useful for such investigations. First, hybrid zones can be used to identify genes and traits under divergent selection by quantifying variation in clinal transitions across them (Endler 1977; Barton and Hewitt 1989; Coyne and Orr 2004; Price 2008). Second, they can be used to associate genetic and phenotypic variation by studying divergent alleles segregating in individuals with mixed genetic backgrounds (Rieseberg and Buerkle 2002; Dalziel et al. 2009).

Most hybrid zones are thought to have formed after periods of geographic isolation followed by secondary contact (Coyne and Orr 2004; Price 2008). At least in North America,
many hybrid zones are assumed to have formed following the retreat of the glaciers present during much of the Pleistocene (Swenson and Howard 2005). During spatial isolation divergent natural or sexual selection may act to fix different beneficial alleles in different populations (termed ‘ecological speciation’; Schluter 2000). If subsequent range overlap brings these diverging populations back together, hybridization can form novel gene combinations that have not previously been under selective scrutiny and can expose incompatibilities between them (Macholan et al. 2007; Presgraves 2010). This can occur if there are negative interactions in hybrids between new ‘derived’ alleles from divergent populations or from ‘derived’ alleles with others that have retained an ‘ancestral’ state (Presgraves 2010). This type of selection against unfit hybrid allelic combinations is known as ‘endogenous’ selection. If, on the other hand, hybrids have intermediate or inferior ecological fitness compared the parental types, this type of hybrid zone implicates adaptation to divergent habitats as a strong reproductive barrier and is termed ‘exogenous’ selection on hybrids (Szymura and Barton 1986; Price 2008).

Identifying the genes or traits under divergent selection can be inferred from multi-locus surveys of molecular markers across hybrid zones (Szymura and Barton 1986). Many well-studied hybrid zones, for instance, are characterized by steep, concordant clines at different loci and diverse traits (Barton and Hewitt 1985). For example, multi-locus genetic studies of a hybrid zone between two subspecies of the rainforest lizard, *Lampropholis coggeri*, is estimated to be only <0.5km wide (Singhal and Moritz 2012). This pattern suggests there is limited introgression across the hybrid zone and strong selection against hybrids within it. These types of hybrid zones, where the amount of gene flow moving into them is high compared to the mixing of the parental populations and selection against hybrids, are termed ‘tension zones’ and are thought of as genetic sinks (Barton and Hewitt 1985). These patterns suggest that differences between the parental taxa are causing premating isolation (e.g. differences in flowering time) or postmating selection against hybrids (e.g. genetic incompatibilities or inferior feed behaviour) and restricting introgression (Szymura and Barton 1986).

Identifying the type and mode of selection in many hybrid zones, however, is unknown in many cases. This is most easily studied in systems where hybrid offspring can be raised under laboratory conditions or easily observed in natural settings (e.g. *Ficedula* flycatchers; Qvarnström et al. 2010). In these cases, simple observational study of the developmental success of hybrids is sufficient to estimate the extent of possible intrinsic incompatibilities causing reduced hybrid fitness. At least in avian systems, however, intrinsic reproductive isolation is
assumed to take a long time to develop between incipient species (Price and Bouvier 2002). This has lead some authors to suggest that other forms of reproductive isolation (i.e. premating or ecologically based post-mating reproductive barriers) may be more important in the evolution of new bird species (Price 2008). However, studies of premating isolation and postmating selection against hybrids can be especially challenging. This is particularly true of migratory birds, where individuals might move over long distances during different parts of their annual cycle. For example, inferior migratory behaviour has been posited as an important selective force in maintaining the hybrid zone between two subspecies of Swainson’s thrush, *Catharus ustulatus* (Ruegg 2008). Only recently have advances in tracking technology allowed for a full study of birds in this system (Delmore *et al.* 2012). Therefore, there have been calls for additional studies of the full annual cycle in organisms that form hybrid zones and also exhibit large seasonal movements (Veen 2013).

Hybrid zones where genetic or phenotypic clines are displaced from the majority of other transitions can also be very informative. For instance, displacement of cline centres amongst genetic markers or traits either indicates that the hybrid zone may have moved (e.g. Krosby and Rohwer 2009) or that there has been introgression between taxa for those genetic markers or traits upon secondary contact (e.g. Brumsfield *et al.* 2001). If hybrid zone movement can be ruled out, this pattern is consistent with a selective advantage of those loci or traits that have introgressed. This pattern can then be used to link these genetic variants to phenotypic and fitness differences, where the traits that distinguish individuals with divergent alleles are present in an otherwise homogenous genetic background (Dalziel *et al.* 2009).

In particular, mitochondrial introgression across taxonomic boundaries has been noted as an especially prevalent form of this type of discordance (Chan and Levin 2005; Toews and Brelsford 2012). ‘Mito-nuclear’ discordance has been observed in many different systems and, given that most phylogenetic studies now incorporate markers in both the mitochondrial and nuclear genomes, it is becoming more readily identified in many systems. However, in most cases, the drivers of this type of discordance are not well understood. The most common explanation for these types of discordant patterns are adaptive (e.g. potential thermal adaptation of the *Phoxinus neogaeus* mitochondria; Mee and Taylor 2012) and sex-biased dispersal (e.g. asymmetric mating of female *Hyla gratiosa* with male *H. cinera*; Lam and Avise, 1986). Therefore, whether they show patterns of discordance or concordance of various traits of molecular markers, data from cases of hybridization in nature can be very informative. In
particular, hybrid zones provide opportunities to understand fundamental questions about how selection acts in nature and what genes and traits are potentially involved in local adaptation and reproductive isolation.

Here I to use three hybrid zones between divergent avian taxa, with the objective to study the effects of hybridization. In particular, my goals are to understand the types of reproductive isolation between incipient species and to quantify the extent and type of variation that may be shared between divergent taxa as a result of hybridization. My research focuses on species in the Setophaga genus of the Parulidae family (i.e. wood warblers; Lovette et al. 2010). This family of mostly migratory songbirds has a long history of study, including MacArthur’s early work on ecological niche divergence (MacArthur 1967) to Rabosky and Lovette’s (2008) more recent treatments using phylogenetic patterns to assess evidence for density-dependent diversification in this group. Hybridization dynamics have also been studied in detail across a number of the taxa in this family. For example, Rohwer (2001) demonstrated compelling evidence of a moving hybrid zone between Townsend’s warblers (S. townsendi) and hermit warblers (S. occidentalis). Similarly, hybridization between blue-winged (Vermivora cyanoptera) and golden-winged warblers (V. chrysoptera) is so extensive and asymmetric that there is serious conservation concern of pure golden-winged warbler populations (Vallender et al. 2007). These patterns contrast the hybrid zone between myrtle (Setophaga coronata) and Audubon’s warblers (S. auduboni), which has been stable in width over many years and has remained in place between the studies of Hubbard (1969) and Brelsford and Irwin’s (2009) more recent treatment.

In chapter two of this thesis I study a potential area of range overlap and hybridization between Townsend’s (S. townsendi) and black-throated green (S. virens) warblers. These two species are phenotypically and genetically divergent groups that occur in western and eastern North America respectively, with potential for range contact in the Rocky Mountains of British Columbia, where other west-east avian pairs come into contact. My goal here is to determine whether these two species interbreed where they co-occur and whether any hybridization between them is a regular occurrence. To do this I examined genetic and phenotypic variation in individuals across the area of putative range overlap.

In chapter’s three and four I examine a possible mechanism of selection against hybrids within such narrow hybrid zones: differences in seasonal migratory behaviour. I explore this in the hybrid zone between Audubon’s (S. auduboni) and myrtle warblers (S. coronata). My goal
here is to estimate the differences between these two parental species in these behaviours and then test how these behaviours might be expressed in hybrids between them. In chapter three I use isotopic data from breeding birds to make inferences about the wintering behaviour of hybrids. In chapter four, I use genetic data and video-based orientation assays of birds on fall migration to estimate the migratory tendency of birds in the hybrid zone. These studies are important, given that most research of avian hybrid zones focuses on breeding individuals. While this research uses indirect methods, it allows me to estimate the behaviour of individuals outside of the breeding season.

Both of these hybrid zones illustrate patterns of concordance between some characteristics whereas discordance in others. As I review in chapter five, the biogeographic patterns associated with discordant molecular markers can reveal novel insights into important evolutionary processes. In this review I focus on examples of discordance between patterns in the mitochondrial genome versus those in the nuclear genome. I provide a synthesis and review of these patterns across all cases that have been examined in animals. In chapter six I address a specific example of such mito-nuclear discordance, where previous research has suggests that mitochondria from *S. coronata* has introgressed throughout much of the range of *S. auduboni* (Brelsford *et al.* 2011; Toews *et al.* 2014). I use genetic, biochemical, and phenotypic variation to show that this shift in mitochondria is correlated with a shift in migratory behaviour and with some aspects of mitochondrial phenotype. Finally, in chapter seven, I use a genome-wide assay of tens of thousands of nuclear polymorphisms to test whether mtDNA is truly an outlier as compared to the nuclear genome. In this case, I use the ‘genotype-by-sequencing’ method (Elshire *et al.* 2011) to assay variation across the nuclear genome.
CHAPTER 2: Hybridization between Townsend’s (Setophaga townsendi) and black-throated green warblers (S. virens) in an avian suture zone

2.1 Introduction:

Determining the frequency with which diverging taxa interbreed in nature can reveal the extent to which they are reproductively isolated (Grant and Grant 1992). At least in birds, rare hybridization events have been noted between many pairs of species (Mayr and Short 1970). These uncommon events usually tell us little about the evolutionary history or taxonomic status of the taxa in question (although see Vallender et al. 2009). In contrast, extensive hybridization in hybrid zones can have a number of important taxonomic and conservation implications (Price 2008). Hybridization can be a source of evolutionary novelty, introducing potentially adaptive alleles to the gene pool of a species (Short 1972). Hybridization between domesticated dogs and grey wolves, for instance, is thought to have introduced a putatively advantageous coat colour allele from canines to their wild relatives, producing a dark colour morph, which has been maintained in natural grey wolf populations in North America (Anderson et al. 2009). In contrast, some cases of hybridization have led to the erosion of differences between species and the loss of unique evolutionary history upon secondary contact (Seehausen 2006). For example, the asymmetric introgression of blue-winged warbler Vermivora cyanoptera alleles into golden-winged warblers V. chrysoptera has left only a few remaining populations that contain genetically pure golden-winged warblers (Vallender et al. 2007). In either scenario, studying the dynamics of hybridization between diverging taxa can lead to an understanding of the genes and traits important in the evolution of reproductive isolation (Payseur 2010).

Areas where numerous different pairs of related taxa come into secondary contact are termed ‘suture zones’ (Remington 1968, Swenson and Howard 2005). We have recently described a suture zone in northeastern British Columbia, Canada, that is home to a number of avian contact zones, some previously unknown to science. This is where the Pacific wren Troglodytes pacificus and the winter wren T. hiemalis overlap in their distribution (Toews and Irwin 2008), where Audubon’s warblers Setophaga auduboni and myrtle warblers S. coronata hybridize (Hubbard 1969, Barrowclough 1980, Brelsford and Irwin 2009) and where a recently

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described hybrid zone between MacGillivray’s warblers *Geothlypis tolmiei* and mourning warblers *G. philadelphia* is located (Irwin et al. 2009).

Another pair of closely related species that has been proposed to come into contact in this area (M. Phinney pers. comm.) are Townsend’s warblers *Setophaga townsendi*, which mostly breed west of the Rocky Mountains, and black-throated green warblers *S. virens*, which generally breed east of the Rocky Mountains (Fig. 2.1). A hybrid zone between Townsend’s warblers and their southern relatives, hermit warblers *S. occidentalis*, has been the subject of detailed study in Washington and Oregon (Rohwer and Wood 1998; Krosby and Rohwer 2009). This is a moving hybrid zone, with phenotypic Townsend’s warblers moving south and replacing hermit warblers, leaving the genetic footprint of hermit warbler mitochondrial DNA in its wake (Krosby and Rohwer 2009). Mitochondrial DNA reveals that Townsend’s and hermit warblers are sister to the group that contains black-throated green warblers and the endangered golden-cheeked warbler *S. chrysoparia* (Lovette et al. 2010) and the two pairs are estimated to have diverged from each other approximately one million years ago (Weir and Schluter 2004; Rabosky and Lovette 2008). Motivated by these interesting hybridization dynamics between Townsend’s and hermit warblers, we wished to investigate whether Townsend’s and black-throated green warblers might also hybridize if they come into contact.

Aside from their mostly geographically separate breeding distributions, Townsend’s and black-throated green warblers differ in a number of phenotypic characteristics (reviewed in Rohwer 1994). Most notably, Townsend’s have a black crown and face patch, whereas black-throated green warblers have an olive green crown and lighter face. Also, Townsend’s have bright yellow on their breast, which extends down to their underparts, whereas black-throated green warblers either have completely white breasts or, more commonly, a pale yellow wash. Morphologically the two overlap in many characters, although Townsend’s generally have a longer wing-chord and tail length (Ridgway 1902; Pyle 1997). The two usually have songs that are easily distinguished, with Townsend’s exhibiting considerable individual and population differences and black-throated greens having quite stereotyped songs across their range (Wright et al. 1998; Morse and Poole 2005). Finally, in allopatry black-throated green warblers nest in territories composed of mixed-wood, primarily deciduous forests, with some coniferous component usually present (Morse and Poole 2005), whereas Townsend’s warblers generally nest in primarily coniferous stands, dominated by spruce and fir (Wright et al. 1998).

In a survey of Townsend’s and hermit warblers, Rohwer (1994) reported finding one phenotypic Townsend’s warbler with a black-throated green mtDNA haplotype, near Valemount,
BC. Given this individual’s proximity to the Rocky Mountains and the breeding range of black-throated green warblers, it was suggested that this was likely a late generation backcross hybrid between the species. At the time, however, the ranges of Townsend’s and black-throated green warblers were not known to come into contact (Rohwer 1994).

In fact, black-throated green warblers are thought to be a recent addition to BC’s avifauna (Campbell et al. 2001). An individual collected near Moberly Lake (north of Chetwynd) in 1965 is the first record of a black-throated green warbler in the province (Salt 1966). Since then, however, the species has become quite common along the eastern slopes of the Rockies, especially near the towns of Chetwynd and Dawson Creek. Some recent observations from breeding bird surveys suggest that some phenotypic black-throated green warblers are now breeding west of the Rocky Mountains, near Prince George, within the range of Townsend’s warblers (J. Bradley pers. comm.).

Given these observations of potential range contact and one possible hybrid, we explored northeastern BC with the goal of determining whether Townsend’s and black-throated green warblers come into contact and, if so, whether there is a hybrid zone between them. We measured phenotypic and morphological differences between the species to determine how to identify intermediate, hybrid individuals. We also assayed variation in three molecular markers, including mtDNA and two nuclear DNA markers, one on a sex chromosome and one on an autosome; these markers have been used to map another Setophaga hybrid zone in the area (between Audubon’s and myrtle warblers; Brelsford and Irwin 2009).

2.2 Material and methods

2.2.1 Field research

During the springs of 2007-2010 we captured Townsend’s and black-throated green warblers for temporary study from allopatric populations (n=33) and from within the putative contact zone (n=138). Especially large samples were collected in 2009 by David Toews (n=114) and in 2007 by Darren Irwin (n=49). We focused efforts along forestry roads west of the town of Chetwynd, BC where both species were thought to occur (Fig. 2.2; M. Phinney pers. comm.). For each individual we took standard measurements, photographs, and a small blood sample, then applied bands and released the bird. We supplemented our black-throated green genetic sampling far from the contact zone by including tissue samples from Chicago, IL (n=16; provided by the Field Museum) of individuals likely on migration (i.e. most were collected
following building collisions); these likely were from breeding locations far east of the contact zone. We aged and sexed all individuals according to Pyle (1997). All of the individuals captured for temporary study were males. Of the 16 museum specimens included 10 were male, 5 were female and 1 was of unknown sex.

2.2.2 Phenotypic and morphometric analyses

Rohwer (1994) reported a number of consistent plumage differences between Townsend’s and black-throated green warblers and how they were manifested in a single hybrid. Here we present an analysis of three of these characteristics, which we found to be the most consistent to score between the species (Fig. 2.3). Crown colour was scored on a scale from 0 to 4 with ‘0’ being a completely green crown (corresponding to black-throated green), ‘1’ having thick black bases to crown feathers, ‘2’ having equal amounts of green and black, ‘3’ having more black than green and ‘4’ being completely black (corresponding to Townsend’s). As some second-year (SY) Townsend’s have a slight olive edging to their crown, which eventually becomes black in after-second-year (ASY) birds, we were conservative in our scoring of potential hybrids and scored these SY individuals as ‘completely black’. We scored breast colour on a scale from 0 to 3 with ‘0’ being completely white or faint yellow wash, ‘1’ having concentrated yellow extending approximately 1 cm from the throat, ‘2’ having bright yellow extending 3/4 of the way down the breast and ‘3’ having bright yellow extending throughout the breast. Finally, we scored the face patch on a scale from 0 to 2 with ‘0’ being faint green or black, ‘1’ being intermediate, with some dark black and faint green feathers, and ‘2’ being completely dark black. Again, as some SY Townsend’s have a slight lighter edging to their feathers, which eventually becomes black in ASY birds, so we also scored these as ‘completely black’ as in the crown. We divided each of the scores by the total number of possible classes for that plumage trait and then, by dividing each by the highest possible score, combined each additively to create a ‘hybrid index’ that ranged between 0 and 1, with black-throated green warblers being ‘0’ and Townsend’s being ‘1’.

We also measured six morphological characteristics (according to Pyle 1997): wing-chord (unflattened), tail, tarsus, beak length, beak depth, and beak width. We log-transformed these six morphological variables and identified the major axis of covariation using a principal components analysis (PCA) as implemented in R (R Development Core Team 2013). We fitted cubic splines to each of the morphological traits and the plumage score in R using a generalized additive model with the ‘identity’ error link function to determine the best smoothing parameter.
To test for differences in the untransformed means of each of these traits we used a Welch’s two-sample $t$-test in R. For this and other analyses we defined the ‘hybrid zone’ as those populations occurring within 130 km of the crest of the Rocky Mountains.

2.2.3 Molecular analyses

Blood samples were taken from the brachial vein and stored in Queen’s Lysis Buffer (Seutin et al. 1991) and left at ambient temperature until returned to the laboratory. DNA was extracted using a phenol-chloroform protocol and resuspended with 50-200 ml of TE (10 mm Tris-HCl, 1 mm EDTA, pH 8.0) depending on the size of the pellet and stored at 4°C. Our choice of molecular markers for distinguishing the taxa was based on limited sequence information from previous surveys (Lovette et al. 1999, Brelsford and Irwin 2009) and the requirement that there was an appreciable frequency difference between allopatric samples.

mtDNA: Lovette et al. (1999) described in detail mitochondrial sequence variation that distinguishes Townsend’s and black-throated green mtDNA haplotypes in a 613 bp region of cytochrome oxidase I (COI). We amplified this region of the mitochondrial genome using the primer pair COIa-3’ and COIf-5’ (Kessing et al. 1989, Lovette et al. 1999). We used a restriction fragment length polymorphism (RFLP) assay to genotype a SNP at 209 bp, where there is a G-A polymorphism, with G common in Townsend’s warblers and A common in black-throated green warblers; the restriction enzyme SmaI cuts only the G variant. PCR reactions included 1xPCR buffer (Invitrogen), 1.5 mM MgCl2 (Invitrogen), 0.2 mM dNTP mix (New England Biolabs), 0.5 uM forward and reverse primer, 0.04 units ul$^{-1}$ Taq DNA polymerase (New England Biolabs), and 2.5 ng ul$^{-1}$ genomic template DNA, in a total volume of 10 ul. 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 5 min at 72°C. We digested 2 ul of the PCR product with 2 units of the restriction enzyme SmaI in its appropriate buffer (New England Biolabs), in a total volume of 6 ul. Products were digested for 2 h at 37°C, and digested DNA was visualized by electrophoresis on 2% agarose gel stained with SYBR Safe (Invitrogen). This digestion cuts the Townsend’s PCR product into two fragments (209 and 404 bp) and does not cut the black-throated green product (one 613 bp fragment). A complicating factor is that this digest also does not cut the hermit warbler product (Lovette et al. 1999); in one case a phenotypic Townsend’s warbler sampled along the Pacific Coast that had only a single fragment was assumed to have hermit warbler mtDNA due to hybridization with them in that area. For purposes of the cline analysis between Townsend’s and black-throated green warblers, we coded this individual as having a Townsend’s mtDNA.
Numt-Dco1: Brelsford and Irwin (2009) and Brelsford et al. (2011) described this ‘nuclear sequence of mitochondrial origin’, an autosomal nuclear marker that is divergent between Setophaga coronata and S. auduboni. We sequenced 1091 bp in this region for one individual (two alleles) from each of our focal species using primers numt_up_2.3 (5’-CCTTCCTCTAATTCCCTACTGTCA) and numt_up_R (5’-CAGAGTGACCCCGAGAAAG) and identified a G-A SNP at 88 bp, with the G variant common in black-throated green warblers; this can be genotyped using the restriction enzyme HpyCH4IV (Genbank accession numbers JF310198-JF310201). The PCR, digestion and visualization protocol are the same as above except the annealing temperature was 53.2°C. This digestion cuts the black-throated green PCR product into two fragments (912 and 179 bp) and cuts the Townsend’s product into three (88, 824 and 179 bp). We only used the presence/absence of the 824 and 912 bp fragments for genotyping and these were easily distinguished on agarose in both homozygotes and heterozygotes.

CHD1Z: Brelsford and Irwin (2009) and Irwin et al. (2009) used sequence variation in the CHD1Z gene, a Z-linked nuclear marker that is used in molecular sexing of birds, to assay variation across the Setophaga coronatal S. auduboni and Geothlypis tolmiei/G. philadelphia hybrid zones. In birds, males are the homogametic sex (ZZ) and therefore have two copies of CHD1Z. We amplified and sequenced a 632 bp fragment in two males from each species (Genbank accession numbers JF310002-JF310009) using primers 2550F and 2718R (Fridolfsson and Ellegren 1999) and found a single T-to-A SNP at 147 bp which creates a cut site in Townsend’s PCR product (with the A variant) for the restriction enzyme AseI. The PCR, digestion and visualization protocol are the same as above except the annealing temperature for the PCR was 53°C. This digestion cuts the Townsend’s PCR product into three fragments (147, 184 and 301 bp) and cuts the black-throated green product into two (184 and 448 bp). We used only the presence/absence of the 301 bp and 448 bp fragments for genotyping. Our sampling included five females and one individual of unknown sex (from museum samples), all of which were coded as having only one CHD1Z allele.

2.2.4 Linkage disequilibrium estimation (LD)

We estimated LD between the two nuclear genetic markers and between each of the nuclear markers and mtDNA haplotypes. Assuming a large population size, no selection and no migration into the hybrid zone the expectation of estimates of LD is that, given enough time, there should be no association between these genetic markers, which are found on different
chromosomes/genomes. Relaxing any of these assumptions may lead to positive LD, which can provide insights into the dynamics of the hybrid zone (Arnold 1993). We performed these analyses with only those individuals that had genetic data for all three of the genetic markers \((n=99)\) and were sampled within 30 km of the centre of the hybrid zone (which is approximately 50 km east of the Rockies; see below).

We estimated the composite digenic gametic linkage disequilibrium between the two nuclear markers using the formula \(D_{AB} = P_{AB} - p_A p_B\), defined as the difference between the frequency of a gamete with upper-case alleles for loci A and B and the product of the frequencies of those alleles \((p_A p_B)\) in the population (Weir 1996). In the current case this is the difference between the frequency of CHD1Z and numt-Dco1 alleles that are common in Townsend’s warblers and are found in individuals in the hybrid zone \((P_{AB})\) and the product of those alleles in this population \((P_A P_B)\); \(D_{AB}\) is the equivalent to \(D_{ab}\) calculated based on alleles common in black-throated green warblers. We also calculated the cytonuclear allelic disequilibrium between each mtDNA haplotype and the two nuclear markers. To do this we used the formula \(D = P_{MA} - P_M P_A\), where disequilibrium is defined the difference between the frequency of a gamete with the mtDNA haplotype and the nuclear allele \((P_{MA})\) and the product of the frequencies of the mtDNA haplotype and nuclear marker \((P_M P_A); \) Asmussen et al. 1987; Arnold 1993). Given that these two nuclear loci are not fixed in black-throated green warblers, the maximum possible LD is likely lower than for markers that are fixed.

### 2.2.5 Cline analyses

The transition of allele frequencies and phenotypic traits across many hybrid zones can be modeled effectively by a three-stepped cline, consisting of a central sigmodal curve bracketed by two exponential tails (Barton and Hewitt 1985; Szymura and Barton 1986). We used the program Cfit6 (Gay et al. 2008) to estimate the parameters for the best fitting clines for the three molecular markers. We did not fit clines for the morphometric and plumage traits. For each of the markers we fit the centre, width and height parameters of a scaled logit cline. We did not fit the exponential tails of the stepped function because estimating these parameters requires dense sampling at the edges of the hybrid zone. To determine whether the best fitting model allowed the clines for different markers to have different centres and slopes, suggesting they are subject to different localized patterns of dispersal or selection, or consisted of clines with identical centres and/or slopes, we ran Cfit for each of the four combinations (i.e. unconstrained, centres constrained, slopes constrained and both constrained). To group populations to generate allele
frequencies we separated groups based on natural breaks in our sampling, with an average population size of 12 individuals ranging from 3 to 26. We used likelihood-ratio tests (LRT) and calculated Akaike information criteria (AIC; Akaike 1974) to determine the best fitting clines with varying sets of constraints on cline centers and slopes.

**2.3 Results**

**2.3.1 Range contact**

Near the towns of Chetwynd and Tumbler Ridge, BC we found an area where Townsend’s and black-throated green warblers occur in sympathy. Our sampling indicates that there are no large gaps in the distribution between the two taxa in this area or further to the west or east. In many cases we captured individuals that were phenotypically Townsend’s and phenotypically black-throated green in the same mist net. We also captured a number of individuals in this area that had mixed plumage traits and were not easily classified as either species (Fig. 2.3).

**2.3.2 Morphometrics and plumage**

Although measurements from the six morphometric traits overlap between the two species, the means of some traits (wing, tail, tarsus and beak width) differed significantly between allopatric populations (Table 2.1). Individuals in the contact zone were on average intermediate between the two parental species for many of the traits. In the PCA, the first and second principal components were highly loaded with beak morphometric traits while PC3 was loaded with wing and tail measurements (Table 2.2). Although sigmoidal clines were not fitted for these traits, the cubic splines showed a clinal transition generally coincident with the molecular traits (Fig. 2.4).

Plumage score, which quantified variation in breast, crown and face colouration, showed distinct differences between the species and was intermediate and combined in complex ways in many individuals in the hybrid zone. The transition in plumage traits occurs over a narrow region approximately 40 km east of the crest of the Rocky Mountains (Fig. 2.4). Most individuals in this area have plumage traits that resemble one or the other parental species; only four of 137 individuals in the overlap area were perfectly intermediate using the plumage score criteria described above. The plumage trait we found most useful in diagnosing the two species and most
variable in the contact zone was the colour of the crown, from black in Townsend’s warblers to green in black-throated green warblers, and a mixture of both in putative hybrids (Fig. 2.3).

2.3.3 Molecular markers

While our allopatric sampling within the range of the Townsend’s warbler was limited, previous studies with greater geographic scope found only one instance of black-throated green mtDNA haplotypes in these populations (Rohwer 1994; Rohwer et al. 2001; Krosby and Rohwer 2009). We found no evidence of mitochondrial introgression of black-throated green haplotypes into the allopatric range of Townsend’s warblers (n = 8). Our sampling in the allopatric range of black-throated greens was more detailed (n = 40) and was supplemented by museum samples. We found only one instance of a mismatch in this allopatric area: one Townsend’s mtDNA haplotype was found in a phenotypically pure black-throated green warbler in Lesser Slave Lake, Alberta (approximately 450 km east of the hybrid zone).

The numt-Dco1 marker was fixed in our sample of Townsend’s warblers but was polymorphic for an alternate allele in black-throated green warblers both inside and out of the contact zone. In black-throated green populations far from the contact zone in Alberta, Saskatchewan and Illinois, the allele fixed in Townsend’s warbler was also found at a frequency of 0.43, 0.38, and 0.28, respectively (Fig. 2.5). The SNP in CHD1Z is also fixed in Townsend’s warblers and is polymorphic for an alternate allele in black-throated green populations in Alberta but, in contrast to numt-Dco1, is fixed for this alternate allele in Saskatchewan and Illinois. If the Townsend’s allele was maintained at the proportion we sampled it in Alberta (0.4), finding no individuals with the Townsend’s allele in either of these populations further east is highly unlikely (i.e. 0 out of 36 CHD1Z alleles sampled, P<0.0001; Fig. 2.5), indicating this Townsend’s allele drops dramatically in frequency between central Alberta and eastern Saskatchewan (Table 2.4).

The estimated linkage disequilibrium (D) between the two nuclear markers in the centre of the zone was 0.05, suggesting that there is a positive non-random association between these two markers. This was comparable to the LD estimated between mtDNA and each of the markers: for CHD1Z the estimate was 0.06 and for numt-Dco1 LD was 0.04.

2.3.4 Hybridization

Within the contact zone, there were often mismatches between mitochondrial haplotypes and plumage score: out of 68 individuals that we classified as black-throated green-like (plumage
score 0-0.2), 10 had a Townsend’s mtDNA, and out of 35 Townsend’s-like individuals (plumage score 0.8-1), 4 had black-throated green mtDNA (Fig. 2.3, 2.6).

Considering the mtDNA together with the nuclear markers suggests even more extensive hybridization. The nuclear markers we used (CHD1Z and numt-Dco1) are fixed in Townsend’s warblers but are polymorphic in black-throated green warblers (at least in populations within 500 km of the hybrid zone; see above). Aside from one instance of a Townsend’s mtDNA in an allopatric black-throated green warbler, it is only in the contact zone that we find novel combinations of Townsend’s mtDNA and black-throated green nuclear markers (Fig. 2.7). Of 53 individuals in the putative hybrid zone with Townsend’s mitochondria, 20 individuals have one or more black-throated green alleles at CHD1Z or numt-Dco1, suggesting a conservative estimate of 38% of individuals as likely hybrids or back-crosses (Fig. 2.7).

To assess whether birds with molecular evidence of hybridization tended to have phenotypes consistent with hybridization, we compared individuals within the hybrid zone that had differing genetic constitutions. As the two nuclear markers are not fixed in black-throated greens (at least near the contact zone) we compared individuals that have only Townsend’s mtDNA and no black-throated green nuclear markers (‘pure Townsend’s’; Table 2.1c) to individuals that have a Townsend’s mtDNA but also have at least one black-throated green nuclear allele from either CHD1Z or numt-Dco1 (‘hybrid Townsend’s’; Table 2.1d). For beak length, PC1 and plumage score, individuals with one or more black-throated green nuclear allele were significantly different from those with all Townsend’s alleles, each in the expected direction of having more black-throated green-like measurements.

2.3.5 Cline analyses

Estimating the centre and width of the best fitting clines allows us to test whether selection is acting differently on the genetic markers we assayed: narrow clines indicate selection is stronger compared to wide clines; the non-coincidence of cline centres indicates that the markers may be subject to different localized patterns of selection. The model in which each of the three markers has its own centre and slope (Table 2.3a) had two additional parameters as compared to models in which each of the markers had the same centre (Table 2.3b) or slope (Table 2.3c) and had four additional parameters as compared to the model where each marker had the same centre and slope (Table 2.3d). Using likelihood-ratio tests and AIC (Table 2.3) we found a model where both cline centers and slopes were unconstrained for the three molecular markers was the best fit to the data (log-likelihood = 449.67; AIC = 917.22) compared to a
model where the centers were constrained (log-likelihood = 453.35; AIC = 920.7; likelihood ratio test: $\chi^2 = 7.36$, DF=2, p=0.02) and a model where both centres and slopes were constrained (log-likelihood = 454.62; AIC = 919.24; likelihood ratio test: $\chi^2 = 9.9$, DF = 4, p=0.04) although the LRT suggests this is not a better fit when just the slopes were constrained (log-likelihood = 452.01; AIC = 918.02; likelihood ratio test: $\chi^2 = 4.68$, DF=2, p=0.1). The model where each cline has its own centre and slope does not provide a better fit to the data when both model selection statistics (AIC and LRT) are controlled for sample size (using AICc; results not shown) and multiple comparisons (LRT Bonferroni-correction $\alpha=0.05/3$; results not shown). Therefore, while the best model is where the clines are considered statistically non-coincident, these differences have low support and variation among markers is relatively small: the cline centres for the molecular markers range from 41 km (COI) to 56 km (numt-Dco1) east of the Rocky Mountains (Table 2.3; Fig. 2.5), and cline widths range from 40 km for COI to 87 km for numt-Dco1 (Table 2.3; Fig. 2.5).

2.4 Discussion

Our results show for the first time that Townsend’s and black-throated green warblers hybridize extensively in the Peace Region of northeastern British Columbia, home to hybrid zones within a number of other pairs of divergent avian taxa. Many of the individuals in this area have plumage traits that resemble one or the other parental species, which is likely why this hybrid zone was not described previously. However, our combined analysis of morphometric, plumage and genetic variation shows that many individuals in this area have a mixture of traits of the two species, a pattern that can only be explained by hybridization in the contact zone.

Clines in various traits are located on the eastern slope of the Rocky Mountains (average cline center for the three markers is 50 km east of the crest), and occur over a very narrow distance (the average width from the three markers is 60 km) in comparison to the estimated single-generation root mean squared dispersal distance (30 km as estimated in hermit and Townsend’s warblers; Rohwer et al. 2001).

Using this assumed dispersal distance and a conservative estimate of the time of range contact (i.e. 1965, Salt 1966), a neutral model of diffusion upon secondary contact ($w = \sqrt{2\pi\sigma^2 t}$, where $w$ is cline width, $\sigma$ is RMS dispersal distance, and $t$ is the number of generations since initial contact) would predict a cline with a width of approximately 360 km after 23 generations (assuming a generation time of two years), much wider than observed (Barton and Hewitt 1985, Barton and Gale 1993). This suggests that some form of pre-mating reproductive isolation or
post-mating selection against hybrids is keeping the zone narrow (Price 2008). The positive values of linkage disequilibrium between each of the genetic markers also support the conclusion that selection may be maintaining the zone; however positive values of LD are also consistent with recent secondary contact.

One potential factor that could be maintaining reproductive isolation in the hybrid zone is pre- or post-mating selection on song, which is an important trait in most songbirds for both male-male territory defence and solicitation of females (Catchpole and Slater 1995). Townsend’s and black-throated green warblers have easily distinguished songs. Like other Setophaga warblers (Wright et al. 1998; Morse and Poole 2005) each species has two stereotyped song classes. Our informal observations in the hybrid zone suggest that song types from both species occur in the contact zone, although this is not always predictive of an individual’s phenotype. We also have observed individuals switching between song types, indicating that some individuals can sing songs of both species. The ability to learn heterospecific song types has been proposed in other avian hybrid zones as a means to defend territories from either species (Price 2008). In a recent analysis of song variation in the MacGillivray’s/mourning warbler hybrid zone, which occurs in the same area, song is also not strongly predictive of phenotype or genotype (Kenyon et al. 2011). In that case there is evidence that the differences observed in allopatry are not maintained in the contact zone and that hetero-specific song learning may be contributing to hybridization (Kenyon et al. 2011). A thorough analysis of song variation across the Townsend’s and black-throated green hybrid zone is warranted.

Another behavioural factor that could be important in this hybrid zone is difference in male-male aggression. Studies of the hermit/Townsend’s warbler hybrid zone found that Townsend’s warblers are much more aggressive than hermit warblers, possibly explaining the hybrid zone movement from Townsend’s into the range of hermit warblers (Pearson 2000; Pearson and Rohwer 2000). We observed many cases of territorial aggression between phenotypic Townsend’s and black-throated green individuals, and informal playback experiments suggest that both species are territorially defensive to each other - both respond to recordings of either species. Study of song playback experiments in this hybrid zone is recommended to determine whether there are differences in recognition and aggression between the two taxa and their hybrids.

That the center of this hybrid zone is coincident with the transition between the mixed-wood boreal forest east of the Rockies and the conifer-dominated cordilleran forests to the west suggests that habitat isolation between the parental species or selection against hybrids with
intermediate or inferior habitat preferences might have a role in maintaining the zone. This ecological transition is more likely to be an important factor in this hybrid zone (as compared to the hybrid zones between Audubon’s/myrtle and MacGillivray’s/ mourning warblers; Brelsford and Irwin 2009, Irwin et al. 2009) because evidence from outside the hybrid zone shows a preference of the parental species for these different forest types (Pearson 1997; Wright et al. 1998; Morse and Poole 2005). Preston et al. (2007) recently combined black-throated green occurrence and remote sensing data to model habitat suitability of this species in northeastern BC. Interestingly, suitability shows a decline coincident with the centres of the clines we studied here. This observation is consistent with the hypotheses that 1) exogenous factors are maintaining the zone or 2) that this area represents a density trough, which are thought to attract endogenously controlled tension zones (Barton and Hewitt 1985). That the two species do come into contact often, however, suggests that pre-mating isolation based on habitat differences alone is unlikely. A more detailed ecological characterization and niche modeling approach in this and other avian hybrid zones is needed to investigate the importance of this ecological transition in maintaining this suture zone (Swenson 2006; Thomassen et al. 2010).

Another selective factor that may be important in maintaining this narrow zone is seasonal migratory behaviour. Migration is under strong genetic influence in most migratory songbirds (Helbig 1996; Irwin and Irwin 2005; Pulido 2007). Townsend’s and black-throated green warblers differ strongly in their migratory routes and wintering ranges (Wright et al. 1998; Morse and Poole 2005), raising the possibility that hybrids might have a suboptimal mixture of migratory traits. If the hybrid zone represents a ‘migratory divide’, with Townsend’s orienting south-southwest in the autumn and black-throated green warblers orienting south-southeast, selection may be acting against hybrid individuals with intermediate and potentially inferior migratory orientation, as has been suggested in the case of willow warblers in central Sweden (Bensch et al. 2009) and a number of other cases (Irwin and Irwin 2005; Irwin 2009; Rohwer and Irwin 2011). Research on the migratory behaviour of birds in and near the hybrid zone would help reveal whether this is a strong source of selection against hybrids.

In the approximately one million years separating these two species from a common ancestor (Lovette et al. 2010), genetic incompatibilities may have accrued between them. Because theoretical analyses have suggested an important role for the Z-chromosome in avian speciation and hybrid incompatibilities, we expected to see a narrower cline in the Z-linked marker, CHD1Z, as compared to the other molecular markers, as some other studies of avian hybrid zones have found (Carling and Brumfield 2009; Qvarnström and Bailey 2009; Backström
et al. 2010). In our case, the CHD1Z cline had an intermediate width compared to the clines of the two other markers, not providing support for this hypothesis, although examination of more molecular markers is needed to evaluate it fully.

The pattern of variation in CHD1Z in the populations of black-throated green warblers we sampled was surprising: in Alberta there is a polymorphism of the Townsend’s and black-throated green alleles, whereas further east the black-throated green allele was fixed (Fig. 2.5). We suggest three possible explanations for this pattern: 1) that the hybrid zone has moved to the west, 2) that a separate mutation that disrupts the restriction digest has become fixed or is at high frequency in the populations east of Alberta, and 3) that there has been introgression through hybridization from Townsend’s warblers at the CHD1Z gene. Given the distance and our survey of other molecular markers we suggest that hybrid zone movement is unlikely. We suggest that introgression from Townsend’s warblers is a more parsimonious explanation than fixation of a third allele over a large fraction of the black-throated green range. Whether this is consistent with a neutral process or the result of positive selection deserves further study. Given this potential introgression and the conservation concern of black-throated green warblers in BC (Cooper et al. 1997) we recommend a detailed spatio-temporal sampling of this hybrid zone and allopatric populations to test for evidence of potential zone movement or more extensive genomic introgression than reported here.

In conclusion, we have provided the first description of a hybrid zone between Townsend’s and black-throated green warblers. The narrowness of this hybrid zone and the similar clines in three molecular markers and a variety of phenotypic traits suggest that the hybrid zone is likely maintained by some form of selection, characteristic of many tension zones observed between other avian taxa (Price 2008). Given this, we suggest the two should continue to be treated as separate taxonomic species. A combination of factors likely maintains the zone: ecological differences and habitat preferences may govern the location, but intrinsic or behavioural incompatibilities likely produce selection against hybrids, maintaining the zone’s narrow width. Further behavioural, ecological and genomic characterization of this and other contact zones located in this small area of northeastern BC is needed to reveal the selective forces maintaining the distinctiveness of these forms despite hybridization.
Table 2.1 Morphometric and plumage measurements for Townsend’s (TOWA; \( n = 8 \)) and black-throated green (BTNW; \( n = 25 \)) warblers in populations in allopatry (a-b) and from a putative hybrid zone (c-d). Morphometric measurements are given in millimetres ±1 standard deviation. The plumage scores range from 0 (phenotypic black-throated green) to 1 (phenotypic Townsend’s). PC1 refers to the first principal component constructed from the six morphometric traits (see Methods). In the hybrid zone ‘pure’ Townsend’s (c) are defined as individuals with Townsend’s mtDNA and no black-throated green nuclear alleles (\( n = 35 \)); ‘hybrid’ Townsend’s (d) are defined as individuals with Townsend’s mtDNA and at least one black-throated green nuclear allele at either \( CHD1Z \) or \( numt-Dco1 \) (\( n = 20 \)). Welch’s two-sample \( t \)-tests are used to test the difference in means; comparisons in bold those that are significant below the critical value of 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Wing</th>
<th>Tail</th>
<th>Tarsus</th>
<th>Beak Length</th>
<th>Beak Depth</th>
<th>Beak Width</th>
<th>Plumage</th>
<th>PC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n = 8 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allopatry</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) TOWA</td>
<td>66.3 (±1.5)</td>
<td>50.9 (±1.3)</td>
<td>18.5 (±0.6)</td>
<td>7.2 (±0.2)</td>
<td>3.2 (±0.2)</td>
<td>3.3 (±0.1)</td>
<td>0.92 (±0.2)</td>
<td>-0.087 (±0.03)</td>
</tr>
<tr>
<td>(b) BTNW</td>
<td>63.6 (±1.4)</td>
<td>49.3 (±1.1)</td>
<td>17.6 (±0.5)</td>
<td>7.0 (±0.3)</td>
<td>3.1 (±0.1)</td>
<td>3.0 (±0.2)</td>
<td>0.02 (±0.1)</td>
<td>0.015 (±0.07)</td>
</tr>
<tr>
<td>( t = 4.51, )</td>
<td>( t = 3.07, )</td>
<td>( t = 3.99, )</td>
<td>( t = 1.63, )</td>
<td>( t = 1.64, )</td>
<td>( t = 5.35, )</td>
<td>( t = 16.07, )</td>
<td>( t = -5.98, )</td>
<td></td>
</tr>
<tr>
<td>( df = 11.4, )</td>
<td>( df = 10.3, )</td>
<td>( df = 11.2, )</td>
<td>( df = 15.8, )</td>
<td>( df = 8.6, )</td>
<td>( df = 28.3, )</td>
<td>( df = 7.6, )</td>
<td>( df = 28.8, )</td>
<td></td>
</tr>
<tr>
<td>( P = &lt;0.001 )</td>
<td>( P = 0.011 )</td>
<td>( P = 0.002 )</td>
<td>( P = 0.122 )</td>
<td>( P = 0.136 )</td>
<td>( P = &lt;0.001 )</td>
<td>( P = &lt;0.001 )</td>
<td>( P = &lt;0.001 )</td>
<td></td>
</tr>
<tr>
<td>Hybrid Zone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(c) Pure TOWA</td>
<td>66.9 (±1.9)</td>
<td>51.6 (±1.8)</td>
<td>18.5 (±0.5)</td>
<td>7.3 (±0.4)</td>
<td>3.0 (±0.1)</td>
<td>3.1 (±0.1)</td>
<td>0.88 (±0.3)</td>
<td>-0.034 (±0.06)</td>
</tr>
<tr>
<td>(d) Hybrid TOWA</td>
<td>66.2 (±2.0)</td>
<td>50.8 (±1.7)</td>
<td>18.3 (±0.5)</td>
<td>7.0 (±0.3)</td>
<td>3.1 (±0.1)</td>
<td>3.0 (±0.1)</td>
<td>0.43 (±0.4)</td>
<td>0.012 (±0.06)</td>
</tr>
<tr>
<td>( t = 1.41, )</td>
<td>( t = 1.72, )</td>
<td>( t = 1.69, )</td>
<td>( t = 3.15, )</td>
<td>( t = -0.42, )</td>
<td>( t = 1.97, )</td>
<td>( t = 4.41, )</td>
<td>( t = -2.86, )</td>
<td></td>
</tr>
<tr>
<td>( df = 37.8, )</td>
<td>( df = 41.4, )</td>
<td>( df = 41.5, )</td>
<td>( df = 45.3, )</td>
<td>( df = 49.5, )</td>
<td>( df = 38.5, )</td>
<td>( df = 29.4, )</td>
<td>( df = 39.0, )</td>
<td></td>
</tr>
<tr>
<td>( P = 0.167 )</td>
<td>( P = 0.093 )</td>
<td>( P = 0.099 )</td>
<td>( P = 0.003 )</td>
<td>( P = 0.6734 )</td>
<td>( P = 0.056 )</td>
<td>( P = &lt;0.001 )</td>
<td>( P = 0.007 )</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2 Factor loadings and variance explained of the first three principal components produced in the PCA of morphological variables.

<table>
<thead>
<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wing</td>
<td>-0.1296</td>
<td>0.2202</td>
<td>-0.5306</td>
</tr>
<tr>
<td>Tail</td>
<td>-0.1471</td>
<td>0.2871</td>
<td>-0.5591</td>
</tr>
<tr>
<td>Tarsus</td>
<td>-0.1411</td>
<td>0.2610</td>
<td>-0.1966</td>
</tr>
<tr>
<td>Beak Length</td>
<td>-0.5492</td>
<td>0.6695</td>
<td>0.4286</td>
</tr>
<tr>
<td>Beak Depth</td>
<td>-0.3090</td>
<td>-0.2288</td>
<td>-0.4170</td>
</tr>
<tr>
<td>Beak Width</td>
<td>-0.7380</td>
<td>-0.5482</td>
<td>0.0978</td>
</tr>
<tr>
<td>% Variation</td>
<td>40.7%</td>
<td>19.5%</td>
<td>15.7%</td>
</tr>
</tbody>
</table>
Table 2.3 Estimates of cline parameters using the program Cfit 6 (Gay et al. 2008) for three molecular markers across a hybrid zone between Townsend’s and black-throated green warblers. (a) the markers each have their own centers and slopes, (b) have their centers constrained, (c) have their slopes constrained and (d) have both their centers and slopes constrained. The number of parameters for each model \((n)\), log-likelihood and the AIC score for each is listed. The best model (i.e. lowest AIC) is highlighted in bold. The estimates of width are given in km and cline centers are in km east from the crest of the Rockies.

<table>
<thead>
<tr>
<th></th>
<th>(a) No constraint</th>
<th>(b) Center constrained</th>
<th>(c) Slope constrained</th>
<th>(d) Centers and slopes constrained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Width</td>
<td>Center</td>
<td>Width</td>
<td>Center</td>
</tr>
<tr>
<td>COI</td>
<td>40</td>
<td>41</td>
<td>40</td>
<td>46</td>
</tr>
<tr>
<td>CHD1Z</td>
<td>54</td>
<td>52</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>numt-Dco1</td>
<td>87</td>
<td>56</td>
<td>70</td>
<td>46</td>
</tr>
<tr>
<td>(n)</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Log-likelihood</td>
<td>-449.67</td>
<td>-453.35</td>
<td>-452.01</td>
<td>-454.62</td>
</tr>
<tr>
<td>AIC</td>
<td>917.2</td>
<td>920.7</td>
<td>918.02</td>
<td>919.24</td>
</tr>
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</table>
Table 2.4 Frequencies of molecular markers in allopatry and at a series of locations across a hybrid zone between Townsend’s and black-throated green warblers. Frequencies are relative to the TOWA allele in COI (mitochondrial), CHD1Z (Z-linked) and numt-Dco1 (autosomal), phenotype and morphometric measurements are averages for each site and locations are averaged distances (km) in relation to the crest of the Rocky Mountains. See caption for Figure 2.1 for site names and locations.

<table>
<thead>
<tr>
<th>Site Name (Distance from Crest)</th>
<th>Phenotype</th>
<th>Morpho PC1</th>
<th>Freq. COI</th>
<th>Freq. numt-Dco1</th>
<th>Freq. CHD1Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – VA (-560 km)</td>
<td>0.98</td>
<td>-0.094</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2 – TO (-320 km)</td>
<td>0.81</td>
<td>-0.077</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3 – HZ (3 km)</td>
<td>0.99</td>
<td>-0.047</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3 – HZ (29 km)</td>
<td>0.80</td>
<td>-0.023</td>
<td>0.71</td>
<td>0.84</td>
<td>0.91</td>
</tr>
<tr>
<td>3 – HZ (35 km)</td>
<td>0.80</td>
<td>0.005</td>
<td>0.92</td>
<td>0.92</td>
<td>0.83</td>
</tr>
<tr>
<td>3 – HZ (44 km)</td>
<td>0.63</td>
<td>-0.002</td>
<td>0.44</td>
<td>0.56</td>
<td>0.72</td>
</tr>
<tr>
<td>3 – HZ (51 km)</td>
<td>0.20</td>
<td>0.003</td>
<td>0.14</td>
<td>0.58</td>
<td>0.55</td>
</tr>
<tr>
<td>3 – HZ (57 km)</td>
<td>0.05</td>
<td>0.012</td>
<td>0.23</td>
<td>0.81</td>
<td>0.65</td>
</tr>
<tr>
<td>3 – HZ (62 km)</td>
<td>0.10</td>
<td>0.027</td>
<td>0.12</td>
<td>0.65</td>
<td>0.50</td>
</tr>
<tr>
<td>3 – HZ (67 km)</td>
<td>0.13</td>
<td>0.035</td>
<td>0.50</td>
<td>0.83</td>
<td>0.50</td>
</tr>
<tr>
<td>3 – HZ (74 km)</td>
<td>0.04</td>
<td>-0.002</td>
<td>0.00</td>
<td>0.50</td>
<td>0.33</td>
</tr>
<tr>
<td>3 – HZ (90 km)</td>
<td>0.11</td>
<td>0.015</td>
<td>0.13</td>
<td>0.69</td>
<td>0.50</td>
</tr>
<tr>
<td>3 – HZ (100 km)</td>
<td>0.03</td>
<td>0.002</td>
<td>0.00</td>
<td>0.38</td>
<td>0.63</td>
</tr>
<tr>
<td>3 – HZ (127 km)</td>
<td>0.03</td>
<td>0.032</td>
<td>0.00</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>4 – LSL (521 km)</td>
<td>0.02</td>
<td>0.021</td>
<td>0.05</td>
<td>0.43</td>
<td>0.40</td>
</tr>
<tr>
<td>5 – HB (~1000 km)</td>
<td>0.03</td>
<td>-0.016</td>
<td>0.00</td>
<td>0.38</td>
<td>0.00</td>
</tr>
<tr>
<td>6 – CH (~3000 km)</td>
<td>**</td>
<td>**</td>
<td>0.00</td>
<td>0.28</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Figure 2.1 Breeding distributions of Townsend’s (*Setophaga townsendi*), black-throated green (*S. virens*) and hermit (*S. occidentalis*) warblers in North America. Adapted from Pearson (1997), Wright *et al.* (1998) and Morse and Poole (2005). Hatched lines denote the hybrid zone between Townsend’s warblers and Hermit warblers in Oregon and Washington. All individuals (*n* = 171) were males captured on their breeding territories except those (*n* = 16) obtained from the Field Museum in Chicago, Illinois, likely on migration. 1, Vancouver and Squamish, BC (VA); 2, Todagin, BC (TO); 3, Chetwynd, BC (Hybrid Zone, HZ); 4, Lesser Slave Lake (LSL); 5, Hudson Bay, SK (HB); Chicago, Il (CH).
Figure 2.2 Distribution of mitochondrial DNA haplotypes in the putative hybrid zone. Townsend’s mitochondrial types are indicated by filled circles; black-throated green with open circles. Inset includes sampling further south close to the town of Tumbler Ridge, 130 km south of Chetwynd. Highway 97 (running southwest across the map) crosses Pine Pass in the Rocky mountains at an elevation of approximately 930 m (this is defined as the crest of the Rockies).
**Figure 2.3** Four male Townsend’s/black-throated green warblers from the contact zone chosen to illustrate plumage variation and the mismatch between plumage traits and molecular markers in this area. Plumage was quantified on a scale from 0 to 4 for crown colour, 0 to 2 for face colour and 0 to 3 for breast colour with lower numbers representing trait classes found in black-throated green warblers and higher numbers representing trait classes found in Townsend’s warblers: (a) a phenotypic Townsend’s (4/4 crown, 2/2 face, 3/3 breast) with a black-throated green mtDNA and, (b) a phenotypic black-throated green (0/4 crown, 0/2 face, 0/3 breast) with a Townsend’s mtDNA, (c) a mixed individual (2/4 crown, 1/2 face, 0/3 breast) with a Townsend’s mtDNA, and (d) a mixed individual (1/4 crown, 1/2 face, 2/3 breast) with a black-throated green mtDNA. Individuals shown have identification numbers (a) IE31T01, (b) GE12D01, (c) IE24T07, and (d) HF20D02.
Figure 2.4 Clines in morphological and plumage traits across a hybrid zone between Townsend’s and black-throated green warblers. Points represent individuals and locations are distances (km) east of the crest of the Rocky Mountains. Plumage score ranges from 0 (phenotypic black-throated green) to 1 (phenotypic Townsend’s). PC1 has the highest loadings from the three measurements of beak (Table 2.2). Lines are fitted by cubic splines in R.
Figure 2.5 Clines in molecular markers across a hybrid zone between Townsend’s and black-throated green wablers. Points are allele frequencies in COI (mitochondrial), CHD1Z (Z-linked) and numt-Dco1 (autosomal) and locations are averaged distances (km) in relation to the crest of the Rocky Mountains. The lines represent the best fitting cline, as estimated by Cfit 6 (Table 2.3a; Gay et al. 2008), and the arrows and gray boxes show its center and width.
Figure 2.6 Relationship between plumage score and mitochondrial type within the contact zone. The phenotypic scale ranges from 0, black-throated green to 1, a phenotypic Townsend’s.
**Figure 2.7** Admixture between nuclear and mitochondrial mtDNA markers in allopatry and in the Townsend’s / black-throated green hybrid zone. Proportion of individuals in Townsend’s (TOWA; \( n = 8 \)) or black-throated green (BTNW; \( n = 40 \)) allopatric populations and in the hybrid zone with either no black-throated green nuclear alleles (filled) or at least one black-throated green allele at either \( CHDIZ \) or \( numt-Dco1 \) (clear). Individuals in the hybrid zone are distinguished by either having a Townsend’s (\( n = 53 \)) or black-throated green (\( n = 81 \)) mtDNA.
CHAPTER 3: Isotopic variation across the Audubon’s-myrtle warbler hybrid zone

3.1 Introduction:

Selection against hybrid offspring between divergent taxa can be a potent form of reproductive isolation (Barton and Hewitt 1985), as demonstrated theoretically and in laboratory experiments (Coyne and Orr 2004). While in some systems the ecological factors that influence hybrid fitness are known (e.g. Vamosi and Schluter 1999), studying the fate of hybrids in wild populations is much more challenging. This is particularly true of migratory birds, a group in which closely related species often differ dramatically in migratory behaviour and wintering ranges (Irwin and Irwin 2005; Price 2008). For instance, hybrids may be ill suited to ecological conditions on their breeding grounds, on their migratory routes or in their over-wintering areas, and these areas may occur over large geographic scales (Delmore et al. 2012). Identifying the relative importance of these different areas to hybrid fitness, while challenging, has important implications for our understanding of hybridization in the wild (Price 2008).

While new tracking technology such as light-level geolocators have improved our ability to follow migratory birds over their annual movements, there are a number of constraints that make their use impractical in some study systems (McKinnon et al. 2013). Alternatively, stable isotopes have been used with some success in other avian systems, particularly in Europe, to indirectly estimate the over-wintering locations of offspring formed in hybrid zones (Inger and Bearhop 2007). For instance, in Phylloscopus willow warblers in Sweden, stable nitrogen and carbon in feathers (Chamberlain et al. 2000) has been combined with band recovery (Bensch et al. 1999), orientation studies (Ilieva et al. 2012) and genetic data to illustrate an excellent example of a ‘migratory divide’ (Bensch et al. 1999; Bensch et al. 2009). In this case these data suggest that the two subspecies migrate to, and winter in, different areas of Africa. Birds in a contact zone between these subspecies are, on average, intermediate in their isotopic signature, although it is not clear whether this represents a mixture of the parental over-wintering locations or a distinct, and potentially intermediate, area in which the hybrids over-winter (Chamberlain et al. 2000; Bensch et al. 2009). In a different European species complex, hybrids between pied and collared Ficedula flycatchers illustrate a different pattern: carbon and nitrogen isotopic data suggest that most of the hybrids winter in a location similar to only one of the parental species

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2 A version of this chapter has been accepted for for publication: Toews, D. P. L., Brelsford, A. and D. E. Irwin. Isotopic variation across the Audubon’s-myrtle hybrid zone. Journal of Evolutionary Biology.
(Veen et al. 2007), although new isotopic data suggests this relationship may be more complex than originally described (Veen in prep.).

There is much evidence that the migratory programme of small songbirds is inherited genetically (Berthold 1996; Helbig 1996; Pulido 2007; Irwin 2009). Therefore, in the case of willow warblers, mixed or intermediate migratory behaviours may be inherited and expressed in hybrids. Such behaviours may be inferior, taking individuals into inhospitable areas, suggesting that this may be an important component of post-mating reproductive isolation in the willow warbler contact zone (Chamberlain et al. 2000; Bensch et al. 2009). Alternately, in the case of the flycatchers, the authors attribute the clustering of hybrids with one of the parental species to a dominant inheritance pattern of migratory behaviour and suggest such a pattern might explain the high over-winter survival of hybrids (Veen et al. 2007). In both cases, estimating these behaviours in the zone of secondary contact using isotopic data proved important for understanding the evolutionary dynamics in the hybrid zones.

Isotopic patterns across avian hybrid zones have not previously been studied in North America, which is in fact very well suited for such studies because of the extensive knowledge of the geographic variation of feather isotopes, especially stable hydrogen (Meehan et al. 2004; Bowen et al. 2005; Hobson et al. 2012). Here we present isotopic data across a narrow and stable hybrid zone between Audubon’s (Setophaga auduboni) and myrtle (S. coronata) warblers in western Canada (Hubbard 1969; Barrowclough 1980; Brelsford and Irwin 2009; note that the American Ornithological Union currently treats these as two subspecies within the broad taxon ‘yellow-rumped warbler’; the International Ornithologists’ Union instead identifies the two as distinct species; here we use the latter treatment). These two migratory songbirds are distinguished by their breeding ranges and distinct plumage patterns (Figure 3.1a): myrtle warblers breed mostly east of the Rockies and have a white throat and a black cheek patch; Audubon’s warblers breed mostly west of the Rockies and have a yellow throat and a grey face (Hunt and Flaspohler 1998). Where the ranges of the two overlap, in the Rocky Mountains between British Columbia and Alberta, they form a narrow hybrid zone (Hubbard 1969; Barrowclough 1980; Brelsford and Irwin 2009). This is also in an area where a number of other avian contact zones and hybrid zones are located (Toews and Irwin 2008; Irwin et al. 2009; Toews et al. 2011). In the Audubon’s / myrtle warbler hybrid zone, hybrids and backcrosses are formed at high frequency and individuals with a wide variety of intermediate plumage patterns are common (Hubbard 1969; Brelsford and Irwin 2009). The narrowness of the hybrid zone, estimated to be 132km wide, combined with the estimated root-mean-squared dispersal distance
of these species (~20 km), suggests that some form of selection against hybrids maintains the hybrid zone, which Brelsford and Irwin (2009) estimate to be moderately strong, with hybrids having 18% lower fitness than parental forms.

Studies of mate pairing, based on plumage and genotype correlations between social mates, indicate that there is little or no assortative mating in this hybrid zone (Brelsford and Irwin 2009). Preliminary studies of song and playback responses (Brelsford 2010) suggest there are not strong differences between the species in vocalizations. There is also no obvious ecological transition that is coincident with the whole length of the hybrid zone, suggesting that habitat selection is unlikely to be a strong reproductive barrier. Taken together these findings imply that none of these traits are likely to serve as sizeable pre-mating reproductive barriers between the two species. Hence post-mating selection on hybrids may be an important form of reproductive isolation maintaining this hybrid zone, but this has been studied in much less detail. For instance, we have no direct data on developmental success, behaviour, or annual survival of hybrids.

An important challenge to understanding hybrid fitness in this system that it is unclear where hybrids spend the winter months and how they move between their breeding and wintering ranges. Field observations illustrate that Audubon’s warblers winter along the Pacific coast from southern British Columbia, Canada, through to Baja California, highland Mexico, and Central America (see overwintering densities from citizen science surveys in Appendix 1). Myrtle warblers winter in the southeastern U.S. concentrating around Louisiana, with wintering populations also occurring along the Pacific coast, along the gulf coast in Central America and in the Caribbean (Hunt and Flaspohler 1998). Limited mark/recapture data involving sites near the hybrid zone are consistent with these observations (Figure 3.1a): recovery data suggest that myrtle warblers banded or encountered near Calgary and Edmonton, Alberta tend to move to and from the southeast U.S. some moving as far as Texas, Louisiana or Georgia to overwinter (Figure 3.1a; Brewer et al. 2006). In contrast, Audubon’s warblers banded or encountered in south-central B.C. and along the Pacific coast travel south through Oregon and some into California. Field data for hybrids is scarce, primarily because hybrids can be difficult to identify and distinguish from the parental species in their basic plumage in the fall and winter. In addition, at present most citizen science surveys and bird-banding stations do not identify hybrids.

Here we analyze variation in stable hydrogen (δ²H) across the hybrid zone between these two species to determine whether there is a change in migratory behaviour across the hybrid
zone and to gain a better understanding of where hybrids are spending the winter. To our knowledge this is the first study to present isotopic variation on such a fine spatial scale in a narrow hybrid zone. We use hydrogen because it forms a strong gradient over much of North America, which varies with patterns of precipitation and is primarily influenced by distance to the ocean and elevation (Bowen et al. 2005). This results in unique values of hydrogen in certain areas, including the areas assumed to be wintering habitats for myrtle and Audubon’s warblers. In particular, areas in the southeastern U.S. have, on average, higher hydrogen values (i.e. more deuterium) as compared to areas along the Pacific Coast (Hobson et al. 2012). More importantly, hydrogen has also been the subject of previous investigations linking environmental hydrogen to hydrogen in feathers of known origin, allowing us to broadly estimate the geographic region where a feather was grown (Hobson et al. 2012).

We first tested whether allopatric Audubon’s and myrtle warblers winter in isotopically distinct areas, as is implied by field observations and banding recovery data. We were interested in testing whether there was evidence of a migratory divide between these two species, whether it was coincident with the center of hybrid zone, and whether any transition in over-wintering behaviour was narrow or gradual. We then studied variation amongst hybrids in the hybrid zone in more detail. There are a number of possible patterns expected from hybrids, depending on their over-wintering location and the distribution of hydrogen in these areas. We have simplified these predictions into three non-mutually exclusive scenarios (Figure 3.2). The first, which we call the ‘additive’ scenario (Figure 3.2a), has been observed in hand-raised blackcap warblers (Sylvia atricapilla; Helbig 1991). These studies found a high variance in hybrid blackcap migratory behaviour and an overall intermediate directionality of their migratory movements. If hybrid Audubon’s and myrtle warblers exhibited such a pattern we would expect hybrids might migrate to and over-winter in areas similar to both parental species as well as areas intermediate between the two. An intermediate route or wintering area between the main parental overwintering concentrations (i.e. coastal California / Baja and the southeastern U.S.) is predicted to be near southern Arizona and New Mexico, western Texas and northern Mexico. While intermediate in geography between the two main overwintering concentrations, these areas in fact have lower isotopic values than is observed in either of the parental taxa (Bowen et al. 2005). Under this scenario we would also expect a correlation between a bird’s migratory behaviour and its genetic background. Under the ‘dominant’ scenario (Figure 3.2b), which has been suggested for flycatchers (Veen et al. 2007) and reed warblers (Yohannes et al. 2011), hybrids winter in areas that overlap with one of the two parental species. Finally, in the ‘novelty’
scenario (Figure 3.2c), hybrids winter in an entirely unique area, which may or may not be intermediate between the two parental species. In these latter two scenarios the relationship between genetic background and migratory behaviour is predicted to be more complex in comparison with the additive scenario.

3.2 Methods:

3.2.1 Sampling and genotyping

During the spring of 2006 and 2007 we captured 70 Myrtle warblers, Audubon’s warblers and hybrids in the hybrid zone and in allopatric populations in eastern British Columbia and western Alberta. We studied territorial individuals along a previously studied transect near Saskatchewan River Crossing, Alberta (sites 1-5 and 7 in Figure 3.1b; this is the same transect as illustrated in panel D of Figure 1 in Brelsford and Irwin 2009) and from each site we sampled 6-16 individuals (average n = 10 birds). We also included samples from a site near the center of the hybrid zone but through a different transect, near Kananaskis, Alberta (site 6 in Figure 1B; panel E in Figure 1 in Brelsford and Irwin 2009). We did this to provide a comparison of variation amongst transects and this was also the site of a series of orientation experiments in 2011 (see Discussion). While sampling additional allopatric populations would be ideal, to address our central questions our sampling emphasized studying individuals within the hybrid zone. For each population we included the relative distance from the center of the hybrid zone, which was estimated from a previous genetic and plumage study (Brelsford and Irwin 2009). These hybrid zone parameters are consistent across space and time (Hubbard 1969; Brelsford and Irwin 2009).

We captured individuals using song playback and mist nets and then took photographs, feather samples and a blood sample (10-40 μL) from each individual. Birds were captured following territory establishment and most prior to nesting at a time when they are most responsive to song playback. To each bird we applied a unique numbered metal leg band before releasing the individual. All animal care was conducted according to the University of British Columbia protocols. We aged and sexed all individuals according to Pyle (1997); most captured birds were males, due to the use of song playback to attract and capture them, but 5 of the 70 individuals were female.

Blood samples, taken using a small needle and capillary tube 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 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 and 1 mM EDTA, at pH 8.0, and stored at 4°C. Genotype information for two of the three nuclear markers (CHD1Z and numt-Dco1) was presented in a previous publication (Brelsford and Irwin 2009), where the full PCR and genotyping protocol can be found. For the third nuclear marker, RIOK2, we used the forward primer ATGGGTGTGGGCAAGAATC and the reverse primer GCTCCTCTTCRTTWGCAACA and used a PCR annealing temperature of 60°C. This amplifies an 850 base pair fragment, which is cut by the enzyme XmnI in Audubon’s warblers. Previous research suggests that all three of the markers are at or near fixation for alternate alleles in the two species, with marker frequencies <0.1 for the myrtle variant in Audubon’s warblers and vice versa of CHD1Z; <0.1 for numt-Dco1 and <0.2 RIOK2 Brelsford and Irwin 2009). Note that RIOK2 and CHD1Z are both Z-linked markers and thus only have a single allele in females. Such linkage can also produce non-independence, especially if there is little recombination between the markers. Given that only 45% of individuals previously studied along this transect have the same allele types (i.e. Audubon’s versus myrtle) for the two markers, there is still much added information with including both Z-linked markers, although excluding RIOK2 or CHD1Z does not alter the conclusions of the study. To generate a genetic hybrid index we added each myrtle allele for the three markers and divided this by the total number of alleles (6 for males, 4 for females) resulting in an index that ranges between 0 (all Audubon’s alleles) to 1 (all myrtle alleles).

3.2.1 Feather isotope analysis

For each bird we determined the stable hydrogen content (δ²H) in one greater covert feather, pulled from the inner side of the prealternate molt-limit. Stable hydrogen here refers to the relative amounts of the two stable forms of hydrogen (deuterium over protium) divided by that ratio in a standard material: \( \delta^2H = \left( \frac{^{2}H}{^{1}H} \right)_{x} - 1 \) \times 1000 where \( x \) is the isotope ratio of the sample and \( S \) is the isotope ratio of the standard. We analyzed feathers at Environment Canada’s Isotope Laboratory at the National Water Research Institute in Saskatoon, SK. We took advantage of the prealternate feather molting pattern in yellow-rumped warblers, where at the end of the winter these birds molt many of their body feathers and 3–4 of their inner-covert feathers on their wintering grounds during a prealternate molt. Therefore, a bird captured on its breeding territory will have freshly molted feathers from the prealternate molt that contain the isotopic signature of its most recent wintering area or an early potion of its migration route (see discussion; Gaddis 2011).
3.2.3 Statistical analyses

To test whether feathers from the two allopatric populations differ in their stable isotope composition we used a two-sample $t$ test in R (R Development Core Team 2013), since distributions appeared roughly normal with similar variances. To study the distribution of isotopic variation amongst hybrid genotypes we used a linear discriminant function analysis to group the individuals based on the isotopic variation in the allopatric sites. We first used the DFA to test the classification of each of the allopatric birds to determine the general rate of misclassification. We then used this function to classify hybrid individuals as either Audubon’s-like or myrtle-like based on their isotope values. This procedure produces posterior probabilities of assignment to each of the groups, with individuals close to the overlap between Audubon’s and myrtle isotope values having similar probabilities of being assigned to either group. To test for a correlation between hybrid index and isotope value we used the binomial classifications from the DFA, which assigned individuals to Audubon’s or myrtle isotopic groups, in a generalized linear model. This was done only for those individuals within the hybrid zone, excluding those from the allopatric populations and from the Kananaskis site. We performed a logistic regression using the “logit” link function in R to test whether the posterior assignment output (response variable) of the DFA was associated with genetic hybrid index (explanatory variable).

We estimated the geographic origin of the feathers by using IsoMAP, a framework that allows for modeling, predicting and analysis of stable hydrogen isoscapes (Bowen et al. 2013; http://www.isomap.org). The system draws on hydrogen analyzed from environmental water collected at stations distributed globally for over 30 years to model fine scale geographic variation in isotopes. We first created a geostatistical precipitation model of environmental hydrogen (the dependent variable) as a function of CRU-derived precipitation data (Mitchell and Jones 2005) and ETOPO elevation data (independent variables; U.S. National Geophysical Data Center 1998) over the years 1980 – 2008 (IsoMAP JobKey: 31437). We included environmental isotope data collected from April – October as previous studies show that growing season correlates more strongly to hydrogen values than other time periods (Bowen et al. 2005; Hobson et al. 2012). This model was then used to estimate spatial variation of hydrogen across the U.S. and Central America within a longitudinal range of -131.5° to -61°W and a latitudinal range of 3.3° to 51.5°N (IsoMAP jobkey: 31439).

In most cases there is not a 1:1 relationship between hydrogen in precipitation ($\delta^2$H$_p$) and organic samples, such as feather keratin ($\delta^2$H$_f$), because of how hydrogen moves through food
webs, and it is therefore important to generate an empirically-based transfer function between the two (Bowen et al. 2014). This is done using known-origin samples, feathers in this case, to estimate environmental isotope equivalents, which can then be used to estimate similar environmental isotope values from feathers of unknown origin (Bowen et al. 2014). To do this we used 209 feather samples previously analyzed by Hobson et al. (2012) where the location of feather growth was known. Our subset of the Hobson et al. (2012) data included only migratory species, of which the majority (>75%) were from species in the Parulidae family (i.e. wood warblers). We then used QGIS (Quantum GIS Development Team 2013) to estimate the environmental equivalent for each of the known-origin samples from the environmental hydrogen surface created by IsoMAP (Appendix 2). IsoMAP assumes a linear relationship between the feather-environmental water isotope function and we used the equation \( \delta^{2}H_{f} = 1.3450* \delta^{2}H_{p} – 20.17 \) (see additional details in deriving this function in Appendix 2). Following Bowen et al. (2014) this can be algebraically rearranged to estimate the environmental hydrogen value from feather isotopes of unknown origin.

From this relationship we estimated the environmental hydrogen equivalents in each of our feather samples. Using IsoMAP, we then generated a geographic likelihood assignment surface for each individual. We used the “individual assignment” function and included each individual’s environmental-equivalent hydrogen value plus the standard deviation of the residuals from the water/feather transformation function (9.96‰). The resulting likelihood surfaces were then standardized for each site. We did this by finding the highest maximum likelihood among individuals within a site and then linearly scaling each individual’s likelihood surface to have this same maximum likelihood in QGIS. The standardized surfaces were then combined, additively, for each individual within a site. We did this standardization because one component of an individual’s likelihood surface includes the absolute number of pixels on the landscape that have similar isotope values. Therefore, if there only a few pixels on the map with a bird’s isotope value it is assigned a high likelihood, whereas if there are a large number of pixels with a similar isotope value, it divides the likelihood among those pixels. In some cases this results in variance in the maximum likelihood amongst individuals within a site and, if not standardized, biases any combination of the surfaces to those individuals having isotopically unique values within the spatial extent of the isotope map. Standardizing the individual likelihoods to have the same maximum likelihood means that individuals are treated similarly in this regard, resulting in a better illustration of the geographic patterns considering all individuals at a site. Therefore, the relative distribution of the resulting likelihood values for any given
standardized map are informative, although the absolute values are not an accurate representation of any individual’s assignment uncertainty. These standardized likelihood surfaces for each site were illustrated with QGIS. To illustrate areas of potential geographic overlap between the assignment of the pure myrtle and Audubon’s sites we also calculated the absolute difference between these two standardized likelihood surfaces.

3.3 Results

Samples from populations outside the hybrid zone (sites 1 and 7 in Figure 3.1) differ significantly in their mean isotope signature ($t_{15} = 4.01, P = 0.001$). This was in the expected direction of the allopatric myrtle population having a significantly higher mean $\delta^2$H (mean $\delta^2$H = -42.6‰) compared to the Audubon’s warbler population (mean $\delta^2$H = -63.6‰). Isotopic variation among individuals and sites within the hybrid zone is more complex and variation in the hybrid sites generally overlaps with that of allopatric populations, with the most overlap with myrtle warblers (Figure 3.3). Sites 3-5 have a similar mean and distribution as compared to allopatric myrtle warblers (site 7), whereas site 2 has a more intermediate mean and high variation, with a mixture of isotopic values observed in the pure Audubon’s and myrtle warbler sites in addition to values well outside the range of both (e.g. -113.6‰ and -9.2‰). Individuals sampled from site 6 (the diamonds in Figure 3.3), along the Kananaskis transect, have overall higher hydrogen values (average -29.5‰) compared to the parental species as well to individuals from other hybrid zone sites.

Isotopic variation within genetic classes is consistent with the geographic patterns: most of the hybrids have distributions of isotopic signatures that are similar to that of pure myrtle warblers (Figure 3.3). The discriminant function analysis (Figure 3.4) has an overall accuracy of assignment for the allopatric birds of 82%. Due to the high hydrogen values, all of the individuals sampled from the Kananaskis transect were assigned to the myrtle group. From the other hybrid zone sites (i.e. sites 2-5), 76% of the individuals were assigned to the myrtle-like isotope group (28 of 37 birds), with an average $\delta^2$H of -39.9‰ (SD = 10.4). The rest were assigned to the Audubon’s-like isotope group (9 of 37 birds), with an average isotope value of -68.8‰ (SD = 18.1). Within the hybrid zone (sites 2-5), there was no significant relationship between genotype and isotopic assignment ($z_{35} = -1.55, P = 0.12$).

The likelihood assignment estimating the growing regions of feathers based on $\delta^2$H differs between the pure Audubon’s and myrtle feathers (Figure 3.4). Feathers from myrtle warblers are assigned to areas that are restricted primarily to the southeastern U.S. and the
Caribbean (Figure 3.5c). In contrast, the likelihood surface for Audubon’s is highest in other regions, although showing altogether more uncertainty with regards to areas of geographic assignment. In this case, birds have high likelihood of wintering in regions including coastal California and the Pacific Northwest, the southwest, as well as a long band that crosses the Great Plains and extends to the Atlantic coast (Figure 3.5b). There are two primary regions where we identified quantitative similarities in the assignment probabilities between these two allopatric groups: a large region in the north (extending from the southern Rocky Mountains north into Canada), and a narrow band extending from the Atlantic, along the southern U.S. and into Central America (Figure 3.5d). The area in the north and northwest are areas where both allopatric groups have extremely low (i.e. near zero) assignment likelihoods. The more southerly, narrow band is instead an area where both groups have similarly low, but non-zero, assignments.

Feathers from individuals in the hybrid zone, sampled from sites 3 - 5 (Figure 3.6b to 3.6d), have the highest likelihood assignment to areas in the southeast U.S. This area extends west to Texas, north to Illinois, east to the Atlantic and south to the Gulf Coast of Central America and into the Caribbean. A smaller proportion of individuals also have areas of assignment further west, including Baja and northwestern Mexico. Feathers from site 6 (Figure 3.6e), sampled from birds in the Kananaskis transect and showing particularly high hydrogen values, have assignments that are shifted even further into the southeast, with the most likely areas of assignment including southern Florida and Cuba. The likelihood surfaces for site 2 (Figure 3.6a) suggest a much more diffuse pattern, with geographic assignments for these birds that include the southeast, the southwest and along the Pacific coast.

3.4 Discussion

Here we present the first study of isotopic variation in the Audubon’s / myrtle warbler hybrid zone. The location of this zone, along the Rocky Mountains of eastern British Columbia and southwestern Alberta, Canada, is in an area where a number of other avian contact zones and hybrid zones are located, and migratory behaviour has been postulated to play a role in maintaining some of them (Toews and Irwin 2008; Irwin et al. 2009; Toews et al. 2011; Rohwer and Irwin 2011). These data from stable hydrogen from birds captured on their breeding territories, but in feathers grown at or near their most recent wintering grounds, provide the first estimates of the over-wintering location of hybrid offspring between these two species. The data suggest that Audubon’s and myrtle warblers, at least in populations near the hybrid zone studied
here, winter in isotopically distinct areas (Figures 3.3 and 3.5). We also found a number of novel and complex patterns in the isotopic data from hybrids. Most hybrids appear to be wintering in the southeastern U.S. overlapping with the known wintering areas for myrtle warblers (Figure 3.6b to 3.6d), although others are also likely wintering in the west (i.e. some individuals from site 2; Figure 3.6a) and some even further to the southeast (i.e. individuals from site 6; Figure 3.6e). Most of these patterns related to where the individuals were breeding in the hybrid zone and there was no detectable correlation with a bird’s genetic background. We interpret these findings below and discuss them within the context of previous research on hybrid zones, speciation and migratory divides.

The result that allopatric Audubon’s and myrtle warblers on either side of the hybrid zone differ significantly in the stable hydrogen composition of their feathers suggests that there is a transition in over-wintering location between these two species and that it is quite narrow. The likelihood surfaces estimating the geographic origin of the feathers grown during the prealternate molt of birds in the allopatric populations generally agree with previous data from band recoveries: feathers from Audubon’s warblers having a high likelihood of originating in coastal California, Baja and southwestern Arizona (Figure 3.5b) and myrtle feathers likely originated in the southeastern U.S. (Figure 3.5c). However, the δ²H likelihood assignment areas are larger than field observations and band recovery data suggest, at least for Audubon’s warblers. For instance, field data suggest that Audubon’s warblers are rarely observed east of Colorado, New Mexico or Texas during winter months (Hunt and Flaspohler 1998; Stephenson and Whittle 2013). This band of high likelihood for Audubon’s feathers originating in the central and eastern U.S. is likely a function of the distribution of δ²H over the landscape (Figure 3.5a) as opposed to being reflective of their true overwintering location. This is also likely true with the area of high likelihood of Audubon’s feathers originating in the southern parts of Central America. While there are records of Audubon’s wintering these areas (Hunt and Flaspohhler 1998), it seems unlikely that individuals from our sample overwintered in these localities, although it cannot be excluded. Future studies sampling individuals captured on the wintering grounds will be important to address such uncertainty (e.g. Bensch et al. 2006).

It is important to note that, while consistent with field observations, the wintering sites of myrtle (and potentially hybrid) birds in our study may be more southerly than is implied by the feather isotope data. This is because myrtle warblers may undergo their prealternate molts during the early stages of migration as opposed to directly in the over-wintering area (Gaddis 2011). Data from a banding station in Oregon suggest that approximately 95% of Audubon’s warblers
had already gone through their prealternate molt as they showed up on spring migration (Gaddis 2011). In contrast, 60% of myrtle warblers had gone through their prealternate molt when captured on migration, although it is not known if these birds in Oregon are also representative of birds further to the east or hybrids (Gaddis 2011). We suspect that myrtle warblers that molt on the wintering grounds (and not on migration) would likely have a higher hydrogen value. This is primarily because hydrogen decreases with latitude in the eastern U.S. (Figure 3.5a). Therefore, if a myrtle warbler began its prealternate molt during the early parts of spring migration, north of its wintering area, it would be expected to have a lower hydrogen value and would appear more ‘Audubon’s-like’ in our analysis. While this would not alter the general conclusions of our study, such a pattern could be confirmed by a more detailed study of molt and isotopic patterns of individuals on spring migration in the eastern U.S.

The data from birds within the hybrid zone suggest that most hybrids likely overwinter in the southeastern U.S. (Figure 3.6). This can be observed in both the spatial patterns of isotopic variation in the hybrid zone and amongst the various hybrid genotypes (Figure 3.2). These patterns can be explained in two ways. First, the individuals from these sites could be growing their feathers in a similar area to myrtle warblers, as we suggest. Alternatively, given the broad regions of high likelihood assignment, hybrids and pure forms could in fact wintering in distinct parts of this area. While unlikely, we cannot exclude these possibilities and such uncertainty may be resolved with additional isotopic data (i.e. carbon, nitrogen and/or oxygen).

There are at least two evolutionary mechanisms that could produce a pattern of most hybrids clustering with one or the other of the parental species. First, dominant inheritance of migratory traits could produce a pattern of most of the hybrids having myrtle-like traits if Audubon’s migratory loci were recessive. Such patterns of migratory dominance have been suggested previously in hybrid pied and collared flycatchers in Europe (Veen et al. 2007) and hybrids between great reed warblers (Acrocephalus arundinaceus) and clamorous reed warblers (A. stentoreus), although in the latter case the number of hybrids sampled was limited (Yohannes et al. 2011). Alternately, the loci responsible for the behaviours may be discordant with the transition in other traits, potentially due to introgression. It has been shown previously that mtDNA has likely introgressed from myrtle into Audubon’s warblers at some point in the past (Mila et al. 2011; Toews et al. 2014) and there is some evidence of nuclear introgression between the species (Brelsford et al. 2011). Whether this clustering pattern is due to dominance or discordance of the responsible loci is not clear, but such a result implies that few loci would be involved, given genetic and phenotypic data do not suggest a general pattern of dominance or
introgression of myrtle genes or traits (Brelsford et al. 2011). Having few responsible loci seems counterintuitive based on the complexities of a multi-faceted trait such as migration, but is not without precedence (Helbig 1996; Sutherland 1998), and future genomic studies will be able to address this more conclusively in the future (Liedvogel et al. 2011). We acknowledge, however, that each of these explanations assumes that the migratory direction and over-wintering location is under genetic control, as previous studies would suggest (Alerstam 2006). However, a more ecological explanation is also possible, where individuals respond to features on the landscape, such as their orientation relative to the Rocky Mountains that cues them to move in various directions. In this case, we would expect to observe greater variation in the stable isotope values within a site, which was instead quite consistent across most of the study sites (i.e. average standard deviation of $\delta^2$H of -11‰ across all sites, excluding site 2). More generally, we suggest there would clearly be a benefit in performing controlled laboratory studies to estimate the extent of genetic control of migration in this or other migratory species in North America, although this would be a logistical challenge.

Among the hybrid zone sites, the clustering of hybrid individuals with the allopatric myrtle site is consistent from sites 3 to 5. In contrast, the individuals sampled at site 6, along the Kananaskis transect (the diamonds in Figure 3.3), while overlapping with the other hybrid zone sites, have on average higher hydrogen values compared to samples taken from the main Saskatchewan River Crossing transect. This may be because these individuals, sampled at breeding populations further south in Alberta, also go further southeast, maybe as far as the Caribbean, for the winter (Figure 3.6e). This preliminary observation could be confirmed with additional analyses of samples taken from other transects across the hybrid zone. Another notable pattern is at site 2, approximately 60 km west from the hybrid zone center, which shows much greater isotopic variation than the other sites (Figure 3.3a and 3.6a). Our sampling here is too limited to test for a bimodal distribution of values, but the values here overlap with what is observed in both allopatric sites as well as some values that are outside the parental ranges. The finding of higher variance in this potential transition area is consistent with previous studies showing much greater variation in hybrid blackcap migratory orientation behaviours compared to the parental species (Helbig 1991). Future sampling along the western side of the hybrid zone could provide a more precise estimate of where the shift in isotopic signatures occurs. Indeed, such an observation raises interesting questions about the dynamics of a potential migratory transition on the edge of the hybrid zone.
Across all of the individuals in the hybrid zone we found no evidence of a strong correlation between the hybrid index and isotopic values (Figure 3.4). Two recent studies in blackcap warblers (Roulhausen et al. 2013; Mettler et al. 2013) also found either non-significant or weak correlations between genetic markers and isotopic data. It should be noted, however, that our results depend in large part in the accuracy of the hybrid index. Similar results were obtained by using the plumage index as opposed to the genetic hybrid index (i.e. most hybrid classes cluster with myrtle warblers; results not shown). Such plumage traits are likely influenced by numerous genetic loci, although the inheritance patterns of these traits are not known. While the three nuclear markers presented here are sufficient to distinguish hybrids and backcrosses from pure individuals, future studies employing genome sequencing (i.e. Davey et al. 2011) would clearly be much better at identifying additional hybrid classes. This method might also have the resolution to potentially identify some of the important genomic regions linked to these divergent migratory patterns (Leidvogel et al. 2011).

These results suggest a combination of our original predictions, where most hybrid sites show a clustering with one of the parental species (the ‘dominance’ prediction; sites 3-5), while one shows potentially a novel isotopic patterns (the ‘novelty’ prediction; site 6) and another shows a high variance and mixture of both parental values (the ‘additive’ prediction; site 2). Taken together, these data suggest that there is likely a transition in migratory behaviour across the Audubon’s / myrtle hybrid zone, but that it is not coincident with the center of the hybrid zone and is instead shifted to the western edge. Migratory divides, where changes in migratory behaviour are coincident with other characteristics, are most notable in willow warblers (Bensch et al. 2009) in Sweden and in Swainson’s thrushes in western Canada (Ruegg, 2008). Migration has been suggested as a potent form of reproductive isolation in these divides: naïve young hybrids may inherit directional cues (Helbig 1991) that cause them to migrate to an intermediate and potentially inferior wintering location or take them over inhospitable areas during migration (Chamberlain et al. 2000). While new technology has expanded the range of patterns expected from such divides (i.e. loop-migration; Delmore et al. 2012), our data from the Audubon’s / myrtle hybrid zone suggests that an intermediate migration route and wintering location of most hybrids is not likely.

While an intermediate route or over-wintering site is unlikely for most hybrids, these data do not rule out the possibility that migration and over-wintering site selection could still impose some fitness consequences for hybrids. For instance, over-wintering in habitat suitable for parental species may still impose a challenge to hybrid individuals, which have a mixture of both
Audubon’s and myrtle genes. It is also important to note that if there is strong selection against hybrids in their first year it would not be identified by this type of analysis, as the current sample is of those individuals that have successfully migrated south-and-back again. Indeed, it may be that some individuals are heading to an intermediate over-wintering location and are encountering less suitable habitat as compared to those further west or east and not returning. Densities of birds in these intermediate and inland areas are lower than those along the coast further east and west (Figure Appendix 1), consistent with the suggestion that these habitats are potentially lower quality. For example, in southern Arizona, Terrill and Ohmart (1984) found that Audubon’s warblers were facultative residents during the winter months: in winters with favorable food availability, more birds remained to overwinter at particular sites. In winters with reduced food availability, however, birds facultatively continued their autumnal migration south to Mexico. This suggests that the winters associated with these inland and higher elevation sites may be more variable than those along the coasts and, consequently, these areas may be less suitable.

In addition to an intermediate over-wintering location, employing an intermediate migratory route could potentially take hybrid Audubon’s and myrtle warblers over inhospitable areas. For instance, an intermediate route might take hybrid warblers over the middle and southern Rocky Mountains or arid regions, such as the deserts of the southwestern U.S. This has been proposed as an important ecological barrier that hybrid Swainson’s thrush may encounter and, as a consequence, has been proposed as an important post-mating reproductive isolation in this and other systems (e.g. Rohwer and Irwin 2011; Delmore et al. 2012). There could also be an interaction with molt timing, where individuals have the migratory behaviour of one species but the timing or molt pattern of the other (Rohwer and Irwin 2011). Finally, hybrids from site 6 heading further into the southeast, potentially into the Caribbean, may experience more extreme weather events during the winter months that could be a potent form of selection. While much of this is speculation, clearly additional technology would be ideal to address more detailed questions such as these. Given that current methods rely on recapturing an individual in subsequent years, inferring the fate of hybrids will necessarily be indirect, until GPS-like tags are small enough to affix to small songbirds.

In conclusion, these isotopic data suggest that myrtle and Audubon’s warblers in populations near the hybrid zone winter in distinct locations. They also suggest that most hybrids between the species over-winter in an area that generally overlaps with myrtle warblers. While there appears to be a transition between the two migratory behaviours, it is shifted approximately
60km to the west of the hybrid zone center. We suggest this pattern is not supportive of a classic migratory divide, where a shift in migration is coincident with transitions in other phenotypic traits and genetic markers (e.g. Delmore et al. 2012) and where there is a greater potential for selection against hybrids employing an intermediate migratory route (Bensch et al. 2009). These patterns should be confirmed by additional studies and we are currently in the process of studying how these isotopic results translate into behavioural patterns in a large-scale orientation study of hybrid myrtle / Audubon’s warblers on fall migration. By integrating patterns of genetics, behaviour, and trace elements our goal is to gain a better understanding of the role of such complex phenotypes in the process of local adaptation and reproductive isolation. They also highlight the importance, and the challenge, of considering an organism’s full annual cycle when studying such hybrid zones.
Figure 3.1  Distribution and band recoveries of Audubon’s and myrtle warblers. (A) Banding
data obtained from Brewer et al. (2006) and the Canadian Bird Banding Office (2013). (B)
Sampling localities across the Audubon’s / myrtle warbler hybrid zone. Site 1 is defined here as
an allopatric Audubon’s population; site 7 is an allopatric myrtle population; sites 2 – 6 are
within 70km of the hybrid zone center. Hybrid zone sites are along the Saskatchewan River
Crossing transect (panel D in Figure 1 of Brelsford and Irwin 2009), except site 6, which is along
the Kananaskis transect (panel E in Figure 1 of Brelsford and Irwin 2009).
Figure 3.2  A simplified schematic of the general predictions for the transition in over-wintering behaviour across a hybrid zone. Each point represents an individual’s estimated wintering longitude with its distance relative to the center of the hybrid zone and coloured by its genetic background. (A) shows the predictions consistent with an ‘additive’ scenario, where hybrids show a high variance, an overall intermediate behaviour and a correlation between their genetic background and over-wintering location. (B) illustrates the ‘dominance’ pattern, where individuals overlap in their over-wintering location with one or the other of the parental species. (C) shows a pattern were hybrids over-winter in an entirely novel areas as compared to either of the parental species.
Figure 3.3  Stable hydrogen and genetic variation across the Audubon’s / myrtle warbler hybrid zone. The filled symbols indicate the two allopatric populations (Audubon’s and Myrtle, left and right respectively); the open circles indicate individuals from sites 2-5 along the Saskatchewan River Crossing transect and the diamonds indicate individuals sampled from site 6 along the Kananaskis transect. The black lines represent the mean for each group and the grey boxes represent the range of variation observed in the two allopatric populations; the darker portion represents the overlap between these two distributions. In (A) the isotopic value of each individual is shown relative to its distance from the center of the hybrid zone (in km). Brelsford and Irwin (2009) estimate the zone to be 132km wide. In (B) the points are grouped by an individual’s genetic hybrid index, derived from three nuclear markers (CHD1Z, RIOK2 and numt). There was no significant relationship between an individual’s genotype and its isotopic assignment for birds in the hybrid sites ($z_{35} = -1.55, P = 0.12$).
Figure 3.4  Posterior probability of individuals assigned as either Audubon’s-like (white) or myrtle-like (black) based on isotopic data from a discriminant function analysis. Each column represents a single individual with the proportion of white or black representing the probability of the DFA assigning its isotopic value as Audubon’s-like or myrtle-like, respectively. Individuals in the hybrid zone are distinguished by the hybrid index, a combination of three nuclear genetic markers. The numbers above each column represents the site that an individual was sampled from (see Figure 3.1).
Figure 3.5  Stable hydrogen isoscapes and likelihood maps from IsoMAP for parental species. (A) Prediction isoscape generated from IsoMAP (Bowen et al. 2013). (B) and (C) are the likelihood assignment surfaces for allopatric Audubon’s and myrtle warblers, respectively. (D) The absolute difference in the standardized likelihood surfaces between myrtle and Audubon’s warblers. Brown areas illustrate areas of isotopic overlap; green areas show areas where the likelihood surfaces differ between the two.
Figure 3.6  Likelihood surfaces for individuals in the hybrid zone. Likelihood assignment surfaces for individuals sampled in the hybrid zone, from site 2 to 6 (A to E respectively). Given these are standardized likelihood surfaces (i.e. each individual has a surface that is scaled to have the same maximum likelihood) the relative likelihood distributions are informative but the absolute values have little meaning (see methods).
CHAPTER 4: Migratory orientation in a narrow avian hybrid zone

4.1 Introduction
The breeding and wintering ranges of many species are separated by thousands of kilometers. Long-distance, seasonal migration exhibited by many taxa moving between these disjunct areas is a complex and energetically demanding task that has been studied for decades (Berthold 1991). However, in many taxa it is still unclear the relevant physiological mechanisms, the important controls and senses involved in navigation and the contribution of different cues to the migratory phenotype (Berthold and Terrill 1991; Alerstam 2006). For instance, one important question involves the relative importance of genetic versus environmental factors in influencing migratory behaviour (Liedvogel et al. 2011). At least in birds, much of our understanding of migratory directionality and navigation comes from studies of blackcap warblers (Sylvia atricapilla) in Europe. In an influential series of studies, Helbig and colleagues (Helbig 1991; Helbig 1996) reared blackcaps that were sampled from populations that exhibit different migratory routes in a common environment. They found that, even in captivity, individuals recapitulated their natural routes, as assayed by Emlen funnels, and this was strongly correlated with an individuals’ genetic background. These studies also found that lab-crossed hybrids between the parental types show an intermediate migratory orientation, on average. However, our understanding of how such genetic mechanisms involved in migratory orientation interact with environmental cues in wild birds under natural settings is unclear.

These questions are relevant because if environmental features or local factors have an important influence on orientation, then the ability of genetic data to predict overall migratory routes in natural populations may be lower than expected. Such observations are particularly pertinent to studies of migratory divides, where populations that differ in migratory directionality come into contact and interbreed (e.g. Irwin and Irwin 2005; Bensch et al. 2009; Rohwer and Irwin 2011). For example, if hybrids between divergent populations have a mixture of alleles responsible for migration and exhibit an intermediate migratory orientation, as has been observed in lab-raised blackcap warblers, such a novel phenotype may be inferior and represent an important fitness detriment. This is because, in a number of systems, intermediate routes have been suggested to take hybrid individuals over unsuitable or inhospitable environments, although empirical examples are rare (Rohwer and Irwin 2011).

To date the only study to directly assay the migratory orientation of wild-caught individuals differing in their genetic constitution across a migratory divide was conducted with willow warblers (Phylloscopus trochilus; Ilieva et al. 2012). In this case, individuals were
captured in SE or SW Sweden during fall migration and their orientation was assayed using Emlen funnels (Emlen 1970). Individuals were genotyped at genetic loci that differ between the two subspecies that are assumed to differ in their fall migratory directionality, based on ring recoveries and isotopic data (Bensch et al. 2009). Consistent with predictions, individuals with genotypes from the subspecies predicted to orient SE (i.e. *P. t. trochilus*) had a mean orientation towards the SE; the individuals with genotypes from the subspecies predicted to head SW (i.e. *P. t. acredula*) oriented SW. While this was one of the first studies to assay migratory orientation of wild individuals across a migratory divide, it had a number of important limitations. First, individuals that differed in their genetic composition were captured in differing locations (i.e. SE versus SW Sweden), thus discerning the effects of geography versus genetics is challenging. Second, there was only a small sample of hybrid individuals, and it was also unclear whether those individuals that had hybrid genotypes were from the contact zone, so it is not possible to infer the behaviour of hybrids more generally.

We attempt to address a number of these limitations here with a large-scale orientation assay of birds sampled on fall migration from a hybrid zone between Audubon’s / myrtle warblers (*s. auduboni* and *S. coronata*) in western North America. The narrowness of the hybrid zone suggests that some form of selection against hybrids maintains the hybrid zone, which Brelsford and Irwin (2009) estimate to be moderately strong. The motivation for studying the migratory patterns of birds in this hybrid zone is primarily because evidence suggests that a lack of assortative mating and other pre-mating reproductive barriers are unlikely to be strong in this system (Brelsford and Irwin 2009; Brelsford et al. 2011), implying a potential role for post-mating selection against hybrids. Band recovery data suggests that, at least in birds outside the hybrid zone, the two forms differ in their migratory movements (Figure 4.1). Therefore, if migratory traits are inherited additively, as the studies of blackcaps suggest, we might expect a correlation between a bird’s orientation and its ancestry. In particular, we expect that individuals more genetically Audubon’s-like will orient SSW, individuals more myrtle-like will orient SE, and hybrids will orient intermediate between these two (i.e. south). However, while these are the expected large-scale differences in these species migratory routes, orientation experiments may instead reflect more local factors. Indeed, a number of studies, primarily using new tracking technology and radar, suggest that the local migratory movements of wild birds can be somewhat idiosyncratic and respond to local factors, such as topological variation (Williams et al. 2001). Therefore, such local orientation patterns may or may not provide a robust signal of migratory
patterns more generally. In this case, we explore the potential influence of landscape topology as possible explanatory variable of local orientation.

To perform this study we used a novel video-based method for assaying the orientation of individuals, first applied by Fitzgerald and Taylor (2008). These orientation cages are similar in many respects to traditional Emlen funnels (Emlen 1970), but where the behavior of birds is scored using video cameras. This method, as compared to the scratch marks quantified with Emlen funnel experiments, has a number of benefits, including: the videos are scored consistently and objectively, a longer period of observation can be obtained for each individual, specific time periods can be analyzed in isolation and any behavioural changes over the course of the trial can be quantified. We designed our study to assay the orientation of individuals on the day they were captured, which previous research suggests is predictive of later orientation (Ilieva et al. 2012). We then genotyped each individual and tested whether there was a correlation between an individual’s orientation and genetic background.

Our sampling design allowed us to study a large number of individuals over the migratory season, most of which were expected to be hatch year individuals (Carlisle et al. 2005). Previous studies drawing upon hybrid zone theory suggests that there is moderately strong selection against hybrids (Brelsford and Irwin 2009). However, is currently unclear whether this is manifested as the reduced development of hybrids or whether hybrids develop normally but are somehow deficient later in life. This design also allowed us to indirectly distinguish between these alternatives and, consequently, the possible timing of selection against hybrids between myrtle and Audubon’s warblers. To do this we compared the proportion of genetic classes based on three molecular markers of those birds captured on migration (mostly hatch year birds) to those same proportions sampled from adult breeding birds (i.e. birds that survived fall migration, over-wintering, spring migration and territory establishment). Extensive sampling of the Audubon’s / myrtle hybrid zone during the breeding season suggests that there is little assortative mating and breeding hybrids are relatively frequent in the hybrid zone (Brelsford and Irwin 2009). In this case, if there is an excess of genetically intermediate individuals on migration and not in the breeding birds, this is consistent with selection against hybrids taking place either during their first migration or during their first winter. If there is no difference in the genetic composition between individuals on migration and older, breeding birds this would be consistent with a number of scenarios, most notably it may be that selection against hybrids takes place before migration (e.g. during development and prior to hatching).
4.2 Methods

4.2.1 Study site and orientation trials

Between August 15th and September 12th of 2011 we captured migratory yellow-rumped warblers \( n = 181 \) near Kananaskis, Alberta, a site at the center of the Audubon’s – myrtle hybrid zone (Brelsford and Irwin 2009). Our sampling was concentrated in two areas: along the southern edge of Barrier Lake (51.023473°N, 115.060799°W) and within the hamlet of Lac Des Arc (51.05357°N, 115.158845°W; Figure 4.1c). We set up mist-nets before dawn and used passive netting along with song playback to increase our likelihood of catching individuals of our target species. Immediately after capturing each individual we took morphometric measurements (i.e. bill, tarsus, wing and tail length), photographs, and a blood sample (10-40 \( \mu \)L), and we applied unique aluminum bands. Birds were then transported from the capture site to the location where the orientation trials were performed (51.028547°N, 115.024170°W). This site is a large, recently clear-cut field (~400m\(^2\); <10 year old) near the Kananaskis Biogeoscience Institute and has clear views in all directions, with the peak of Mount Baldy visible approximately 2.5km to the southwest.

Each individual was placed into one of 12 outdoor holding / orientation cages and given water and small mealworms throughout the day. The cages were a modified design based on Fitzgerald and Taylor (2008), who also used them to study orientation in yellow-rumped warblers (Figure 4.2). The cages were leveled, oriented with a compass and spaced approximately 3-5 meters apart. The cage frames were made out of pine boards, with the top and bottom of the cages made from composite plywood. The perch was made with a 9” plastic embroidery hoop, placed horizontally such that birds could perch on the hoop, and four 7/16” dowels that held up the hoop. The wood pieces were joined with non-magnetic brass screws and stainless steel staples were used to affix screen mesh to the top and sides of the cage, as research has suggested that birds may use magnetic cues for navigation (Alerstam 1993; Muheim et al. 2006). During the day the sides of the cages were covered with a blue, opaque tarp for sun shelter, although part of the tarp was rolled up to provide sufficient airflow (Figure 4.2a). Using the same cages to both hold the individuals during the day and run the orientation trials in the evening reduces the stress inherent in moving individuals shortly before the trials, as is necessary with Emlen funnels (Emlen 1970). If individuals did not immediately fly to the perch, showed any signs of stress (e.g. panting), or were not eating properly they were released during the day (approximately 15% of birds).
Before we started the orientation trials we removed the food and water dishes and closed the tarp around the sides of the cages, allowing individuals to see only out of the top of the cage, which had a full view of the sky. Through a hole in the bottom of each cage we attached a D-Link Wireless Network Camera (DCS-932L) pointing directly up, with the top of the camera oriented northwards. The cameras use an infrared LED light for illumination during low light conditions. We have no evidence to suggest that the birds can see into the infrared spectrum, although given the light was applied consistently across all of the trials in the unlikely event that there was an effect of the light it would not explain any differences found between birds. The cameras were set such that video image was recorded with right side of the video representing the west side of the cage and the left side of the video representing the east side of the cage. We recorded 320p x 240p 30 frame-per-second video from each cage simultaneously using the D-ViewCam software on a PC laptop via a D-Link router. We began recording each individual’s behaviour approximately one hour before sunset and ran the trials until approximately 30 minutes past sunset. Each evening we ran the trials until the last individual stopped moving, after which we released all of the individuals. During the trials we recorded the wind speed, direction and amount of cloud cover (on a scale from 0 to 8). We only tested each bird once and we recorded the behaviour of 1 to 10 individuals each evening, averaging 6 birds per evening, with 124 individuals for which we collected complete orientation data.

4.2.2 Video analysis

From the D-ViewCam software we exported each video in “.asf” format, noting the start and end time of each trial. To analyze the data we used radR, an open source platform developed for acquiring and analyzing radar data (Taylor et al. 2010) that was more recently adapted to analyze video files. In brief, radR uses contrast to score individual pixels, and then uses movement, area and intensity to define an object. For our analysis we sampled the videos at 3 frames per second. For each object in a sample we extracted the X and Y coordinates of its centroid, as weighted by the area of the object. Given that the light conditions change over the evening, we used three groups of parameters that we found accurately and consistently identified the bird as the primary object relative to the background given the changing light conditions. We defined the center of the video at the center of the circular perch and created an exclusion zone within the perch, such that the only region that the program would identify the bird was from the perch outwards (Figure 4.3). We did this to reduce points where the individual was flying
between sides of the perch; this also makes our results more comparable to previous funnel studies, which only record jumps where the individual contacts the side of the funnel (Fitzgerald and Taylor 2008). From this data radR generates a list of time-stamped X and Y coordinates for points where it identified an object.

Using R (R-core Development Team 2013) we then applied a number of additional filters, primarily to remove noise (i.e. objects that were not the bird) and remove times when the bird was not moving (i.e. sitting on the perch). To remove potential noise, we first removed all data where there were three or more objects identified at a single time-stamp, as three or more objects were invariably an artifact of background noise. Sometimes radR identifies the bird as two separate objects, primarily when the individual is over top of one of the four perch dowels, such that parts of the bird stick out on either side of the dowel. For time-stamps with two objects, we averaged the XY values that were less than a given threshold (less than 500 pixels apart for the current analysis) to include only those times that an individual was above the dowels (i.e. the dowels are slightly less than 500 pixels wide in the video image) and visually inspected the data to confirm this was accurate. To ignore points where the bird was sitting motionless on the perch we removed consecutives points in time that had a lower XY distance than a predetermined threshold, which we calculated by studying the movements of individuals sitting quietly on the perch (50 pixels for the current analysis). Each of the points that passed these filters were then transformed into an angle relative to North and given a timestamp (in seconds) relative to the time of sunset. In this case, angles were relative to north (0°) clockwise, with E=90°, S=180° and W=270°.

From these data we calculated three behavioural traits for each individual: the mean orientation, rho and activity. We calculated mean orientation using the “circular statistics” package in R (Lund Agostinelli 2007). We used the mean of angles observed over the entire observational period, although we found restricting the time range to only those times when the birds were more active did not meaningfully change our results. We used the same R package to estimate rho, an estimate of angular variance that varies between 0 and 1 (i.e. a measure of the concentration of points). Finally, we used the total number of objects estimated over the trial as the total activity for each individual simply by summing the number of objects identified by radR within the time period.

To assess robustness of estimating the mean orientation for each individual using the data generated from radR we chose five orientation videos at random and scored them by eye, blind to the output from radR. For this we visually estimated the angle of the bird (if present in the
frame) every 30 seconds, over the entire video, and also recorded whether the bird was on the perch or mid-flight. We then calculated the difference in angle between this estimate and that obtained from radR for the full dataset and also from only those points where the bird was observed to be in flight.

### 4.2.3 Molecular analysis

Blood samples, taken using a small needle and capillary tube 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 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 and 1 mM EDTA, at pH 8.0, and stored at 4°C. Genotype information for two of the three nuclear markers (CHD1Z and numt-Dco1) was presented in a previous publication (Brelsford and Irwin, 2009), where the full PCR and genotyping protocol can be found. For the third nuclear marker, RIOK2, we used the forward primer ATGGGTGTTGGCAAAGAATC and the reverse primer GCTCCTCTTCTTWGCAACA and used a PCR annealing temperature of 60°C. This amplifies an 850 base pair fragment, which is cut by the enzyme XmnI in Audubon’s warblers. To generate a genetic hybrid index we added each myrtle allele for the three markers and divided this by the total number of alleles (6 for males, 4 for females) resulting in an index that ranges between 0 (all Audubon’s alleles) to 1 (all myrtle alleles).

We were also interested in comparing the distribution of genotypes that we observed during migration to those birds breeding in previous seasons. Most of the individuals that we captured on migration in 2011 were born recently (i.e. hatch year birds) and we were interested if our sample from migration had a similar distribution of genetic hybrid indices as compared to the genetic class proportions from breeding birds sampled throughout the hybrid zone. For this comparison we used the dataset from Brelsford and Irwin (2009), who genotyped individuals with the identical protocol and markers described above (ROIK2 was also assayed by Brelsford and Irwin 2009 but not reported). We included birds from their study that were captured on their breeding territories, were within 50 km of the hybrid zone center and for which there was complete genotype information. We calculated the genetic class proportions for each of the five transects separately to capture some of the possible geographic and temporal variation (Kananaskis transect: n = 129; Cassiar transect: n = 49; Jasper transect: n = 64; Pine Pass transect: n = 38; Saskatchewan River Crossing transect: n = 79). We then estimated 95%
confidence intervals for the genotype proportions of breeding individuals in Kananaskis \((n = 129)\) as well as our sample from autumn migrants \((n = 166)\) using Agresti-Coull binomial confidence intervals for proportions using the ‘binom’ package in R (Agresti and Coull 1998).

### 4.2.4 Combining genotype and migratory behaviour

Finally, we were interested in testing whether birds tended to orient in a specific direction and whether there was association between genetic background and orientation direction. From the individuals where we had both orientation and genotype information we performed our statistical analysis with two subsets of the data: (1) including all individuals and (2) removing birds with low orientation concentration \((\rho < 0.1)\) and overall activity (total activity < 500). We found that the two methods produced largely similar results and thus only report the results from the subset of individuals that were estimated to be orienting strongly.

To test whether there was a significant mean orientation of all of individuals considered together, regardless of their genetic background, we used a Rayleigh test. This is a procedure to test whether the distribution of circular angles are significantly different from random, with the alternative being that the distribution is clumped in certain direction(s). The test statistic is \(r\), or \(\rho\), and is the magnitude of the mean vector (Fitzgerald and Tayler 2008). To test whether the mean orientation of individuals differed from 1) the angle at sunset or 2) the angle of the major valley axis, we generated 95\% confidence intervals for the mean using a bootstrap maximum likelihood estimates from a von Mises distribution (the circular equivalent of a normal distribution; Fisher 1993). Using a compass, we estimated the angle of sunset during the study period, which was on an average of 267°. Using Google Earth™ we estimated the angle of the valley axis along the Kananaskis trail as it exited the Rocky Mountains, which is approximately 23°. Based on the genetic data we then divided the individuals into 5 genetic groups, those with all Audubon’s alleles \((h\text{-}index = 0;\ \text{Group } A)\), those with mostly Audubon’s alleles \((h\text{-}index > 0 \text{ but } < 0.5; \ \text{Group AH})\), those with mixed genotypes \((h\text{-}index = 0.5; \ \text{Group H})\), those with mostly myrtle alleles \((h\text{-}index > 0.5 \text{ but } < 1; \ \text{Group MH})\) and those will all myrtle alleles \((h\text{-}index = 1; \ \text{Group M})\). Given that all but one of these groups did not show significant mean orientation (see results) we did not compare the distributions between the groups.
4.3 Results

4.3.1 Molecular data

Of the 181 individuals captured, we obtained reliable genotypes from 166 of those, including 124 individuals with orientation data. Based on the three genetic markers, yellow-rumped warblers migrating through the Kananaskis area have a mixture of myrtle and Audubon’s alleles, suggesting a mixture of hybrids and pure-type individuals. Daily allele frequencies varied over the study period although the frequency was usually between 0.3 to 0.6 per day (relative to the myrtle alleles) and an average over the study period of 0.4 (Figure 4.4a). Our molecular sexing of individuals suggests that we captured an excess of males over the study, with an average proportion of males to females of 0.73, likely due to our use of song playback during mist netting (Figure 4.4b).

Compared to the genotype proportions of individuals breeding in the hybrid zone our study of migrants found many more genetically intermediate birds (i.e. genetic hybrid index class = 0.5; Figure 4.5a). In particular, in Kananaskis, 17.8% of birds that we found breeding in this area had a hybrid genetic class of 0.5, compared with 33.1% of individuals from our sample of migrants, with non-overlapping confidence intervals for these proportions (Figure 4.5b and 4.5c). The highest proportion of intermediate individuals was found along the Cassiar highway in northwestern B.C. with the genetic class = 0.5 individuals making up 26.5% of the sample, still much lower than from our sample of migrants.

4.3.2 Orientation trials

For the orientation trials, initial observations of yellow-rumped warbler’s behaviour over the evening suggested that their activity begins to increase approximately one hour before sunset. During this time of increased activity their behavior also changed, from primarily sitting on the perch and flying to the bottom of the cage, to performing more short flights from the perch to the top of the cage, consistent with zugunruhe (i.e. migratory restlessness; Emlen 1970). The activity of the birds, as estimated by the number of objects identified by radR over a given time interval (i.e. the number of times the bird was recorded moving), increased over this period, peaking 20-30 minutes before sunset (Figure 4.6). Following sunset their activity sharply declined, such that we recorded virtually no movements after 40 minutes following sunset. At the end of most evenings individuals usually stopped moving within 5 – 10 minutes of each other with remarkable consistency.
Our data filtering appeared to be effective at removing most of the noise from the data, and our data from a random selection of videos analyzed by eye was very consistent to the output from radR. When we compared all of the points observed by eye, including those where the individual was sitting on the perch, the resultant mean angle was within +/- 22° relative to the output from radR after filtering. If we included only those points where the individual was in flight (by excluding those times we observed by eye the individual was on the perch) the resultant mean angle was within +/- 11° of the radR output after filtering. The decrease in difference between the estimates based on different methods likely relates to the fact that some of the filters applied in R were designed to remove potential perch points.

Of those 124 individuals that had genotype and orientation data, we found that 96 birds showed individual evidence of strong orientation (i.e. within-individual rho >0.1; total activity >500). Of the five genetic groups, our sample contained n = 5 for Group A (i.e. genetically Audubon’s), n = 33 for Group AH, n = 31 for Group H, n = 18 for Group MH and n = 9 for Group M (i.e. genetically Myrtle). While there is quite a lot of spread in terms of orientation of individuals, when all of the individuals grouped together we found there was a significant mean orientation towards 26° or NNE (Figure 4.7a; n = 96, among-individual rho = 0.386, P < 0.001), with a 95% confidence interval between 2° and 51°. This was significantly different than the estimated the angle of sunset during the study period (267°) but not the angle of the valley axis (approximately 23°). Each of the groups separately had mean orientations similar to N or NE, although only Group M (h index = 1) showed evidence of significant mean orientation: Group A = 49° (n = 5, rho = 0.591, P = 0.18), Group AH = 18° (n = 33, rho = 0.284, P = 0.07), Group H = 52° (n = 31, rho = 0.291, P = 0.07), Group MH = 354° (n = 18, rho = 0.265, P = 0.29), and Group M = 15° (n = 9, rho = 0.733, P < 0.01).

4.4 Discussion
These behavioural assays of migratory behaviour using video-based orientation experiments in the Audubon’s / myrtle hybrid zone is the first study of its kind in wild hybrids in an avian hybrid zone. Our application of diagnostic molecular markers in the hybrid zone is also novel in that it allows us to track allele frequency changes on a very fine, day-to-day resolution. In addition, our use of video-based orientation trials allowed us to both sample a large number of individuals over the migratory period while also gathering high resolution and objective orientation data for each individual, a benefit over previous Emlen funnel methods (Emlen 1970). Our results suggest that 1) there is a mixture of Audubon’s-like, myrtle-like and hybrid individuals that moved through our sampling area on migration, 2) there is evidence of an excess
of genetically intermediate individuals as compared to previous studies of breeding birds in this hybrid zone, and 3) that regardless of an individual’s genotype there is a general tendency is for individuals to orient NNE, surprising for birds on fall migration. Below we discuss and interpret our results in the context of hybrid zones, migration ecology and reproductive isolation.

Genotyping individuals on migration is not a common practice and, in those studies that have incorporated molecular markers into studies of migratory birds, the goal is usually to more confidently assign breeding origins and/or investigate migratory connectivity between breeding, stopover, and wintering regions (e.g. Paxton et al. 2013). In contrast, here we used genetic data of birds on migration to better understand the correlation between genetic background and orientation behaviour. This was motivated by studies of blackcap warblers raised in a common environment (Helbig 1991), which suggest that genetic ancestry can be a strong predictor of an individual’s orientation during times of migration. Aside from small day-to-day fluctuations and a general tendency for more Audubon’s alleles on average, we found no evidence of a temporal trend in allele frequencies over our sampling period. In their study of Wilson’s warblers (Cardellina pusilla) during spring migration, Paxton et al. (2013) found a marked change in mtDNA haplotype composition over the migratory period at a banding station in southwestern Arizona, which they attribute to temporal variation in movement of different populations (i.e. southern populations move through first during spring). While our sample of yellow-rumped warblers did not show any obvious trend in marker frequency, the migratory period for yellow-rumped warblers is longer than our sampling effort, extending into October for parts of British Columbia and Alberta (Hunt and Flashpohler 1998). It is possible that later in the fall migration warblers breeding further in the North may move through the Kananaskis area, increasing the frequency of myrtle-type alleles (the more northerly distributed of the two species). Another consideration is that it is also possible that there may be age related timing differences in migration timing that we did not capture. For instance, hatch-year yellow-rumped warblers have been found to migrate earlier than older birds (Carlisle et al. 2005), although the absolute difference in timing is small (~4 days).

We found that the proportion of individuals assigned to the various genetic classes was different than would be expected based on previous studies of breeding birds. In particular, we found an excess of individuals with an intermediate genetic composition (i.e. genetic hybrid index = 0.5). These individuals made up a larger proportion of the sample as compared to birds in Kananaskis or from other transects throughout the hybrid zone (Figure 4.5a). There are a number of possible explanations for this observation. First, this may reflect seasonal fluctuations
in allele frequencies, which may change based on a variety of factors that we did not measure. It is important to note that the genetic data from the breeding birds was collected 4-6 years prior to collecting our data on migratory individuals. While we cannot rule out chance seasonal variation, the fact that the distribution of genotype classes was consistent across a variety of geographic localities of breeding birds sampled in different years suggests these proportions are generally conserved across space and time in the hybrid zone. It may also be that genetically intermediate birds are more likely to migrate through the Kananaskis area. For instance, it may be that birds that are genetically more Audubon’s or myrtle-like head away from the mountains as soon as they begin migration, whereas hybrids simply follow the Rocky Mountains south. While possible, this scenario does not necessarily fit with our data from isotopes or orientation, which suggest that individuals have similar migratory and overwintering tendencies regardless of their genotype.

An alternative explanation is that there may be selection against genetically intermediate individuals during migration or over their first wintering period. This finding is consistent with Brelsford and Irwin’s (2009) conclusion of little-to-no assortative mating on the breeding grounds and selection against hybrids. However, it has been unclear whether the inferred selection occurred during development of hybrid individuals, or whether it occurs later in life. Our data suggests there is an excess of intermediate individuals, implying that hybrids develop normally, but that there is a reduction in these individuals during their first year. This is in contrast to another well-studied avian hybrid zone, between Pied and Collared flycatchers, where intrinsic incompatibilities are thought to contribute to much of the fitness reduction in hybrids. In this case, interspecific hybrids develop at a much lower rate and have very low fertility as compared to pairings between pure types and are at a much lower frequency in the population (Qvarnstrom et al. 2010; Saetre and Saetre 2010). More generally, while a number of studies of small songbirds suggest that there is high mortality during their first year of life, this is primarily due to predation and starvation (e.g. Sullivan 1989) and these types of selection are not necessarily expected to affect certain genetic classes over others, although this has not been tested. The factors that might be acting as a potential selective agent against Audubon’s / myrtle hybrids clearly deserves further study.

The orientation assays of individuals on migration revealed a number of novel findings. First, we found that individuals were mostly active during 1.5 hour period around sunset. The fact that movement consistently stopped approximately 40 minutes after sunset suggests that yellow-rumped warblers, at least in our study, exhibit behaviours consistent with crepuscular
migratory activity as opposed to nocturnal movements, as has been suggested in a number of sources (Hunt and Flashpohler 1998). More importantly, we found that migratory orientation was directed towards the NNE (26°) regardless of the genotype class of individuals. In their study of migratory willow warblers (Phylloscopus trochilus) in Sweden, Ilieva et al. (2012) found that groups differing in their genetic composition differed significantly in their orientation, in the expected directions based on ringing recovery and isotope data (Bensch et al. 2009). In their case, however, groups of differing genetic constitutions were also sampled in different sites, confounding geography and genetics. Our site in the Audubon’s/myrtle hybrid zone sampled a wide variety of genetic classes, allowing for a robust test of a correlation between genetics and behaviour. In our case, however, we found little predictive power of an individual’s genotype and their migratory orientation.

The observation that, on average, groups of individuals with differing genetic constitutions in the hybrid zone exhibited a similar orientation is consistent with the results from isotope values in feathers sampled from breeding birds: most hybrid zone individuals overlap in the isotopic distribution of myrtle warblers (Toews et al. in review). However, the observation that most of these birds were orienting northwards was a surprising result, given that these warblers are expected to be heading south for the winter months. One possible explanation is that individuals were exhibiting phototaxis, flying towards the light of the setting sun, as has been reported in other orientation studies (Sandberg, 1991; Åkesson, 1994; Ilieva et al. 2012). In this case the sun was in the west, significantly different from the mean angle of the birds tested, thus would not entirely explain results reported here. We suggest another possible explanation: that the majority of the individuals were first moving NE out of the valleys in the Rocky Mountains to later turn south. Indeed, the orientation of the valley near the capture location and orientation experiment area is approximately 23° NNE, very close to (and not significantly different from) the observed orientation of the birds when grouped together. While previous orientation studies have tested the effect of ecological barriers on migratory behaviour (i.e. water bodies; Sandberg and Moore 1996; Ilieva et al. 2012), this is one of the first studies to assay orientation in and around mountainous areas. Using high-resolution radar technology, Williams et al. (2001) found evidence that nocturnal migrants responded to local topological features by changing their orientation during fall migration, especially those birds migrating below 300m, as is assumed with yellow-rumped warblers. Given the type of data these methods collect, however, it is challenging to assign these types of observations to specific species or even species groups (Williams et al. 2001). For the warblers in our study, it may be that individuals have a memory.
of the axis of the valley, which they are recapitulating in the orientation cage. We recommend that future orientation studies should consider including additional orientation localities in valleys of varying direction. It would also be useful to assay individuals each evening over a longer period (i.e. 1-2 weeks) to test whether this orientation is maintained or dissipates with time. This could provide a robust test of the role of topological features in influencing migratory movements and an important confirmation of our results. More generally, our orientation data suggest that tracking studies using recent technology such as radio towers and geolocator tags may be appropriate for studying large-scale migratory movements (Taylor et al. 2011; Delmore et al. 2012; Veen 2013).

It may also be, given the limited power of our genetic markers, that majority of the individuals we sampled were multi-generation hybrids and that the NNE orientation behaviour is in fact maintained throughout the migratory period and highly deleterious for these birds. Northwestern migration has been observed in German blackcap warblers, raised in the laboratory setting, that were sampled from the contact zone between SE and SW migrants (Helbig 1991; Mettler et al. 2012; Rolshausen et al. 2013). In this case, however, the authors suggest that this behaviour may be adaptive, in that a new (more northerly) over-wintering habitat recently became available and colonized. A similar situation is very unlikely for hybrid myrtle and Audubon’s warblers in North America and it is more likely that, if such an orientation is maintained, that the behaviour would be maladaptive for hybrids. While speculative, it may be that it such an inverted migratory directionality in wild hybrids is more common that currently appreciated. Such questions highlight the need for additional studies of this and other taxa with possible orientation divides (Rohwer and Irwin 2011).

Taken together, we suggest that data from isotopes and the orientation assays are consistent with the migratory behavior of birds in the hybrid zone being influenced by both the local landscape topology and, to a lesser degree, genetics. The isotopic patterns from birds breeding in Kananaskis suggest that most of these individuals winter in the far southeastern U.S. or Caribbean, regardless of their genotype. It seems that if individuals were moving towards the wintering sites along the Pacific Coast that they would show a SW orientation. This is primarily because there are a number of less-circuitous routes via valleys that warblers could travel through towards the interior of British Columbia and Washington. Based on isotopic data, we would predict that the birds near Yard Creek and some of those sampled near Golden, B.C. would show a SW orientation pattern. This is because these are the only sites where the isotopic values were more suggestive of a western overwintering location (Toews et al. in press). Indeed,
the observation that individuals near Golden have much variation in isotopic values and genetic hybrid index, suggests it would be an ideal site to study fall migratory orientation in the future. For example, is there a correlation between an individual’s genotype and orientation, or are birds also responding to more local landscape features, as we suggest for our data from birds sampled near Kananaskis?

In conclusion, we found no evidence of a correlation between an individual’s genetic background and their fall migratory orientation, at least in birds near the hybrid zone. Combined with inferences from isotopes about wintering locations, we suggest that these data imply that at least these facets of migratory behaviour (i.e. orientation and over-wintering location) are unlikely to have a strong link with selection against hybrids. In contrast, our comparison of the genetic composition between breeding birds versus birds on migration illustrates an excess of genetically intermediate individuals, at least during their first year of life. This presents the exciting possibility that hybrids develop properly, but have phenotypes that have negative fitness consequences during their first year of life. Confirming this observation, in addition to identifying the potential selective agents, will be important for understanding the potential mechanisms of post-mating reproductive isolation in this hybrid zone and in birds more generally.
Figure 4.1  Distribution and band recoveries of Audubon’s and myrtle warblers. (A) Banding data obtained from Brewer et al. (2006) and the Canadian Bird Banding Office (2013). (B) Sites of capture for migratory yellow-rumped warblers with the site of the orientation assays (C).
Figure 4.2  Holding and orientation cages as modified from Fitzgerald and Taylor (2008).

(A) Orientation cage with tarp sheild

(B) Orientation cage without tarp sheild

(C) Measurements

- 10.5” (diameter)
- 7” (plastic hoop)
- 21” (7/16” doweling)
- 23” (square)
- 31”
Figure 4.3  Example of the radR interface and the perch exclusion zone. The image shows two frames from a video taken from the bottom center of the cage. The exclusion zone is the area within the circular perch.

(A) Bird identified by radR during flight; centroid XY point recorded

(B) Bird identified by radR within perch exclusion zone; XY point not recorded
Figure 4.4  Allele frequency and sex-ratio fluctuations over the fall migratory period for the three nuclear genetic markers. (A) Allele frequency is relative to the myrtle allele (i.e. 0 – all Audubon’s alleles; 1 – all myrtle alleles). (B) Proportion of daily sample that were male, as determined by molecular sexing.
Figure 4.5  Genetic classes across various hybrid zone transects. (A) Proportion of total sample assigned to one of nine genetic hybrid classes from various transects in the Audubon’s/myrtle hybrid zone. Samples from the present study (birds on fall migration) are found to the far right. (B) The same data, but only for birds sampled in Kananaskis and distinguished as on migration (filled circles) or breeding (open circles; note that these points have been shifted slightly to avoid overlap). Error bars show Agresti-Coull 95% confidence intervals. (C) The difference between the proportions of migrating and breeding birds in Kananaskis.
Figure 4.6  Activity of birds over the evening during the orientation trials. Each point is the number of objects identified by radR over a 5-minute time period with the time relative to sunset. The grey points are the raw data from all individuals. The connected, filled circles are the averaged points over a 20-minute window. Peak activity occurs approximately 20 minutes before sunset.
Figure 4.7  Mean orientation of all individuals. (A) grouped together and (B-F) as distinguished by genetic hybrid index.
CHAPTER 5 – The biogeography of mitochondrial and nuclear discordance in animals

5.1 Introduction

It is now commonplace for studies of molecular biogeography to employ a diverse suite of genetic markers, including loci both in the mitochondrial genome (mtDNA) and throughout the nuclear genome (nuDNA). This variety of genetic information is, in many cases, now complemented with broad taxon sampling encompassing a large geographic scope. In most studies that employ a diverse array of genetic markers and a robust sampling effort, the patterns observed between different genetic marker types generally align (Avise 1994). This is true for comparisons between species as well as phylogeographic structure that arises within species – the localities that harbor deep splits between mtDNA clades also have corresponding differences in the nuclear genome (Zink and Barrowclough, 2008). This observation is one reason the “barcoding of life” project has proved successful: clades identified in mtDNA are generally concordant with other phenotypic and genetic information (e.g. 94% of taxonomic bird species in North America have concordant mtDNA clusters; Kerr et al. 2007). However, concordant patterns between mtDNA and nuclear DNA are not always observed (Funk and Omland 2003; Chan and Levin 2005). In fact, the number of studies that report discordant patterns between mtDNA and nuclear markers, while not large, is increasing, especially within the last decade, as more researchers have been able to use both types of markers in combination.

Discordance between mtDNA and nuDNA can be most broadly defined as a significant difference in the patterns of differentiation between these two marker types. Most commonly these conflicts can be in the overall amount of differentiation or in how these markers reconstruct relationships among groups. This type of discordance is expected because the mitochondrial genome is haploid and uniparentally inherited in most animals (but see Hoeh et al. 1991) and therefore has a fourfold smaller effective population size (Hudson and Turelli 2003; Zink and Barrowclough 2008). This means that mtDNA will complete the process of lineage sorting, where ancestral polymorphisms are lost over time, faster than nuDNA, as this rate is inversely proportional to the effective population size (Funk and Omland 2003). While the inheritance properties of mtDNA make it more likely than any single nuclear marker to accurately reflect recent divergence (Zink and Barrowclough 2008), studies that rely solely on mtDNA to infer phylogenetic relationships risk generating gene trees that do not represent the true relationships

3 A version of this chapter has been published: Toews D. P. L. and A. Brelsford. 2012. The biogeography of mitochondrial and nuclear discordance in animals. Molecular Ecology 16: 3907-30.
amongst taxa (Edwards and Bensch 2009). The prevalence of incomplete lineage sorting in contributing to discordant patterns between mtDNA and nuDNA has been discussed extensively (Funk and Omland 2003; Zink and Barrowclough 2008; McKay and Zink 2010) and the primary resolution is that, where feasible, researchers should include multiple independent loci to generate robust phylogenetic relationships (Edwards and Bensch 2009).

Even if numerous nuclear loci are employed, mito-nuclear discordance can also arise if there are differences in how selection acts on the mitochondrial genome as compared to the nuclear genome or if there is biased movement of either marker type driven by demographic asymmetries, such as sex-biased dispersal (Rheindt and Edwards 2011). For instance, 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 (Bazin et al. 2006; Meiklejohn et al. 2007; Edwards 2009; Ballard and Melvin 2010; Scott et al. 2011). If selection for mtDNA variants varies geographically, then discordant patterns between mtDNA and nuDNA can arise (Irwin 2012). In addition to differences in the adaptive landscapes for nuDNA and mtDNA, demographic asymmetries can also create discordant patterns and distributions of these different marker types. For instance, female-biased dispersal or disparities in range size or abundance between hybridizing groups can promote the dispersal of mtDNA in the absence of concordant movement of nuDNA (Funk and Omland 2003).

Distinguishing between incomplete lineage sorting and these other types of discordance can be difficult (McKay and Zink 2010). One important distinction, however, is that discordance that arises from incomplete lineage sorting is not expected to leave any predictable biogeographic pattern (Figure 5.1; Funk and Omland 2003). Therefore, in cases where there are strong geographic inconsistencies between patterns in mtDNA and nuDNA, incomplete lineage sorting can usually be ruled out. This type of discordance, referred to more generally as biogeographic discordance, can result from clines in mtDNA being displaced from nuclear DNA in both their location and/or their width (Figure 5.2). Biogeographic discordance can be extensive, such as the complete replacement of mtDNA of one species by another (i.e. ‘mitochondrial capture’), or more limited, where mtDNA haplotypes show a higher frequency in a given population than would be expected from nuDNA markers.

Two general situations can lead to biogeographic discordance between mtDNA and nuDNA: following isolation and hybridization or in situ (i.e. secondary versus primary contact). Most of the taxa that display patterns of biogeographic mito-nuclear discordance are groups that
were isolated for long periods of time and are either currently in secondary contact or have experienced range contact at some point in their past. During this period of isolation, it is assumed that divergent groups accumulated mutations in both their mitochondrial and nuclear genomes, which increased to high frequency via selection, drift or some combination of the two (i.e. “genetic draft”; Hudson and Turelli 2003). Upon secondary contact these groups formed hybrid zones, interbreeding to varying extents, and mtDNA-nuDNA discordance was promoted by divergent patterns of gene flow between the two genomes.

It has also been suggested that mito-nuclear discordance can arise in the absence of geographic isolation, where mitochondrial types show strong frequency differences between localities that potentially arose in the face of gene flow (Irwin 2002; Ribeiro et al. 2011). In these cases, patterns in the nuclear genome, combined with the biogeographic history of the taxa, suggests a narrow mtDNA divide that may not be the product of geographic isolation followed by secondary contact. This pattern is consistent with a scenario where selection favors one mitochondrial variant over another in a given area; in some cases, these differences may be associated with important environmental characteristics (Cheviron and Brumfield 2009; Irwin 2012).

In many cases discordant biogeographic patterns have been used to infer the potential drivers of discordance. For situations where the mtDNA of one taxon shows complete fixation in another or where a mtDNA cline center is displaced and/or wider as compared to nuDNA, a number of processes have been inferred: 1) adaptive introgression of mtDNA; 2) demographic disparities; 3) sex-biased asymmetries; 4) hybrid zone movement; 5) Wolbachia infection; and 6) human introductions. Adaptive processes can create discordance if selection favors mutually beneficial mitochondrial variants and promotes introgression upon secondary contact. Demographic disparities can generate discordance if there are large differences in population or range size between two taxa, especially if there is the potential for very small population sizes to influence mtDNA frequency by sampling effects (i.e. genetic drift), promoting asymmetric introgression (i.e. Currat et al. 2008). A subset of more general demographic differences, systems with female-biased dispersal propensity, behavioural differences in mating likelihood, and differential production of offspring can promote mtDNA introgression due to its matrilineal inheritance. Hybrid zone movement can also create discordance when the majority of nuclear markers (in addition to phenotypic traits) shift their geographic location, leaving a wake of mtDNA behind (Rohwer et al. 2001). In insects, Wolbachia infection is a potentially important driver of discordance, where mating incompatibilities can arise between individuals with and
without this cytoplasmic endosymbiotic parasite and mtDNA hitchhikes (i.e. infected males mated with uninfected female are incompatible, whereas infected females mate with uninfected males suffer less fitness loss; Jiggins 2003). It has also been recognized that human actions can facilitate secondary contact and generate some of the demographic asymmetries outlined above by moving individuals (i.e. Perry et al. 2001) or by facilitating interaction via habitat alteration, potentially generating discordance.

Biogeographic discordance can also occur if secondary contact and hybridization generates more structuring in mtDNA and/or narrower geographic clines as compared to nuDNA. These are likely produced by either nuclear introgression and/or sex-biased asymmetries. Sex-biased asymmetries in this context can be driven by male-biased dispersal, mating behavior or sex-biased offspring production. This latter scenario (sex-biased offspring production) has been the focus of theoretical and empirical investigations as it is a specific prediction of “Haldane’s rule” (reviewed in Coyne and Orr 2004). This theory posits that, following secondary contact and interbreeding between divergent taxa, if one sex suffers a fitness loss it will more often be the heterogametic sex. It follows that in those systems that where females are the heterogametic sex (i.e. ZW systems), as in Aves and Lepidoptera, mtDNA will be less likely to introgress between divergent groups as compared to other taxonomic groups with XY sex determination (such as mammals), and subsequently mtDNA will have a narrow cline (Coyne and Orr 2004).

Given the increase in interest and the availability of molecular markers, biogeographic patterns of mito-nuclear discordance are being identified more readily in many systems (Edwards and Bensch 2009). However, in most cases the processes driving such patterns are still unknown. Here we attempt to address some of these knowledge gaps by reviewing recent progress in our understanding of mito-nuclear discordance in animal taxa. Our goals in this synthesis are to: 1) review known cases of biogeographic mito-nuclear discordance in animal systems 2) to summarize the geographic patterns in each instance and 3) to identify common drivers of discordance in various groups. Our treatment differs from previous articles in both scope and inclusiveness, as the primary criterion for our survey is only that the systems display a strong biogeographic signal of mito-nuclear discordance (see Box 1 for a discussion of previous treatments of mito-nuclear discordance). We focus on biogeographic discordance because these cases are much more likely to be associated with other complementary historical, biological and ecological information that can be used to reveal the underlying processes driving discordance.
5.2 Methods

Our survey consisted of two approaches. First, we collected all papers where authors explicitly reported mito-nuclear or cyto-nuclear discordance in animals. We used discordance that has occurred between divergent genera, species, subspecies and, in some cases, distinct genetic clusters within a taxon. We restricted our survey to animals to maintain reasonable bounds on the extent of our review and also because the mitochondrial genome can behave very differently in other taxonomic groups, such as plants (Galtier 2011). Second, to provide a more objective metric for the prevalence of biogeographic mito-nuclear discordance, we searched 100 randomly chosen studies published in *Molecular Ecology* for articles referring to mitochondrial markers and microsatellites, AFLPs or nuclear introns. For those studies that were relevant (i.e. empirical, in animal systems, not asexual hybrids and presented both marker types) and had sufficient sampling to identify potential discordance, we looked for congruence between the geographic patterns of mtDNA versus nuDNA makers. We did this to evaluate the extent of mito-nuclear discordance in the absence of an author’s explicit discussion of discordance.

Biogeographic mito-nuclear discordance is best illustrated by studies that report geographic clines for mtDNA along with multiple nuclear markers and other phenotypic traits (Figure 5.2). A number of studies do not report these clinal data, so for some cases in our literature survey we identified mtDNA-nuDNA discordance from maps, figures and supporting text. From these data we extracted two important characteristics for our synthesis: first, we estimated the geographic extent of mitochondrial and nuclear discordance; and second, we determined the average frequency of mtDNA haplotypes throughout the area of discordance. In the case of secondary contact, the geographic extent of discordance is defined as the percentage of the range for which the mtDNA of taxon 1 is observed within the range of taxon 2 (relative to the overall range of taxon 2) as defined by nuclear markers or, in a small number of cases, phenotypic characteristics (Figure 5.2). The frequency of mtDNA throughout the area of discordance is quantified as the average haplotype frequency of the foreign mtDNA in the region between the contact zone inferred from nuDNA and where the native mtDNA haplotype becomes fixed (in the cases where introgressed mtDNA is not at complete fixation; Figure 5.2).

There are two points that should be noted in defining these groups based primarily on differences in the nuclear genome. First, most studies use only a small number of nuclear loci (i.e. <10) to assay the nuclear genome, especially studies employing intron sequencing or allozyme variation, and therefore may not be representative of the rest of the genome. Secondly, nuclear markers can be discordant amongst themselves, as a result of drift or of different patterns of
dispersal, selection or demography. Only in cases where mtDNA was a clear outlier to the general pattern of other nuclear markers did we include it in our survey. Quantifying this type of discordance amongst nuclear markers was beyond the scope of the review, however given the number of studies now employing numerous loci in a robust geographic framework there is a need for future syntheses to quantify these patterns.

We used three bins to categorize both the geographic extent of discordance and the frequency of discordant haplotypes: <50%, 50-95%, and >95%. These large bins allowed us to reliably quantify data reproduced in different forms in various papers. We use these biogeographic patterns to gain insight into some of the factors that may be promoting mito-nuclear discordance. In the case of mtDNA introgression following secondary contact, the extent and frequency of discordant haplotypes can indicate the relative importance of selection or neutral processes in driving discordant patterns (Rheindt and Edwards 2011). For instance, it is less likely that neutral genetic drift would explain the distribution of the mtDNA of taxon 1 at near fixation (>95% frequency) across more than half of the range of taxon 2, unless historical bottlenecks during or following introgression were frequent.

We supplement this coarse quantitative evaluation of inferred drivers of discordance with information provided by the authors in the text of each study. For the systems where mito-nuclear discordance is identified by less structuring of mtDNA and/or wider geographic clines compared to nuDNA, or where mtDNA and nuDNA were both structured but the taxon boundaries differed between marker types, we classified each study (based on the interpretations of the authors of each study) as having evidence that supports one or more of the following scenarios (discussed in detail above): 1) selective introgression of mtDNA; 2) demographic disparities (including genetic drift); 3) sex-biased asymmetries; 4) hybrid zone movement; 5) Wolbachia infection; and 6) human introductions. Cases in which mtDNA was more structured and/or had narrower geographic clines compared to nuDNA were classified as being driven by either 1) nuclear introgression and/or 2) sex-biased dispersal, mating or offspring production (discussed in detail above). Genetic drift is ubiquitous in finite populations, and can interact with many of the aforementioned processes to increase discordance between mtDNA and nuDNA.

While drift alone can also produce geographically discordant patterns (e.g Petit and Excoffier 2009), authors of studies documenting mito-nuclear discordance typically propose other explanations. When authors did cite genetic drift as a likely explanation there was usually complimentary evidence of historical bottlenecks producing small populations sizes and therefore we included these cases under the broad category of ‘demographic disparities’.
We excluded studies based on a number of criteria, especially in cases where discordance was suspected but was based on limited sampling. We also excluded studies where mtDNA-nuDNA discordance was generated by asexual offspring produced by sexual parental species. In this case discordance is brought about when the female parental species always contributes its mtDNA to the asexual hybrid offspring (reviewed in Avise 1994). Dealing with potential cases of incomplete lineage sorting was more difficult. For instance, sharing of similar mtDNA haplotypes across the range of two taxa (i.e. >95% geographic extent and >95% haplotype frequency) can be the result of introgression and complete fixation of a foreign mtDNA or the result of incomplete lineage sorting. We excluded situations where morphology or traditional taxonomy was used to infer this type discordance. In fact, only in a few cases where phenotypic data showed a strong biogeographic signal, such as mtDNA discordance with morphology across a hybrid zone, were phenotypic characters included in our survey as proxy for patterns in the nuclear genome. This is because phenotypic convergence can create the impression of discordance where this is most likely the result of imperfect taxonomy (Funk and Omland 2003). We included cases where there are strong differences in nuDNA markers but much less differentiation in mtDNA if there was additional information supporting the discordance, such as evidence for current or past potential for hybridization. This is because it is expected that the mitochondrial genome will show either greater or comparable levels of divergence and structuring compared to markers in the nuclear genome (Zink and Barrowclough 2008). In these cases significantly less mtDNA divergence compared to nuclear differentiation can implicate mitochondrial introgression between taxa (e.g. Cathey et al. 1998, Bachtrog et al. 2006; Irwin et al. 2009).

However, given the ability for high-resolution nuclear genetic markers such as AFLPs, microsatellites and full genome sequencing to detect very subtle and potentially very recent reductions in gene flow, it is becoming increasingly difficult to determine whether mtDNA homogeneity between groups divergent in nuclear DNA is a result of hybridization or of the high power of multilocus nuclear data sets to detect differentiation (Edwards and Bensch 2009). These types of discordant patterns, where there is low but detectable amounts of nuDNA differentiation, were not included in our survey because incomplete lineage sorting of mtDNA could not be dismissed, even though many of these had excellent taxonomic and genetic sampling. Several examples of such discordant patterns can be found between sympatric colour morphs of the rockfish _Sebastes inermis_ (Kai et al. 2002), in different host races of the leaf miner _Phytomyza glabricola_ (Scheffer and Hawthorne 2007) and the grasshopper _Hesperotettix viridis_
(Apple et al. 2010), between two subspecies of willow warbler *Phylloscopus trochilus* in Sweden (Bensch et al. 2009), and between various populations of the dispersal limited newt *Calotriton asper* (Milá et al. 2009).

**5.3 Results and discussion:**

**5.3.1 Prevalence of mito-nuclear discordance**

The initial studies that identified mito-nuclear discordance were in systems where early genetic tools were more developed compared to other taxa: mitochondrial introgression between two species of fruit fly (*Drosophilia pseudoobscura* and *D. persimilis*; Powell 1983) and between two subspecies of the house mouse (*Mus domesticus* and *M. musculus* Ferris et al. 1983). Following these early discoveries, the number of cases slowly increased until, around 2001, methods for assaying numerous individuals for their nuclear genotype became more widely available to researchers and, subsequently, the number of cases increased dramatically (Figure 5.3). For instance, between 2001 and 2011 on average over eight studies have been published per year documenting mito-nuclear discordance in animals.

In total we identified 126 cases in animal systems where there is strong evidence of discordance between the biogeographic patterns identified in mitochondrial DNA and those observed in the nuclear genome. The majority of cases (97%) are those where discordance likely arose following geographic isolation and secondary contact (Appendix 4). Of those, 109 (89%) found evidence that mtDNA was an outlier, showing little affinity to boundaries identified by nuclear DNA. In the thirteen remaining cases, mtDNA showed a general geographic concordance with the nuclear genome, but had a narrower cline and/or more geographic structure (Appendix 5). In seven studies there is evidence for extensive introgression of mtDNA between numerous pairs of taxa within a single species complex (see Box 2). Interestingly, in four systems there is evidence of strong mtDNA structuring that has likely arisen *in situ* in the absence of geographic isolation and maintained in the face of nuclear gene flow (see Box 3).

Our random survey of 100 articles in *Molecular Ecology* found 75 relevant studies and 61 of those that included both mitochondrial and multi-locus nuclear data that were presented in a way that would show major discrepancies between the marker types. Of the 61 systems, 11 had discordant biogeographic patterns between mitochondrial and nuclear DNA (18%). While in all cases the authors noted discordance, most mentioned it in passing and did not make it a focus of the paper. While this survey method does not avoid publication bias, it does try to control for author bias, and the finding of 18% of studies employing both marker types identifying mito-
nuclear discordance is interesting. This is a large figure, especially when combined with the number of systems with discordance documented in the last two decades (Appendix 4), the suggestive cases requiring further confirmation and those likely caused by incomplete lineage sorting not included in our survey (e.g. McKay and Zink 2010). This suggests that discordance between the mitochondrial and nuclear genomes is a prevalent and important phenomenon.

In general, the prevalence of discordance was not disproportionate among any taxonomic group, although mammals and fish have a higher frequency of reported discordance compared to other groups (Figure 5.4). This may represent a propensity for these taxonomic groups to exchange mtDNA and/or a publication bias for studies investigating these species. It has been suggested that birds, which have a ZW sex determination system, would be less likely to exchange maternally inherited mtDNA upon secondary contact owing to Haldane’s rule (Chan and Levin 2005). We find limited support for this prediction: while more than half of the cases of narrow mtDNA compared to nuDNA clines were in avian systems (Figure 5.4; Appendix 5), there are also a number of cases showing strong evidence of mtDNA introgression in birds (Appendix 4), a pattern not appreciated in previous treatments (e.g. Petit and Excoffier 2009).

5.3.2 Extent and type of discordance following secondary contact

When populations experience periods of geographic isolation and subsequent secondary contact, patterns of genetic variation in the mtDNA and the nuclear genome can become dissociated. The most common form of mito-nuclear discordance is asymmetric movement of mtDNA, with our survey finding only four papers reporting bidirectional movement of mtDNA (Appendix 4; see also Chan and Levin 2005; Currat et al. 2008). In those cases where there was no directionality in the movement of mtDNA this was because clines in mtDNA were wider than nuDNA (e.g. Tamiasciurus squirrels; Chavez et al. 2011). Some authors suggest that this pattern may be due to the fact that, in those cases, mtDNA harbors fewer genetic incompatibilities as compared to the nuclear genome and thus there are fewer barriers to introgression (Colliard et al. 2010).

However, in the majority of studies, sampling suggests that asymmetric discordance does not extend far beyond the current area of sympatry – most studies found that foreign mtDNA did not extend beyond 50% of the range of the native taxon (Figure 5.5). For example, in a hybrid zone between the Guatemalan black howler monkey, Alouatta pigra, and the Mantled howler, A. palliata, hybrids are rare, but the majority have A. pigra mtDNA (Cortes-Ortiz et al. 2007). In
this case, the authors suggest that this is caused by a combination of biased mating and post-
zygotic selection on offspring, where hybrids between female A. palliata and male A. pigra do
not develop. In some cases where human-mediated introgression has been implicated,
discordance also does not appear to be extensive. For instance, in lakes where rainbow trout
(Oncorhynchus mykiss) have been introduced and allowed to hybridize with native cutthroat
tROUT (O. clarkii), mtDNA shows a bias towards O. mykiss haplotypes, beyond what would be
expected based on nuclear genotypes, but not to complete fixation (Metcalf et al. 2008).

In those cases where foreign mtDNA haplotypes are found deep within the range of a
second taxon, they are likely to be at a high frequency (Figure 5.5). For instance, we did not find
any studies that identified a foreign mtDNA present at <50% frequency throughout the entire
range of another taxon. In those cases where low frequency mtDNA haplotypes (i.e. 0-50%) did
extend over a large area (50-95% geographic extent) it was inferred to be caused either by hybrid
zone movement (e.g. phenotypic Townsend’s warblers, Setophaga townsendii, moving into the
range of hermit warblers, S. occidentalis; Kroby and Rohwer, 2009), large asymmetries in range
size (e.g. introgression of the Woodhouse toad’s mtDNA, Bufo woodhousii, into the Arizona
toad, B. microscaphus, which has a nested range within B. woodhousii; Malmos et al. 2001), or
differences in population sizes (e.g. the Russian sturgeon, Acipenser gueldenstaedtii,
introgressing mtDNA into the critically endangered Adriatic sturgeon, A. naccarii; Ludwig et al.
2003). In many cases authors could neither confirm nor rule out demographic explanations for
observed discordance, where mtDNA introgression occurred because of variation in range sizes
or preceding a population bottleneck and range expansion, however most offered more specific
explanations, such as adaptive introgression or sex-biased asymmetries.

Most studies identifying a large geographic extent of discordance found that, throughout
the area of discordance, foreign mtDNA was either at or near fixation, completely replacing the
mtDNA of the native taxon. In many of these cases authors ascribed this to a hypothesized
selective advantage of the introgressed mitochondrial type (Figure 5.6a). For example, the
replacement of the northern redbelly dace’s (Phoxinus eos) mtDNA with that of the finescale
dace (P. neogaeus) across most of its range is thought to be promoted by P. neogaeus, with a
more northerly distribution, having a mitochondria being better adapted to cold temperatures
(Mee et al. 2012). The finding of myrtle warbler (Setophaga coronata) mtDNA throughout the
majority of the range of the Audubon’s warbler (S. auduboni), with considerably less nuclear
introgression, also suggests the potential for adaptive introgression, which authors suggest may
be correlated to a shift in migratory strategy (Brelsford et al. 2011; Milá et al. 2011; Toews et al.
The repeated waves of introgression of common collared lizard (*Crotaphytus collaris*) mtDNA into populations of the Great Basin collared lizard (*C. bicinctores*) is a pattern that would not be expected under a neutral scenario and, as the authors suggest, is consistent with *C. collaris* mtDNA having a selective advantage (McGuire *et al.* 2007). In one of the few studies to test for the selective advantage of mtDNA, Aubert and Solignac (1990) found that experimental laboratory conditions favoured the *Drosophila simulans* mtDNA over *D. mauritiana* haplotypes, consistent with the pattern of introgression documented in natural populations.

In contrast to adaptive introgression, sex-biased mating or offspring production (the most common explanation for discordance; Figure 5.7), while in some cases is implicated in large-scale discordances, is more often associated with cases of less extensive discrepancies (Figure 5.6b). For example, a classic study by Lamb and Avise (1986) found that the mtDNA of the barking tree frog, *Hyla gratiosa*, was disproportionately found in hybrids with the American green tree frog, *H. cinerea*. This was supported by behavioural observations, which suggested that *H. cinerea* males were more likely to act as satellites and tended to intercept *H. gratiosa* females, facilitating the movement of mtDNA from the latter to the former. Recently, Ng and Glor (2011) identified strong discordance between Bayesian clustering assignments from nuclear markers, mtDNA haplotypes and dewlap colouration between two subspecies of *Anolis* lizards in the Caribbean. In one of their transects, they found the mtDNA of *Anolis distichus ravitergum* throughout populations that were otherwise *A. d. ignigularis* in their nuclear genome, which they attributed to evidence of asymmetric mating and/or survival of offspring (Ng and Glor 2011).

### 5.3.3 Untangling processes driving discordance from biogeographic patterns

One difficulty in interpreting the differences in the extent and frequency between sex-biased gene flow versus adaptive introgression is that, in many cases, authors base their interpretation of the drivers of discordance on biogeographic patterns themselves. Indeed, the fact that many authors presented more than one explanation for discordant patterns in a given system (i.e. areas of overlap in Figure 5.7) suggests that more data is required. Ideally, experimental manipulations or other types of complementary data could elucidate the evolutionary processes generating discordant genetic patterns. For instance, sex-biased dispersal, mating and offspring production is a common explanation for mito-nuclear discordance, but behavioral and crossing data have only been collected for a handful of systems. For example, in a genetic survey of the dark-spotted frog (*Rana nigromaculata*), Liu *et al.* (2010) found that many populations are fixed for the eastern golden frog’s (*R. plancyi*) mtDNA. Data combined
from the field and lab suggest that all F1 males were sterile, but that females were partially fertile, consistent with Haldane’s rule and with patterns of mito-nuclear discordance in natural populations (Liu et al. 2010). Unfortunately, these data are difficult to collect in many systems, and researchers instead rely on more general patterns. For example, many authors have suggested that sex-biased dispersal may explain the lower propensity of mtDNA to introgress between species of birds as compared to species of mammals, which commonly have female-biased and male-biased dispersal, respectively (Greenwood 1980). However, assuming that these dispersal patterns are largely conserved within these groups, why does asymmetric discordance between closely related species seem so common, as our survey suggests? These observations suggest either that the dispersal characteristics between closely related species are more labile than has been previously appreciated or that other processes are driving discordant patterns (i.e. the invasion and introgression hypothesis as outlined by Petit and Excoffier 2009).

Explanations invoking adaptive introgression between related species present another paradox: the conservation of mitochondrial genes, the interplay between mitochondrial and nuclear gene products, and the central role of the electron transport chain in oxidative phosphorylation and basic metabolic functioning suggest that 1) functional mutations would only rarely have a selective advantage and 2) introgression between taxa with divergent mitochondrial clades would be unlikely. Our survey, in addition to previous work (Ballard and Whitlock 2004; Ballard and Melvin 2010), suggests that selection on the mitochondrial genome may commonly drive introgression, although few studies have tested for it. Surprisingly, of the few studies to assay potential functional differences between mitochondrial types that could influence introgression, most have found only limited support (e.g. Salvelinus by Blier et al. 2006; Myodes by Boratynski et al. 2011). For example, testing for differences in basal metabolic rate between sympatric individuals of the bank vole (Myodes glareolus), some with northern red-backed vole (M. rutilus) mitochondria, Boratynski et al. (2011) found that mitochondrial type explained very little variation in basal metabolic rate. Blier et al. (2006) also found little evidence to suggest that functional differences in enzyme activity could explain the introgression of Arctic charr (Salvelinus alpinus) mtDNA into brook charr (S. fontinalis). Combining these types of approach with more sensitive techniques assaying mitochondrial function directly, such as respiration in isolated mitochondrial preparations (Scott et al. 2009), will be valuable in determining if introgressed mitochondria differ phenotypically from the native type and, ideally, how this might affect whole-animal fitness (reviewed by Dalziel et al. 2009 and Ballard and Melvin 2010). In this way cases of mitochondrial introgression could potentially be used to link the effects of
specific mtDNA mutations in introgressed genetic variants to phenotypic differences (Dalziel et al. 2009; Scott et al. 2011).

\subsection{Conclusions and future research}

Our survey extends previous reviews of mito-nuclear discordance by focusing on the biogeographic differences that can be recovered between these divergent genomes. The prevalence of this type of discordance confirms that this is a prevalent and important phenomenon shaping genetic variation in natural populations. While some have lamented the fact that such processes may muddy the phylogenetic waters, it is clear that most authors now recognize the potential pitfalls from inferring relationships from only a small portion of the genome (Edwards and Bensch 2009). We suggest the next generation of multilocus studies should focus on the drivers of discordance rather than simply documenting discordance in and of itself. While improved sequencing technologies will greatly aid in identifying mito-nuclear and nuclear-nuclear discordances, testing various alternative explanations (including a null model of simple genetic drift) for observed patterns will be one of the biggest challenges. Gathering phenotypic and environmental data from natural populations will be a first step, with subsequent experimental and genetic crosses ideal where feasible. Therefore, these natural systems provide a number of unique research opportunities, which include quantifying the relative importance of introgression from independent evolutionary lineages in providing genetic variation for adaptive evolution, linking genotype to phenotype in introgressed populations and individuals and, more generally, addressing fundamental questions about how natural selection and demographics act and interact in nature.
BOX 1 – Previous treatments of mitochondrial discordance and introgression

Discordance between mtDNA, nuDNA and other taxonomic characters has been the focus of a number of previous reviews and discussions. In an early synthesis, Avise (1994) highlighted a number of systems that were among the first to report strong discordance between nuDNA markers and mtDNA, owing to hybridization and introgression. Funk and Omland (2003) extended this in a more general review by attempting to quantify the prevalence, causes and consequences of genealogical polyphyly at the species level. Patterns of biogeographic discordance and asymmetric introgression of mtDNA were considered along with a number of other processes contributing to discordant phylogenetic patterns, such as imperfect taxonomy, inadequate phylogenetic information and incomplete lineage sorting (Funk and Omland 2003).

McKay and Zink (2010) further explored Funk and Omland’s (2003) analysis by trying to distinguish, in each case of polyphyly in avian systems, whether imperfect taxonomy, incomplete lineage sorting, or gene flow was responsible for the discordance. While some authors suggest that taxonomy may not be the ideal guide for studies of mtDNA paraphyly (i.e. Rheindt and Edwards 2011), McKay and Zink (2010) reported that approximately 14% of 856 avian species examined showed evidence of paraphyly and that imperfect taxonomy was the most prevalent explanation for the discordances (55.7% of cases) as compared incomplete lineage sorting (15.6%) or introgression (5.7%), although in many cases they suggest that the latter two processes could not be distinguished (21.3%; McKay and Zink 2010).

By more specifically examining the correspondence between estimates of divergence in mtDNA and nuDNA in avian systems, Zink and Barrowclough (2008) suggested that, in most cases, there was little conflict between the estimates obtained from nuDNA and mtDNA. For instance, in most of the cases they reported that mtDNA divergence was greater than estimates from nuDNA, as would be expected from the differences in effective population size. This result was interpreted as an affirmation of mtDNA, “under siege” in phylogeography, to recover robust relationships (Zink and Barrowclough 2008). In their reply, Edwards and Bensch (2009) suggest that this conclusion may be true in many cases, but argue that there is much value at little cost if mtDNA data is supplemented by additional loci in the nuclear genome.

Models of mito-nuclear discordance have been derived analytically (Chan and Levin 2005) and from simulations (Currat et al. 2008). Chan and Levin (2005) modeled the propensity for incomplete pre-mating barriers (i.e. strong mating preference) to promote mtDNA introgression between taxa as compared to post-mating barriers (i.e. strong selection against hybrids). They found that post-mating barriers to reproduction were better at preventing...
introgression, an observation that has generally been borne out in empirical systems (e.g. Lamb and Avise 1986), although in many cases there is little information on the barriers acting in any given system. The simulations performed Curra et al. (2008), which were supplemented by a review of empirical examples by Petit and Excoffier (2009), focused on mtDNA introgression following invasion and hybridization with a related taxon. They found that gene flow was consistently in the direction of the native taxon towards the colonizer and, counter-intuitively, genetic markers with lower intraspecific gene flow were more likely to introgress between taxa (Curra et al. 2008; Petit and Excoffier 2009). They suggest this is because these low-dispersing genetic markers are less likely to be swamped out by gene flow of from other populations of the colonizing taxon. From this result they predicted that gene flow of mtDNA in systems with male-biased dispersal will be higher as compared to those systems with female-biased dispersal (Curra et al. 2008; Petit and Excoffier 2009). Unfortunately in the cases reviewed by Petit and Excoffier (2009) the dispersing sex is confounded with taxonomy (i.e. birds versus mammals), sex-determining system and, combined with limited data on dispersal bias, it is currently difficult to determine how prevalent this process is in natural systems. However, these non-intuitive results were not predicted based on previous surveys of empirical systems, suggesting that such simulation studies present a promising line of inquiry into the drivers of discordance in the future.

BOX 2 – Extensive discordance in various groups

A number of studies evaluating mtDNA and nuclear variation in numerous taxa have reported rampant discordance between marker types, where there is evidence for extensive introgression of mtDNA between numerous pairs of taxa within a single species complex. Such patterns have been reported in: Laupala crickets (Shaw 2002), Neodiprion sawflies (Linnen and Farrell 2007), Bufo Toads (Fontenot et al. 2011), Brienomyrus electric fishes (Sullivan et al. 2004), Ohomopterus carabid beetles (Sota 2002), Lycaeides butterflies (Gompert et al. 2008), and Tibetan megophryid frogs (Chen et al. 2009). Most studies show numerous cases of discordance between phylogenies generated from mtDNA and nuDNA in the absence of specific cases of hybridization (i.e. Shaw 2002). In a particularly well developed case, Linnen and Farrell (2007) report that mitochondrial gene flow was consistently higher than nuclear gene flow across 120 pairwise species comparisons in Neodiprion sawflies. They suggest that shared hosts and/or pheromones facilitate hybridization between species and that differences in abundance between pairs could promote mitochondrial introgression (Linnen and Ferrell 2007).
In another interesting case, Gompert et al. (2008) identified a single mitochondrial haplotype ("h01") that is distributed among various taxonomic groups of Lycaeides butterflies. This pattern is not concordant with other mtDNA clades or nuclear DNA but was positively associated with the presence of the endosymbiotic bacterium Wolbachia. In general, however, it is not clear why these taxonomic groups tend to show a propensity to introgress mtDNA compared to others, aside from incomplete reproductive barriers facilitating hybridization. Indeed, identifying any unifying characteristics across these disparate taxonomic groups displaying extensive discordance will be important for future studies.

**BOX 3 – Mitochondrial structure in the absence of geographic isolation?**

The vast majority of studies that identify mitochondrial DNA structure and/or divergence also have complementary historical and biogeographic evidence that imply divergent mitochondrial haplotypes evolved during periods of geographic isolation (Appendix 4 and 5). Interestingly, four recent studies have identified mtDNA divergence where long periods of allopatry are less likely, all of which are in avian systems. This pattern has been observed between highland and lowland mtDNA clades of the rufous-collared sparrow (Zonotrichia capensis; Cheviron and Brumfield 2009), arid and mesic clades of the karoo scrub-robin (Cercotrichas coryphaeus; Ribeiro et al. 2011), eastern and western clades of the greenish warbler (Phylloscopus trochiloides; Irwin et al. 2005), and between tree-nesting and ground-nesting host parasites of the greater honeyguide (Indicator indicator; Spottiswoode et al. 2011). While empirical examples of such phenomena are rare, simulation studies suggest that such phylogenetic breaks can arise in the absence of barriers to gene flow if dispersal and population sizes are low (Irwin 2002) or if selection favors different mtDNA haplotypes in different environments (Irwin 2012). In three of these four cases the molecular markers used to assay the nuclear genome were microsatellites. It is important to note that due to their complex mutational patterns microsatellites may imply higher gene flow than might otherwise be estimated from other nuclear markers with a simpler mutation mechanisms, such as SNPs (Brito and Edwards 2009). While differences in the biogeographic patterns between microsatellites and mtDNA are not necessarily expected, it would be valuable in the future for some of these cases to be confirmed with additional multi-locus nuclear markers.

Of the four cases, some period of allopatry is possible in the greenish warblers, but is not implied by phenotype or nuclear AFLPs (Irwin et al. 2001). In this case it is thought that an ancestral population, which occurred near India, expanded its range northward as a ‘ring’ around
the Tibetan plateau (Irwin et al. 2005). During this time reproductive isolation evolved between northern ends of eastern and western groups and, in parallel, mtDNA structure around the southern side of the ring arose in the face of nuclear gene flow. The southern break between the two clades currently occurs in the Lāhul Valley, in the Himachal Pradesh province of India, in populations that are otherwise similar in phenotype and in their nuclear genome (Irwin et al. 2005). Future genomic studies employing many more nuclear genetic markers will hopefully be able to determine the role that geographic isolation did or did not play in generating the genetic patterns in this system.

In the karoo scrub-robin, genetic patterns in mtDNA and microsatellites are quite discordant – over its range in southern Africa, the species shows little to no variation in nuclear DNA, but displays a strong east-west genetic break in mitochondrial DNA (Ribeiro et al. 2011). The authors report that mtDNA variation is explained best by environmental data, such as annual precipitation, as opposed to geographic distance. Whether mitochondrial variation can be attributed to adaptation to different climactic regimes, or whether other historical explanations can explain the observed patterns, will require further confirmation.

One of the most striking cases of discordance in the absence of geographic isolation can be found in haplotype variation in the rufous-collared sparrow along an elevational gradient in South America (Cheviron and Brumfield 2009). Cheviron and Brumfield (2009) sampled individuals in populations at varying elevations in addition to populations at similar elevations at varying distances. Surprisingly, they report that the frequency of a mtDNA clade that is primarily found at high elevation, showing more genetic structure than implied by geographic variation in microsatellites along control transects. The authors hypothesize that this high-elevation haplotype may be adaptive for lower temperatures and oxygen levels in these populations, but have not yet tested these predictions.

Finally, a recent study of the greater honeyguide, an avian brood parasite in Africa, demonstrates strong mtDNA structure between individuals with ecologically distinct host species (ground-nesting birds versus tree-nesters) with little to no structure in microsatellites (Spottiswoode et al. 2011). By comparison, other taxa closely related to the greater honeyguide show much more nuclear divergence for the same mtDNA distance. The authors suggest this is due to an ancient switch and subsequent adaptation to a new host species, which is faithfully parasitized by females (a behavior that the authors suggest may be encoded by genes on the W chromosome), but where there is random mating by males between the types, facilitating nuclear
gene flow. The current phylogeographic distribution of the mitochondrial clades suggests that these patterns likely arose in sympatry without a long period of geographic isolation.
Figure 5.1  A scenario illustrating different biogeographic patterns expected under incomplete lineage sorting versus hybridization and introgression. The left panel is consistent with a pattern of incomplete lineage sorting of mtDNA, where two mtDNA clades are distributed between two nuclear clusters (as illustrated with the phylogenetic tree), Taxon A (blue) and B (white), with no discernible geographic pattern in the distribution of mtDNA clades amongst the nuclear groups. This pattern is also consistent with complete introgression of mtDNA that is maintained at low frequency across the range of both taxa. The right panel illustrates a pattern that is consistent with partial introgression of mtDNA from Taxon A into Taxon B (as distinguished by nuDNA markers), where individuals in the range of Taxon B have mtDNA from Taxon A at the range edge.
Figure 5.2  A hypothetical scenario with a range map and geographic clines of alternative means to quantify biogeographic patterns of discordance. The top panel shows the ranges of two taxa, Taxon A (blue) and Taxon B (white), as distinguished by nuclear genetic markers, where the mtDNA of Taxon A can be found within the range of Taxon B (red area); this demonstrates the geographic extent of discordance. The bottom panel depicts this mito-nuclear discordance measured as relative to frequencies of nuclear markers (blue) and mtDNA (red) of Taxon A.
Figure 5.3  The number of papers that report mito-nuclear discordance between 1983 and 2012 (filled bars). The cumulative number of papers (secondary axis) from 1983 is also shown (grey line).
Figure 5.4  The number of papers reporting mito-nuclear discordance following secondary contact distinguished by taxon group. The filled portion of the bars represent in number of cases where mtDNA was less structured than nuDNA. Cases where mtDNA cline was more structured than nuDNA are indicated by the open portion of the bars.
Figure 5.5  The number of cases of mito-nuclear discordance following secondary contact (where mtDNA is less structured than nuDNA) distinguished by the geographic extent of discordance and the frequency of discordance haplotypes. The area of the circles is proportional to the number of cases observed in each bin, displayed in the center of the circle.
Figure 5.6  The number of cases of mito-nuclear discordance following secondary contact (where mtDNA is less structured than nuDNA) distinguished by the geographic extent of discordance and the frequency of discordance haplotypes for those cases that report only (a) adaptive introgression or (b) asymmetric dispersal, mating or offspring production. The area of the circles is proportional to the number of cases observed in each bin, displayed in the center.
Figure 5.7  A Venn diagram illustrating the prevalence of and overlap between different explanations for observed patterns of mito-nuclear discordance. The size of the circle is proportional to the number of cases within each category. It should be emphasized that overlap between the circles illustrates cases where authors offered more than one explanation for the observed discordance and is usually based on speculation as few studies have measured adaptation, dispersal, or mating frequency driving discordant patterns.
Figure 5.8  Examples of systems where mitochondrial structure is inferred to have arisen in the absence of geographic isolation (A) greenish warbler (*Phylloscopus trochiloides*) (B) karoo scrub-robin (*Cercotrichas coryphaeus*) (C) rufous-collared sparrow (*Zonotrichia capensis*) and (D) greater honeyguide (*Indicator indicator*). Photographs reproduced with permission from Darren Irwin (A), Angela Ribeiro (B), James Lowen (C) and Warwick Tarboton (D).
CHAPTER 6: Migration, mitochondria and the yellow-rumped warbler

6.1 Introduction

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, 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). While many authors have noted these 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; three are designated as separate species by the International Ornithological Council but all are considered subspecies of a single species by the American Ornithologists’ Union; Figure 6.1a): 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; *S. a. auduboni*, the Audubon’s warbler, which breeds west of the Rocky mountains and winters in the southwestern U.S. Mexico, and central America; *S. a. nigrifrons*, the black-fronted warbler, which is a resident year-round in Mexico; and finally, *S. goldmani*, the Goldman’s warbler, which consists of a small population of resident birds confined to Guatemala (Hubbard 1970).

Studies of multi-locus 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. a. nigrifrons*) separate these individuals from the rest of the Audubon’s group in the U.S. (i.e. *S. a. 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 Figure 6.1c 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).

While 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 behaviour, from resident birds in the south to migratory birds in the north. While 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 behaviour and variation in mitochondrial phenotype. This ‘migration adapted mitochondrion’ hypothesis posits that in the past, natural selection favoured 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 behaviour, we predicted that the mtDNA transition would also be located at the transition in migratory phenotype. While stochastic processes alone could generate such a
correlation, preliminary studies suggest that no other phenotypic trait (e.g. colour),
environmental factors (e.g. habitat characteristics) or demographic parameters (e.g. population
size) co-vary with mtDNA. To determine the location and shape of the mtDNA cline we
genotyped individuals across two transects (depicted in Figure 6.1b) 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 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 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).
6.2 Methods:

6.2.1 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 U.S. We captured territorial individuals along two broad transects (Figure 6.1b; Table 6.1): 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 the present 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 (see below). Due to logistical constraints, specimens were destroyed following blood and tissue sampling. All animal care and experimentation was conducted according to the University of British Columbia protocol #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 1x PCR buffer (Invitrogen), 1.5 mM MgCl₂ (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 *XbaI* cuts only the C variant. We digested 2 µl of the PCR product with 2 units of the restriction enzyme *XbaI* 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 SYBR Safe (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 (Szymura and Barton 1986). The estimated center and width of a particular genotypic transition (i.e. Toews et al. 2011) can then be compared to those of other traits (i.e. migratory behaviour). 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.

### 6.2.2 Feather isotope analysis

For a subset of birds (*n* = 110) we determined the stable hydrogen value (δ²H) 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. 2011; Hobson et al. 2012), although there is some local variation that likely relates to elevational differences. We sampled feathers from 7-9 randomly chosen individuals from each of the 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 unique feather molting pattern in Audubon’s warblers: in the fall a bird will molt most of its feathers during a pre-basic 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 pre-alternate 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 pre-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 colour-banded yellow-rumped warblers studied over multiple years indicate this is true of them (A. Brelsford, unpublished 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 Appendix 3). Using these regressions, we then estimated wintering location latitude from the isotopic composition of pre-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 behaviour). 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.

In order 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 co-variance (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 behaviour.
6.2.3 mtDNA sequencing

We sequenced mtDNA from six individuals: three of each mtDNA type with two individuals <250km from the center of the mtDNA contact zone and one >400km. 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 #HQ896034) as a reference. We then translated the mtDNA sequences into corresponding amino acid sequences to determine the number of non-synonymous 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 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).

6.2.4 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 (see below). 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 3mm deep into the tissue. Half of the tissue collected was flash frozen in liquid N₂ for enzyme assays while half was used to immediately assess mitochondrial respiration (see below). These individuals were later genotyped as having either a myrtle (n = 9) or black-fronted (n = 15) mito-type using the RFLP method described above.

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 below). 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 (see below) 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 N₂ cooled mortar and pestle. Approximately 100 mg of tissue was quickly weighed and homogenized on ice in 6 volumes of ice-cold homogenization buffer (25 mM K₂HPO₄, 5mM MgCl₂, 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, California) 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 minute incubation period. The assay conditions necessary to measure maximal enzyme activities were as follows (in mM unless otherwise indicated): complex I: 25 K₂HPO₄, pH 7.2, 5 MgCl₂, 2.5 mg/mL bovine serum albumin, 0.1 DCIP, 0.2 NADH, 0.065 Coenzyme Q₂, 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.
6.2.5 Mitochondrial respiration

Muscle samples were immediately placed into an ice-cold relaxing solution (in mM): 20 taurine, 0.5 DTT, 6.56 MgCl$_2$, 50 potassium methane-sulfonate, 20 imidazole, 5.77 Na$_2$ATP, 15 creatine phosphate, 2.77 CaK$_2$EGTA, 7.23 K$_2$EGTA. Muscle fiber bundles were then mechanically separated using tweezers under a dissecting microscope. Bundles were placed into a 2mL centrifuge tube filled with the relaxing solution containing saponin (50 ug/ml) and gently mixed on ice using a rocking plate for 30 minutes. This was followed by three 10 minute 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$_2$PO$_4$, 2.77 CaK$_2$EGTA, 7.23 K$_2$EGTA and 1g 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 2mL 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 minutes 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 5mM 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 5mM of ADP in yellow-rumped warbler muscle tissue. Measurements were taken only after consumption rate had stabilized and we took measurements for 1 – 2 minutes of the trace. We then added 0.5 mM of rotenone, inhibiting complex I, and re-oxygenated 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 antimycin-A, an inhibitor of complex III, and again re-oxygenated 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 one minute) and 1 hour 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.

6.3 Results:

6.3.1 Sampling and cline analysis

Yellow-rumped warblers in the southwestern U.S. are most abundant in an archipelago of high-elevation coniferous forests between 2000 – 3500m 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 213km (Figure 6.2a), which is similar to a previous study of populations along this transect, although slightly narrower (37.17°N and 297km 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, 743km, although it had a similar center at 35.94°N (Figure 6.2a).

6.3.2 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 $\delta^2$H content. We found that while there was large variation in $\delta^2$H 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*(‰ $\delta^2$H)+30.66 best explained variation in latitude with isotope values ($R^2 = 0.61; df = 46,
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 (Figure 6.2b). For 10 individuals the deuterium content of the winter feathers fell outside the range of the summer feathers (-34.7 to -119.7‰ δ²H) by <15‰ δ²H; 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, while the rest of the sites towards 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 – 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 versus migrant) and this is broadly coincident with the mtDNA cline centers from both transects (the triangles in Figure 6.2b). 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 6.2). 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 6.2b.

6.3.3. mtDNA sequencing

Accession numbers for sequences can be found in Appendix 6. 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; Figure 6.3). 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) 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).

### 6.3.4 Mitochondrial enzyme assays and respiration

We found no significant difference in overall maximal enzyme activity ($V_{\text{max}}$) in complex I between individuals with myrtle or black-fronted type mitochondria in the center of the contact zone ($P = 0.179$, df = 21; Figure 6.4a). While complex I had the most fixed AA differences between the mito-types in the mtDNA genes sequenced (see above), 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 (Figure 6.4b), 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 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; Figure 6.5a) and State III respiration rate following ADP addition also did not differ ($P = 0.175$, df = 22; Figure 6.5a). 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; $P = 0.376$, 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 acceptor control ratio (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; Figure 6.5b). 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).
6.4 Discussion:

In an effort to understand the drivers of mitochondrial introgression in the yellow-rumped warbler system we have assembled a diverse dataset that includes genetic, biochemical and phenotypic variation obtained from several natural populations. We sampled individuals in an area in the southwestern U.S. that is a cryptic transition zone between the myrtle and black-fronted mtDNA (i.e. introgressed versus ancestral mitochondrial types, respectively). We found that this transition in mtDNA is broadly coincident with a shift in migratory behaviour and also with some aspects of mitochondrial phenotype. We discuss and interpret the findings for this novel dataset 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 un-sampled 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. While 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 Figure 6.1b). 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.

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, $\sigma$ is root-mean-squared dispersal and $t$ is number of generations since contact. Assuming a dispersal distance of 20km and a generation time of two 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 (unpublished 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 or 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 U.S. and those in Mexico (i.e. across the traditional taxonomic boundary between S. a. auduboni and S. a. 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 is 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 behaviours 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 much better predictor of migratory distance. While finer-scale sampling along both transects would have been ideal, our estimates suggest that the shift in migratory behaviour occurs between 35-36°N (Table 6.1), very near the centers of the mtDNA transitions (35.94-37.4°N; Figure 6.2). The fact that this transition in migratory behaviour 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‰ δ²H (Figure S3; deuterium ratios increase towards 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 U.S. (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 the present study.

The mtDNA sequencing data suggests that there are numerous amino acid differences between the two mitochondrial types (Figure 6.3); 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 (Figure 6.3) 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. While a previous multi-locus 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 mitochondria 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) have a more efficient mitochondria as a result of increased coupling, measured here as the ratio of state III to state II consumption rates or the acceptor control ratio (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. While 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 (Figure 6.5a).

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. While 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. While 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 transition zone in the southwestern U.S. 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. While 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.
Table 6.1 Sampling localities, sample sizes and frequency of black-fronted type mitochondrial DNA (*Setophaga auduboni nigrifrons*) in populations along the eastern and western transects. Numbers of sampling locations correspond to those shown in Figure 6.1.

* 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.

<table>
<thead>
<tr>
<th>Site (abbreviation)</th>
<th>Avg. Lat.</th>
<th>Avg. Long.</th>
<th>Transect</th>
<th>n for mtDNA genotype (n for isotopes)</th>
<th>Frequency of S. a. nigrifrons mtDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Apache National Forest, AZ (ANF)</td>
<td>33.95</td>
<td>-109.42</td>
<td>Western</td>
<td>11 (8) N.S.</td>
<td>0.91</td>
</tr>
<tr>
<td>2) Coconino National Forest, AZ (CNF)</td>
<td>35.00</td>
<td>-111.51</td>
<td>Western</td>
<td>8 (7) N.S.</td>
<td>1.00</td>
</tr>
<tr>
<td>3) Kaibab National Forest, AZ (KNF)</td>
<td>36.53</td>
<td>-112.21</td>
<td>Western</td>
<td>36 (9)*</td>
<td>0.97</td>
</tr>
<tr>
<td>4) Dixie National Forest - Pine Valley, UT (DNF1)</td>
<td>37.37</td>
<td>-113.46</td>
<td>Western</td>
<td>8 (0)</td>
<td>0.50</td>
</tr>
<tr>
<td>5) Dixie National Forest – Navajo Lake, UT (DNF2)</td>
<td>37.59</td>
<td>-112.81</td>
<td>Western</td>
<td>44 (8)*</td>
<td>0.32</td>
</tr>
<tr>
<td>6) Fish Lake National Forest, UT (FLNF)</td>
<td>38.61</td>
<td>-111.70</td>
<td>Western</td>
<td>16 (8)*</td>
<td>0.13</td>
</tr>
<tr>
<td>7) Uinta National Forest, UT (UNF)</td>
<td>40.42</td>
<td>-111.64</td>
<td>Western</td>
<td>12 (8)*</td>
<td>0.00</td>
</tr>
<tr>
<td>8) Lincoln National Forest, NM (LFNF)</td>
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<td>-105.76</td>
<td>Eastern</td>
<td>13 (8) N.S.</td>
<td>0.85</td>
</tr>
<tr>
<td>9) Santa Fe National Forest, NM (SFNF)</td>
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<td>-106.50</td>
<td>Eastern</td>
<td>11 (8)*</td>
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<tr>
<td>10) Carson National Forest, NM (CNF)</td>
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<td>-106.22</td>
<td>Eastern</td>
<td>10 (7)*</td>
<td>0.30</td>
</tr>
<tr>
<td>11) Rio Grande National Forest – Stunner, CO (RGNF1)</td>
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<td>-106.59</td>
<td>Eastern</td>
<td>15 (8)*</td>
<td>0.27</td>
</tr>
<tr>
<td>12) Rio Grande National Forest – Posos, CO (RGNF2)</td>
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<td>-106.56</td>
<td>Eastern</td>
<td>10 (8)*</td>
<td>0.40</td>
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<td>13) Gunnison National Forest, CO (GNF)</td>
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<td>-106.73</td>
<td>Eastern</td>
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<tr>
<td>14) White River National Forest CO, (WRNF)</td>
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<td>-106.03</td>
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<td>11 (8)*</td>
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<tr>
<td>15) Arapaho National Forest, CO (ANF)</td>
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<td>-106.06</td>
<td>Eastern</td>
<td>7 (7)*</td>
<td>0.00</td>
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</table>
Table 6.2 Results of an ANCOVA 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 (Akaike 1974), is shown in bold, although the small difference between the top three models suggest they are statistically indistinguishable.

<table>
<thead>
<tr>
<th>Model</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtDNA type + breeding latitude + mtDNA type × breeding latitude</td>
<td>70.89</td>
</tr>
<tr>
<td>breeding latitude + mtDNA type</td>
<td><strong>69.42</strong></td>
</tr>
<tr>
<td>breeding latitude</td>
<td>70.80</td>
</tr>
<tr>
<td>mtDNA type</td>
<td>162.61</td>
</tr>
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</table>
Figure 6.1  The distribution of the currently classified species in the yellow-rumped warbler (*Setophaga spp.*) complex and sampling scheme. (A) 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 U.S. 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.
Figure 6.2 Clines in mtDNA and isotope variation across two transects in cryptic contact zone in the southwestern U.S. between myrtle-type and black-fronted type mtDNA. (A) Points are allele frequencies in the mitochondrial ATPase gene, locations are averaged distances (in degrees latitude) and colours 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 an individual with black-fronted mtDNA and open circles are individuals with a myrtle-type mtDNA. The arrows indicate the centers of the clines for mtDNA for the two clades shown in (A).
**Figure 6.3** Sequence divergence and the number of fixed amino acid changes between the two mitochondrial clades in the contact zone. Three individuals were sampled from each mitochondrial type, both from the western transect.
Figure 6.4 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.
Figure 6.5  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). (A) Rates are shown for state II (background respiration rate without ADP), state III (maximally stimulated rate with ADP), as well 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 bars indicate standard error.
CHAPTER 7: Genomic variation in yellow-rumped warblers

7.1 Introduction:

Local adaptation is the process by which, over many generations, organisms evolve towards an optimal phenotype within a given environment via natural selection. Selection can act upon variation that is either already present in the population (i.e. ‘standing variation’) or new genetic variants that arise ‘de novo’ (Barrett and Schluter 2008). The two types of variation differ in a number of ways, but most notably is the time span over which evolution takes place: adaptation of a population from standing variation will likely be faster as it does not have to wait for new mutations (Barrett and Schluter 2008). The standing variation upon which selection can act is also not only restricted to the population at hand – research into the role of hybridization between divergent lineages has illustrated that this is also an important process in generating biological variation (Rieseberg 1997; Mallet 2007). Hybridization between domesticated dogs and grey wolves, for instance, is thought to have introduced a putatively advantageous coat colour allele from canines to their wild relatives, producing a dark colour morph, that has been maintained in natural grey wolf populations in North America for many years (Anderson et al. 2009). This is similar to selection acting on standing variation in a population but from a larger genetic pool (i.e. from independent evolutionary lineages).

In some cases hybridization has also been suggested to result in the formation of entirely novel taxa (Jacobsen and Omland 2011). In particular, homoploid hybrid speciation has been suggested for a number of species of plants and, rarely, in some vertebrates (Rieseberg 1997; Hermansen et al. 2011; Brelsford et al. 2011; Elgvin et al. 2011; Trier et al. 2014). For example, such a scenario has been suggested for the Italian sparrow (Passer italiae), which has been proposed as a hybrid species between house sparrows and Spanish sparrows (Trier et al. 2014). In this case, Italian sparrows are intermediate for many genetic and phenotypic characteristics between house sparrows and Spanish sparrows (Elgvin et al. 2011). There is also evidence that there is appreciable levels of reproductive isolation between Italian sparrows and its putative parental species: Italian sparrows form a narrow hybrid zone in northern Italy where they come into contact with house sparrows, and, in the south of Italy, they also show little evidence of admixture with Spanish sparrows (Trier et al. 2014). Understanding the relative frequency of hybrid speciation in natural systems, however, is unclear, especially because there are so few empirical examples, at least in animals (Coyne and Orr 2004).
Brelsford et al. (2011) posited that hybridization might be important in the generation of genetic and phenotypic variation in the yellow-rumped warbler complex. This complex consists of three distinct nuclear groups, which generally align with phenotypic patterns (Brelsford et al. 2011): one group that aligns with myrtle warblers (S. coronata) from eastern North America, another group that aligns to Audubon’s and black-fronted warblers (S. auduboni auduboni and S. a. nigrifrons) from western North America and Mexico, and another distinct cluster that aligns with Goldman’s warblers (S. goldmani) from Guatemala. Brelsford et al. (2011) suggested that the intermediate genetic patterns of Audubon’s warblers as compared to myrtle and black-fronted warblers might be consistent with a type of hybrid origin of this taxon. This mixing occurs both in the nuclear genome and is also suggested from the distribution of mitochondrial DNA. In this case, mitochondrial DNA from myrtle warblers is found throughout the range Audubon’s warblers and is a particularly conspicuous suite of linked genes that has likely introgressed between the taxa (Brelsford et al. 2011; Mila et al. 2011). Detailed study of the mtDNA contact zone in the southwestern U.S. presented by Toews et al. (2014), suggests that mtDNA covaries with differences in migratory behaviour and some aspects of mitochondrial respiration. Questions remain, however, as to how general this pattern of introgression is and whether mitochondria have introgressed in concert with other loci in the nuclear genome.

Hybrid speciation in this case would also require some level of reproductive isolation between Audubon’s warblers and its parental taxa (i.e. myrtle and black-fronted warblers). Research into the narrow hybrid zone between Audubon’s and myrtle warblers in the Rocky Mountains suggests some level of reproductive isolation between them, and analysis of linkage disequilibrium is consistent with roughly 18% selection against hybrids (Brelsford and Irwin 2009). However, previous studies have not tested for evidence of possible reproductive isolation between Audubon’s and black-fronted warblers, which is important for understanding the possible hybrid origin of Audubon’s warblers.

Here we use genomic data to test for evidence of reproductive barriers between Audubon’s warblers and other groups in this complex. In particular, we use data from genetic markers with known chromosomal locations to gain insights into the patterns of divergence across the genomes of these groups. These data allow for robust estimates of genome-wide divergence and also have the resolution to identify smaller genomic regions that may be more highly differentiated as compared to the rest of the genome. Such differentiated regions between closely
related species may represent loci important in the evolution of reproductive isolation and/or local adaptation and, depending on their distribution and number, may be sufficient to produce substantial reproductive isolation in hybrids (Feder et al. 2013; 2012; Nosil and Feder 2013). While this data provides primarily a genic view of the speciation process, at least in birds most speciation events are assumed to involve some period of allopatry and, subsequently, genetic divergence (Price 2008).

We first use this genomic data to quantify the distribution of genetic variation in the entire yellow-rumped complex. We then tested whether Audubon’s were genetically intermediate between myrtle and black-fronted warblers, as previous studies of AFLPs have suggested (Brelsford et al. 2011). Next, we tested for differences within Audubon’s warblers by studying individuals that differ in their mitochondrial genome. In this case, we were interested in identifying if other nuclear markers have introgressed from myrtle warblers as extensively as mtDNA. Next, we tested for genomic evidence of reproductive barriers between the taxa in this group. We use a number of lines of evidence to identify barriers to gene flow. First, we used patterns of isolation-by-distance (IBD) to make inferences regarding gene flow between populations. Here we assumed that identifying IBD across known taxonomic boundaries was consistent with gene flow between populations close to a parapatric range boundary. Second, we estimated $F_{ST}$ for loci across the genome and tested for evidence of highly divergent ‘islands’ of differentiation in various comparisons. For this we used a method outlined by Renaut et al. (2013) to test for evidence of biased clustering of high $F_{ST}$ markers.

7.2 Methods

7.2.1 Sampling

We sampled 93 yellow-rumped warblers from across their range during the breeding season using song playback and mist nets. Detailed sampling information can be found in Table 7.1. Samples were obtained from two periods: between 2003-2006 samples were collected by Borja Mila in Guatemala (site 1), Mexico (sites 2 and 3) and Maine (19). Samples from Slave Lake (site 18) were collected by Alan Breldford in 2005 and samples for Oregon (site 16), Idaho (site 17) were obtained from the Burke Museum. In 2010 and 2011 David Toews collected detailed sampling of Audubon’s warblers (sites 4 to 15). Blood samples, taken using a small needle and capillary tube 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 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 and 1 mM EDTA, at pH 8.0, and stored at 4°C. For our analyses we grouped individuals in two different ways. In some cases we split the groups by population (the numbered sites in Figure 7.1a) whereas in others we grouped them into one of five groups (the colours in Figure 7.1a): Goldman’s (site 1), black-fronted (sites 2 and 3), Audubon’s with the ancestral mtDNA (individuals from sites 4-17 with black-fronted mtDNA), Audubon’s with introgressed mtDNA (individuals from sites 4-17 with the myrtle mtDNA) and myrtle warblers. We distinguished Audubon’s warblers with differing mitochondrial DNA using a RFLP digest as described in Toews et al. (2014).

7.2.2 Molecular analysis

To generate genomic data we used a reduced genome “genotype-by-sequencing” method adapted from Elshire et al. 2011 for the Illumina platform. We first standardized the concentration of all of the DNA samples to 20ng/µL. From each diluted sample we then took 5µL to add to a digestion mixture. This included: 6µL of common adaptors (0.4ng/µL; see Elshire et al. 2011 for sequences) and 6µL of barcoded adaptors (0.4ng/µL; see Elshire et al. 2011 for sequences), 20 units of the high fidelity PstI restriction enzyme (New England Biolabs) and 2µL of the provided buffer (10X). The barcodes we used to identify each individual later in the analysis were variable in length, between 4-8bp, and every pair differed by at least 3bp. This mixture was then incubated at 37°C for 2 hours. Following the digestion, to each sample we added 640 units of T4 DNA ligase (New England Biolabs), with 5µL of the provided buffer (10X) and 23.4µL of UltraPure water. We then incubated this ligation reaction for 1 hour at 22°C and then inactivated the enzyme by incubating the mixture for 65°C for 10min.

We cleaned this reaction using AMPure XP beads (Beckman-Coulter) to remove unused enzyme and small DNA fragments. In a new plate we added 15µL of the ligation mixture to 23µL of beads and mixed thoroughly with a pipette. The samples were then placed onto a magnetic plate and washed twice with 200µL of 70% ethanol. The beads were then removed from the magnetic plate and resuspended in 40µL of 1X TE. The samples were again placed on the magnetic plate and the solution was removed and added to a new plate, carefully avoiding the beads. We then performed a PCR for each sample separately: each 25µL reaction was prepared on ice and included: 0.5 units of PhusionTaq (New England Biolabs), 5µL of 5x Phusion Buffer, 0.5µL of 10uM dNTPs, 0.125µL of forward and reverse GBS primers (200uM; see Elshire et al.
2011 for sequences), 18µL of UltraPure water and 1µL of the cleaned DNA fragments from the ligation reaction. For the PCR we used the following thermocycling profile: 98°C for 30 seconds follow by 20 cycles of: 98°C for 10 seconds, 65°C for 30 seconds and 72°C for 30 seconds. This was followed by an extension of 72°C for 5 minutes. We then quantified the product of this amplification and visualized it on a 2.5% agarose. Each sample was then added to a pool and 25µL was run in one of three lanes of a 2% agarose gel. We then used a gel extraction kit (QIagen) to isolate the final libraries within a size range of 300-400bp. This size range was then confirmed using a high sensitivity Bioanalyzer chip (Agilent Technologies) and quantified using qPCR. The final libraries were sequenced using paired-ends in one lane of an Illumina HiSeq 2000.

### 7.2.3 Data analysis

We used the programs “GBSBarcode splitter” and “AdapterRemoval” (version 1.5; Lindgreen 2012) for the first stages of the analysis. We first de-multiplexed the sequencing reads, separating each individual by their unique barcodes. In this case we did not allow for any mismatches in the barcode sequence. This was conservative, as the barcodes we used differed by a minimum of 3bp. Any additional barcode or adapter sequence was then trimmed and each of the paired sequencing reads were aligned and, if there was overlap, collapsed into a single read. This type of overlap occurs in paired-end sequencing when the DNA insert size is smaller than approximately 100bp and is known as “read through” (Lindgreen 2012). It is important to collapse “read through” for genotype calling later in the analysis, where separate reads from the same DNA molecule should not be considered independent. Paired reads that had no overlap were kept as separate reads.

We then used BOWTIE2 (version 2.1; Langmead and Salzberg 2012) to map each of the individual reads to a build of the Zebra finch genome (Warren et al. 2010). For this we used the “very sensitive local” set of alignment presets. For SNP discovery and variant calling we used GATK (DePristo et al. 2011) and followed the Van der Auwera et al. (2013) set of GATK “best practices” as a guideline. We then applied additional filters using the program “VCFtools” (Danecek et al. 2011). First, we coded genotypes with a Phred-scaled quality lower than 20 as missing data, which corresponds to a genotyping accuracy of at least 99%. Then we excluded loci with more than 50% missing data, set the minimum call rate per individual as 20% and included sites with a minimum allele frequency of at least 2%.
To visualize the data and test for population clustering we used a principal components analysis (Patterson et al. 2006) using the “SNPRelate” package (Zheng et al. 2012) in R (R Development Core Team 2013). We retained the number of eigenvectors for which there were significant differences using an ANOVA in R, using the five groups (Goldman’s, black-fronted, Audubon’s with black-fronted-type mtDNA, Audubon’s with myrtle-type mtDNA and myrtle) as our categorical variable. Patterson et al. (2006) suggest the Tracy-Widom distribution may be the most appropriate distribution to test the significance of eigenvectors, although in practice the $F$ distribution used in the ANOVA produces similar results, which we use here.

To test how differentiation amongst populations relates to the distance between them we used a pairwise isolation-by-distance (IBD) comparison. Between each population we calculated the weighted mean $F_{ST}$ (Weir and Cockerham 1984) using the program “VCFtools” (Danecek et al. 2011). Given that some Audubon’s warbler populations can have mixtures of two mtDNA types (i.e. myrtle versus black-fronted mtDNA), and we were interested in nuclear differences associated with differing mtDNA types, we distinguished comparisons of Audubon’s warbler populations that were on the same side of the mtDNA transition versus those across the mtDNA transition. The center of the mtDNA cline was based on Toews et al. (2014) and grouped sites 4-6, 9 and 10 on the southern side of the mtDNA transition and sites 7, 8 and 11-17 on the northern side of the mtDNA clines (Figure 7.1). For each pairwise comparison we calculated the great circle distance between each using the “r.distearth” function in the “fields” package in R (Fields Development Team 2006).

We first tested for IBD by including only those comparisons amongst Audubon’s warbler populations (sites 4 – 17), which is our most well sampled group. In this case we tested for evidence of IBD by using a Mantel test. To explicitly examine whether differences across the mtDNA contact zone type might explain some of the residual variation in $F_{ST}$, after controlling for the distance between the populations, we performed an analysis of covariance (ANCOVA) using the “step” function in R. We included three variables: pairwise distance, $F_{ST}$ and whether the populations were on the same or opposite sides of the mtDNA transition zone. This procedure removes variables in a stepwise manner and determines, with AIC, which is the best model given the data.

We then tested whether there was evidence of IBD across known taxonomic boundaries. In this case, a restriction in gene flow will result in between-group $F_{ST}$ estimates that are higher
than that predicted based on the within-group IBD relationship; a finding of IBD in these cases is consistent with gene flow across the taxonomic boundary. We only tested for this between groups where we had multiple populations: all of the comparisons with Audubon’s warblers (i.e. Audubon’s versus black-fronted, myrtle and Goldman’s populations) and the comparisons between black-fronted and myrtle warbler populations. Because these comparisons are partial distance matrices (i.e. no within-group comparisons) it is not possible use a simple Mantel test. Therefore, we used a related type of permutation test: we randomly rearranged the rows and columns of the distance matrix associated with each pairwise comparison and then estimated the correlation with the $F_{ST}$ estimates. We conducted 10000 permutations then calculated where the observed correlation fell within this distribution.

To compare patterns of divergence across the genomes between each of the five groups we used “VCFtools” (Danecek et al. 2011) to estimate weighted $F_{ST}$ (Weir and Cockerham 1984) for each locus between each of the five main groups (i.e. ten comparisons). We tested for clustering of high $F_{ST}$ loci within the genome using the method described by Renaut et al. (2013). For each comparison we identified the loci within the top 3% of the distribution of $F_{ST}$ estimates. Using a simple quantile to estimate outliers allows for a robust comparison amongst groups that might vary in their genome-wide distribution of divergence (Renaut et al. 2013). We then used a narrow window and counted the number of high $F_{ST}$ markers within each. We explored a variety of window sizes (i.e. 500Kb to 2Mb) and found that the results were relatively insensitive to varying this parameter within that range; hence we only report results from implementing a window size of 1Mb (for scale, the longest chromosome in the Zebra finch, chromosome 2, is approximately 156Mb). To test whether clusters of highly differentiated markers could be due to chance sampling we used a permutation test: for each window we counted the number of total markers, randomly sampled that same number of markers from across the genome and then counted the proportion of markers in the sample that were identified as within the genome-wide top 3% of $F_{ST}$ estimates. For each window we conducted 1000 permutations and estimated statistical significance if the observed proportion was higher than 99% of the observations from the random permutations (i.e. a critical value of 0.01). This procedure allowed us to objectively quantify the number of clusters with high $F_{ST}$ between each of these comparisons. In this case, we were interested in testing how such clustering might relate to the history of gene flow between these groups.
7.3 Results
Following filtering, we identified 42,255 polymorphic SNPs associated with known locations in the Zebra Finch genome. For our PCA, we included 4197 additional SNPs located on unknown chromosomes, random chromosomes or linkage groups (LG2, LG5 and LG22). We did not have any fragments that aligned to the Zebra finch’s mitochondrial genome, which was expected given that *PstI* is not predicted to cut there, at least based on the sequence from the Zebra finch.

The PCA revealed strong evidence of genetic differentiation amongst a number of the previously identified taxonomic groups. The ANOVA identified PC1, PC2 and PC3 to be highly significant when considering the *a priori* grouping of individuals as categorical variables in the model (PC1: $P < 0.001$; PC2: $P < 0.001$; PC3 $P < 0.001$) whereas PC4 was not (PC4: $P = 0.35$). This was confirmed by visual inspection of the cumulative explained variance - there was little additional variance explained with PC4 (data not shown). PC1 and PC2 (collectively explaining 8.2% of the variation) split individuals into three distinct clusters: Goldman’s warblers, the most divergent along PC1, myrtle warblers, the most divergent along PC2, and Audubon’s / black-fronted warblers (Figure 7.2a). PC3, which explains 2.0% of the variation, separates Audubon’s warblers from black-fronted warblers (Figure 7.2b). At this scale there was no evidence of differentiation within Audubon’s warblers: individuals with myrtle mtDNA (red diamonds in Figure 7.2a and 7.2b) overlap with individuals with Audubon’s with black-fronted mtDNA (red circles in Figure 7.2a and 7.2b).

Relationships between geographic distance and genetic differentiation varied within and between these groups (Figure 7.3). Comparisons with Goldman’s warbler populations showed the highest level of differentiation across all of the comparisons (circles filled or outlined with purple in Figure 7.3) with pairwise $F_{ST}$ estimates ranging from approximately 0.2 to 0.3. The next most divergent group of comparisons involved those with myrtle warblers (circles filled or outlined with blue in Figure 7.3). Pairwise comparisons of myrtle populations with Audubon’s and black-fronted warbler populations had $F_{ST}$ estimates ranging between 0.05 and 0.1. While low, we also found evidence of genetic differentiation between Audubon’s and black-fronted warbler populations (circles filled with yellow and outlined in red in Figure 7.3). This differentiation was over-and-above what is observed from differences associated with distance alone (i.e. black-fronted – Audubon’s comparisons generally have a higher $F_{ST}$’s than comparisons between Audubon’s warbler populations for a given distance).
We found strong evidence for isolation-by-distance for those comparisons among Audubon’s warbler populations \((r = 0.37; P = 0.03; \text{Figure 7.3}; \text{Figure 7.4a})\). In contrast, we found no evidence that Audubon’s warbler populations, across the mtDNA transition, were more differentiated for a given distance that those populations residing on the same side of the mtDNA clines (in \text{Figure 7.3} and \text{Figure 7.4a} solid red circles were populations on the same side of the mtDNA transition; red circles with a black outline represent population comparisons across the mtDNA divide). This was confirmed by the results of the analysis of covariance test. Here were tested for variables that explain variation in \(F_{ST}\) between Audubon’s warbler populations on the same side versus different side of the mtDNA contact zone. The best model, as chosen by AIC, included only the distance between the populations (model with just distance: \(\text{AIC} = -932.0\)) and not whether the populations were on similar or different sides of the mtDNA contact zone (model that included only whether a population was on the same or different side of the mtDNA contact zone: \(\text{AIC} = -921.2\); model with distance and the side of the mtDNA contact zone: \(\text{AIC} = -931.2\)). We also found evidence of a geographic component of pairwise \(F_{ST}\) estimates across one of the known taxonomic boundaries: between Audubon’s warblers and myrtle warblers there is a significant and positive relationship with distance \((P < 0.05; \text{Figure 7.4b})\). This was not true of the other comparisons (Audubon’s and black-fronted \(P > 0.05\), \text{Figure 7.4c}; Audubon’s and Goldman’s \(P > 0.05\), \text{Figure 7.4c}; black-fronted and myrtle \(P > 0.05\), data not shown).

Levels of differentiation across the genome varied widely between the comparisons (\text{Figure 7.5}). Each of the comparisons with Goldman’s warblers (\text{Fig. 7.5a to 7.5c}) showed high levels of differentiation across the genome and almost all chromosomes had at least one SNP that was completely fixed for alternate alleles in each comparison (usually many more). In contrast, comparing myrtle warblers with Audubon’s or black-fronted warblers (\text{Fig. 5D-F}) suggests a more heterogeneous pattern: clusters of highly differentiated regions, separated by regions of low average \(F_{ST}\), scattered in distinct areas. For example, chromosome 2 shows little evidence of high \(F_{ST}\) clusters in these comparisons, while chromosome 8 has a clear high divergence region. Much less differentiation was observed when comparing black-fronted and Audubon’s warblers overall (\text{Fig. 5g}) and few markers have \(F_{ST}\) values above 0.5. This was similar when comparing Audubon’s warblers with differing mitochondrial genomes (myrtle or black-fronted type mtDNA): we found no detectable SNPs with even moderate levels of differentiation across the genome.
Our permutation test was designed to objectively identify regions of clustering of high $F_{ST}$ markers in discrete windows (Figure 7.6 and 7.7). In agreement with the qualitative observations, comparisons with myrtle warblers showed much more evidence of clusters of highly differentiated markers (i.e. 49 – 62 clusters; Fig. 7.7). This is also true when comparing myrtle and Goldman’s warblers, which is not directly obvious in Figure 7.5. We found many of the clusters between myrtle and Goldman’s warblers occurred in a similar genomic location as the comparisons between myrtle and Audubon’s / black-fronted warblers (Figure 7.6). For instance, 29% of windows with evidence of clustering between myrtle-Goldman’s warblers were also found to have clustering in comparisons between myrtle-Audubon’s warblers (with the myrtle mtDNA type). The other comparisons, not including myrtle warblers, showed many fewer high $F_{ST}$ clusters (i.e. between 8 to 17 clusters). In particular, we found very little evidence that there were highly divergent regions between Audubon’s and black-fronted warblers (Figure 7.6).

7.4 Discussion

Here we have provided a comprehensive and broad scale genomic study of yellow-rumped warblers from across their range. To our knowledge it is one of the first next generation sequencing datasets for a North American Passerine. These data have allowed us to address a number of important questions relating to the evolutionary and biogeographic history of this species group and, more generally, has important implications for the role of hybridization in generating genetic and phenotypic variation in this group. These results suggest that the yellow-rumped warbler complex consists of a number of highly differentiated groups that have varying levels of gene flow and genomic differentiation. Below we discuss how these results relate to our original objectives and, more generally, how they fit into the context of previous studies exploring patterns of genomic differentiation and hybrid speciation.

The finding of very low levels of genetic differentiation between the black-fronted and Audubon’s warblers has implications for the possibility of the Audubon’s warbler as a hybrid species (Brelsford et al. 2011). Homoploid hybrid speciation has been suggested for a number of species of plants and in some vertebrates (Rieseberg 1997; Hermansen et al. 2011; Brelsford et al. 2011; Elgvin et al. 2011; Trier et al. 2014). Brelsford et al. (2011) posited that the intermediate genetic patterns of Audubon’s warblers as compared to myrtle and black-fronted warblers might be consistent with a type of hybrid origin of this taxon. These new genomic data reveal a number of important patterns. First, it shows Audubon’s are much more closely related to black-fronted warblers than they are to myrtle warblers, as patterns in AFLPs originally
suggested (Figure 7.2). While Audubon’s warblers are intermediate between the two putative parental species, they are much more closely aligned to black-fronted warblers than to myrtle warblers (Figure 7.2).

Hybrid speciation also requires some level of reproductive isolation between Audubon’s warblers and the parental taxa (i.e. myrtle and black-fronted warblers). The narrow hybrid zone in the Rocky Mountains suggests some level of reproductive isolation between Audubon’s and myrtle warblers (Brelsford and Irwin 2009). This new genomic data is consistent with strong isolation between these taxa. In particular, the finding of numerous clusters of high $F_{ST}$ markers suggests appreciable levels of isolation across the genome. However, patterns in in the southern range of the Audubon’s warbler, across the boundary with black-fronted warblers, are more complicated. Using AFLPs, Brelsford et al. (2011) found there was some genetic separation of these two taxa but also much overlap. The current data are able to detect more subtle genetic structure within the Audubon’s/black-fronted group. For instance, while not clearly separated by AFLPs, the present genomic data suggest that black-fronted and Audubon’s warblers are genetically distinguishable, which is clear from the genomic variation summarized by PC3 (Figure 7.2b). However, these differences translate into very low estimates of $F_{ST}$ across the genome ($F_{ST}$ between Audubon’s and black-fronted warblers is estimated to be between 0.015-0.017; Figure 7.7) and very few clusters of the highest 3% of $F_{ST}$ markers (i.e. <20). These clusters also show low levels of divergence between the two. For instance, the top 3% of markers between Audubon’s (with myrtle mtDNA) and black-fronted warblers have $F_{ST}$ estimates above 0.15; between myrtle and Audubon’s (with myrtle mtDNA) the top 3% are above an $F_{ST}$ of 0.34.

While this differentiation is low between Audubon’s and black-fronted warblers, it is over-and-above what is expected based on differences due to distance alone (Figure 7.3). This suggests that there is likely some reduction in gene flow between these two taxa, beyond geographic differences alone, but given the low levels of differentiation any barrier them is likely to be weak. While rare, it is possible to have the evolution of reproductive isolation even with low levels of genetic divergence. For example, reproductive isolation in the absence of appreciable genetic differences has been note in indigobirds in Africa (Sorensen et al. 2003), one of the best examples of ‘sympatric speciation’ in birds. However, such cases are very rare, especially in avian systems. Given that this data provides a primarily genic view of reproductive barriers between these taxa, clearly a more detailed study of the Audubon’s – black-fronted
contact zone, combined with controlled playback studies, would be ideal to estimate the extent of possible isolating barriers between these groups.

Whether or not hybridization with myrtle warblers may not have resulted in the evolution of reproductive isolation, it has likely increased the overall diversity in the Audubon’s/black-fronted warbler complex. For instance, across taxonomic boundaries we did not find evidence of isolation-by-distance (Figure 7.3c and 7.3d) except for comparisons between Audubon’s and myrtle warbler populations (Figure 7.3b). Such a pattern is consistent with gene flow between myrtle and Audubon’s warblers, where populations closer in proximity to their parapatric range boundary are more genetically similar than those at further distances. In this case, gene flow near the range boundary is not surprising given that these two taxa hybridize extensively where they co-occur (Hubbard et al. 1969; Brelsford and Irwin 2009). Brelsford et al. (2011) also found Audubon’s warblers closer to the myrtle / Audubon’s hybrid zone showed evidence of introgression.

However, we found little evidence that introgression of nuclear markers from myrtle warblers that had introgressed as far as the mtDNA contact zone: there were no markers that showed high levels of divergence in Audubon’s warblers in the western U.S. that differed in their mitochondrial genome (i.e. Audubon’s with black-fronted type mtDNA versus myrtle type; \( F_{ST} = 0.002 \)). This result is important in that it implies the transition zone between the introgressed, myrtle-type mtDNA and the ancestral, black-fronted-type mtDNA is unlikely to be a general genomic transition area or a tension zone (Toews et al. 2014). The yellow-rumped warbler system was included in the 11 cases of mitochondrial introgression reviewed by Toews and Brelsford (2012) where introgression of mtDNA was at-or-near fixation across >50% the range of a ‘native taxon’ (i.e. mitochondrial introgression is not restricted to an area of secondary contact nor completely introgressed). Of these studies, to our knowledge this is first to employ next generation sequence data to confirm mtDNA as an outlier as compared to markers in the nuclear genome (Toews and Brelsford 2012). As a more robust confirmation of this result, it would be ideal to assay additional genetic markers, especially targeting those genes that code for nuclear encoded mitochondrial gene products.

Across the complex more generally, the amount and extent of genomic divergence differed widely between the various comparisons. Across all of the comparisons, Goldman’s warblers showed very high levels of differentiation across the genome. This is likely function of
a current, and presumably historical, small population size and the limited range of this taxon (Brelsford et al. 2011). Under such a scenario, genetic drift is predicted to have a strong influence on the levels of differentiation between populations. Indeed, this differentiation is distributed broadly across the genome, at least in comparisons with black-fronted and Audubon’s warblers, consistent with the effects of drift (Feder et al. 2013). In these comparisons the number of high $F_{ST}$ clusters is very low (Figure 7.7), while genome-wide divergence is high. In contrast, there are many high $F_{ST}$ clusters when comparing myrtle warblers with Audubon’s, black-fronted or Goldman’s warblers (i.e. there are >49 clusters across the genome for each of these comparisons; Figure 7.7). This clustering is especially clear when comparing myrtle warblers with Audubon’s warblers: the majority of the genome shows low levels of differentiation, which separate more restricted regions of loci with high $F_{ST}$ values (Figure 7.5d to 7.5f; Figure 7.6).

Under a model of secondary contact and gene flow, it is possible that these clusters of highly divergent markers represent regions of the genome that are less prone to introgression. In this case, secondary contact and gene flow might also reduce genome-wide divergence such that the detectability of diverged regions is increased. Therefore, these regions may be good candidates for those housing genes potentially involved in reproductive isolation and/or local adaptation (Feder et al. 2012; 2013). These observations could be confirmed with additional studies in the myrtle / Audubon’s hybrid zone. For instance, do these regions of increased divergence between myrtle and Audubon’s warblers also correlate to narrower clines across the hybrid zone between them? In their genomic study of a hybrid zone between the white-collared (Manacus candei) and the golden-collared manakins (M. vitellinus), Parchman et al. (2013) found significant correlations between a marker’s $F_{ST}$ values and estimates of cline width (Parchman et al. 2013; Yeaman 2013). In this case, these markers are likely good candidates for regions underlying local adaptation and/or reproductive isolation. We suggest detailed study of genomic variation across the myrtle and Audubon’s hybrid zone would be very informative in this regard.

As suggested above, it may be that gene flow following secondary contact has influenced some of the patterns of divergence between those taxa in secondary contact. However, interpreting the clusters of highly differentiated regions as a result of gene flow following secondary contact may not be the most plausible explanation for these data. This is because there are also numerous high $F_{ST}$ clusters between myrtle and Goldman’s warblers. Given the current
allopatric distribution of these taxa and the genome-wide distribution of $F_{ST}$ values, there is little evidence that these two taxa have experienced much if any gene flow following their initial isolation. An alternative explanation for the clustering of high $F_{ST}$ markers is that they may instead represent diverged regions that are unique to myrtle warblers, regardless of secondary contact and introgression from other taxa. For instance, selection may have driven the evolution of certain regions of the myrtle warbler’s genome, which are detected as clusters regardless of the genome-wide distribution of divergence between groups. Supporting this interpretation is the observation that many of the clusters between myrtle and Goldman’s warblers occur in a similar genomic location as those comparisons between myrtle and Audubon’s warblers (e.g. Figure 7.6).

Similar geographic patterns of genomic divergence have been noted *Heliconius* butterflies and *Helianthus* sunflowers (Renaut et al. 2013; Martin et al. in press). In *Heliconius*, hybridizing parapatric pairs show the lowest levels of divergence across the genome, as compared to sympatric or allopatric pairs (Martin et al. in press). This pattern is consistent across the genome, except near loci previously shown to correlate with wing pattern differences, which have high levels of divergence (Martin et al. in press). However, the finding of strong genomic heterogeneity between allopatric pairs also implies that such patterns are possible in the absence of gene flow (Martin et al. in press). In *Helianthus* sunflowers, Renaut et al. (2013) found that those populations in known to hybridize did not differ in the number and size of divergent regions as compared those allopatric or pairs known to have limited gene flow. These examples both imply that the evolution genomic heterogeneity between groups can and does occur in allopatry, as we suggest here for the clustering patterns between comparisons with myrtle warblers.

There is clearly much power in the genotype-by-sequencing approach employed here, although there are some important considerations with regards to our analysis that should be noted. First, the number of high $F_{ST}$ clusters should not be treated as absolute, as this is partially related to the window size we used and, in some cases, a single large peak in the $F_{ST}$ values would be counted as multiple clusters (e.g. Figure 7.6). The relative distribution of the number of clusters between the various comparisons, however, was robust to differences in window size. Furthermore, undoubtedly there are some differentiated regions of the genome that the GBS analysis did not detect. To some extent this is expected, given that the method is designed to represent only a fraction of the genome and this is an unavoidable cost associated with tractably
sequencing common genomic regions across many individuals (Elshire et al. 2011). However, it is notable that some previously identified markers known to be fixed for alternate alleles in Audubon’s and myrtle warblers, including CHD1Z, were not represented in our survey (Brelsford and Irwin 2009). This implies that there could still be many more small clusters of high differentiation that our current analysis did not have the resolution to detect. Given that many of the short sequencing reads could not be mapped directly to the Zebra finch genome, we suggest that sequencing the full genome of a New World warbler would aid future genomic studies such as ours.

In conclusion, while there is evidence of introgression between Audubon’s warblers, myrtle and black-fronted warblers there is less evidence that strong reproductive isolation has evolved between them, at least between Audubon’s and black-fronted warblers. This does not rule out a creative role of hybridization in this instance (Jacobson and Omland 2011), but it does suggest that it may not have been involved in the evolution of barriers to gene flow. More generally, empirical genomic studies, such as the current one, will have important implications for our understanding of the general patterns involved in divergence, hybridization and speciation (Seehausen et al. 2014). In particular, systems that have experienced periods of allopatric isolation followed by secondary contact will be important systems for future studies of genomic evolution (Feder et al. 2013), as this is likely an important mode of speciation in many taxa (Price 2008). Indeed, inferring historical processes from genetic data has been a central goal of evolutionary biology and genomic data are becoming an increasingly important tool for addressing these and other relevant questions.
Table 7.1 Sampling localities, sample sizes and taxa sampled throughout the range of the yellow-rumped warbler complex.

<table>
<thead>
<tr>
<th>Site</th>
<th>Avg. Lat.</th>
<th>Avg. Long.</th>
<th>n</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Guatemala</td>
<td>15.53</td>
<td>-91.52</td>
<td>5</td>
<td>Goldman’s</td>
</tr>
<tr>
<td>2) Durango, Mexico</td>
<td>24.54</td>
<td>-104.60</td>
<td>3</td>
<td>Black-fronted</td>
</tr>
<tr>
<td>3) Chihuahua, Mexico</td>
<td>28.61</td>
<td>-106.06</td>
<td>11</td>
<td>Black-fronted</td>
</tr>
<tr>
<td>4) Apache National Forest, AZ</td>
<td>33.95</td>
<td>-109.42</td>
<td>5</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>5) Coconino National Forest, AZ</td>
<td>35.00</td>
<td>-111.51</td>
<td>5</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>6) Kaibab National Forest, AZ</td>
<td>36.53</td>
<td>-112.21</td>
<td>5</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>7) Fish Lake National Forest, UT</td>
<td>38.61</td>
<td>-111.70</td>
<td>5</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>8) Uinta National Forest, UT</td>
<td>40.42</td>
<td>-111.64</td>
<td>5</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>9) Lincoln National Forest, NM</td>
<td>32.90</td>
<td>-105.76</td>
<td>5</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>10) Santa Fe National Forest, NM</td>
<td>35.84</td>
<td>-106.50</td>
<td>5</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>11) Carson National Forest, NM</td>
<td>36.69</td>
<td>-106.22</td>
<td>5</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>12) Rio Grande National Forest – Stunner, CO</td>
<td>37.38</td>
<td>-106.59</td>
<td>5</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>13) Rio Grande National Forest – Poso, CO</td>
<td>37.98</td>
<td>-106.56</td>
<td>5</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>14) Gunnison National Forest, CO</td>
<td>38.86</td>
<td>-106.73</td>
<td>5</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>15) Arapaho National Forest, CO</td>
<td>40.29</td>
<td>-106.06</td>
<td>5</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>16) Oregon</td>
<td>44.11</td>
<td>-120.16</td>
<td>4</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>17) Idaho</td>
<td>43.91</td>
<td>-114.94</td>
<td>3</td>
<td>Audubon’s</td>
</tr>
<tr>
<td>18) Slave Lake, AB</td>
<td>55.49</td>
<td>-114.85</td>
<td>2</td>
<td>Myrtle</td>
</tr>
<tr>
<td>19) Maine</td>
<td>45.16</td>
<td>-69.34</td>
<td>5</td>
<td>Myrtle</td>
</tr>
</tbody>
</table>
Figure 7.1  Distribution of classified groups in the yellow-rumped warbler (*Setophaga* spp.) complex and sampling. Myrtle warbler (blue); Audubon’s warbler (red); black-fronted warbler (yellow); and Goldman’s warbler (violet). The hatched areas distinguish birds with the myrtle-type from those with black-fronted type mtDNA. (B) Sampling localities in North and Central America (see Table 1 for location information). We distinguish Audubon’s warbler populations, sites 4-6 and 9-10, as on the southern side of the mtDNA transition area based on Toews *et al.* (2014).
Figure 7.2  Principal components analysis based on 42,255 polymorphic SNPs. The colours represent the four groupings presented in Figure 7.1. Audubon’s warblers are distinguished by their mtDNA types: Audubon’s with myrtle type mtDNA are shown as red diamonds; Audubon’s with black-fronted type mtDNA are shown as red circles.

(A) Principal components analysis showing the first three components. The first component (PC1) explains 5.1% of the variance, the second component (PC2) explains 3.1% of the variance, and the third component (PC3) explains 2.0% of the variance.

(B) Close-up of the first and second components, showing the distribution of the four groups: Myrtle, Goldman’s, Black-fronted, and Audubon’s.
**Figure 7.3** The relationship of population pairwise $F_{ST}$ estimates relative to distance between all of the sampled populations (i.e. isolation-by-distance). Each point represents a comparison between two populations, with sampling information for each in Figure 7.1 and Table 7.1. The two colours for each circle (outline or fill) represent one of the two pairwise comparisons (blue – myrtle; yellow-black-fronted; red – Audubon’s; violet – Goldman’s). Thus a myrtle population compared to an Audubon’s population would be coloured blue and red. The population comparisons amongst Audubon’s warblers are distinguished by populations on different sides of the mtDNA transition in southern Utah and Colorado (black circles with red fill) versus populations on the same side of the transition (solid red). The comparison between the black-fronted warbler populations is not shown.
Figure 7.4  Relationship of population pairwise $F_{ST}$ estimates relative to distance between all of the sampled populations (i.e. isolation-by-distance). Each point represents a comparison between two populations, with sampling information for each in Figure 7.1 and Table 7.1. The two colours for each circle (outline or fill) represent one of the two pairwise comparisons (blue – myrtle; yellow- black-fronted; red – Audubon’s; violet – Goldman’s). (A) Shows comparisons amongst only Audubon’s warbler populations, where there is a significant relationship between the distance between sampled populations and the $F_{ST}$ estimates between them (slope = $6.3 \times 10^6$; $P < 0.001$). Populations across the mtDNA contact zone are identified with black outline, populations on the same side have a red fill and outline. (B) Comparisons between Audubon’s and myrtle warbler populations (slope = $6.1 \times 10^6$; P < 0.05). (C) Comparisons between Audubon’s and black-fronted warbler populations (P > 0.05). (D) Comparisons between Audubon’s and Goldman’s warbler populations (P > 0.05).
Figure 7.5  $F_{ST}$ estimates for each locus relative to its position in the Zebra finch genome. Comparisons are between individuals grouped into the five categories (myrtle, black-fronted, Goldman’s, Audubon’s with myrtle mtDNA and Audubon’s with black-fronted type mtDNA). For space, two of the ten comparisons are not shown: Goldman’s versus Audubon’s with myrtle mtDNA and black-fronted versus Audubon’s with myrtle mtDNA.
Figure 7.6  Examples of divergence and clustering on chromosome 7 (A), 8 (B) and 9 (C). The small filled points show the $F_{ST}$ values for comparisons between myrtle and Audubon’s warblers (with myrtle mtDNA) and open points show comparisons between myrtle and Goldman’s warblers. The coloured circles show those windows that had a significant number of high $F_{ST}$ markers (positioned at 0 indicates no significance; at 1 indicates significance): blue circles show the myrtle-Goldman’s comparisons; red circles show the myrtle-Audubon’s comparisons. This illustrates that some of those windows where there is significant clustering in one of the comparisons also show clustering in the others.
**Figure 7.7** The circles illustrate the number of significant clusters of high $F_{ST}$ markers for each of the 10 comparisons. Individuals are grouped as either myrtle (blue), black-fronted (yellow), Goldman’s (violet), Audubon’s with myrtle mtDNA (red with black dot) and Audubon’s with black-fronted mtDNA (red without black dot). The two colours for each circle (outline or fill) represents one of the two groupings in the comparisons. The secondary axis (+) shows the genome-wide $F_{ST}$ between individuals grouped into the five categories.

![Graph showing the number of significant clusters of high $F_{ST}$ markers for each comparison. The x-axis represents different comparisons of grouped individuals, and the y-axis represents the number of significant clusters. The graph includes circles of different colours and outlines to represent the groupings.]
CHAPTER 8: General conclusions

Throughout this thesis I have drawn inferences from biogeographic patterns of genetic and phenotypic variation to better understand ecological and evolutionary processes shaping natural populations. My research has focused on a group of birds in the Setophaga genus, which have had a rich history of study (Hubbard 1969; Barroclough 1980; Brelsford and Irwin 2009; Lovette et al. 2010). For each of these research chapters I have focused on the process and effects of hybridization between divergent and, in some cases, independently evolving lineages. In chapters two, three and four I focused on the characteristics of hybrids in two narrow hybrid zones. In chapter two, I showed that there is strong evidence that Townsend’s warblers and black-throated green warblers hybridize where they co-occur. These two species were not previously known to hybridize extensively and this study provides an important foundation for future studies of potential reproductive barriers between these two taxa. In this case, I suggest an important avenue of future research would be to investigate potential pre-mating reproductive barriers between these taxa, which may result in assortative mating. Research into the potential role of song variation across this contact zone will be particularly informative (Kenyon et al. 2014), especially given the large differences in vocalizations between these species and the known role of song differences in avian contact zones (Toews and Irwin 2008; Kenyon et al. 2011). Also, studying the development and behaviour of hybrids in this system would be particularly informative. More generally, studies of avian contact zones have provided important insights into both taxonomic relationships (e.g. Toews and Irwin 2008) and evolutionary dynamics (Brelsford and Irwin 2009; Price 2008). Given that this area of overlap in northeastern B.C. is shared with a number of other avian species, information regarding the evolution of reproductive barriers in this group could be more broadly applicable to other taxa in this area.

One suggested reproductive barrier between incipient avian species are differences related to seasonal migratory behaviours (Rohwer and Irwin 2011). I attempted to investigate these behaviours in chapters three and four in the hybrid zone between Audubon’s and myrtle warblers. In this case, I studied variation in orientation and isotopic patterns across the hybrid zone to make inferences about the migration routes and wintering locations of these birds. The isotope data from chapter three suggests that the pure forms of these two species winter in isotopically distinct areas, consistent with band recovery data (Brewer et al. 2006). I also found that hybrids between these two taxa exhibit a wide range of variation in these behaviours, although most appear to be wintering in the southeastern U.S. overlapping with what has been
observed in pure myrtle warblers. The results of the orientation experiments suggest that individuals orient in a more myrtle-like direction, although the observation that the mean orientation across individuals was towards the northeast was not expected. Taken together, these results suggest that migratory behaviour is much more complicated in wild populations than some of the results of lab-raised blackcap warblers might suggest (Helbig 1991).

The finding that there are many genetically mixed individuals leaving the hybrid zone on fall migration provides important evidence that hybrids in this system develop properly, at least through to the beginning of their first year. It is notable that the proportion of genetically intermediate individuals (e.g. genetic index = 0.5) was much larger in the sample of birds of fall migration as compared to breeding birds. This suggests that any fitness deficits in hybrids may be observable within their first year, following fall migration and prior to their first breeding. I suggest two possible future studies that could confirm and extend these studies of migratory patterns in yellow-rumped warblers. First, additional genetic markers could be used to assay individuals leaving on fall migration near the hybrid zone, such as the GBS approach I used to assay genomic variation across the entire species complex. This data would allow a more robust classification of individuals as F1’s or backcrosses. This would provide a more powerful comparison of the genetic composition of birds leaving on migration to those breeding in the following year. Second, it would be useful to study the genetic composition of yellow-rumped warblers on spring and fall migration in various regions throughout North America. One method to address this would be to genotype yellow-rumped warblers killed from building strikes on migration. Birds that strike windows on migration make up a large fraction of many tissue collections in museums, therefore genetic samples are readily available in many cases. This would allow for an objective estimation of the regions used on migration by myrtle warblers, Audubon’s warblers and, more importantly, the hybrids between them.

In chapters five and six I addressed another effect of hybridization, specifically focusing on the effects of introgression following secondary contact. My review of mito-nuclear discordance in chapter five suggests that mitochondrial introgression in particular is a prevalent and important phenomenon in animal taxa. In total, I identified 126 cases in animal systems with strong evidence of discordance between the biogeographic patterns obtained from mitochondrial DNA and those observed in the nuclear genome. In most cases, authors attributed these patterns to a variety of scenarios, including: adaptive introgression of mtDNA, demographic disparities
and sex-biased asymmetries, with some studies also implicating hybrid zone movement, human introductions and Wolbachia infection in insects. For those cases where foreign mtDNA haplotypes were found deep within the range of a second taxon, I found that those mtDNA haplotypes were more likely to be at a high frequency and commonly driven by sex-biased asymmetries and/or adaptive introgression. In many cases, I found that authors presented more than one explanation for discordant patterns in a given system, which indicates that likely more data are required in many cases. Ideally, to resolve this issue, I suggest that future work shift focus from documenting the prevalence of mito-nuclear discordance towards testing hypotheses regarding the drivers of discordance. Indeed, there is great potential for certain cases of mitochondrial introgression to become important natural systems within which to test the effect of different mitochondrial genotypes on biochemical phenotypes.

I attempted such a study in my examination of mitochondrial introgression in the yellow-rumped warbler complex. In this case, I found evidence 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, within otherwise phenotypic Audubon’s warblers, myrtle mtDNA comes into contact with another clade that occurs primarily in the Mexican black-fronted warbler. Both northern forms exhibit seasonal migration, whereas black-fronted warblers are assumed to be nonmigratory. I 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 Audubon’s warblers with myrtle-type mtDNA have a significantly greater acceptor control ratio of mitochondria, suggesting it may be more metabolically efficient.

There are a number of important future studies that could extend this research. First, as I suggest in chapter six, the difference in the acceptor control ratio appears to be driven mostly by differences in the background or state II respiration rate. This suggests that proton leak may be lower in the myrtle-type mitochondria. Using sensitive electrodes it is possible to measure proton conductance between these two mitochondrial types (e.g. Brand et al. 2006). This would be useful in confirming any potential differences in proton leak between these two mitochondrial types. Second, while migratory differences were broadly correlated with the geographic location
of the shift in mitochondrial DNA, there are other possible environmental factors that could be examined. For instance, elevational segregation of individuals differing in their mitochondrial types in the transition zone is one important abiotic covariate that should be investigated further. Finally, while I observed significant differences in the respiration of these to mitochondrial types, these differences were small and, given that I was studying wild birds, the research was necessarily correlative. Therefore, to robustly address cause and effect one would need to bring birds into a common environment and potentially study whole-organism measures of performance and/or respiration. In particular, this would allow a stronger test of whether the differences noted in mitochondrial respiration scale up to differences in migratory ability. Such studies would be very informative in this regard, although logistically challenging.

The survey of genomic variation in the complex in chapter seven identified a number of important findings. First, the results suggest that there is mixed support for the hybrid origins of the Audubon’s warbler. This interpretation is mostly drawn from the observation that there is very low genomic differentiation between Audubon’s and one of the putative parental species, black-fronted warblers. While this does not rule out the fact that there may be appreciable levels of reproductive isolation between these groups, it does suggest that, if present, any barriers are likely weak. More generally, the results suggest that the yellow-rumped warbler complex consists of a number of independently evolving lineages, some of which would likely be recognized taxonomically as full species under most species concepts. I suggest genetic patterns observed here are consistent with the yellow-rumped warbler being provisionally divided into three separate species: *Setophaga coronata*, the myrtle warbler; *S. auduboni*; the Audubon’s warbler; and *S. goldmani*, the Goldman’s warbler. Given the distinct plumage (Hubbard 1969; Mila et al. 2011), morphological (Hubbard 1969; Mila et al. 2011) and bioacoustic patterns (Mila unpublished) of Goldman’s warblers, we suggest that, in combination with the current genetic data and previous studies (Mila et al. 2007; Brelsford et al. 2011), this taxon likely represents a distinct species under most species concepts. As Brelsford (2011) points out, this has important taxonomic and conservation implications: this may be one of the only bird species largely restricted to Guatemala. The numerous highly differentiated genomic regions between Audubon’s and myrtle warblers also suggest there is considerable reproductive isolation between these taxa across the genome. This is consistent with studies of the hybrid zone between them, which is stable, narrow and likely maintained by some form of selection against hybrids (Brelsford and Irwin 2009). In contrast, given the very low levels of differentiation across the
genome, we suggest that the black-fronted warbler should be treated as a separate subspecies of Audubon’s warblers (S. a. nigrifrons). This is consistent with current designations from both classification committees, which distinguish the two as subspecies (i.e. the International Ornithologists’ Union and the North American Classification Committee).

I also found that, at least in this survey of nuclear markers, that there are no detectable differences in the nuclear genome between Audubon’s warblers that differ in their mitochondrial genomes (i.e. myrtle versus black-fronted mtDNA). While this does not rule out the effects of other loci not included in our genomic assay, it strongly suggests that introgression of mitochondria from myrtle warblers has occurred in the absence of many other genes in the nuclear genome, at least to the point where the mtDNA transition occurs. These results generally confirm the results of Brelsford et al. (2011). In this case, however, my use of genomic markers associated with known locations in the Zebra finch genome allowed me to quantify the amount and extent of divergence throughout the genomes of these groups. Not surprisingly, as AFLPs suggest (Brelsford et al. 2011), Goldman’s warblers appear to be strongly differentiated from all of the other groups in the complex. This is likely due to an extended period of geographic isolation coupled with reduced population size of this group.

A novel finding from this genomic survey was the identification of clustering of highly differentiated markers between myrtle warblers and the other groups. Given that this clustering was observed between groups that are currently in contact and are known to hybridize extensively (i.e. myrtle and Audubon’s warblers) it is possible that gene flow following secondary contact is contributing to this observation. However, the fact that these clusters also appear between comparisons between myrtle and Goldman’s warblers is less consistent with this scenario: there is no biogeographic or genetic evidence that these two taxa have experienced gene flow since their initial isolation. Therefore, I suggest that the clustering of high $F_{ST}$ markers is more consistent with diverged regions that are unique to myrtle warblers, regardless of secondary contact and introgression from other taxa. This observation may be related to the unique biogeographic and natural history and of myrtle warbler, at least compared to other members of this group. For instance, myrtle warblers are the only members of this group that occur throughout the Boreal forest, and have a breeding range that extends far into north, including the Yukon and Alaska (Hunt and Flaspohler, 1998). Myrtle warblers also exhibit longer seasonal migratory movements as compared to Audubon’s, black-fronted or Goldman’s.
warblers and there are no known myrtle populations that are nonmigratory, which is not true of the latter groups. Myrtle warblers also have the most unique plumage patterning of the complex, with a white throat that contrasts with the yellow plumage of the other members of the group. Therefore, these genomic regions are good candidates for identifying regions that may contain loci responsible for these or other traits in myrtle warblers.

In conclusion, each of these studies suggest that the effects of hybridization and the patterns from hybrid zones can be useful for studying a number of important evolutionary processes, including the evolution of local adaptation and reproductive isolation. While in many cases it would be ideal to conduct experimental manipulations to address some of the outstanding questions raised by this and other systems, in some cases logistical challenges restrict such endeavours. Here I have correlated natural variation in phenotypic traits, such as mitochondrial respiration and migratory directionality, with genetic markers in wild-caught individuals. While I draw upon a number of different types of data in each case, I have primarily relied upon assays of natural variation to draw inferences. Throughout this thesis I have demonstrated that in many cases, in the absence of experimental data, such observational studies can be an important first step for understanding ecological and evolutionary processes acting in natural populations. In pursuit of such goals, adopting an integrative approach and incorporating a variety of data types will be important to tackling relevant biological questions. This presents a novel and powerful toolbox for future studies of biogeography, evolutionary biology and natural history.
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Appendix 1:
Frequency of all checklists within a given grid cell that identified Audubon’s (A) or myrtle (B) warblers between the months of December – February from 2004-2014. It is important to note that subspecies information is not required for eBird observation submissions. Therefore, the approval of submissions is based solely on whether a “yellow-rumped warbler” is expected in a given area and “Audubon’s” or “myrtle warbler” entries are not usually flagged and vetted for accuracy. This is important because, in basic plumage, the two subspecies can look similar. Thus, the maps should be treated with caution, although they generally agree with those found in curated bird guides.

(A) December - February eBird records for Audubon's warblers

(B) December - February eBird records for myrtle warblers

Image provided by eBird (www.ebird.org) and created 31-Dec-2013
Appendix 2:
Regression of known-origin feather samples from Hobson et al. (2012) with environmental hydrogen. The open points are the values associated with the known-origin samples and the X’s indicate the hydrogen values from the current study (i.e. feathers from unknown locations). The diamond with the whiskers illustrates the estimated breakpoint in the segmented regression (plus confidence intervals). IsoMAP assumes a linear regression for a transfer function between feather hydrogen and environmental hydrogen equivalents. However, as illustrated by the LOESS smoothing function (solid line), a simple linear model does not represent this relationship well. To test this and estimate the best transfer function we used a segmented regression using the ‘segmented’ package in R, which estimates a continuous two-part linear regression as well as the breakpoint between the two functions. This procedure estimates a breakpoint at $-53.6\%$, where below this values are best represented by the function $\delta^{2}H_{f} = 0.5765 \times \delta^{2}H_{p} - 61.34$, higher values represented by $\delta^{2}H_{f} = 1.345 \times \delta^{2}H_{p} - 20.17$. The Davies tests the null hypothesis that the difference between the slopes is zero, which is strongly rejected ($P<0.001$).
Appendix 3:
Panel (A) and (B) illustrate the regression of deuterium values used to estimate wintering latitudes (western and eastern transects, respectively). Points are the deuterium values for each individual at a given capture location. These regressions were used as predictors for the feathers grown during on the breeding grounds at an unknown location. Panel (C) illustrates the values of deuterium for feathers grown on the wintering grounds, illustrating that individuals are wintering at isotopically similar locations regardless of breeding latitude.
Appendix 4:
Cases of mitochondrial-nuclear discordance following secondary contact where clines in mtDNA are wider than nuDNA. In cases of asymmetric discordance, Taxon 1 is the “foreign mtDNA” whereas Taxon 2 is the “native mtDNA”.

<table>
<thead>
<tr>
<th>Taxon 1 (&quot;foreign&quot;)</th>
<th>Common Name</th>
<th>Inferred Process(s)</th>
<th>Geographic Extent</th>
<th>Haplotype Frequency</th>
<th>Reference</th>
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<td>Odocoileus virginianus</td>
<td>White-tailed and mule deer</td>
<td>sex-biased</td>
<td></td>
<td></td>
<td></td>
<td>Cathey et al. 1998</td>
<td></td>
<td></td>
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<tr>
<td>O. hemionus</td>
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<td>Phocoenoides dalli</td>
<td>Porpoise</td>
<td>sex-biased</td>
<td></td>
<td></td>
<td></td>
<td>Willis et al. 2004</td>
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<td>Phocoena phocoena</td>
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<tr>
<td>Taxon 1 (&quot;foreign&quot;)</td>
<td>Taxon 2 (&quot;native&quot;)</td>
<td>Common Name</td>
<td>Inferred Process(s)</td>
<td>Geographic Extent</td>
<td>Haplotype Frequency</td>
<td>Reference</td>
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<tr>
<td><em>Pipistrellus pipistrellus</em></td>
<td><em>P. pygmaeus</em></td>
<td>Common and soprano pipistrelles</td>
<td>sex-biased, demographic</td>
<td>•</td>
<td>•</td>
<td>Hulva et al. 2010</td>
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<tr>
<td><em>Praomys daltoni</em> &quot;Clade A&quot;</td>
<td><em>P. daltoni</em> &quot;Clade C2&quot;</td>
<td>Dalton's mouse</td>
<td>sex-biased, demographic</td>
<td>•</td>
<td>•</td>
<td>Bryja et al. 2010</td>
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<td><em>Sorex araneus</em></td>
<td><em>S. granarius</em></td>
<td>European common shrew</td>
<td>adaptive, sex-biased, demographic</td>
<td>•</td>
<td>•</td>
<td>Yannic et al. 2010</td>
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<tr>
<td><em>Spermophilus erythrogenys</em></td>
<td><em>S. major</em></td>
<td>Red-cheeked and russet ground squirrel</td>
<td>none given</td>
<td>•</td>
<td>•</td>
<td>Spiridonova et al. 2006</td>
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<td><em>Tamias ruficaudus</em></td>
<td><em>T. amoenus</em></td>
<td>Red-tailed and yellow pine chipmunk</td>
<td>adaptive, demographic</td>
<td>•</td>
<td>•</td>
<td>Reid et al. 2010</td>
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<td><em>Tamiasciurus douglasi</em></td>
<td><em>T. hudsonicus</em></td>
<td>Douglas and American red squirrels</td>
<td>sex-biased, demographic</td>
<td>•</td>
<td>•</td>
<td>Chavez et al. 2011</td>
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<tr>
<td><em>Thomomys bottae ruidosae</em></td>
<td><em>T. b. actuosus</em></td>
<td>Pocket gophers</td>
<td>demographic, HZ movement</td>
<td>•</td>
<td>•</td>
<td>Ruedi et al. 1997</td>
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<tr>
<td><em>Ursus arctos</em></td>
<td><em>U. maritimus</em></td>
<td>Brown bear and polar bear</td>
<td>sex-biased</td>
<td>•</td>
<td>•</td>
<td>Edwards et al. 2011</td>
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**REPTILES**

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<tr>
<th>Common Name</th>
<th>Inferred Process(s)</th>
<th>0-50%</th>
<th>50-95%</th>
<th>&gt;95%</th>
<th>0-50%</th>
<th>50-95%</th>
<th>&gt;95%</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Anolis distichus ravitergum</em> A. d. ignigularis</td>
<td><em>Anolis</em> lizards</td>
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<td>•</td>
<td>•</td>
<td></td>
<td></td>
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<td>Ng &amp; Glor 2011</td>
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<td><em>Crotaphytus collaris</em> C. bicinctores</td>
<td>Common and Great Basin collared lizard</td>
<td>adaptive, demographic</td>
<td>•</td>
<td>•</td>
<td></td>
<td></td>
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<td>McGuire et al. 2007</td>
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<td><em>Crotaphytus collaris</em> C. reticulatus</td>
<td>Common and reticulate collared lizard</td>
<td>adaptive</td>
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<td>McGuire et al. 2007</td>
</tr>
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<td><em>Ctenosaura pectinata</em> &quot;Colima–Balsas clade&quot;</td>
<td>Mexican black iguana</td>
<td>demographic</td>
<td>•</td>
<td>•</td>
<td></td>
<td></td>
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<td>Zarza et al. 2011</td>
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<td><em>C. pectinata</em> &quot;north C &amp; D clade&quot;</td>
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<td><em>Emys orbicularis</em></td>
<td><em>E. o. fritzjuergenobsti, E. o. galloitalica</em></td>
<td>European pond turtle</td>
<td>sex-biased, demographic</td>
<td>•</td>
<td>•</td>
<td></td>
<td></td>
<td>Pedall et al. 2010</td>
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<tr>
<td><em>Podarcis spp.</em> (not sampled) P. hispanicus, P. liolepis</td>
<td>Iberian wall lizard</td>
<td>adaptive</td>
<td>•</td>
<td>•</td>
<td></td>
<td></td>
<td></td>
<td>Renoult et al. 2009</td>
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<td>Taxon 1 (&quot;foreign&quot;)</td>
<td>Taxon 2 (&quot;native&quot;)</td>
<td>Common Name</td>
<td>Inferred Process(s)</td>
<td>Geographic Extent</td>
<td>Haplotype Frequency</td>
<td>Reference</td>
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<tr>
<td>Sceloporus tristichus &quot;northern clade&quot;</td>
<td>S. tristichus &quot;southern clade&quot;</td>
<td>Fence lizard</td>
<td>HZ movement</td>
<td>・</td>
<td>・</td>
<td>Leache &amp; Cole 2007</td>
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| Carcinus maenas  
C. aestuarii | Mesobuthus gibbosus  
"Anatolian type"  
M. gibbosus "greek type" | Green crabs  
Blue and Mediterranean mussel | sex-biased  
adaptive | ・ | ・ | ・ | Darling 2011  
Gantenbein & Largiader 2002 |
| Mytilus edulis  
M. galloprovincialis | Mytilus edulis  
M. trossulus  
O. propinquus  
Strongylocentrotus pallidus  
S. droebachiensis | Blue and bay mussel  
Rusty northern and clearwater crayfish  
Sea urchins | adaptive, sex-biased  
sex-biased, human  
sex-biased, demographic | ・ | ・ | ・ | Rawson & Hilbish 1998  
Quesada et al. 1999  
Perry et al. 2001  
Addison & Hart 2005 |
Appendix 5:
Cases of mitochondrial-nuclear discordance following secondary contact where clines in mtDNA are narrower than nuDNA.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Animal Group</th>
<th>Common Name</th>
<th>nuDNA Introgression</th>
<th>Sex-biased</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Rana blairi</em></td>
<td>Amphibian</td>
<td>Plains and northern leopard frog</td>
<td>•</td>
<td>•</td>
<td>Di Candia <em>et al.</em> 2007</td>
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<tr>
<td><em>R. pipiens</em></td>
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<tr>
<td><em>Hippolais icterina</em></td>
<td>Bird</td>
<td>Icterine and melodious warbler</td>
<td>•</td>
<td></td>
<td>Secondi <em>et al.</em> 2006</td>
</tr>
<tr>
<td><em>H. polyglotta</em></td>
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</tr>
<tr>
<td><em>Larus spp.</em></td>
<td>Bird</td>
<td><em>Larus</em> Gulls</td>
<td>•</td>
<td>•</td>
<td>Crochet <em>et al.</em> 2003</td>
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<tr>
<td><em>Parus minor</em></td>
<td>Bird</td>
<td>Japanese and great tit</td>
<td></td>
<td>•</td>
<td>Kvist &amp; Rytkonen 2006</td>
</tr>
<tr>
<td><em>P. major</em></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Passerina cyanea</em></td>
<td>Bird</td>
<td>Indigo and lazuli bunting</td>
<td></td>
<td>•</td>
<td>Carling &amp; Brumfield 2008</td>
</tr>
<tr>
<td><em>P. amoena</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phylloscopus collybita</em></td>
<td>Bird</td>
<td>Iberian and common chiffchaff</td>
<td>•</td>
<td></td>
<td>Bensch <em>et al.</em> 2006</td>
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<tr>
<td><em>P. c. brehmii</em></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td><em>Setophaga petechia</em></td>
<td>Bird</td>
<td>Yellow warbler</td>
<td>•</td>
<td></td>
<td>Gibbs <em>et al.</em> 2000</td>
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<tr>
<td>“west”</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Basley &amp; Bernatchez 2001</td>
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<tr>
<td><em>S. petechia</em> “east”</td>
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<tr>
<td><em>Coregonus clupeaformis</em></td>
<td>Fish</td>
<td>Lake whitefish</td>
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<td><em>Limenitis arthemis</em></td>
<td>Insect</td>
<td>Admiral butterflies</td>
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<td>Mullen <em>et al.</em> 2009</td>
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<td><em>astyanax</em></td>
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<td><em>L. a. arthemis</em></td>
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<tr>
<td><em>Macaca mulatta</em></td>
<td>Mammal</td>
<td>Rhesus and long-tailed macaque</td>
<td>•</td>
<td>•</td>
<td>Bonhomme <em>et al.</em> 2008</td>
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<td><em>M. fascicularis</em></td>
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<tr>
<td><em>Microtus arvalis</em></td>
<td>Mammal</td>
<td>Common vole</td>
<td>•</td>
<td></td>
<td>Braaker &amp; Heckel 2009</td>
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<tr>
<td><em>Pongo pygmaeus</em></td>
<td>Mammal</td>
<td>Orang-utan</td>
<td>•</td>
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<td>Nietlisbach <em>et al.</em> 2012</td>
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<tr>
<td><em>Sorex antinorii</em></td>
<td>Mammal</td>
<td>Valais shrew</td>
<td>•</td>
<td></td>
<td>Yannic <em>et al.</em> 2012</td>
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</table>
### Appendix 6:
Accession numbers, primer information and PCR conditions for mtDNA sequencing.

<table>
<thead>
<tr>
<th>Genes (percent coverage)</th>
<th>Primer Names</th>
<th>JE14T03 (S)</th>
<th>JE20T02 (S)</th>
<th>JE30T01 (S)</th>
<th>JF05T01 (N)</th>
<th>JF11T02 (N)</th>
<th>JF14T03 (N)</th>
<th>Annealing Temperature</th>
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<tbody>
<tr>
<td>ND1 (100%)</td>
<td>L3722-H5201</td>
<td>KC977564</td>
<td>KC977565</td>
<td>KC977566</td>
<td>KC977567</td>
<td>KC977568</td>
<td>KC977569</td>
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<tr>
<td>ND2 (96%)</td>
<td>Metb-TRPc</td>
<td>KC991020</td>
<td>KC991021</td>
<td>KC991022</td>
<td>KC991023</td>
<td>KC991024</td>
<td>KC991025</td>
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<tr>
<td>COX1 (41%) - COX2 (20%)</td>
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<td></td>
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<tr>
<td>ATP8 (100%)</td>
<td>L7525-H8628</td>
<td>KF010613</td>
<td>KF010614</td>
<td>KF010615</td>
<td>KF010616</td>
<td>KF010617</td>
<td>KF010618</td>
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<td>ND2 (96%)</td>
<td>L8929-H9855</td>
<td>KF010619</td>
<td>KF010620</td>
<td>KF010621</td>
<td>*</td>
<td>KF010622</td>
<td>KF010623</td>
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<tr>
<td>ATP6 (100%) - ATP8 (100%)</td>
<td>L9700-H10343</td>
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<td>KF005587</td>
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<td>KF005589</td>
<td>KF005590</td>
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<td>COX3 (100%) - ND3 (37%)</td>
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<td>ND4 (95%)</td>
<td>KF010607</td>
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<td>KF010609</td>
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<td>ND4 (95%)</td>
<td>KF010624</td>
<td>KF010625</td>
<td>KF010626</td>
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<td>KF010628</td>
<td>KF010629</td>
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<td>ND5 (48%)</td>
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<td>KF010626</td>
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<td>KF010628</td>
<td>KF010629</td>
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<td>Cytb (97%)</td>
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