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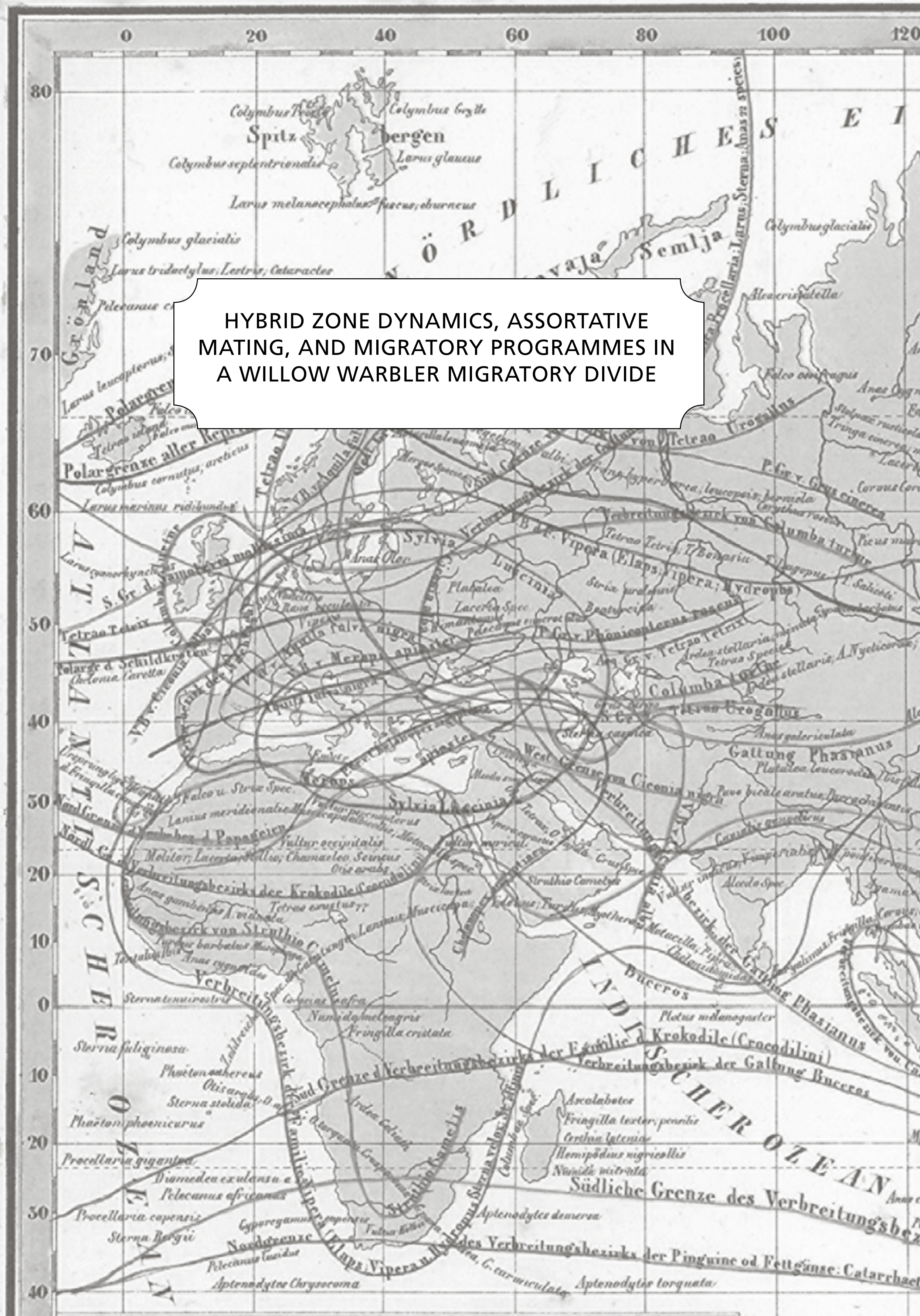
Keith W. Larson

Department of Biology, Evolutionary Ecology, Lund University 2012

HYBRID ZONE DYNAMICS, ASSORTATIVE MATING, AND MIGRATORY PROGRAMMES IN A WILLOW WARBLER MIGRATORY DIVIDE



Hybrid zone dynamics, assortative mating, and migratory programmes in a willow warbler migratory divide

Keith W. Larson

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Faculty opponent:
Priv.-Doz. Dr. H. Martin Schaefer
University Freiburg, Biology I
Freiburg, Germany

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Keith W. Larson 2012
e-mail: keith.w.larson@gmail.com

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Nature, rightly questioned, never lies.
Manual for Scientific Enquiry, Sir John F. W. Herschel (1859).

Organization
LUND UNIVERSITY
Department of Biology, Section for Evolutionary Ecology, Ecology Building
SE-223 62 Lund, SWEDEN

Author(s)
Keith W. Larson

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Abstract

In this thesis I will present and contrast the two willow warbler subspecies (*Phylloscopus trochilus trochilus* and *P. t. acredula*) or migratory phenotypes in the context of their migratory divide and hybrid zone in central Sweden. In the first paper I investigate the role of population abundance in determining the location of the hybrid zone. Specifically, is there a region of low abundance associated with the hybrid zone? Further, is the hybrid zone located on an environmental gradient which might suggest that breeding ground environmental conditions are responsible for the lower abundance? This lower abundance may reflect the unsuitability of habitats along the environmental gradient for either parental or hybrid offspring. In my second paper, I ask if there are population specific differences in their wintering moult ecology that can be elucidated from diet derived stable isotope patterns in their winter moulted primary flight feathers? The third paper addresses the important question, does assortative mating lead to reproductive isolation or do these very similar subspecies hybridize and produce offspring? In my fourth paper, I ask does local adaptation to environmental conditions, such as temperature extremes and the short growing season, in mountain populations of willow warblers explain the apparent distribution of the "northern-allele" for the AFLP derived genetic marker WW1? Finally, in the fifth paper, I conduct a detailed analysis of phenotypic traits at 50 sites across the hybrid zone, including 35 sites visited more than once. Here I ask, does lower abundances in the west of the hybrid zone predict the zone to be wider in the west than in the east? Further, using data from repeated visits to sites across the zone, we predict low repeatabilities for migratory associated traits that would suggest that high annual turn-over in migratory phenotypes occupying the zone.

To better understand the hybrid zone dynamics it will be essential to develop genetic markers that allow one to separate each parental migratype, hybrids, and backcrosses. Once markers allow the identification of hybrid offspring, orientation experiments should be conducted to elucidate migratory directional preferences that would support our hypothesis that hybrids take an intermediate migratory direction to their parental migratypes.

Key words: migratory divide, phenotype, willow warbler, hybrid zone, tension zone, *Phylloscopus trochilus*, migratype, assortative mating, migration

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List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I Reduced abundance in a migratory songbird hybrid zone: evidence for selection against hybrids outside the breeding area (*submitted Journal of Evolutionary Biology*)

II Inferring the ecology of winter moult by stable isotope analyses across the wings of willow warblers (*manuscript*)

III No evidence for assortative mating between two distinct migratypes across a willow warbler migratory divide (*manuscript*)

IV Climate exerts direct selection on genomic region in a mountain living bird (*manuscript*)

V Phenotypic turn-over and hybrid zone dynamics in the willow warbler migratory divide (*manuscript*)

Introduction

The journey begins looking for the ground nest for a small eight to ten gram song bird breeding in the mountain birch forests in Jämtland, Sweden. Each May migrants pour into the Scandinavian Peninsula having endured a long migration from their wintering grounds in Africa. Here at my study sites in the province of Jämtland we find that some of our birds had spent the winter in the arid savannah of West Africa, while others had been somewhere on the east side of the African continent. These birds from West Africa migrated north through the Iberian Peninsula across the densely populated landscapes of Western Europe, and likely crossed the narrow straits of the Baltic Sea between Denmark and Sweden. The birds that wintered in eastern Africa migrated north on the east side of the Mediterranean Sea through the Balkans and central Europe, passed through Finland and into Sweden crossing the Baltic Sea. Thus this region represents a migratory divide and hybrid zone for two subspecies with different migratory programmes making it an ideal region to study evolution in action.

Each individual's journey shaped by its evolutionary trajectory through the past, migration routes reflecting the post-glacial expansion routes out of their ancestor's glacial refugium and optimized over generations. Generation-after-generation migration routes refine as dispersal, genetic drift, mutation, and adaption work in concert to optimize fitness. For example, early in the Holocene the Scandinavia Peninsula and continental Europe were continuous landscapes because of lowered sea levels. Similarly, the Baltic Sea ranged from a fresh water lake to a brackish sea due to changes in sea level and isostatic rebound of the earth's plates as the ice sheets retreated. These changing conditions mean that where once the willow warbler migrated across a continuous terrestrial landscape they today cross the Öresund Strait in to southern Sweden from Denmark and in the north they cross the Baltic Sea to reach the Peninsula.

The accumulated knowledge of their ecology is a reflection of the efforts of a long succession of biologists, naturalists, bird watchers, and those who found bird migration a phenomenon worth studying. I have spent the past five summers hiking around the forests on central Sweden listening for the song of one of the most abundant song birds of Northern Europe, the willow warbler (*Phylloscopus trochilus*). Observing, capturing and measuring the variability of hundreds of individuals each summer, my aim to unravel one part of the story of the willow warbler's migration and breeding biology. Specifically, what are the causes and consequences of the divergence in migratory programs on their breeding dynamics where they meet in a narrow migratory divide?

Linnaeus recognized the willow warbler as two species, *Motacilla trochilus* and *M. acredula*, based on colouration and size (Linnaeus 1759). This determination was not made from individuals in the hand captured in a mist-net, but from published descriptions of specimens from England and examining others from the Uppsala region of Sweden (Figure 1). For the next 150 years, naturalists across Europe described many willow warbler species, subspecies, and varieties, largely based on colouration, measurements, and geographic location, each claiming priority in a quest to describe the diversity of life (Ticehurst 1938). By 1928, Danish ornithologist, Finn Salomonsen observed differences in their migratory behaviours based on wing length and timing of passage (Salomonsen 1928). As our knowledge accumulated about the distribution of these taxa, their biology, migratory tendencies, it became clear that they more likely reflected a cline in these previously described traits. Today we recognize them as two subspecies, *P. t. trochilus* and *P. t. acredula* based largely on differences in their migratory programs (Hedenström and Pettersson 1987, Bensch 1999, Chamberlain *et al.,* 2000).

By the 1980's, hundreds of thousands of willow warblers from both subspecies, reflecting both migratory programs, were ringed across Sweden. Detailed analysis of ringing recoveries further elucidated the dramatic differences in

Figure 1. Colour variability in willow warblers across a latitudinal cline. Typical grey "*acredula*" like willow warbler with more dull plumage from the north (left), contrasting with brighter yellowish plumage colouration of the southern "*trochilus*" subspecies (right). Despite variation in several traits, such as colour, we cannot separate individuals to subspecies in the hand. Photograph taken by Max Lundberg in Ljungdalen, Sweden, on 19 May 2011.

the migratory directions and putative wintering grounds for each subspecies (Hedenström and Pettersson 1984, 1987) (Figure 2). Most importantly this analysis showed that the subspecies' migratory programs resulted in a migratory divide in central Sweden, with the southern *trochilus* migrating southwest through Western Europe and the Iberian Peninsula to winter in West Africa. To the north, *acredula*, migrated south-southeast through central Europe and the Balkan Peninsula to winter in East to South Africa.

Later investigations into the migratory behaviours sought to quantify genetic differences between the two subspecies, built on the knowledge that these small passerines undergo a phenomenal migration each year to Africa and back based on their endogenously controlled migratory programs (Berthold and Quer 1981, Helbig 1996). Results of genetic analyses revealed that despite the clear differences in their endogenous migratory programs, both subspecies were panmictic for mitochondrial and several nuclear DNA regions, supporting a more recent divergence (Bensch 1999, Bensch *et al.,* 2009a).

The migratory divide in central Sweden thus represents a secondary contact zone where these previously allopatric or isolated subspecies that had diverged in migratory behaviour, meet, potentially mate and produce hybrid offspring. In this thesis I will present and contrast the two willow warbler subspecies or migratory phenotypes in the context of their migratory divide and hybrid zone in central Sweden. In the first paper I investigate the role of population abun-

Figure 2. Range map for the willow warbler depicting both the Eurasian breeding ground and the sub-Saharan African wintering grounds. The latitudinal migratory divide across Scandinavia is approximated with a black dotted line. The migratory routes of the southern willow warbler, *P. t. trochilus*, and northern, *P.t. acredula*, are generalized and depicted with arrows to indicate migration routes and west versus eastern wintering grounds in Africa of both subspecies.

dance in determining the location of the hybrid zone. Specifically, is there a region of low abundance associated with the hybrid zone? Further, is the hybrid zone located on an environmental gradient which might suggest that breeding ground environmental conditions are responsible for the lower abundance? This lower abundance may reflect the unsuitability of habitats along the environmental gradient for either parental or hybrid offspring. In my second paper, I ask if there are population specific differences in their wintering moult ecology that can be elucidated from diet derived stable isotope patterns in their winter moulted primary flight feathers? The third paper addresses the important question, does assortative mating lead to reproductive isolation or do these very similar subspecies hybridize and produce offspring? In my fourth paper, I ask does local adaptation to environmental conditions, such as temperature extremes and the short growing season, in mountain populations of willow warblers explain the apparent distribution of the "northern-allele" for the AFLP derived genetic marker WW1? Finally, in the fifth paper, I conduct a detailed analysis of phenotypic traits at 50 sites across the hybrid zone, including 35 sites visited more than once. Here I ask, does lower abundances in the west of the hybrid zone predict the zone to be wider in the west than in the east. Further, using data from repeated visits to sites across the zone, we predict low repeatabilities for migratory associated traits that would suggest that high annual turn-over in migratory phenotypes occupying the zone.

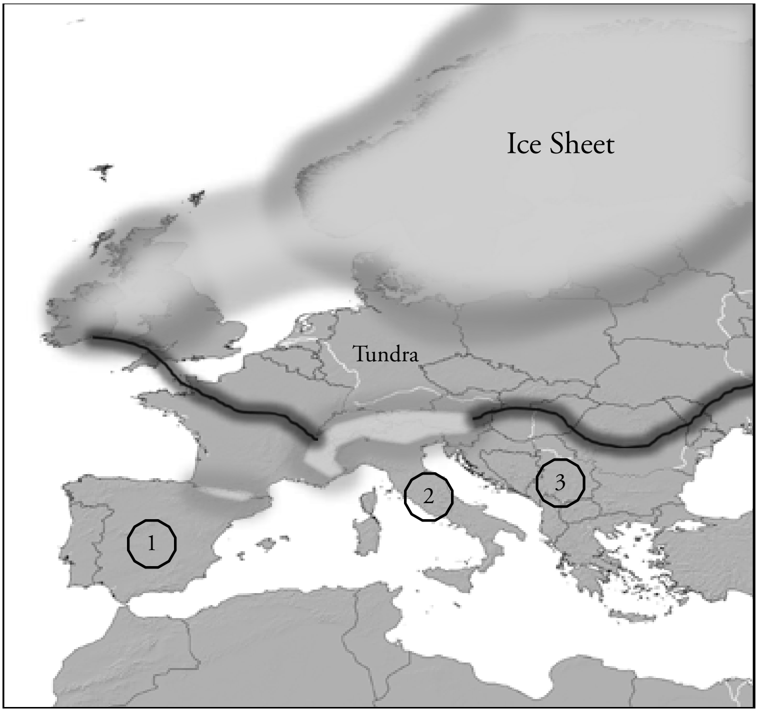

Figure 3. Maximum extent of the ice sheets during the Last Glacial Maximum, approximately 21,000 years ago. Black lines represent approximation of the southern-most limits of the tundra and steppe habitats characterized by permafrost. The hypothesized peninsular glacial refugia (circled numbers) are located on the (1) Iberian, (2) Apennine, and (3) Balkan Peninsulas. Note that the lower sea levels of this period are not illustrated. Map redrawn from (Taberlet *et al.,* 1998, Hewitt 1999).

Background

Hybrid zones and the legacy of the ice ages

The range of any species often includes subspecies, populations, ecotypes, or otherwise phenotypically varying forms that result from genetic differentiation through drift, point mutation, and local adaptation, resulting in population divergence (Hewitt 1988). In species with diverse geographic forms, range expansion can lead to secondary contact. If reproductive isolation is incomplete between divergent forms, a hybrid zone or narrow region of parapatry develops where genetically distinct populations, meet, mate, and produce hybrids (Barton and Hewitt 1985, 1989). Since the development of theoretical models to understand the role of hybrid zones in reproductive isolation and speciation, biologists have turned to them 'as natural laboratories for studying evolution in action' (Hewitt 1988, Harrison 1993).

Secondary contact as the result of range expansion by allopatric forms of a species has occurred repeatedly as climates oscillate between cold and warm phases (Taberlet *et al.,* 1998, Hewitt 1999, 2004, 2011). In particular, range limits for species with northern distributions have expanded and contracted with the ebb and

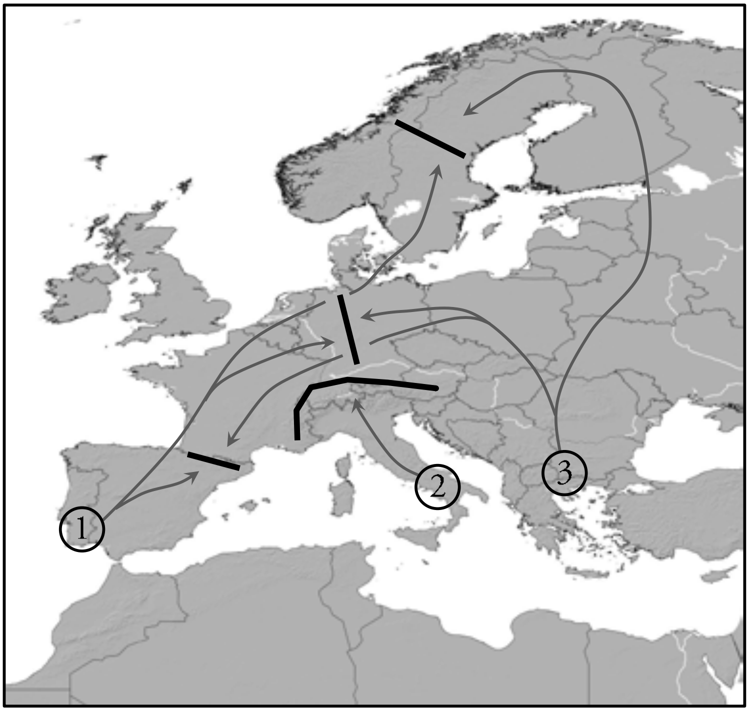

Figure 4. Post-glacial expansion routes after the Last Glacial Maximum. Black lines represent main European suture or secondary contact zones and dark grey lines with arrows the hypothesized expansion routes from the (1) Iberian, (2) Apennine, and (3) Balkan Peninsulas. Map redrawn from (Taberlet *et al.,* 1998, Hewitt 1999).

flow of continental ice sheets during the Quaternary Period. Deep divergence in DNA for many taxa suggest that repeated periods of allopatry plays an important role in speciation (Hewitt 2004). During cold phases extensive glaciers covered high latitudes and mountain ranges, populations contracted to lower latitudes where suitable climate conditions and land surfaces emerged due to lower sea levels (Hewitt 2011). These range contractions resulted in one or more refugia for retreating populations (Taberlet *et al.,* 1998, Hewitt 2011).

During the last glacial maximum (approximately 18 to 21 thousand years ago) terrestrial species often used peninsular refugia across southern Europe (i.e., Iberian, Appenine, and Balkan Peninsulas) (Figure 3). In Europe, glaciers extended south to Germany (52° N) and permafrost and periglacial conditions extended from France to the Caspian Sea and eastward (Hewitt 1999). As the glaciers retreated, previously allopatric populations expanded north and frequently met in secondary contact (Taberlet *et al.,* 1998, Hewitt 1999). Across Europe several regions contain hot spots of secondary contact for a diversity of species (Taberlet *et al.,* 1998, Hewitt 1999). When numerous secondary contact zones overlap in geographically distinct regions, they are described as 'suture zones' (Taberlet *et al.,* 1998, Hewitt 1999, Swenson and Howard 2004) (Figure 4).

One such suture zone occurs on the Scandinavian Peninsula in Northern Europe. Here the brown bear (*Ursus arctos*), meadow grasshopper (*Chorthippus parallelus*), common shrew (*Sorex araneus*), willow warbler (*Phylloscopus trochilus*), and other species expanded both from Denmark to the south and Finland in the northeast (Taberlet *et al.,* 1998, Bensch 1999, Hewitt 1999). The southern willow warbler subspecies, *P. t. trochilus*, may have reached Scandinavia as early as 11,5000 years ago when the ice sheet had contracted to the mountainous regions of the northwest and forest conditions returned to the peninsula (Ericsson and Tyrberg 2004). Pollen evidence for key habitat components such as birch and willow and lower sea levels around the peninsula suggest suitable habitats for this long-distance migratory passerine (Backéus 1999). It is unknown when the northern subspecies, *P. t. acredula*, arrived in northern Scandinavia, but suitable habitat conditions likely existed for at least several thousand years. By 7,500 years ago, pollen evidence suggests that extensive broadleaf and coniferous forests covered the peninsula, except in high elevation habitats where glacial and periglacial conditions persisted (Backéus 1999). Although we do not know exactly when the two subspecies of the willow warblers came into secondary contact, willow warblers (not the subspecies) were described as common across the whole of Sweden by the early 19th century (Nilsson 1817).

Migratory Divides

Bird migration is the regular spatial and temporal movements of a population in response to gradients in environmental conditions. At northern latitudes, migration occurs with the onset of the boreal winter when food shortages and inhospitable conditions result in the regular movement of individuals from their breeding to their southern wintering grounds (Newton 2007).

The subset of population-specific phenotypic traits that characterize a migratory strategy, namely, the onset, duration, and direction of migration define the migratory phenotype, or "migratype" (see Paper 2). When phenotypically divergent migratory forms of a species meet in secondary contact, a migratory divide develops, where selection maintains phenotypic differences. In the central European avian suture zone, several species of migratory birds meet and form migratory divides as a result of post-glacial expansion by divergent populations from southern peninsular glacial refugia (Møller *et al.,* 2011). Detailed studies of ringing recoveries from a diversity of species from white storks to wagtails revealed a general SW- and SE-orientation of autumn migrants that winter in West and East Africa (Helbig 1996). Divergence in migratory direction and location of wintering grounds likely reflects both the post-glacial expansion routes from southern European refugia and avoidance of ecological barriers such as The Alps, Mediterranean Sea, and the Sahara Desert at their widest stretches. For example, evidence from ringing recovery data and orientation experiments revealed significant differences in migratory direction of Eurasian blackcaps (*Sylvia atricapilla*) across a migratory divide in central Europe (Helbig 1991a, 1992) (Figure 5).

In the Scandinavian suture zone, the willow warbler secondary contact zone is defined by their migratory divide (Hedenström and Pettersson 1987, Bensch 1999, Chamberlain *et al.,* 2000). Within this region, willow warblers represent two subspecies with different migratory traits. Salomonsen (1928) was the first to observe these differences in the timing of spring migrants returning to breed in Fennoscandia. Later work at bird observatories across Sweden further identified differences in the arrival dates by spring migrants, with *acredula* arriving on average 12 days later than *trochilus* (Hedenström and Pettersson 1984). Analysis of extensive ringing recoveries throughout Europe, with only a few from Africa, revealed the migratory divide placing it on the map in central Sweden at approximately 60° N (Hedenström and Pettersson 1987, Bensch *et al.,* 1999a, Chamberlain *et al.,* 2000).

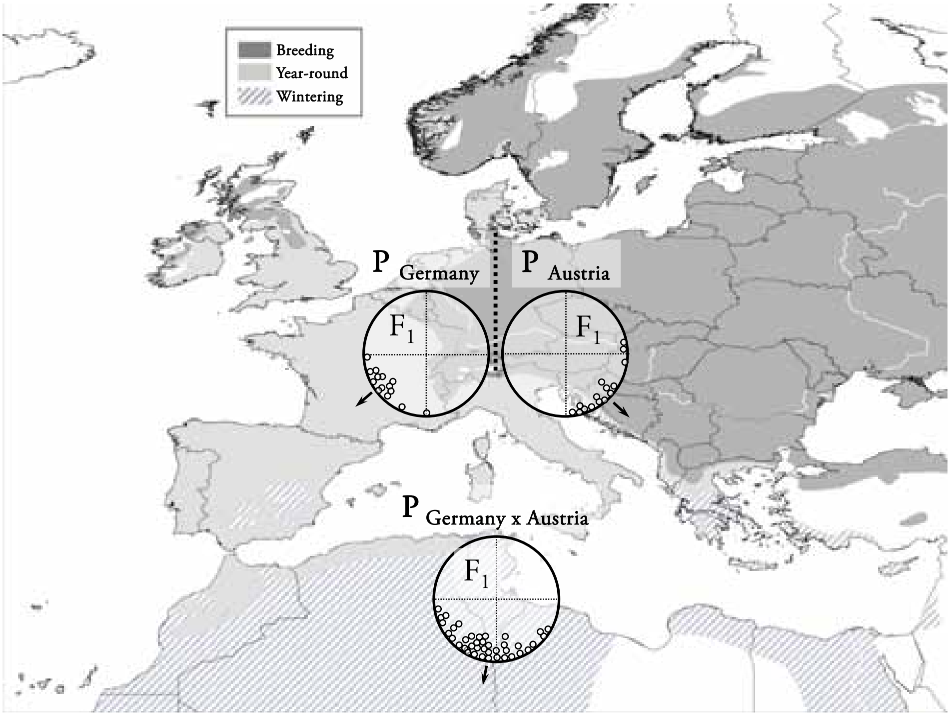

Figure 5. Eurasian Blackcap (*Sylvia atricapilla*) selection and cross-breeding experiments across the central European migratory divide (depicted as dotted line). Parental (P) German populations migrate southwest to the Iberian Peninsula and Western Africa, while Austrian populations migrate southeast through the Balkan Peninsula to Eastern Africa. Laboratory selection and cross-breeding experiments between the German and Austria populations lead to F1 hybrid offspring showing a mean intermediate migratory direction relative to parental populations during orientation experiments, selectively bread young (upper circles) show the same migratory direction as their respective parental phenotype. Black arrows represent mean direction of selectively (upper circles) and cross-bred (lower circle) offspring during autumn orientation experiments. Modified from (Helbig 1991).

Hybrid Zone Theory

As previously mentioned, hybrid zones represent regions where genetically distinct populations meet, mate and produce hybrid offspring (Barton and Hewitt 1985, 1989). In these zones, phenotypic stability is maintained despite neutral gene flow (Barton and Hewitt 1989). The strength of selection and dispersal capacity of the parental populations determines the shape and width of the cline. One of three theoretical scenarios can be invoked to explain the existence of steep phenotypic clines: (1) The neutral diffusion hypothesis (Endler 1977, Barton and Gale 1993), (2) the bounded hybrid superiority hypothesis (Moore 1977, Moore and Buchanan 1985), and (3) the tension zone hypothesis (Barton and Hewitt 1985).

(1) The neutral diffusion hypothesis predicts that in the absence of barriers to gene flow, steep character clines that developed during previous periods of allopatry will decay over time leading to panmixia. The resulting clines are wide relative to root-mean-square (RMS)

dispersal distance. This measure of dispersal is the displacement from the natal site to the first breeding location of an individual (Ruegg 2008). The RMS is the square root of the sum of the individual dispersal distances squared, divided by the number of individuals (Moore and Buchanan 1985).

(2) The bounded hybrid superiority hypothesis predicts that hybrid zones that fall on ecotones (i.e. an environmental gradient between two habitats) may result in hybrid populations with greater fitness than either parental population, i.e. ecological selection favours hybrids and discriminates against parental forms within the hybrid zone. Under this model the width of the zone is correlated with the width of the ecotone or ecological gradient (Moore and Price 1993).

(3) The tension zone hypothesis predicts that the hybrid zone represents a balance between dispersal of parental populations into the center of the zone and selection against hybrids (Barton and Hewitt 1985). Multiple coincident trait clines (which are narrow relative to RMS dispersal distance) characterize these tension zones. The result is often low population density in the center of the zone or a region of low abundance (Barton and Hewitt 1985, Barton and Gale 1993). In a tension zone the will shift towards regions of low dispersal, low hybrid fitness, and low population density. In cases where the one of the parental forms is not equally fit, the zone will move in the direction of the fitter phenotype (Barton 1979, Barton and Hewitt 1985, Barton and Gale 1993). Further, in the tension zone model, selection can be either intrinsic which leads to reduced hybrid fitness, or extrinsic, where ecological conditions act as the agent of selection against hybrids (Bridle *et al.,* 2001, Phillips *et al.,* 2004, Alexandrino *et al.,* 2005).

Regardless of the mode, selection must be strong enough to overcome the homogenizing effect of gene flow (Endler 1973). This is especially true for organisms such as birds that often have very large dispersal distances. In migratory divides this selection could be pre-zygotic (breeding) in that assortative mating leads to reproductive isolation between parental phenotypes. Assortative mating can be either ecological or non-ecological, e.g. by differential timing of arrival on the breeding grounds between two distinct migratory populations or sexual selection. Post-zygotic selection occurs either intrinsically through lower hybrid fitness, such as hybrid dysfunction. For example, pied and collared flycatchers (*Ficedula hypoleuca* and *F. albicollis*, respectively) in a hybrid zone on the island of Gotland, F_1 hybrid males experience reduced fecundity while surviving females are infertile (Veen *et al.,* 2001). Alternatively, post-zygotic selection can occur when environmental conditions provide the mode of selection that reduces hybrid fitness. In this case, over time as a tension zone moves in the direction that minimizes its width, the zone can become trapped on environmental gradients or ecotones, where neither parental form is fit and low population or a density trough forms (Barton and Hewitt 1989, Barton and Gale 1993, Moore and Price 1993).

Based on previous analyses, post-zygotic selection for willow warblers is hypothesized with hybrid offspring having an intermediate migratory direction to the parental migratypes (Bensch *et al.,* 1999a, 2009a). This intermediate migratory program would result in a non-adaptive intermediate migratory route not taken by either of the parental populations, which would lead directly over The Alps, Mediterranean Sea, and Saharan Desert. Presumably the lack of stopover sites and adaptations for long-distance non-stop flight and the necessary fat storage adaptations would severely limit the fitness of migrating willow warbler hybrid offspring. This is indeed plausible as in cross-breeding experiments carried out on European blackcaps, F_1 hybrids demonstrated intermediate migratory directional preferences under experimental conditions (Helbig 1991b).

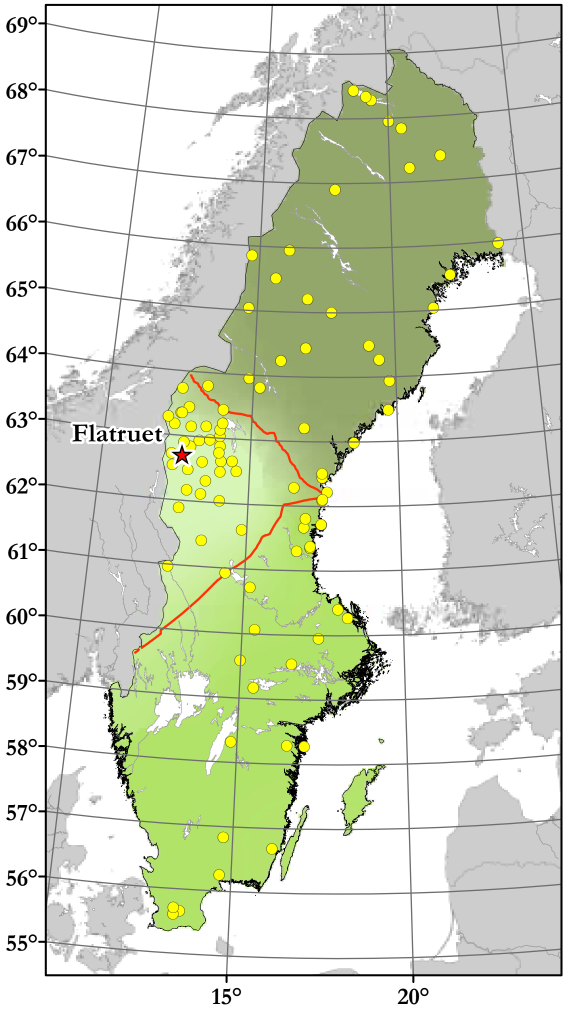

Figure 6. The map depicts the breeding range across Sweden with the *acredula* subspecies (dark olive) in the north and *trochilus* subspecies (light green) in the south. Red lines approximate the north and south boundary of the migratory divide that creates a hybrid zone in central Sweden. Yellow circles are study sites sampled between 1996 and 2011, with the red star Flatruet (62.7° N, 12.7° E, 846 meters ASL) the study site where intensive studies on assortative mating were carried out in 2011 and 2012.

Methods

Field studies

During the breeding seasons of 2008 to 2010 we intensively sampled territorial male willow warblers throughout the hybrid zone (61.4° N and 64.1° N). Specifically, we sampled 50 sites and invested repeated efforts at 36 sites to assess the genotypic and phenotypic variation of local populations and to characterize the phenotypic distribution across the zone. For the 2011 and 2012 breeding seasons we focused on a single site in the center of the hybrid zone to establish a colour-ringed population that allowed us to assess assortative mating. From late May to mid-July we carried out territory mapping and nest searching activities at the Flatruet breeding site (62.7° N, 12.7° E, and 846 meters above sea level) in the north-to-south center of the hybrid zone (Figure 6). We selected this study site based on a careful assessment of our dataset collected between 2008 to 2010 which indicated this site was not only geographically close to the center of the previously identified genotypic and phenotypic trait clines (Bensch *et al.,* 1999a, 2009a), but also had a similar frequency of both willow warbler migratypes.

We captured all birds using mist-nets with an audio-lure of a conspecific song that was presented to all individuals over the entire period of our study, 1996 to 2011. We followed previous established protocols (Bensch *et al.,* 1999a, 2009a, Chamberlain *et al.,* 2000) to collect four morphological measurements (i.e. wing chord, whole tail, tarsus, and bill-head), assess plumage colouration, sample blood for subsequent genetic analyses and collect the first primary flight feather from each wing to assess wintering ground stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes for all individuals captured. Methods of analyzing these isotopes from feathers are well described (Chamberlain *et al.,* 2000, Bensch *et al.,* 2006) and DNA genotyping of the previously identified bi-allelic AFLP molecular markers (WW1 and WW2) (Bensch *et al.,* 2002, 2009a).

Over the 2011 and 2012 breeding seasons we identified all willow warbler territories held within our Flatruet study site, attempted to capture all males and their social mates and locate their nests (Figure 6). In this study, we captured males and a few females (exhibiting solicitation behaviour in response to audio-lure) using mist-nets and the same conspecific song recording. Typically, we trapped most females at the nest during the chick rearing period when females and males are feeding young and give contact calls when approaching the nest. Each individual received a unique combination of metal and colour-rings to facilitate resighting throughout the breeding season. We identified social mates as females by the presence of a brood patch, during incubation, brooding, or feeding nestlings at the nest attended by their respective male partners. Confirmation of pairs occurred on breeding territories by visual observation of colour-rings or by direct capture at the nest.

Stable Isotopes

Stable isotope analysis of consumer tissues reveals dietary history and both abiotic and biotic processes encountered while foraging (Kelly 2000, Rubenstein and Hobson 2004). For example, $\delta^{13}C$ values in plants increase with altitude due to plant responses to the stress of decreases in temperature (Kelly 2000, Graves *et al.,* 2002, Hobson *et al.,* 2003). These values also increase in xeric (arid) habitats compared to mesic (moist) habitats (Smith and Epstein 1971), and decrease with increased latitude (Rubenstein and Hobson 2004). Importantly, food web $\delta^{13}C$ values reflect landscape carbon pools, for example the differences between plant photosynthetic pathways (C_3, C_4, and CAM) where C_4 plants have mean $\delta^{13}C$ values of approximately 12 ‰ compared with 27 ‰ for C_3 plants (West *et al.,* 2006) (Figure 7). This means that animal diets derived from C_4 grasses can be distinguished from those based on C_3 plants, such as shrubs, trees, and grasses. In addition, increased $\delta^{13}C$ values in C_3 plants are strongly correlated with water-use efficiency adaptations, especially in arid regions (Hobson 1999, Rubenstein and Hobson 2004). Stable-nitrogen isotope values increase predictably at each trophic level and have higher values in arid compared to moist habitats (Heaton 1987, Hobson 1999, Rubenstein and Hobson 2004).

Inert tissues such as feathers have isotopic profiles that reflect the individual's diet over the period of feather growth and become fixed once fully grown. Isotopic information derived from feathers thus matches a species' moult or feather replacement schedule which is highly predictable for most species (Holmgren and Hedenström 1995). For willow warblers, feathers collected during the breeding season reflect environmental conditions, habitats and diet encountered during the winter period.

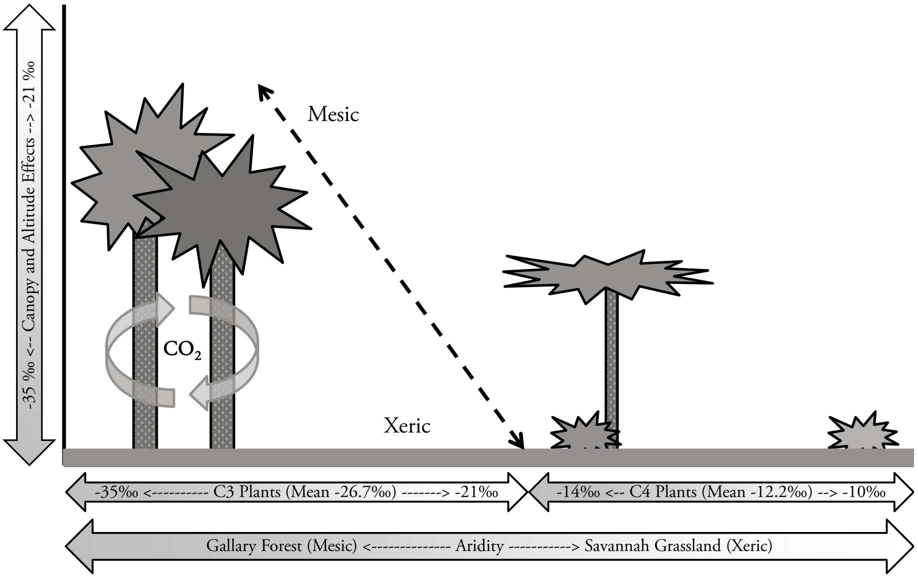

Figure 7. Stable isotope analyses as a useful tool to gain insight into the animal's ecology inferred from diet and habitat use. The effect of temperature (aridity), canopy and altitude on δ^{13}C values within C_3 and C_4 plant communities. Very low δ^{13}C values (< –27 ‰) result from CO_2 recycling from plant respiration and decomposing C_3 plants in forest ecosystems.

Results and Discussion

Paper I

Reduced abundance in a migratory songbird hybrid zone: evidence for selection against hybrids outside the breeding area

It is expected that abundances should be lower in hybrid zones compared to adjacent regions (Barton 1986, Ruegg 2008). For example, Ruegg (2008) showed that Swainson's thrush (*Catharus ustulatus*) had lowered abundance inside the hybrid zone that coincides with a migratory divide. Alternatively, dispersal into a hybrid zone might compensate for the loss of fitness that penalizes mixed pairs and the local reduction in reproductive output that results from hybridization might not result in lower population abundances in hybrid zones (Barton and Hewitt 1985, 1989). Finally, selection pressures in migratory populations may take place in both temporal and spatial contexts across the annual cycle. For example, lower hybrid zone abundance may result from factors that select against hybrids on the breeding grounds or result from an interaction between intrinsic and extrinsic factors during migration or on the wintering grounds. In this case, the endogenously controlled intermediate migratory direction may result in individuals encountering environmental conditions, e.g. long-distance over water crossings, that neither parental population experiences during migration.

In this study, we first tested the prediction that these tension zones are often associated with a region of low abundance using a nationwide abundance dataset encompassing the entire breadth of a migratory divide and hybrid zone for willow warblers in Sweden. Second, if detected, we predict that the region of lowest

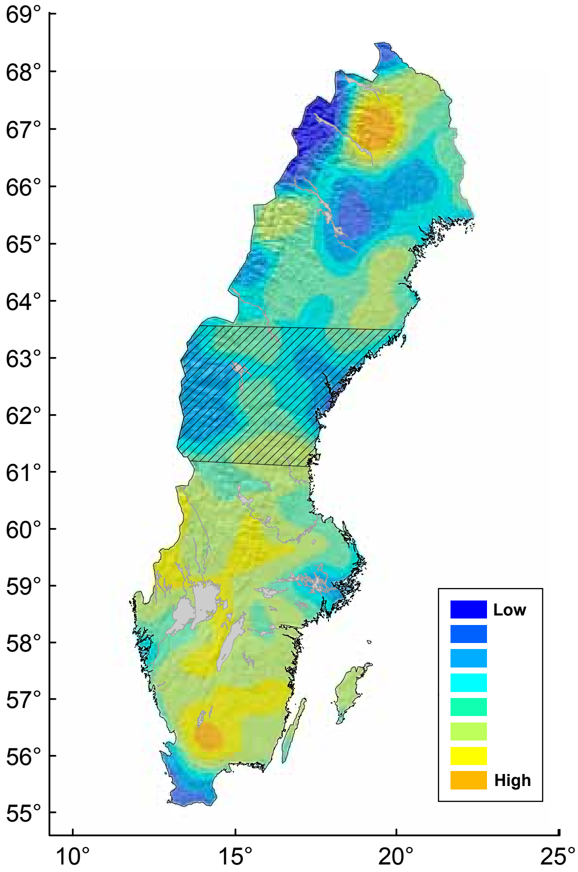

Figure 8. Willow warbler relative abundance calculated from the Swedish Breeding Bird Survey dataset (1996 to 2009), (A) spatially interpolated relative abundance, the cross-hatched region represents the independently derive hybrid zone, between 61.4° and 64.1° N latitude (Bensch *et al.*, 2009), (B) mean relative abundance by latitude. We used the cubic spline method (Schluter, 1988) to fit a line (black dashed) to the latitudinal mean relative abundances. The thick dashed red box represents the hybrid zone as defined in (Staffan Bensch *et al.*, 2009). The vertical thin dotted black line in the middle of the hybrid zone defines the cline centers for four traits identified in Bensch *et al.* (2009).

abundance will coincide with the four previously identified trait cline centers associated with the hybrid zone. Third, we test the tension zone prediction that the position of the hybrid zone has stabilized on an environmental gradient associated with lowered abundance by analyzing climate and habitat. Our results reveal lower abundance for willow warblers in the hybrid zone where the region of the lowest abundance is coincident with the center of four previously described genotypic-phenotypic trait clines (Figure 8). Finally, neither the location nor the orientation of the hybrid zone is significantly associated with environmental gradients. These results support the hypothesis that selection against hybrid migratypes in the willow warbler likely takes place outside the breeding grounds.

Paper II

Inferring the ecology of winter moult by stable isotope analyses across the wings of willow warblers

We present a comparison of feather stable isotope (δ^{13}C, δ^{15}N) patterns representing the habitat and diet conditions for two subspecies of Swedish willow warblers that breed in parapatry, but winter in different regions of sub-Saharan Africa. Previous analyses have shown that on average winter moulted innermost primary flight feathers (P1) show subspecific differences in δ^{15}N values, although individuals show substantial variation for both δ^{13}C and δ^{15}N within subspecies. We examined whether correspond-

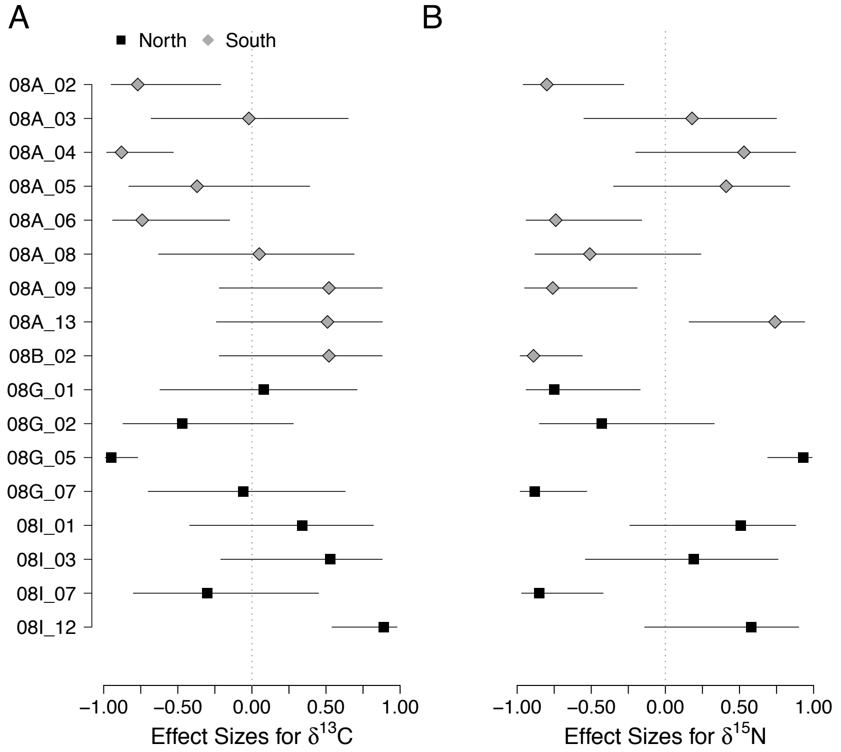

Figure 9. Effect sizes (correlation coefficient) plotted for individuals (i.e. 08A_02 with 08 referring to sampling year 2008, A as site index, and 02 as individual code) with 95% confidence intervals for both (A) $\delta^{13}C$ and (B) $\delta^{15}N$ and primary feather position (1 to 9) for willow warbler winter grown feathers in Africa.

ing variation in the timing of the winter moult that would be reflected by consistent repeatabilities for $\delta^{13}C$ and $\delta^{15}N$ values for individual birds, could explain some of this isotopic variation. Specifically, if $\delta^{13}C$ or $\delta^{15}N$ values correlate with feather position (which reflects the moulting sequence) we can infer that individuals either shift diet, habitat, or location. Further, differential subspecific adaptations to winter precipitation patterns across Africa might result in a variable degree of site fidelity or itineracy during moult when energetic demands may require resource tracking. We found no consistent trend in isotopic values from innermost to outermost primary flight feather, thus inter-individual variation in the timing of moult does not explain the subspecific isotopic variation for P1. Patterns in wing feather $\delta^{13}C$ and $\delta^{15}N$ values indicated that 41% of the individuals from both subspecies shifted their diet or habitats during winter moult (Figure 9). Importantly, despite well-documented itineracy in willow warblers during the winter, 59% of the individuals had isotope signatures consistent with stable use of habitats or diets during winter moult. Repeatability analyses of individuals suggest that both subspecies initiate moult in similar habitats from year-to-year while feeding on isotopically similar diets.

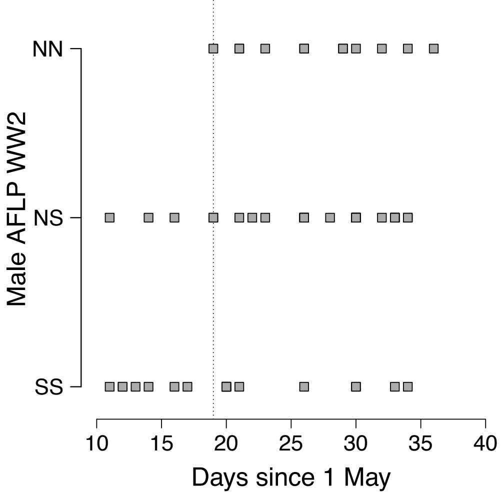

Figure 10. Capture date for all males captured at both Flatruet ($n = 27$) and Ljungdalen ($n = 29$) sites combined with pair counts for AFLP WW2 genotypes (SS homozygotes for the southern allele, NN homozygotes for the northern allele, SN heterozygotes) ($n = 56$, $R = 0.37$, $P = 0.003$, $LCL = -0.16$, $UCL = 1.00$). Capture dates are presented as the number of days after 1 May.

Paper III

No evidence for assortative mating between two distinct migratypes across a willow warbler migratory divide

We investigated the occurrence of assortative mating in two willow warbler subspecies that form a migratory divide and hybrid zone in central Sweden. For willow warblers, documentation of actual hybridization across the migratory divide or the potential magnitude thereof is unknown. In order to assess the role of assortative mating for limiting hybridization we conducted nest searching in the central part of the hybrid zone to identify and assess social pairings. Here we located all territories within our study site and identified nests. Our results suggest there is no assortative mating across the willow warbler migratory divide based on a comparison of plumage colour, morphology, stable nitrogen-isotopes, and one AFLP marker, despite the apparent nine day difference in arrival between the two migratypes (Figure 10). This work lends further support for the hypothesis that both willow warbler migratypes frequently hybridize across the migratory divide. As a major factor maintaining the hybrid zone, we instead speculate that selection is acting on the expression of the genetic migratory program where hybrid offspring presumably take an intermediate migratory route resulting in lower survival. Hybrid offspring with an intermediate migratory program would have to overcome significant ecological barriers that are avoided by the parental migratypes (Hedenström and Pettersson 1987). During their first autumn migration, the intermediate willow warbler migratypes would have to undertake extensive flights across The Alps and over both the Mediterranean Sea and Sahara desert at their widest stretches with minimal stopover opportunities, which are likely to result in increased hybrid mortality. Without selection against the many hybrid forms, the observed steep trait clines would have dissolved in only a few hundred years (Bensch *et al.,* 1999b, 2009b).

Paper IV

Climate exerts direct selection on genomic region in a mountain living bird

Local adaptation is an important process contributing to population differentiation which can occur in continuous or isolated populations connected by various amounts of gene flow (Via 2009). The willow warbler is the most common songbird in Scandinavia. It has a continuous

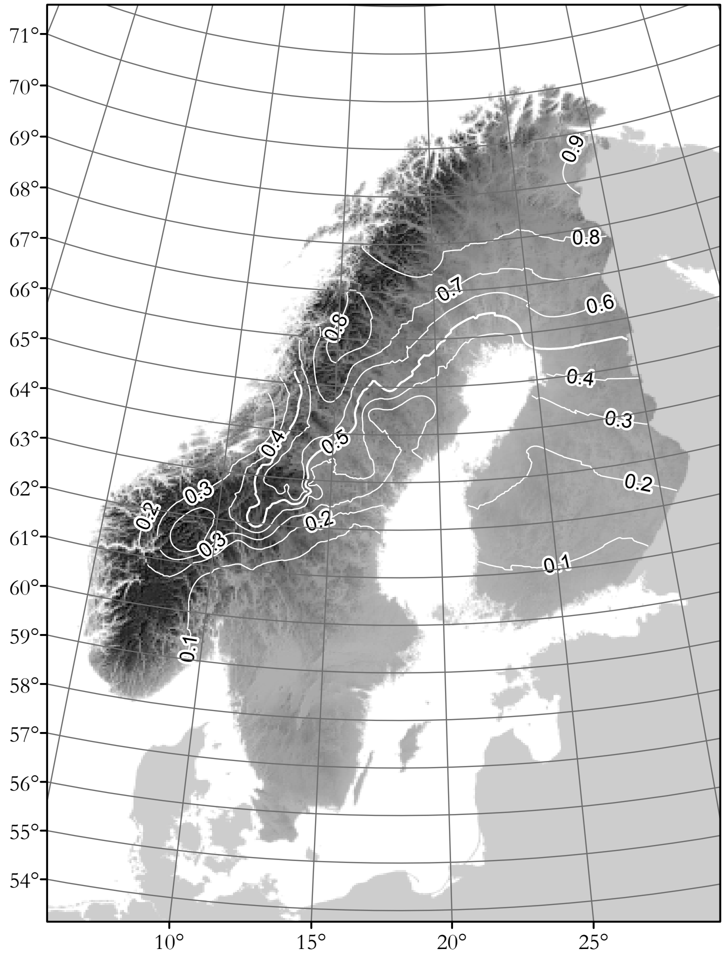

Figure 11. Spatially interpolated observed 0.1 northern-allele frequency contours for the locus WW1 overlaid on altitude hill shade background. The 0.5 isocline represents the center of the cline. The background represents a digital elevation model (DEM) with hill shading for study area.

breeding distribution in forest habitats from the south to north, east to west, and from sea level to the tree line. Therefore it constitutes an ideal species for study of environmental gradients and the associated locally adapted genes or genomic regions. In this study, we sampled 2,285 individuals at 126 sites across Fennoscandia to study the geographic and climatic variables that explain the allele frequency distribution for two alleles of the AFLP derived marker WW1. Our results demonstrate that 1) allele frequency patterns clearly differ between mountain and lowland populations, 2) these allele differences coincide with extreme temperature conditions and short growing season in the mountains, and milder conditions in coastal areas, and 3) the northern-allele or “altitude variant” of WW1 is found in mountain populations for both subspecies (Figure 11). Finally, our results suggest that climate exerts direct selection on the genomic region associated with this allele, making this marker suitable for monitoring climate change as a force of selection on mountain bird populations. One prediction from this study is that future climate warming will cause the southern-allele to expand at the expense of the northern-allele.

Paper V

Phenotypic turn-over and hybrid zone dynamics in the willow warbler migratory divide

In this study we characterize a willow warbler (*Phylloscopus trochilus*) migratory divide and hybrid zone spatially, both its geographic location and shape. Based on earlier findings of lower abundances in the west of the hybrid zone, we predict the zone to be wider in the west than in the east. Further, using data from repeated visits to sites across the zone, we predict low repeatabilities for migratory associated traits that would suggest that high annual turn-over in migratory phenotypes occupying the zone. The results of our analysis demonstrate that the hybrid zone is approximately 80 km wide in east along the Baltic Sea 300 km wide in the mountainous west. These differences likely reflect differences in landscape heterogeneity. For example, weather in the east is milder and less stochastic and habitats relatively homogeneous, while in the mountainous west, habitats are heterogeneous, winters are long, and temperatures more extreme. In the future it will be essential to develop genetic markers that allow for the identification of the two subspecies, hybrids, and backcrosses. Only then will we be able to elucidate differences in hybrid zone dynamics related to the region of low abundance in the west and areas of high abundance in the east. Once markers allow for the identification of hybrid offspring, orientation experiments could elucidate migratory directional preferences. These results would either support or refute our hypothesis that an intermediate migratory programme results in lower hybrid fitness.

Future Directions

To better understand the hybrid zone dynamics it will be essential to develop genetic markers that allow one to separate each parental migratype, hybrids, and backcrosses. This is especially important given our results that demonstrate that the hybrid zone varies in width from east to west associated with an environmental gradient in the west of the zone. Once markers allow the identification of hybrid offspring, orientation experiments should be conducted to elucidate migratory directional preferences, that would support our hypothesis that hybrids take an intermediate migratory direction to their parental migratypes (Helbig 1991a, Bensch 1999, Bensch *et al.,* 2009a). Additionally, we found that the northern-allele frequencies for the genetic marker AFLP-WW1 represent local adaptation to mountain environments across Fennoscandia. To understand the function of the genes associated with this allele experiments in cold tolerance and diet choice may identify mechanisms and physiological pathways associated with these adaptations.

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I started this journey tracking migrant birds in Nevada when I encountered Anne Bellamy at Great Basin National Park. Soon thereafter, I followed her advice and climbed a ridge on the western edge of the Great Salt Lake where I found not only an amazing migration of raptors, but the field biologists that counted and trapped them. I climbed the ridge many times that fall helping to count while I learned to identify each species by their unique flight characteristics. From that autumn onwards I spent my life watching, counting, ringing, and following birds on their epic migrations across the globe.

Over the next decade I migrated from one field project to another traversing the continents and oceans, working with countless field biologists who like me could not imagine a better life. Somewhere along the line I met Robert Frey, certainly the best bird bander I had ever met, who became a life-long friend. He turned me on to keeping a field notebook and Grinnell Journal. Keeping a journal inspired within me a deep sense of happiness describing the world around me. I soon found that this process revealed to me the patterns in nature that inspires all of my research interests. For me the fieldwork is where the patterns are revealed and the questions are inspired.

As my experience grew and thirst for knowledge expanded, I sought out books and travel narratives from the great naturalists. Along the way, I found a book about keeping a Grinnell Journal. It presents a methodology for natural history observation. Looking to expand my biological education, I found the author at The Evergreen State College in Washington State. Steven Herman quickly became my friend and mentor. My studies with Steve at Evergreen eventually lead me to my PhD here in Lund.

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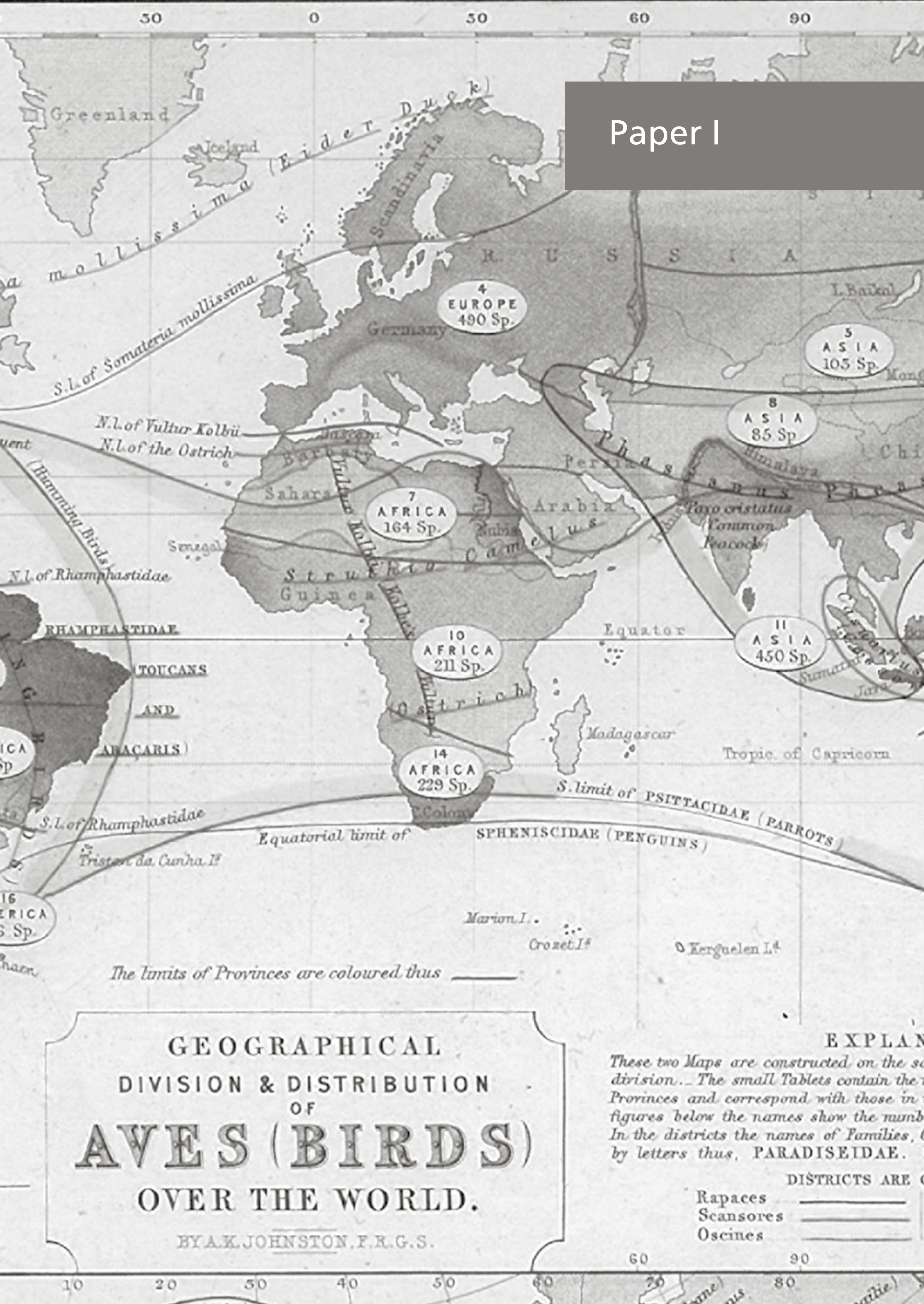
Paper I


GEOGRAPHICAL
DIVISION & DISTRIBUTION
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AVES (BIRDS)
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L. Baikal
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Arabia
Nubia
Sahara
Barbary
Senegal
Guinea
Madagascar
Equator
Tropic of Capricorn
Somateria mollissima (Eider Duck)
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N.L. of Vultur Kolbii
N.L. of the Ostrich
Vultur Kolbii
Struthio Camelus
Ostrich
Phasianus
Pavo cristatus (Common Peacock)
Casuarius
Sumatra
Java
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ARAÇARIS)
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Equatorial limit of SPHENISCIDAE (PENGUINS)
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C. Colony
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Reduced abundance in a migratory songbird hybrid zone:

evidence for selection against hybrids outside the breeding area

Keith W. Larson[1], Susanne Åkesson[1], Staffan Bensch[1],
Henrik G. Smith[1], David P. L. Toews[2], and Åke Lindström[1]

[1] Department of Biology, Ecology Building, Lund University, SE-223 62 Lund, Sweden
[2] Department of Zoology and Biodiversity Research Centre, University of British Columbia,
Vancouver, BC, V6T 1Z4, Canada

Abstract

Hybrid zones can result from the secondary contact between previously isolated populations and are often "tension zones." Maintenance of these tension zones occurs as a balance between dispersal of parental populations into the zone and selection against hybrids within the zone. In this study, we first test the prediction that these tension zones are often associated with a hybrid sink using a nationwide abundance dataset encompassing the entire breadth of a migratory divide and hybrid zone for willow warblers in Sweden. Second, if detected, we predict that the region of lowest abundance will coincide with the four previously identified trait cline centers associated with the hybrid zone. These four traits include morphology, colouration, stable nitrogen-isotopes, which is a proxy for the subspecific wintering grounds, and an AFLP molecular marker associated with the migratory direction. Third, we test the tension zone prediction that position of the hybrid zone has stabilized on an environmental gradient associated with lowered abundance by analyzing climate and habitat. Our results reveal lower abundance for willow warblers in the hybrid zone where the region of the lowest abundance is coincident with the center of four previously described genotypic-phenotypic trait clines. Finally, neither the location nor the orientation of the hybrid zone is significantly associated with environmental gradients. These results support the hypothesis that selection against hybrid migratypes in the willow warbler likely takes place outside the breeding grounds.

Keywords: migratory divide, migratype, hybrid zones, tension zones, migration, willow warbler, *Phylloscopus trochilus*

Introduction

Hybrid zones often result from the secondary contact between previously isolated populations where the two populations meet and interbred (Barton and Hewitt, 1989; Harrison, 1993). Barton and Hewitt, (1985, 1989) posit that most hybrid zones are in fact "tension zones", where parental dispersal into the zone is balanced with selection against hybrids within the zone. Tension zones result from either high rates of dispersal by parental populations balanced by strong selection against hybrids or low dispersal and weak selection (Barton and Gale, 1993; Brelsford and Irwin, 2009). The former scenario results in a zone populated by primarily pure parental forms at the center, whereas in the later, individuals resulting from generations of hybridization occupy the center (Brelsford and Irwin, 2009). Selection against intermediate genotypes minimizes the width of tension zones, whereas temporal factors affect the location of tension zones (Barton and Hewitt, 1985, 1989). For example, tension zones form at the origin of secondary contact and they can move due to selection and anisotropic dispersal (Barton and Hewitt, 1985, 1989). Finally, tension zones will often float until trapped in a region of low abundance or along an environmental gradient (Barton and Hewitt, 1985, 1989) or reflect the original location of secondary contact which may be coincident with an environmental gradient (Bridle *et al.*, 2001).

Most studies of hybrid zones typically focus on introgression, zone widths, asymmetries in trait clines and the frequency of mixing (Szymura and Barton, 1986, 1991; Pearson and Rohwer, 2000; Bronson *et al.*, 2003; Payseur, 2010), and identifying the agents of selection. However, few studies have addressed the population level consequences of secondary contact and hybridization (Ruegg 2008) where selection against hybrid offspring results in a "hybrid sink" or lowered population density (Barton, 1980, 1986; Barton and Hewitt, 1985, 1989). Because hybridization is often associated with reduced fitness, it is expected that abundances should be lower in the hybrid zone compared to the adjacent regions (Barton, 1986; Ruegg, 2008). For example, Ruegg (2008) showed that Swainson's thrush (*Catharus ustulatus,* Nuttall, 1840) had lowered abundance inside the hybrid zone that coincides with a migratory divide. Alternatively, dispersal into a hybrid zone might compensate for the loss of fitness that penalizes mixed pairs (Barton and Hewitt, 1985, 1989). Hence, the local reduction in reproductive output that results from hybridization might not result in lower population abundances in hybrid zones (Barton and Hewitt, 1985, 1989). Finally, selection pressures in migratory populations may take place in both temporal and spatial contexts across the annual cycle. For example, lower hybrid zone abundance may result from factors that select against hybrids on the breeding grounds or result from an interaction between intrinsic and extrinsic factors during migration or on the wintering grounds.

Since the last glacial maximum, willow warblers (*Phylloscopus trochilus*, Linnaeus, 1758) have expanded their range north and subsequently around the Baltic Sea from their glacial refugium (Bensch *et al.*, 1999, 2009). During expansion around the Baltic Sea, each terminal form breeding in peripheral allopatry developed unique migratory programs (Bensch *et al.*, 2009; Irwin, 2009). In central Sweden these two "subspecies" form a breeding season migratory divide and hybrid zone (Salomonsen, 1928; Hedenström and Pettersson, 1987; Bensch *et al.*, 1999, 2009). Although overlap in the morphological and colour traits precludes subspecific identification in the field, the two subspecies correspond to migratory phenotypes (hereafter "migratypes") that differ in their migratory directions and wintering locations (Bensch *et al.*, 1999, 2009; Chamberlain *et al.*, 2000). *Phylloscopus t. acredula* (Linnaeus, 1758) in the north of Sweden migrate towards the SSE to winter in East and Southern Africa whereas *P. t. trochilus* (Linnaeus, 1758) in the south of Sweden migrate towards the SW to winter in West Africa (Hedenström and Pettersson, 1984, 1987; Chamberlain *et al.*, 2000; Bensch *et al.*, 2006). The changes in the migratory program occur over a relatively short (< 300 km) distance (Bensch *et al.*, 1999, 2009). Despite the rapid geographic change in migratory programs between the two migratypes, previous molecular work demonstrates no subspecific differences in mitochondrial DNA (mtDNA) and neutral nuclear microsatellites (Bensch *et al.*, 1999, 2009). However, steep north-south clines in genotypic and phenotypic traits do characterize the hybrid zone, i.e., colouration, morphology, stable nitrogen-isotopes that serves as a proxy for wintering grounds, and an ALFP derived molecular marker that coincides with migratory direction (Bensch *et al.*, 1999, 2002, 2009).

These four trait clines associated with the migratory divide are substantially narrower than expected given the assumed natal dispersal distances and time since secondary contact, suggesting reduced hybrid fitness (Bensch *et al.*, 1999, 2009). The lack of apparent genetic (i.e., mtDNA and neutral nuclear DNA) and phenotypic (e.g., song or habitat preferences) differences that could result in pre-breeding mating isolation supports the hypothesis that selection against hybrid offspring occurs outside the breeding grounds. Thus, a post-breeding reduction in hybrid fitness would result in lower population density within the hybrid zone. Inspection of the Swedish breeding bird atlas suggests a region of low willow warbler abundance in central Sweden coincident with the migratory divide (Svensson *et al.*, 1999). Lower abundance in the hybrid zone may result from the maladaptive migratory behaviour of hybrids or local habitat characteristics within the hybrid zone (Barton, 1986). Together this suggests that either frequent hybridization reduces local reproductive output through reduced hybrid fitness or that the migratory divide coincides with suboptimal habitats, or both.

Because the hypothesized selection against hybrid willow warblers would take place outside the breeding grounds, one would not expect to find an association between the migratory divide and climate or habitat gradients. However, it is possible that a region of low population density (typically associated with an environmental gradient) has captured the willow warbler hybrid zone (Barton and Hewitt, 1985, 1989). Bensch *et al.*, (2009) noted an apparent perpendicular environmental gradient to the hybrid zone (a west to east gradient of mountains to lowlands) compared to the north-south clines in the examined genotypic and phenotypic traits. Despite these observations, landscape heterogeneity may result in complex and less apparent climatic and biotic patterns that require detailed analyses before one can rule out an association of the hybrid zone with an environmental gradient.

In this study, we compare the breeding season abundance of the willow warbler, inside and outside a well-defined subspecies migratory divide and hybrid zone and assess which selection pressures may shape the observed patterns. First, we determine whether the *a priori* identified hybrid zone between 61.4° and 64.1° N latitude (Bensch *et al.*, 2009) coincides with a region of low abundance as suggested by Svensson *et al.,* (1999). Second, if a region of low abundance does exist in the hybrid zone, we predict that the region of lowest abundance will coincide with the four trait (i.e., colouration, morphology, stable isotopes, and AFLP molecular marker) cline centers at 62.7° N latitude, identified in Bensch *et al.*, (2009). Third, we combine our abundance data and environmental variables to indirectly test whether environmental factors explain the observed patterns of abundance. In this context, we predict that if environmental selection occurs in the hybrid zone there should be a north-south environmental gradient in habitat or climate coincident with reduced abundance in the hybrid zone. Alternatively, habitat and climate clines occurring outside or perpendicular to the hybrid zone would support the hypothesis that post-breeding selection against hybrids maintains the narrow hybrid zone and thus contributes to the reduced abundance in this region.

Materials and Methods

Relative Abundance Data

We explored willow warbler abundance across Sweden using data from the "fixed route" scheme within the Breeding Bird Survey (BBS) (Lindström *et al.*, 2007). This survey of 716 routes systematically distributed in a 25 km grid to cover the entire 450,000 km² of Sweden began in 1996. Observers count all birds seen and heard along each 8 km line transect represented by a 2 x 2 km square. Although the aim of the survey is to census each route once every year, the number of routes surveyed annually has varied among years, from 48 routes in 1996 up to 585 in 2008. From 2003 onwards, observers surveyed at least 400 routes annually. Willow warblers show no subspecific differences in song (Irwin et al. unpubl. data), thus limiting detections during the annual BBS to only species-level identification across their entire range.

We estimated the relative abundance for each route by totaling the number of observations, adjusted for effort (percentage of route completed during the survey), and then averaged the total adjusted observations across all years of the survey included in our study (1996–2009). Next, we created an interpolated relative abundance surface for the entire country using ordinary kriging, a geospatial modeling technique that estimates the values between known points as a continuous surface (ESRI, 2010). Separately, we calculated means and standard errors for all routes located on a line of latitude from 55° to 70° N. To visualize these results, we plotted the relative abundance against latitude and fit a cubic spline through the points using the program Cubic Splines (Schluter 1988).

Climate and Habitat Data

We compiled a set of climate and satellite remote sensing variables to characterize the environmental heterogeneity across Sweden (Table 1). These included 19 bioclimatic variables derived from the WorldClim database (Hijmans *et al.*, 2005), which contain estimates of annual means, seasonal extremes and degrees of seasonality in temperature and precipitation. The additional 13 variables derived from climate and habitat data include annual aridity and potential evapotranspiration (Zomer *et al.*, 2008), elevation data (Hijmans *et al.*, 2005), surface moisture and roughness (Long *et al.*, 2001), tree cover (DeFries *et al.*, 2000; Hansen *et al.*, 2002), and greenness (Pettorelli *et al.*, 2005; Zhang *et al.*, 2005). All variables have a spatial resolution of 30 arc seconds (1 km²). We extracted values for all environmental layers from each BBS route polygon using the Spatial Analyst extension within ArcMap 10 (ESRI, 2010).

Statistical Analysis

We first performed a Pearson correlation analysis on all environmental variables (Table 1) to check for the presence of highly (> 95%) correlated variables (Buermann *et al.*, 2008; McCormack *et al.*, 2010). Seven of the bioclimatic variables were highly correlated with other variables and were thus excluded from further analyses. For example, maximum temperature of warmest month and maximum temperature of warmest quarter were highly correlated and the later was dropped. Next, we conducted a principal components analysis (PCA) on the remaining 25 environmental variables (Table 1) reducing it to four principal components (PCs) using the Varimax orthogonal rotation method. Sixty-six routes were excluded from the analysis due to missing environmental data, e.g., those along the national boundary and shorelines, on islands in the Baltic Sea and lakes across Sweden. We then used general linear mixed models (GLMM) to test the predictions that, (i) relative abundance was lower in the hybrid zone than in regions to the south and north of the zone, (ii) hybrid zone abundance was coincident with an environmental gradient, and (iii) the environmental characteristics of the hybrid zone differed from those

Table 1: Overview of climate and remote sensing datasets used in this study

Data record	Instrument	Ecological attributes	Variables derived
Scatterometer-Backscatter *	Satellite-QSCAT	Surface moisture + roughness (forest structure)	QSCAT (August)
DEM	Space shuttle radar	Digital elevation model	Elevation, elevation STD, solar radiation (August)
WorldClim †	Station-network	Bioclimatic variables	Bio1-Bio19, annual aridity, annual potential evapotranspiration
NDVI ‡	Satellite-AVHRR	Vegetation photosynthetic productivity or 'greenness'	Growing season, NDVI mean, NDVI max, NDVI std
TCCF §	Satellite-MODIS	Percentage tree cover by forest type	Broadleaf, needleleaf
VCF ¶	Satellite-MODIS	Proportional estimate of woody vegetation cover	Tree cover

* QSCAT annual mean and standard deviation (QSD) are based on monthly data from the year 2001 with complete data coverage (Long *et al.,* 2001).

† WorldClim data are based on monthly climatologies of the last five decades (Hijmans *et al.,* 2005). The bioclimatic variables are: annual mean temperature (Bio1), mean diurnal temperature range (Bio2), isothermality (Bio3), temperature seasonality (Bio4), maximum temperature of warmest month (Bio5), minimum temperature of coldest month (Bio6), temperature annual range (Bio7), mean temperature of the wettest quarter (Bio8), mean temperature of the driest quarter (Bio9), mean temperature of the warmest quarter (Bio10), mean temperature of the coldest quarter (Bio11), annual mean rainfall (Bio12), rainfall of the wettest month (Bio13), rainfall of the driest month (Bio14), rainfall seasonality (Bio15), rainfall of driest quarter (Bio16), rainfall of wettest quarter (Bio17), rainfall of the warmest quarter (Bio18), rainfall of the coldest quarter (Bio19), annual aridity (Zomer *et al.,* 2008), annual potential evapotranspiration (Zomer *et al.,* 2008).

‡ NDVI dataset are generated from 2001 data. The layers are: length of 'green' or growing season (NDVI growing season), annual production of vegetation (NDVI maximum), annual mean production of vegetation (NDVI mean), and annual variation on productivity of vegetation (NDVI stdev) (Zhang *et al.,* 2005, Pettorelli *et al.,* 2005)

§ TCCF dataset are generation from 1992–1993 data. The layers include percent broadleaf and needle leaf tree cover (Defries *et al.,* 2000)

¶ VCF dataset are generated from 2001 data. The layers are percent bare ground, herbaceous and woody ground cover (Hansen *et al.,* 2002)

of the regions outside of the zone. We used a spatial regression model to account for the spatial autocorrelation between route locations by including the "power" spatial covariance structure and partitioned the BBS abundance dataset into three regions using the hybrid zone (61.4° to 64.1° N latitude) (Bensch *et al.*, 2009) for the middle region. The model also included environmental explanatory variables summarized by our PCA. To meet the model assumptions, we used a square-root transformation for the relative abundance data. The full model included all the PCs and their second-order interactions with our "region" variable. We then used the Akaike information criterion (AIC) model selection to fit a model with the fewest explanatory variables (Burnham and Anderson, 2002).

To assess spatial trends in the environmen-

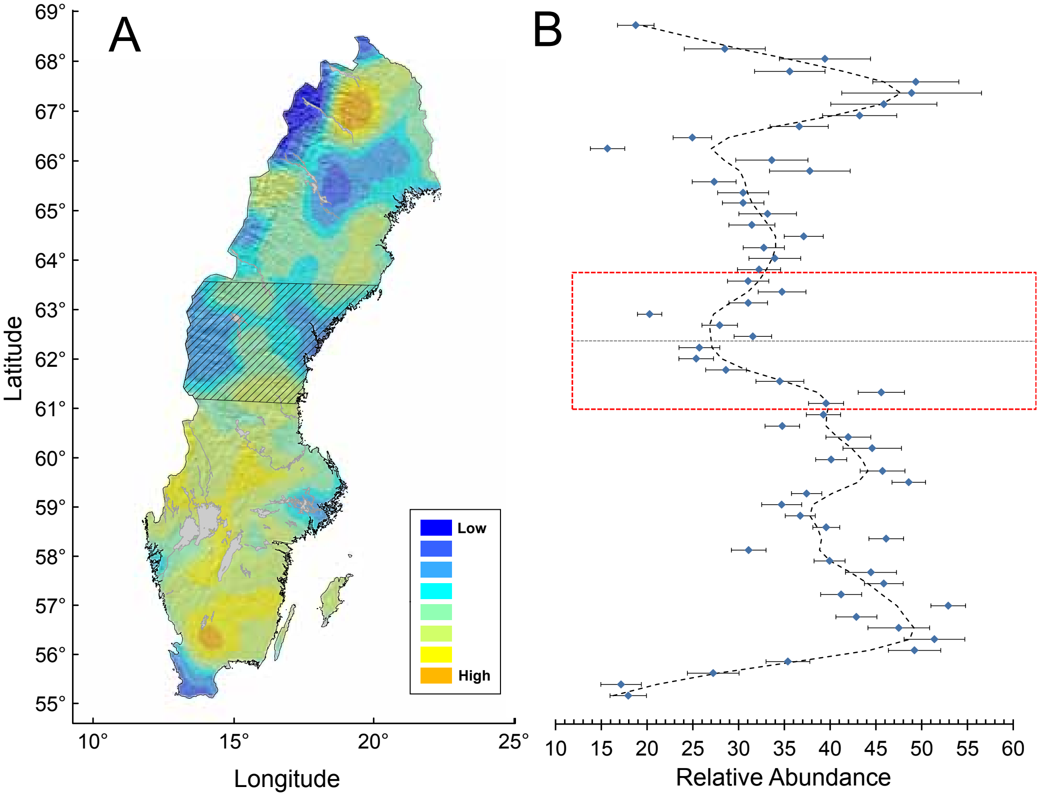

Figure 1. Willow warbler relative abundance calculated from the Swedish Breeding Bird Survey dataset (1996 to 2009), (A) spatially interpolated relative abundance, the crosshatched region represents the independently derive hybrid zone, 61.4° and 64.1° N latitude (Bensch *et al.*, 2009), (B) mean relative abundance by line of latitude. We used the cubic spline method (Schluter, 1988) to fit a line (black dashed) to the latitudinal mean relative abundances. The thick dashed red box represents the hybrid zone as defined in (Staffan Bensch *et al.*, 2009). The vertical thin dotted black line in the middle of the hybrid zone defines the cline centers for four traits (i.e., δ15N, AFLP markers, and morphometrics) identified in Bensch *et al.* (2009).

tal data, we constructed a general linear model (GLM) to evaluate the relationship between each of the PC and latitude and longitude for each of the three regions. We excluded elevation as a covariate because it is highly correlated with both longitude and latitude across all three regions.

We performed all statistical analyses with SAS for Windows 9.2 (SAS Institute Inc., 2008) and spatial analyses with ArcMap 10 (ESRI, 2010), including the Geospatial and Spatial Analyst extensions.

Results

Observers detected willow warblers on 707 routes of the 716 BBS routes surveyed (Figure S1). The mean relative abundance for all years surveyed (1996–2011) for all of the routes ranges from zero to 150 individuals per route (Figure 1A). The pattern of abundance confirms that willow warblers are widespread and abundant across Sweden. Willow warbler abundance differs significantly between the hybrid zone and the regions outside the zone ($F_{1,\,112} = 7.97$, $P = 0.01$). Further, the latitude of lowest abundance within the hybrid zone (62.53° N; Figure 1B) coincides closely with the latitude cline centers found by Bensch *et al.*, (2009).

The four principal components used in the PCA explain 79% of the total environmental variation (Table S1, Figure 3 A–D); PCs 1 through 4 explain 36%, 25%, 8%, 6%, and 5% of the variation, respectively. The full GLMM model ($t_{204} = 35.31$, $P < 0.001$) found that both environmental factors and the *a priori*-defined regions explained the patterns of abundance across Sweden. We removed PC2 and the interactions between PC1, PC2, PC3, and PC4 with the Region parameter using an AIC delta value of four as cut-off (Burnham and Anderson, 2002). Region ($F_{2,193} = 7.45$, $P < 0.001$), PC1 ($F_{1,222}= 3.58$, $P = 0.06$), PC3 ($F_{1,382} = 42.94$, $P < 0.001$), PC4 ($F_{1,614} = 8.91$, $P = 0.003$), PC5 ($F_{1,380} = 14.79$, $P = 0.0001$), and the interaction term PC5 and Region ($F_{2,414} = 3.63$, $P < 0.03$) best explain patterns of reduced abundance. Residuals from the final model show the same pattern in the *a priori*-defined hybrid zone (Figure 2B). To further test the relationship between hybrid zone abundance and the selected PCs, we reran the model with the Region parameter restricted to the hybrid zone. In the hybrid zone only PC3 ($F_{1,\,114} = 16.13$, $P < 0.001$) and PC4 ($F_{1,\,137} = 7.20$, $P = 0.01$) are significantly associated with abundance.

To determine if any environmental gradients explained the patterns of abundance within the north-south oriented hybrid zone, we regressed each of the selected PCs separately against latitude and longitude. In the hybrid zone, PC3 is significantly associated with longitude ($R^2 = 0.24$, $F_1 = 45.55$, $P < 0.001$; Figure 3A), but not with latitude ($R^2 = 0.01$, $F_1 = 1.78$, $P = 0.18$; Figure 3B). Similarly, PC4 is significantly associated with longitude ($R^2 = 0.11$, $F_1 = 17.90$, $P < 0.001$; Figure 3C), but not with latitude ($R^2 = 0.00$, $F_1 = 0.01$, $P = 0.93$; Figure 3D).

Discussion

Our finding of lower hybrid zone abundance for willow warblers in the absence of a latitudinal environmental gradient supports the hypothesis that selection against hybrid offspring occurs outside the breeding grounds. The presence of such a gradient would make it difficult to discern whether the lower observed hybrid zone abundance resulted from reduced hybrid fitness versus the movement of the zone across the landscape until trapped by a region of low abundance (Barton and Hewitt, 1985, 1989). Our results suggest that although willow warbler abundances are to some extent constrained by habitat quality, non-breeding grounds factors further limit abundances in the hybrid zone. Our results contrast with the only other study looking at patterns of abundance and the role of environmental gradients in a hybrid zone associated with a migratory divide. In that study, the authors found lower local abundance in a Swainson's thrush hybrid zone, but the region overlapped an environmental transition from wet coastal to drier interior climates in the Pacific Northwest (Ruegg, 2008). Thus, our study is the first to demonstrate reduced abundance in a migratory divide not attributed to other factors aside from presumed migratory differences between parental and hybrid migratypes.

Selection against willow warbler hybrids outside the breeding grounds may result from a migratory program that includes an intermediate direction to the parental migratypes (Bensch *et al.*, 1999, 2009). In this case, selection would act against the hybrid migratory program because such a route would intersect three prominent ecological barriers (the Alps, the Mediterranean Sea, and the Sahara Desert) at much wider points than those of their parental populations (Bensch *et al.*, 1999, 2009; Chamberlain *et al.*, 2000). Physiological adaptations for longer nonstop flights required for crossing extended ecological barriers such as larger fuel stores, which are unlikely to develop *de novo* in hybrids, be-

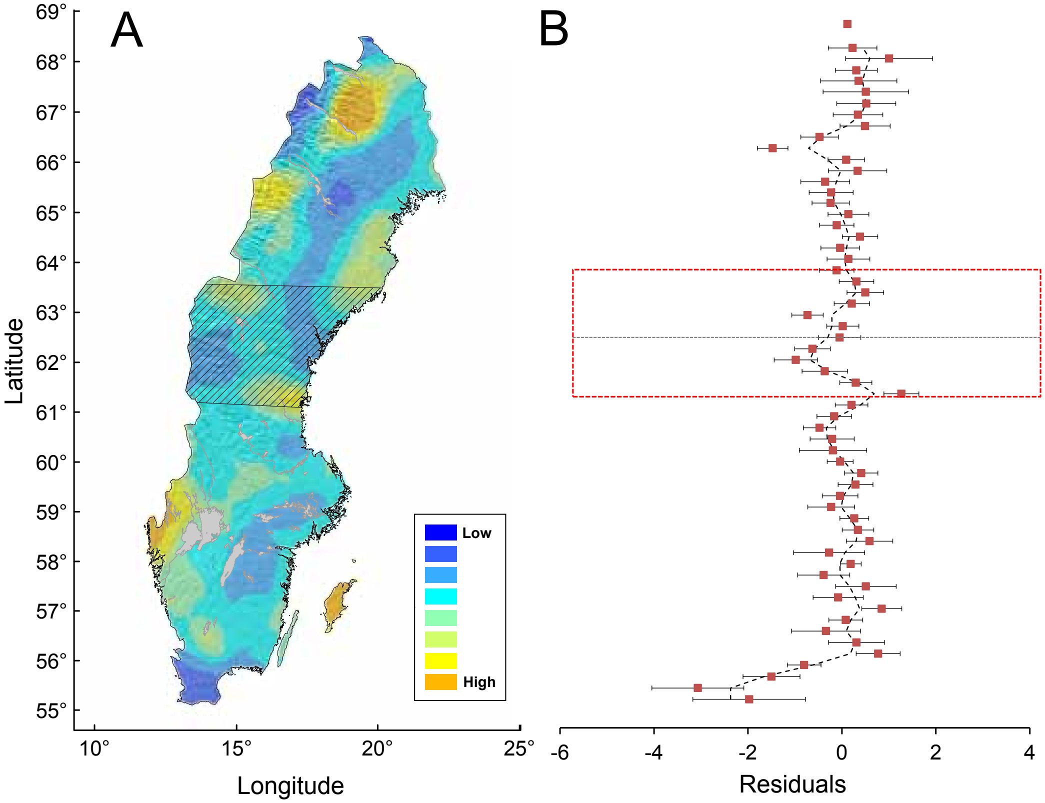

Figure 2. Willow warbler mean (A) spatially interpolated residual abundance and (B) residual abundance modeled with region and environmental PCs, with standard errors summarized by line of latitude. We used the cubic spline method (Schluter 1988) to fit a line (black dashed) to the latitudinal mean relative abundances. The thick dashed red box represents the independently derived hybrid zone as defined in Bensch *et al.,* (2009) between 61.4° and 64.1° N latitude and is derived from two quantitative traits (δ^{15}N and body size) and two biallelic loci (AFLP-WW1 and AFLP-WW2). The vertical thin dotted black line in the middle of the hybrid zone defines the cline centers for four traits (i.e. δ15N, AFLP markers, and morphometrics) identified in Bensch *et al.,* (2009).

cause neither of the parental migratypes undertake such flights (Bensch *et al.*, 2002). This hypothesized selection mechanism against willow warbler hybrids is consistent with cross-breeding and selection experiments with Central European populations of the European Blackcap, *Sylvia atricapilla* (Linnaeus, 1758) that also form a migratory divide (Helbig, 1991a; b; Berthold *et al.*, 1992). Results showed that F_1 hybrids migrated in an intermediate migratory direction relative to their parental migratypes (Helbig, 1991a; b; Berthold *et al.*, 1992). Accordingly, a combination of endogenous (i.e. genetically controlled intermediate migratory direction) and exogenous (i.e. negative environmental conditions encountered during fall migration outside the breeding grounds) factors may act to reduce hybrid fitness.

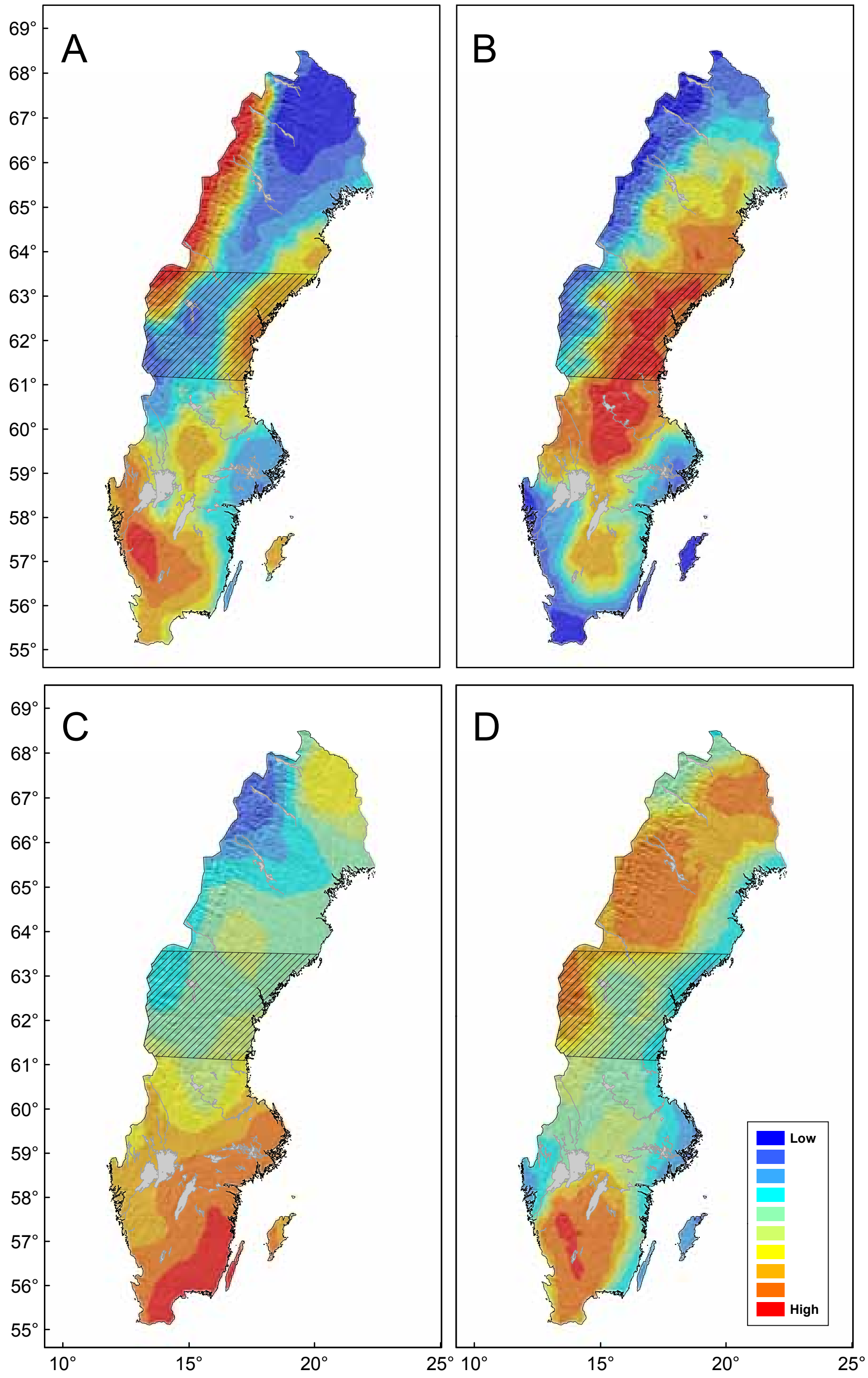

Figure 3. The four principal components (PC1, PC2, PC3, and PC4) from the willow warbler abundance GLMM. Only PC3 and PC4 significantly explained the variance in our abundance model. These PCs summarize the 25 environmental layers by a principal components analysis using a Varimax rotation, explaining 79% of the environmental variability.

The "Tension Zone" Model

To explore the relationship between the hybrid zone and abundance in willow warblers, we used a single-locus tension zone model

$$s = K \cdot \left(\frac{\sigma}{w}\right)^2$$

to compare our estimate of abundance in the hybrid zone to that expected from reduced hybrid fitness. Here we assume that 1) most selection against hybrids takes place in the first annual cycle and 2) our estimate of abundance takes place after selection (given the timing of the BBS). To estimate this reduction in hybrid fitness we use the single-locus tension zone model where s is the selection coefficient associated with reduced fitness, w is the width of the hybrid zone, σ is the standard deviation of parent offspring distance (a measure of dispersal) and K is a multiplier that depends on the type of selection (Barton and Gale, 1993). We use $K = 8$ because this is the relevant multiplier for selection against heterozygotes (with intermediate and potentially inferior migration) and set $w = 296.8$ km. Next we calculated two estimates for σ: 46.3 km derived from ringing recovery data (Paradis *et al.*, 1998) and 26.3 km estimated from linkage disequilibrium (LD) between two genetic markers in the hybrid zone (see electronic supplementary material for this calculation). This yields respective s values of 0.20 and 0.06 for ringing recovery and LD dispersal estimates. Comparing the estimated mean relative abundance outside the hybrid zone (37.4), we estimate abundance in the hybrid zone to be 30.6 using the estimate of dispersal from ring recovery data and 35.6 using the LD estimate of dispersal. Both estimates are higher than the abundance estimate at the center of the zone (22.5). That this model does not fully reflect all of the observed patterns in abundance is not surprising because these model estimate selection needed to maintain a hybrid zone of a given width at a single locus. In our case, multiple isolating factors likely contribute to reduced abundance in the hybrid zone and, immigration into the zone can compensate for the reduction caused by reduced hybrid fitness. Future analyses should incorporate additional diagnostic genetic markers between these two subspecies to refine estimates of LD and dispersal.

Conclusions

We present results from the novel use of a long-term breeding survey data coupled with data on the orientation, location, and width of a hybrid zone for willow warblers to elucidate the role of selection acting on the breeding grounds. Since the last glacial maximum, range expansion by two subspecies of willow warblers from their glacial refugium resulted in secondary contact in central Sweden. Here they form a migratory divide and hybrid zone, which corresponds with a region of low abundance oriented on a north-south latitudinal gradient coincident with the transition of four previously described traits (Bensch *et al.*, 1999, 2009). The environmental gradients in this region are oriented east-to-west, i.e. perpendicular to the hybrid zone, which suggests a limited role of breeding ground environmental factors in the observed patterns of lower abundance. Further, the overall observed abundance of willow warblers in the hybrid zone is slightly lower than that expected from the reduced hybrid fitness calculated from a simple single-locus tension zone model. This discrepancy calls for obtaining improved estimates of dispersal, selection, and frequency of hybridization, as these variables are essential to understand hybrid zone dynamics. Although our results suggest that selection occurs outside the breeding season, we cannot rule out untested factors such as interspecific competitors, predators, or pathogens in affecting local abundance. Further investigations into the mechanisms that maintain the clines should focus on factors the might reduce hybrid fitness on and off the breeding grounds, such as assortative mating, orientation experiments of juveniles of southern, hybrid, and northern migratypes, and identification of genomic regions that are under differential selection in the two subspecific migratypes.

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Supplementary Material

Our estimate of dispersal from linkage disequilibrium (LD) in the selection equation relies on previous data from the molecular markers WW1 and WW2 (Bensch *et al.,* 2009). We used the relationship

$$D = \frac{s^2(1+r)}{rw^2}$$

where D is the composite digenic LD between two diagnostic genetic markers, r is the recombination rate between them (here assumed to be 0.5), and w is the width of the clines (227.6 km - an average of the cline widths of WW1 and WW2), which can be rearranged to estimate dispersal, s (Barton and Gale 1993). We estimated linkage disequilibrium using the formula

$$\overline{D} = \frac{1}{n} n_{AB} - 2\overline{p}_A \overline{p}_B$$

where n is the number of individuals, A and B represents WW1 and WW2 alleles and P_A and P_B are the allele frequencies of “northern” alleles at the two loci. For this analysis we used only individuals that had been typed for both WW1 and WW2. We included individuals in populations occurring at a range of distances between the centers of the two markers. This analysis is complicated by the fact that the WW1 and WW2 are not perfectly coincident – the centre of WW1 is estimated to be 106.7 km north of WW2. This likely explains negative values of LD close to the centre of the hybrid zone. For our estimate of LD we use only populations within 90 km, which gives an LD = 0.04 and a dispersal estimate of 26.3 km.

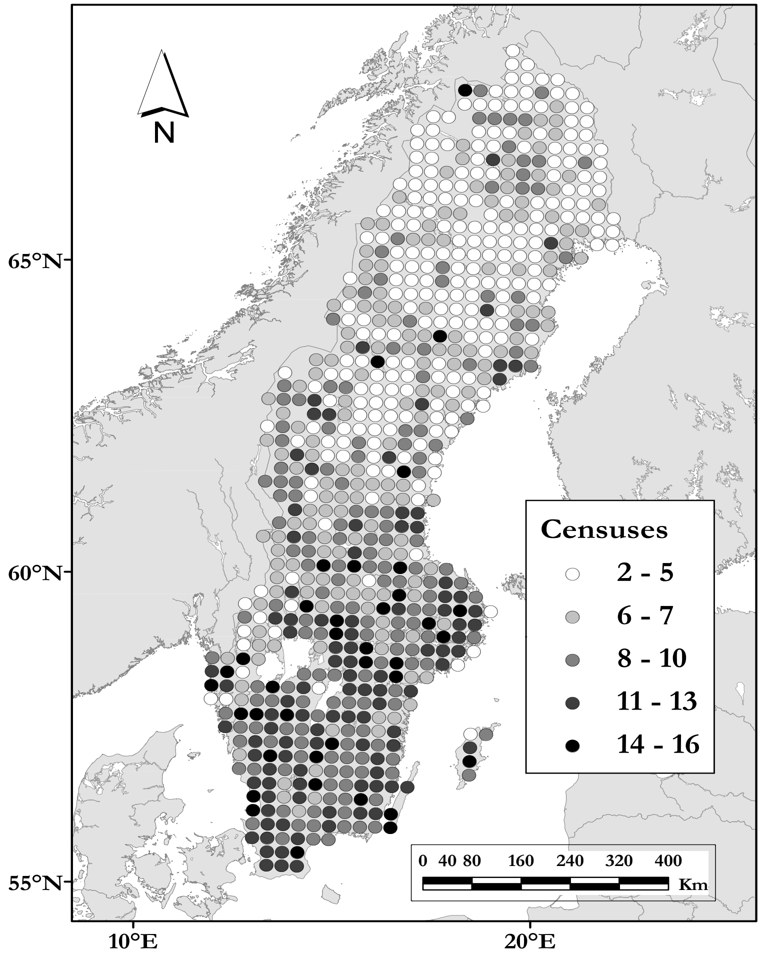


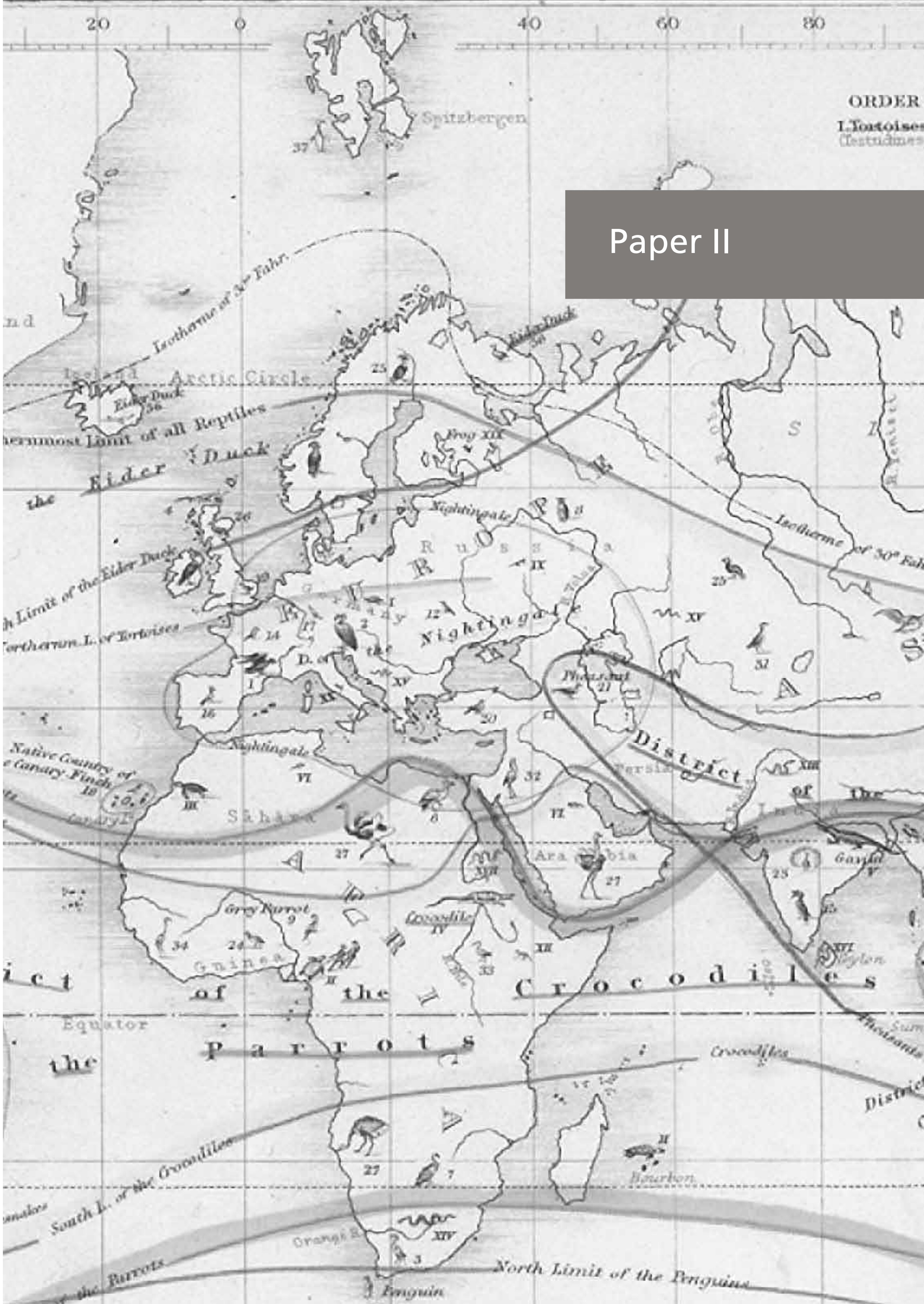
Figure S1. Location and census effort for the 716 Swedish Breeding Bird Survey routes, each route is an 8 km line transect, shaped as a 2 x 2 km square, and centered on a 25 km grid of the country. The survey starts at 04.00h local time at a date when all breeding birds of the area are supposed to have arrived. This means mid-May in southern-most Sweden and late June/early July in the northernmost routes at high altitude. All birds seen and heard along the route are counted and reported per kilometer. Some routes contain obstacles such as stretches of the sea, lakes, rivers and large fields of growing crops, which means that the entire route cannot be surveyed. The percentage of the route completed is noted each year for calculating effort and abundances. For a given route this percentage is normally stable between years.

Table S1. Proportional Eigen-vectors for each of the first five principal components (PC) that explain 79% of the total variation within the 25 selected environmental variables

	PC1	**PC2**	**PC3**	**PC4**	**PC5**
Pct Explained	35.79%	24.94%	7.99%	5.72%	4.91%
Biological Interpretation	North-south seasonal temperature gradient	Northwest-southeast dominate airflow	Warm, low elevation, coniferous forests	Regions of stable vegetation productivity	Moist broadleaf forest
Correlations[1]					
Longitude	**-0.67*****	**-0.83*****	**0.13***	-0.01	-0.02
Latitude	**-0.28*****	**-0.14***	**-0.53*****	**0.15***	**0.19****
Relative Abundance	0.08	-0.05	**0.37*****	**0.11****	**0.12****

[1] Bold significant ($P < 0.05$) correlations; asterisks indicate levels of significance

Paper II


20
0
40
60
80
ORDER
I. Tortoises
(Testudines
Spitzbergen
Isotherme of 32° Fahr.
Arctic Circle
Iceland
Eider Duck
Northernmost Limit of all Reptiles
the Eider Duck
South Limit of the Eider Duck
Northern L. of Tortoises
Frog
Nightingale
Russia
Germany
Nightingale
Isotherme of 30° Fahr.
Pheasant
District
Persia
Native Country of the Canary Finch
Sahara
Arabia
Grey Parrot
Crocodile
Guinea
Ceylon
Gavial
District of the Crocodiles
Equator
the Parrots
Pheasants
Crocodiles
Bourbon
South L. of the Crocodiles
Orange R.
North Limit of the Penguins
Penguin

Inferring the ecology of winter moult by stable isotope analyses across the wings of Willow Warblers

Keith W. Larson[1], Miriam Liedvogel[1], Staffan Bensch[1],
Susanne Åkesson[1], Leonard I. Wassenaar[2] and Keith A. Hobson[2]

[1] Department of Biology, Centre for Animal Movement Research, Ecology Building,
Lund University, SE-223 62 Lund, Sweden
[2] Environment Canada, 11 Innovation Blvd., Saskatoon, SK S7N 3H5, Canada

Abstract

We present a comparison of feather stable isotope (δ^{13}C, δ^{15}N) patterns representing the habitat and diet conditions for two subspecies of Willow Warblers (*Phylloscopus trochilus*) that breed in parapatry, but winter in different regions of sub-Saharan Africa. Previous analyses have shown that on average winter moulted innermost primaries (P1) show subspecific differences in δ^{15}N values, although individuals show substantial variation for both δ^{13}C and δ^{15}N within the subspecies. We examined whether corresponding variation in the timing of the winter moult as reflected by consistent intra-wing correlations for individual's δ^{13}C and δ^{15}N values, could explain some of the previously observed isotopic variation. Further, differential subspecific adaptations to winter precipitation patterns across Africa might result in a variable degree of site fidelity or itineracy during moult. We found no consistent trend in isotopic values from innermost to outermost primaries, thus inter-individual variation in the timing of moult does not explain the subspecific isotopic variation for P1. Patterns in wing feather δ^{13}C and δ^{15}N values indicated that 41% of the individuals from both subspecies shifted their diet or habitats during winter moult. Importantly, despite well-documented itineracy in Willow Warblers during the winter, 59% of the individuals had isotope signatures consistent with stable use of habitats or diets during winter moult. Repeatability analyses suggest that individuals of both subspecies initiate moult in similar habitats from year-to-year while feeding on isotopically similar diets.

Keywords: migratype, stable carbon-isotopes, stable nitrogen-isotopes, migratory divide, connectivity, diet, habitat, moult

Introduction

The subset of population-specific phenotypic traits that characterize a migratory strategy, namely, the onset, duration, and direction of migration define the migratory phenotype, or "migratype". In the boreal forest of Scandinavia, the Willow Warbler (*Phylloscopus trochilus*) is one of the most abundant long-distance Palaearctic-Afrotropical migratory birds (Ulfstrand and Högstedt 1976, Cramp and Brooks 1992). Two migratypes meet in Scandinavia forming a migratory divide with the southern *P. t. trochilus* wintering in West Africa while the northern *P. t. acredula* winters in East to South Africa (Hedenström and Pettersson 1987, Chamberlain *et al.,* 2000, 2000, Bensch *et al.,* 2006). On either side of the migratory divide, the two subspecies of Willow Warblers show clear differences in population-specific migratory behaviour. Differences in winter grown feather isotopic values between migratypes provide otherwise inaccessible insights into adaptations and the ecology during the moulting period in Africa, an important but difficult part of the annual cycle to study. Here we analyze the wing feather stable isotope (δ^{13}C, δ^{15}N) patterns from the sub-Sahara African wintering areas for these two Willow Warblers subspecies.

Researchers regularly use stable isotope analysis to better understand ecologies and life histories for both sedentary and migratory organisms (Rubenstein and Hobson 2004, Hobson and Wassenaar 2008, Inger and Bearhop 2008). Stable isotope analysis of consumer tissues reveals dietary history and the temporal and spatial patterns related to both abiotic and biotic processes encountered while foraging. For example, stable carbon-isotope (δ^{13}C) values in plants increase with altitude due to plant responses to the stress of decreased temperatures (Kelly 2000, Graves *et al.,* 2002, Hobson *et al.,* 2003). These values also increase in xeric (arid) habitats compared to mesic (moist) habitats (Smith and Epstein 1971), and decrease with increased latitude (Rubenstein and Hobson 2004). Importantly, food web δ^{13}C values reflect landscape carbon pools, for example the differences between plant photosynthetic pathways (C_3, C_4, and CAM) where C_4 plants have mean δ^{13}C values of approximately –12 ‰ compared with –27 ‰ for C_3 plants (West *et al.,* 2006). This means that diets derived from C_4 grasses are distinguishable from those of C_3 plants, such as shrubs, trees, and grasses. In addition, increased δ^{13}C values in C_3 plants are strongly correlated with water-use efficiency adaptations, especially in arid regions (Hobson 1999, Rubenstein and Hobson 2004). Stable-nitrogen isotope (δ^{15}N) values increase predictably at each trophic level and have higher values in arid compared to moist habitats (Heaton 1987, Hobson 1999, Rubenstein and Hobson 2004). Further, anthropogenic land-use practices such as the use of nitrogen fertilizers influence landscape nitrogen-isotope values (Rubenstein and Hobson 2004).

Inert tissues such as feathers have isotopic profiles that reflect the individual's diet over the period of feather growth and become fixed once fully grown. Isotopic information derived from feathers thus matches a species' moult or feather replacement schedule which is highly predictable for most species (Holmgren and Hedenström 1995). For birds that moult in sub-Saharan Africa, the east-west moisture gradient and differences in precipitation patterns north and south of the equator may make δ^{13}C and δ^{15}N isotopes useful indicators of moult provenance (Chamberlain *et al.,* 2000, 2000, Evans *et al.,* 2003, Møller and Hobson 2004, Pain *et al.,* 2004). The Willow Warbler annual cycle includes two complete moults, one after breeding in Eurasia and the other while wintering in Africa (Underhill *et al.,* 1992). If we assume that differences in feather isotope values indicate different isotopic landscapes (isoscapes) or provenance where feathers are grown, a comparison of feather isotope values between both subspecies may reveal differences in climate, habitat, and diet on their respective wintering grounds. In our case, the two Willow Warbler subspecies make an excellent model to study and contrast possible different behaviours and adaptations in a Palaearctic-Afrotropical migratory songbird.

In the spring of 2008 we collected Willow Warblers in both the south and north of Sweden for gene expression analyses as they returned to their breeding grounds from their wintering grounds in Africa. We salvaged all wing feathers from these birds and determined $\delta^{13}C$ and $\delta^{15}N$ values for each primary feather in order to investigate the temporal and spatial differences of their wintering localities and potential differences in winter moult ecologies. We know from previous work that $\delta^{15}N$ values differ between West African wintering *trochilus* and East to South African wintering *acredula*, however there is a lot of variation within each subspecies (Chamberlain *et al.,* 2000). Part of this variation could be caused by individual differences in the location, timing, and duration of their winter moult. The time span between the shedding of the innermost and outermost primary feather is at least four weeks, but might be substantially longer in slower moulting individuals (Underhill *et al.,* 1992, Bensch and Grahn 1993). If the isotopic values are found to change consistently across the wing (from lower to higher or vice versa), part of the within-subspecies variation in isotopic values as measured for the innermost primary could be caused by a corresponding variation in the timing of moult.

When comparing the subspecies, we predict that because *trochilus* arrives to winter in West Africa at the end of the rainy season we should see $\delta^{13}C$ and $\delta^{15}N$ values that reflect increasingly arid conditions and the physiological responses to these conditions, i.e. increasing $\delta^{13}C$ and $\delta^{15}N$ values from the replacement of the innermost to the outermost primary. These patterns are predicted to contrast with *acredula* which arrives in East to South Africa at the onset of the rainy season where increasingly wet conditions should result in stable or decreasing $\delta^{13}C$ and $\delta^{15}N$ values across all nine primaries.

Many Palaearctic-Afrotropical migrants use an "itinerancy" strategy on their sub-Sahara African wintering grounds, rather than being stationary or territorial (Moreau 1972, Salewski and Jones 2006). Itinerancy likely reflects the necessity to track highly seasonal resources that increase with the rainy seasons and decrease steadily after the rains end. In the case of itinerant winter residents, individuals may stay in one location to moult or change locations or shift diet in response to changes in resource availability. We know that some Willow Warblers likely move during their winter moult (Underhill *et al.,* 1992), but it is not known whether this is more common among *trochilus* or *acredula*. We predict greater variability in feather $\delta^{13}C$ and $\delta^{15}N$ values for *trochilus* as moult maybe more protracted and occur over larger isotopic gradients as they track resources following the moisture gradient south. Whereas, patterns of $\delta^{13}C$ and $\delta^{15}N$ variability across the wing were predicted to be less in *acredula* as individuals might show greater site fidelity due to improving conditions and stability of resources and moult duration being shorter.

Finally, Bensch *et al.,* (2006) found that there is significant between year repeatability in both $\delta^{13}C$ and $\delta^{15}N$, showing that to some extent these birds return to the same or ecologically similar moulting areas from year to year. We used a separate dataset of spring individuals captured across Sweden in two or more years to compare between-year repeatabilities of feather stable isotope values with repeatabilities from our wing feathers that represent the beginning (innermost primary) and end (outermost primary) of the moult sequence of the same individual. By comparing the within year variation (innermost to outermost primary) with the between year variation (only the innermost primary), we examine to what extent the unexplained between year variation can be accounted for by within year variation. Further, low between-year or within-wing (same year) repeatabilities would suggest low winter habitat or site-fidelity and/or switching diet, while high repeatabilities, high habitat or site-fidelity and/or a similar diet.

Table 1. Willow Warbler sample size and locations for Sweden by region, coordinates are in decimal degrees.

Site Name	Region	N	Latitude	Longitude	Elevation (m)
Anjan	North	4	63.73	12.54	439
Tångböle	North	4	63.35	12.59	520
Stensoffa	South	8	55.70	13.46	17
Vomb	South	1	55.67	13.55	26

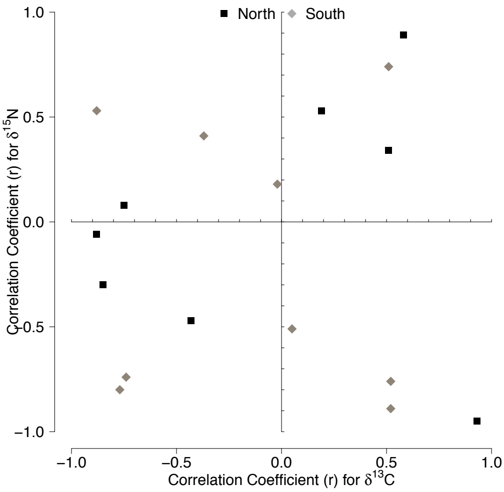


Figure 1. Pearson's correlation coefficient (r) for $\delta^{13}C$ and $\delta^{15}N$ and feather position.

Methods

Background and Sampling

Passerines typically replace all of their flight feathers and body feathers on an annual basis after breeding and before migration to the wintering grounds or after arrival on the wintering grounds (Svensson 1992, Pyle 1997). Flight feather replacement occurs in a fixed sequence that coincides with the replacement of the entire feather coat (Ginn and Melville 1983). For the Willow Warbler, the post-breeding moult in adults is quite rapid which can result in periods of flightlessness (Haukioja 1971). In addition, all individuals have a second, more protracted complete moult on their African winter-grounds (Underhill *et al.,* 1992). Both complete moults begin with innermost primary (P1) and ends with outermost full-sized primary (P9) (Norman 1990, Bensch and Grahn 1993).

We collected 17 adult Willow Warblers upon return to Sweden from the wintering grounds in Africa in the spring of 2008 from both sides of the migratory divide, i.e. nine "southern" and eight "northern", for a separate study on the genetic differences of the two migratypes (Table 1 and Figure 1). From the same individuals we salvaged the wings for stable isotope analysis. We used the tips for each of the nine full-sized primary feathers from the left wing of each individual for isotopic analyses. From a separate study on the migratypic differences of *trochilus* versus *acredula*, we collected adult Willow Warbler first primaries from males grown on their wintering grounds in Africa captured on their breeding grounds in Sweden, between 1996 and 2010. Fifty-eight of these individuals were recaptured during this period and were sampled for both $\delta^{13}C$ and $\delta^{15}N$, see methods described in Chamberlain *et al.,* (2000) and Bensch *et al.,* (2006).

We collected Willow Warblers following the ethical guidelines under a permit from Malmö/Lund djurförsöksetiska nämnd, permit number M94-08. Approval for permission for capturing birds came from Swedish Environmental Protection Agency and the Swedish Ringing Centre at the Natural History Museum in Stockholm.

Laboratory Analysis

We prepared 1.0 ± 0.1 mg of feather material removed from the distal end (the tip) of each primary feather on the left wing from each individual. All samples were loaded into tin cups and analyzed for $\delta^{13}C$ and $\delta^{15}N$ via continuous-flow isotope-ratio mass spectrometry (CFIRMS). Specifically, between 0.5 ($\delta^{13}C$) and 1.0 mg ($\delta^{15}N$) of material was combusted online using a Eurovector 3000 (Milan, Italy - www.eurovector.it) elemental analyzer. The resulting CO_2 and N_2 analyte gas from the samples was separated by Gas Chromatography (GC) and introduced into a Nu Horizon (Nu Instruments, Wrexham, UK - www.nu-ins.com) triple-collector isotope-ratio mass-spectrometer via an open split and compared to a pure CO_2 or N_2 reference gas. Stable-nitrogen and carbon isotope ratios were expressed in delta (δ) notation, as parts per thousand (*‰*) deviations from the primary standards of atmospheric nitrogen and VPDB (Vienna Pee Dee Belemnite carbonate) standards, respectively. Using previously calibrated internal laboratory C and N standards (powdered keratin, peagrain and gelatin), within run precisions for $\delta^{15}N$ and $\delta^{13}C$ were better than ±0.15*‰*.

Statistical Analysis

We used a general linear mixed model (GLMM) approach to test whether our samples demonstrated the previously described regional differences in winter grown primary feather $\delta^{15}N$ between *acredula* in the north and *trochilus* in the south (Chamberlain *et al.,* 2000). Model structure included $\delta^{13}C$ and $\delta^{15}N$ separately as the dependent variables with region as fixed factors and individual as a random factor. To determine if feather isotope values were correlated with primary position across all nine primary feathers from each individual, we calculated a Pearson's correlation coefficient (r) for feather position and each isotope ($\delta^{13}C$ and $\delta^{15}N$) separately. We plotted r for both $\delta^{13}C$ and $\delta^{15}N$ with a = 0.05 levels of significance to identify relationships between stable isotope values and feather position and fitted line with a Bonferroni correction (n = 8, a = 0.006, r = –0.86, 0.86) for multiple comparisons to provide conservative estimates of significance for each correlation. In addition, we plotted the effect size (r) with corresponding confidence intervals for each individual to evaluate the strength of the correlation between feather position and feather isotopes ($\delta^{13}C$ and $\delta^{15}N$) and the confidence in interpreting the effect (Nakagawa and Cuthill 2007). Next, we calculated the repeatability or intraclass correlation coefficient (R) for both isotopes measured across the tips of (i) all nine primaries, and (ii) separately for P1 and P9. In the latter test, the first and last primaries represent the start and end points of an individual's moult. For this repeatability analysis we used a two-way ANOVA (Shrout and Fleiss 1979, Lessells and Boag 1987), as implemented in the *psych* R package (Revelle 2012), with feather position as a fixed effect to subdivide the total variability into three components, (i) between-individual, (ii) between-feather position, and (iii) the residual to determine the intraclass correlation coefficient (Shrout and Fleiss 1979). For comparison, we calculated between-year repeatabilities with a separate Swedish Willow Warbler dataset (1996 and 2010). Here we used only the first primary (P1) for between-year samples from the same individual. Between-year samples (n = 58) allowed us to compare annual differences in habitat use or diet. Finally, we then compared the repeatabilities to determine whether R was lower or higher within the same moult episode (P1/P9, wing dataset) compared to the same feather (P1, 1996 to 2010 dataset) in different moult episodes (i.e. between years).

All analyses were performed in R version 2.14 (R Development Core Team 2012).

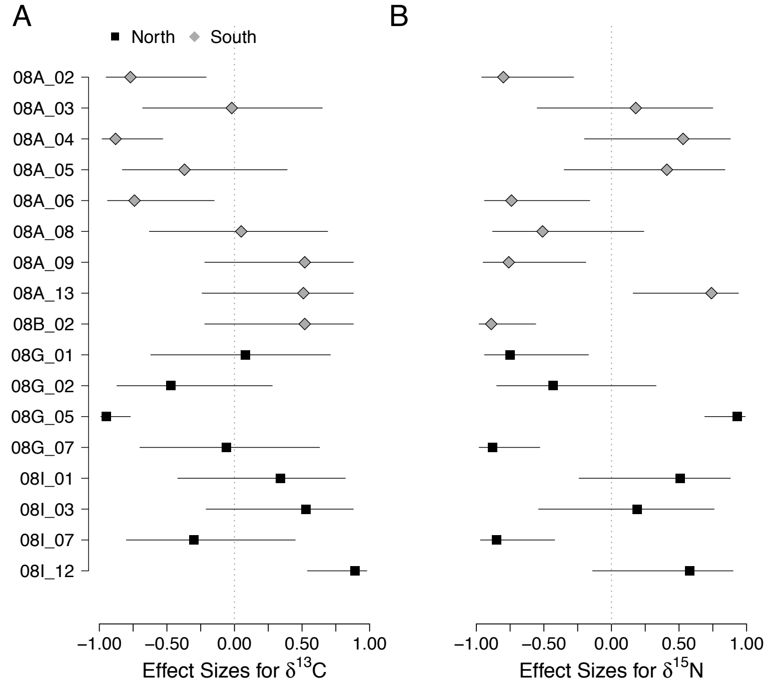


Figure 2. Effect sizes (correlation coefficient) with 95% confidence intervals for both (A) δ^{13}C and (B) δ^{15}N and primary feather position (1 to 9) for willow warbler winter grown feathers in Africa.

Results

We found significant differences in winter grown primary feather δ^{15}N (T_{15} = –2.90, SE = 0.90, P = 0.01), but not δ^{13}C (T_{15} = –0.16, P = 0.87) between southern *trochilus* that winters in West Africa and northern *acredula* which winters in East or South Africa. The mean estimates for δ^{15}N values (±SD) were 7.4 ± 1.1 ‰ for *trochilus* and 10.0 ± 2.4 ‰ for *acredula.* The corresponding estimates for δ^{13}C were –21.28 ± 1.3 ‰ for *trochilus* and –21.27 ± 1.4 ‰ for *acredula*. Fourteen individual's showed significant δ^{13}C (Table S1) and/or δ^{15}N (Table S2) correlations across the primary feathers (Figure 1), demonstrating consistent changes in isotopic values from the innermost to the outermost feather. Seven of these individuals revealed significant negative and two positive correlations for δ^{15}N and four showed significant negative and one positive for δ^{13}C; one of these individuals was significant for both isotopes. Of these 14 individuals, inspection of effect sizes and confidence intervals (Tables S1 and S2, Figure 2 A–B) demonstrated that only seven of these individuals had confidence intervals that did not overlap zero, supporting that these individuals from both subspecies likely changed their diet or habitats during moult.

Repeatability analyses for all nine primaries were significant and similar in both the north and south, but when we compared the innermost (P1) to the outermost (P9) primary only δ^{15}N was significant for both regions while δ^{13}C significant only for the north (Table 2). For the separate dataset representing between-year captures of the same individuals, repeatability analysis of the innermost primary (P1) for both δ^{13}C and δ^{15}N were significant (Table 3). There

Table 2. Repeatability analysis for two subspecies of Willow Warblers in the north and south of Sweden reporting the intraclass correlation coefficients (R) for δ^{13}C and δ^{15}N values in the tips of primary flight feathers by feather position

Region	**Isotope**	DF	SS	MS	*F*	*P*	*R*	lower CI	upper CI
Only primaries (P1 and P9)									
North	δ^{13}C	7	75.34	10.76	5.47	0.020	0.69	0.05	0.93
South	δ^{13}C	8	25.38	3.17	1.89	0.194	0.31	–0.40	0.79
North	δ^{15}N	7	93.93	13.42	4.70	0.029	0.65	–0.03	0.92
South	δ^{15}N	8	19.11	2.39	5.11	0.017	0.67	0.07	0.92
All 9 primaries (P1-P9)									
North	δ^{13}C	7	129.45	18.49	18.72	<0.001	0.66	0.42	0.90
South	δ^{13}C	8	120.05	15.01	14.70	<0.001	0.60	0.36	0.86
North	δ^{15}N	7	366.32	52.33	32.57	<0.001	0.78	0.57	0.94
South	δ^{15}N	8	80.15	10.02	30.54	<0.001	0.77	0.57	0.93

Table 3. Willow Warbler repeatability analysis reporting the intraclass correlation coefficients (R) for δ^{13}C and δ^{15}N values in primary flight feathers (P1) of individuals captured in different years (n = 58). Data collected between 1996 and 2011.

Isotope	**DF**	SS	MS	*F*	*P*	*R*	**lower CI**	**upper CI**
δ^{13}C	57	259.17	4.55	2.45	<0.001	0.42	0.18	0.61
δ^{15}N	57	321.84	5.65	6.11	<0.001	0.72	0.57	0.82

were wide confidence intervals with moderate effect sizes for within-year (both for comparing P1 and P9). When we analysed the effect sizes for the repeatability of between-year samples (P1 only) for δ^{15}N, we found a strong effect size with narrow confidence intervals (Figure 3). The same analyses for δ^{13}C resulted in moderate effect size with wider confidence intervals (Figure 3).

Discussion

Here we analyze wing feather stable isotope (δ^{13}C, δ^{15}N) patterns from two Willow Warbler subspecies with distinctly different wintering areas to investigate population-specific adaptations and ecology during the wintering (moulting) period in Africa, which is an important little known part of the annual cycle. We found that in 41% of the birds the primary feather δ^{13}C and δ^{15}N values changed significantly across the wing from the innermost to the outermost primary feather suggesting a shift in diet, habitat, or location during the winter moult. Contrary to our predictions that *trochilus* in West Africa would likely show increasing δ^{13}C and δ^{15}N values from the innermost to the outermost feather in response to conditions becoming drier during the moulting period; the observed changes were neither consistent among individuals nor subspecies.

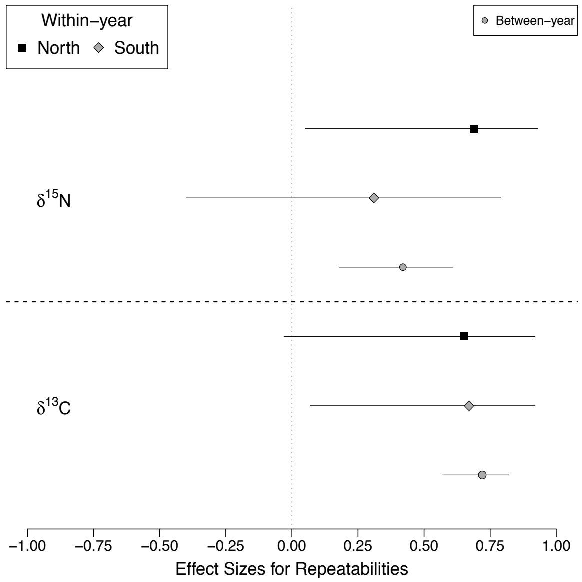


Figure 3. Effect sizes (correlation coefficient) with 95% confidence intervals for both $\delta^{13}C$ and $\delta^{15}N$ repeatability analysis for willow warbler winter grown feathers in Africa. Within-year samples, (A) Primaries 1 to 9, (B) primaries 1 and 9, and between-year samples (C) P1 only.

Willow Warblers occur in a wide range of habitats across sub-Saharan Africa (Herremans 1997, Salewski and Jones 2006, Jones *et al.,* 2010). The use of these habitats varies temporally with changes in densities recorded throughout the wintering period and across their range, suggesting intra-continental movements or 'itineracy' (Jones *et al.,* 2010). Many species of Palaearctic-Afrotropical migrants, including Willow Warblers, track the rains associated with the Intertropical Convergence Zone (ITZ) as it shifts south after the summer rains and then moves back north in the spring (Moreau 1972, Jones *et al.,* 1998, Salewski *et al.,* 2002). Presumably as local habitats dry out and food resources become scare, many individuals have to intensify their foraging movement in order to track available resources at different times (Jones *et al.,* 1998, Salewski and Jones 2006).

In West Africa, the drying out occurs between December and March coincident with *trochilus* winter moult (Williamson 1962, Underhill *et al.,* 1992, Salewski *et al.,* 2004). For *trochilus* the winter moult of the outermost primary occurs about a month later than the innermost primary as habitats become dryer. Given these conditions, one might expect $\delta^{13}C$ and $\delta^{15}N$ values to increase if they follow the general patterns for how these isotopes respond to increasingly arid climate conditions (Rubenstein and Hobson 2004). Although isotopic patterns changed across the wing for six individuals, the direction of change was both towards higher and lower values. Hence, this suggests that the starting date of moult cannot explain the between-individual-variation in isotope values of the innermost primary.

Phylloscopus t. acredula arrives in East Africa at the onset of the rainy season as the ITZ moves south, where they encounter improved conditions and a flush of food resources (Moreau 1972, Jones 1995). For these populations the moult period typically occurs between December and April (Williamson 1962, Underhill *et al.,* 1992). In a study to investigate the timing and extent of moult in southern African *acredula* Underhill *et al.,* (1992) found fewer individuals than expected had completed their moult, a suggestion that the Willow Warblers also shift locations during the moulting period. For *acredula* one might expect δ^{13}C and δ^{15}N values to decline given the increased precipitation during the moult period. However, changes in isotopes occurred in in both directions for five individuals. Further, we could find no information available in the literature on possible differences in primary growth rates or in feather quality between the two subspecies during the winter that may impact feather δ^{13}C and δ^{15}N values.

The changes in δ^{13}C and δ^{15}N for both subspecies in our study may reflect a diet shift or that they supplemented their normal diet of insects with other seasonally abundant resources. For example, Biebach *et al.,* (1986) noted that Willow Warblers, despite being primarily foliage-gleaning insectivores, consumed berries and other plant materials in Africa. How much feather δ^{13}C and δ^{15}N values reflect a shift in diet in these arid regions is unknown. Likewise, the time-integration for δ^{13}C and δ^{15}N into the diet items consumed during feather growth are unknown, e.g. berries or insect biomass might have grown months previous to consumption. However, well-established patterns of δ^{15}N in animal tissues based on switching diet or trophic position do exist. Repeatability analysis of the innermost and outermost winter grown primaries (Table 3) resulted in similar repeatabilities to our between-year (same-individual) estimates for both δ^{13}C and δ^{15}N. This suggests that individuals are as faithful to the area where they start moult between years as they are to the area where they complete moult within a year. Alternatively, the birds' restrict their movements to habitats with similar isotopic conditions and prey base. These results also indicate that primary feather position was not an important consideration for isotopic studies in Willow Warblers.

Conclusions

Our stable isotope analyses revealed that for nearly 41 percent of the individual's sampled, primary feathers acquired on the wintering grounds in Africa showed changes in isotopic values indicative of a shift in diets or change in habitats exploited during moult. These isotopic changes were not consistent among either migratype, suggesting no phenotypic differences in moulting strategies on the wintering grounds in Africa. For example, five individuals of both *trochilus* and *acredula* showed significant correlations between δ^{15}N and feather position suggesting that individuals may shift their diets or change habitats during moult. The significant correlation with δ^{13}C for two other individuals suggests a change in habitat or region. These individuals may also have shifted their winter quarters in Africa, a behaviour described as 'itinerancy', in response to changes in environmental conditions. This behaviour is thought to be an adaptation to the dramatic seasonal changes that occur south of the Saharan desert as the Intertropical Convergence Zone moves south during the period Palaearctic migrants over winter in Africa (Moreau 1972, Jones 1995, Fry 2008). Finally, despite their well-documented itineracy on the winter grounds almost 60 percent of the individuals we sampled showed stable isotopes signatures consistent with stationary behaviour or diet consistency during moult.

Acknowledgements

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Supplementary Material

Tables S1 and S2, see next page.

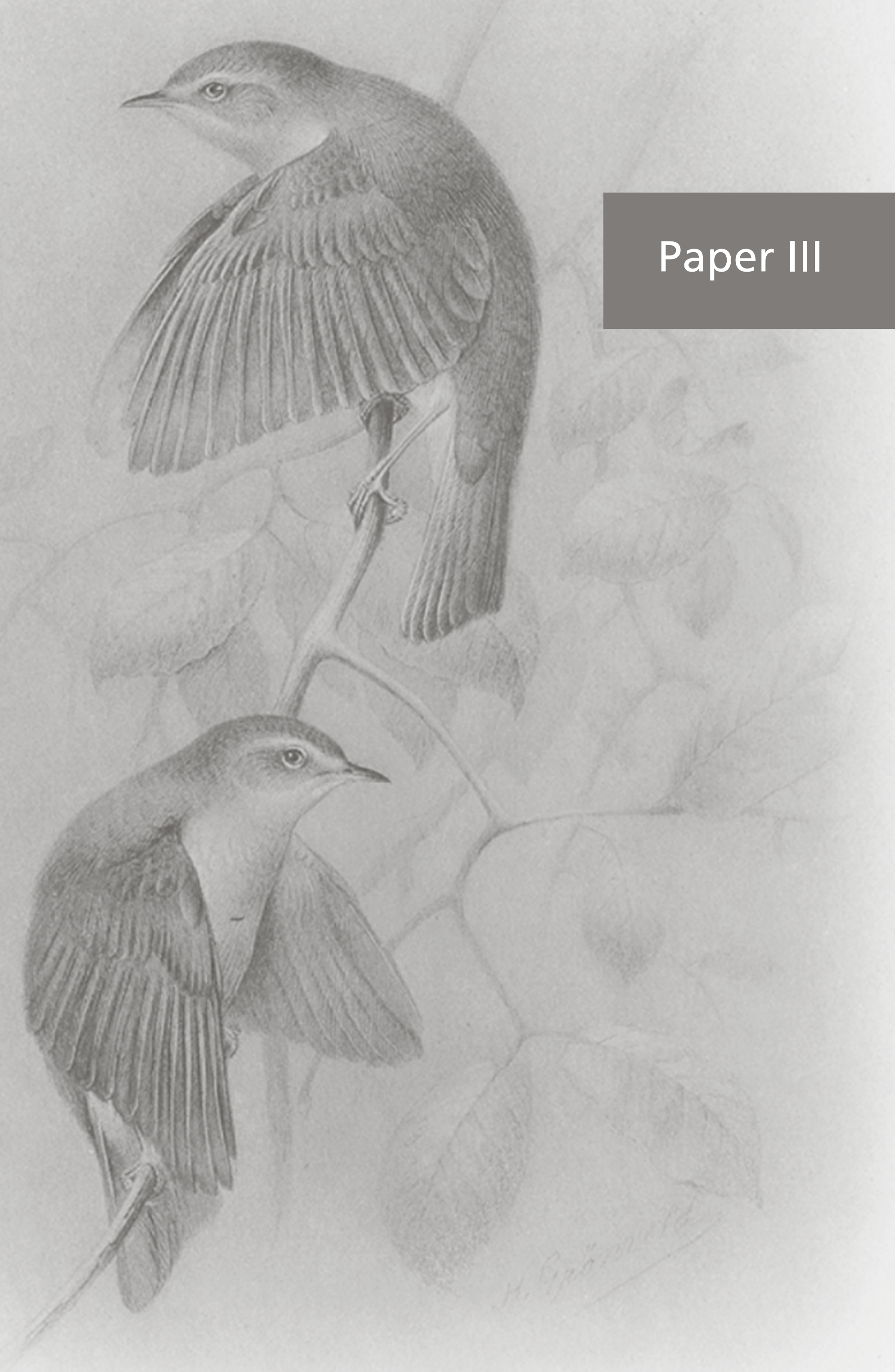
Table S1. Stable carbon-isotope ($\delta^{13}C$) values (‰) for the tip of each full-sized primary (9) sampled from Willow Warblers captured in the south and north of Sweden during the spring of 2008 with feathers moulted on their African wintering grounds. Pearson's correlation coefficient (r) given with *p*-value and 95% confidence intervals, * = individuals with significant p-values and wide confidence intervals, ** = individuals with significant p-values and narrow confidence intervals.

Individual	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**Mean**	**SD**	*r*	*p*	**lower CI**	**upper CI**
South															
08A_02*	–18.67	–19.16	–20.38	–20.96	–21.61	–21.65	–21.31	–20.80	–21.28	–20.65	1.07	–0.77	0.016	–0.95	–0.21
08A_03	–20.06	–20.30	–21.21	–22.90	–22.87	–21.13	–20.68	–20.58	–20.69	–21.16	1.04	–0.02	0.953	–0.68	0.65
08A_04*	–16.54	–15.60	–16.61	–19.65	–20.98	–21.18	–21.70	–21.18	–21.33	–19.42	2.46	–0.88	0.002	–0.98	–0.53
08A_05	–20.33	–20.28	–20.58	–20.80	–21.24	–20.94	–20.54	–21.04	–20.42	–20.69	0.34	–0.37	0.332	–0.83	0.39
08A_06*	–21.28	–21.24	–21.22	–21.20	–21.47	–21.23	–21.89	–21.89	–21.60	–21.45	0.28	–0.74	0.023	–0.94	–0.15
08A_08	–23.86	–23.95	–24.00	–24.16	–24.93	–24.93	–24.48	–24.17	–23.10	–24.18	0.57	0.05	0.894	–0.63	0.69
08A_09	–21.30	–21.44	–21.49	–21.49	–21.10	–20.84	–20.78	–21.58	–20.76	–21.20	0.33	0.52	0.150	–0.22	0.88
08A_13	–21.34	–24.13	–23.03	–21.77	–21.40	–21.57	–21.45	–21.32	–21.59	–21.96	0.97	0.51	0.164	–0.24	0.88
08B_02	–20.87	–20.95	–20.76	–20.90	–21.02	–20.84	–21.11	–20.64	–20.11	–20.80	0.29	0.52	0.152	–0.22	0.88
North															
08G_01	–20.32	–20.02	–19.92	–20.05	–20.23	–20.73	–20.91	–19.87	–19.58	–20.18	0.42	0.08	0.836	–0.62	0.71
08G_02	–19.35	–19.38	–19.70	–19.94	–20.43	–20.08	–20.81	–20.90	–19.24	–19.98	0.63	–0.47	0.199	–0.87	0.28
08G_05*	–21.20	–21.29	–21.85	–22.22	–22.34	–22.42	–22.69	–22.75	–22.73	–22.17	0.59	–0.95	0.000	–0.99	–0.77
08G_07	–21.61	–21.42	–21.84	–21.68	–21.79	–21.49	–21.88	–21.62	–21.54	–21.65	0.16	–0.06	0.878	–0.70	0.63
08I_01	–18.30	–18.53	–19.55	–20.18	–20.96	–21.08	–21.50	–17.42	–13.18	–18.97	2.58	0.34	0.376	–0.42	0.82
08I_03	–22.30	–22.20	–22.18	–22.14	–21.55	–20.85	–23.10	–20.75	–20.90	–21.77	0.81	0.53	0.142	–0.21	0.88
08I_07	–21.60	–21.77	–22.17	–22.21	–22.24	–22.47	–22.19	–21.89	–21.89	–22.05	0.27	–0.30	0.429	–0.80	0.45
08I_12**	–24.12	–24.08	–24.00	–24.20	–24.16	–22.87	–22.51	–22.12	–22.30	–23.37	0.90	0.89	0.001	0.54	0.98

Table S2. Stable carbon-isotope ($\delta^{15}N$) values (‰) for the tip of each full-sized primary (9) sampled from Willow Warblers captured in the south and north of Sweden during the spring of 2008 with feathers moulted on their African wintering grounds. Pearson's correlation coefficient (*r*) given with p-value and 95% confidence intervals, * = individuals with significant p-values and wide confidence intervals, ** = individuals with significant p-values and narrow confidence intervals.

Individual	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**Mean**	**SD**	***r***	***p***	**lower CI**	**upper CI**
South															
08A_02*	7.35	7.27	7.23	7.07	7.13	7.38	6.98	6.88	6.72	7.11	0.22	–0.80	0.010	–0.96	–0.28
08A_03	5.72	5.76	5.51	6.12	5.80	5.34	5.47	5.78	6.20	5.74	0.28	0.18	0.644	–0.55	0.75
08A_04	7.33	7.45	7.30	7.19	7.56	7.54	8.12	7.71	7.44	7.52	0.27	0.53	0.139	–0.20	0.88
08A_05	9.45	9.81	9.70	9.08	8.60	9.34	10.38	9.67	10.48	9.61	0.59	0.41	0.273	–0.35	0.84
08A_06*	7.71	7.76	7.95	7.73	7.69	7.24	6.64	6.42	7.31	7.38	0.54	–0.74	0.021	–0.94	–0.16
08A_08	8.26	8.10	8.07	8.70	8.98	9.44	7.84	7.26	6.05	8.08	1.00	–0.51	0.163	–0.88	0.24
08A_09*	8.04	7.73	7.55	6.77	6.99	6.84	7.09	6.78	7.07	7.21	0.46	–0.76	0.018	–0.95	–0.19
08A_13*	6.96	6.00	5.91	6.48	7.06	7.27	8.32	7.85	7.45	7.03	0.81	0.74	0.022	0.16	0.94
08B_02**	6.90	6.73	6.97	6.67	6.76	6.59	6.58	6.42	6.30	6.66	0.21	–0.89	0.001	–0.98	–0.56
North															
08G_01*	11.64	11.83	11.66	11.30	11.32	11.29	10.91	10.77	11.40	11.35	0.34	–0.75	0.020	–0.94	–0.17
08G_02	9.50	9.55	9.22	8.84	8.89	9.14	9.80	9.13	8.51	9.18	0.40	–0.43	0.249	–0.85	0.33
08G_05**	7.70	7.80	8.29	9.65	10.25	13.67	14.66	13.48	13.76	11.03	2.85	0.93	0.000	0.69	0.99
08G_07**	8.70	8.70	8.36	8.11	8.47	8.13	6.88	7.21	7.16	7.97	0.70	–0.88	0.002	–0.98	–0.53
08I_01	14.07	13.91	14.07	14.26	13.99	13.51	13.77	14.83	15.13	14.17	0.51	0.51	0.165	–0.24	0.88
08I_03	10.93	10.85	11.31	11.28	11.96	13.41	8.12	12.96	12.03	11.43	1.52	0.19	0.616	–0.54	0.76
08I_07**	8.16	8.29	8.43	8.53	8.10	7.65	7.60	7.51	7.38	7.96	0.43	–0.85	0.004	–0.97	–0.42
08I_12	6.44	6.18	6.33	6.49	6.34	8.65	7.73	7.13	7.07	6.93	0.82	0.58	0.105	–0.14	0.90

Paper III



No evidence for assortative mating in a willow warbler migratory divide

Keith W. Larson[1], Miriam Liedvogel[1], Arzu Gursoy[2], Max Lundberg[1],
Leonard I. Wassenaar[3], Keith A. Hobson[3], Staffan Bensch[1], Susanne Åkesson[1]

[1] Department of Biology, Lund University, 22362 Lund, Sweden
[2] Department of Biology, Faculty of Arts and Sciences, University of Ondokuz Mayis, Samsun, Turkey.
[3] Environment Canada, 11 Innovation Blvd., Saskatoon, SK S7N 3H5, Canada

Abstract

Hybrid zones can be maintained by pre-zygotic (assortative mating), post-zygotic (hybrid fitness), or a combination of these selection factors. We investigated the occurrence of assortative mating in two willow warbler subspecies that form a migratory divide and hybrid zone in central Sweden. The two subspecies represent two distinct migratypes defined by clear differences in their migratory phenotypes (i.e. migratory direction and distance) resulting in distinct geographically separate wintering areas in Africa. Despite near panmixia for mtDNA and microsatellite genetic markers for the two migratypes, we have found narrow coincident trait clines in the hybrid zone including morphology, stable nitrogen-isotopes (proxy for wintering grounds), and one AFLP marker (allele frequency coincides with migratory direction). For willow warblers, documentation of actual hybridization across the migratory divide or the potential magnitude thereof is unknown. In order to assess the role of assortative mating for limiting hybridization we conducted nest searching in the central part of the hybrid zone to identify and assess social pairings. Our results suggest there is no assortative mating across the willow warbler migratory divide based on a comparison of plumage colour, morphology, stable nitrogen-isotopes, and one AFLP marker, despite a difference in arrival dates for males of the two migratypes. This work lends further support for the hypothesis that both willow warbler migratypes freely hybridize across the migratory divide. We speculate that such post-zygotic selection is acting on the expression of the genetic migratory program where hybrid offspring take an intermediate migratory route resulting in lower survival.

Keywords: pre-zygotic selection, post-zygotic selection, reproductive isolation, willow warbler, migratype, hybrid zone, *Phylloscopus trochilus*

Introduction

Migratory divides are regions where two migratory phenotypes, or "migratypes", of the same species or sister taxa meet (Chamberlain *et al.*, 2000; Dallimer *et al.*, 2003; Irwin and Irwin, 2005). Migratory divides result from the dispersal and range expansion of two allopatric populations that then meet in secondary contact (Rohwer and Irwin, 2011). Examples of migratory divides based on secondary contact include, the willow warbler (*Phylloscopus trochilus*) (Hedenström and Pettersson, 1984; Hedenström and Petterson, 1987; Bensch *et al.*, 1999; Chamberlain *et al.*, 2000) and Swainson's thrush (*Catharus ustulatus*) (Ruegg, 2008). Alternatively, migratory polymorphisms can arise from a single migratory population where some individuals develop a novel migratory program, e.g. European blackcaps (*Sylvia atricapilla*) (Berthold *et al.*, 1992; Rolshausen *et al.*, 2009). Avian migratypes differ in their endogenous controlled migratory programs, including the direction of travel, distance traveled, and potentially the timing of other migration associated behaviours (e.g., post-breeding moult, pre-migration fattening) and cues used for navigation (Åkesson and Hedenström, 2007). In several species of passerines it has been shown the migratory program is genetically controlled (reviewed in Liedvogel *et al.*, 2011). These studies include selection and cross-breeding experiments with European blackcaps (Helbig, 1991a; b) orientation experiments with wheatears (*Oenanthe oenanthe*) (Ottosson *et al.*, 1990; Sandberg *et al.*, 1991), and displacement experiments with starlings (*Sturnus vulgaris*) and chaffinches (*Fingella coelebs*) (Perdeck, 1958). Hence, these studies support the prediction that the migratory program is under genetic control, although only a few components of it have been studied in a restricted number of species.

The study of migratory divides requires both an assessment of (i) the strength of selection on phenotypic and genotypic traits and (ii) the role of pre- or post-breeding selection in maintaining the divide. Assortative mating provides an important mechanism for reproductive isolation between two parapatric populations (Huber *et al.*, 2007; Hendry *et al.*, 2009). Differences in habitat, diet, behaviour, and breeding phenology all have the ability to raise reproductive barriers (Patten *et al.*, 2004; Bearhop *et al.*, 2005; Huber *et al.*, 2007). For example, in the European blackcap the timing of arrival to the breeding grounds promotes assortative mating between the two migratypes with different wintering grounds, i.e., UK and the Iberian Peninsula to NW Africa (Bearhop *et al.*, 2005; Rolshausen *et al.*, 2010). Post breeding selection may take place through the reduction in hybrid fitness. In cross-breeding experiments with European blackcaps, F_1 hybrid offspring demonstrated an intermediate migratory direction to the two parental migratory phenotypes (Helbig, 1991a; b). In this case, selection would act against the hybrid migratory program because their intermediate southern migratory route would necessitate crossing two prominent ecological barriers (i.e., the Mediterranean Sea and Sahara desert) at much wider points than their parental populations where presumably they lack physiological adaptations for long-distance flights without stop-over points to refuel (Helbig, 1991a; Bensch *et al.*, 2009).

In central Sweden two distinct willow warbler migratypes meet and form a migratory divide. Each migratype likely possesses a distinct endogenously controlled migratory program, including both migratory direction and distance. In southern Sweden *P. t. trochilus* migrates SW in the autumn to winter in West Africa, whereas in the north, *P. t. acredula* migrates SSE to winter in East to South Africa. Ringing recoveries (Hedenström and Pettersson, 1987; S. Bensch *et al.*, 1999; Chamberlain *et al.*, 2000) and stable nitrogen-isotopes (δ^{15}N) (Chamberlain *et al.*, 2000; Bensch, Bengtsson, *et al.*, 2006) identify both the migratory direction and wintering grounds for the two migratypes.

Despite the clear distinction in migratory behaviour, molecular analyses reveal almost complete panmixia (Fst ≈ 0) in neutral markers, i.e., mtDNA and microsatellites, suggesting recent divergence (S. Bensch *et al.*, 1999, 2009). In contrast to the high level of genetic similarity, phenotypic traits including plumage colouration, morphology and δ^{15}N (proxy for wintering grounds) and one genetic marker associated with migratory direction, present steep clines (< 300 km) between the *acredula* in the north and *trochilus* in the south (Chamberlain *et al.*, 2000; Bensch *et al.*, 2002; Bensch, Bengtsson, *et al.*, 2006; Bensch *et al.*, 2009). The strength of selection maintaining these clines can be estimated with cline models using dispersal distance and the time since secondary contact (Barton and Hewitt, 1985; Gay *et al.*, 2008; Bensch *et al.*, 2009). Although dispersal in passerines is notoriously difficult to determine (Paradis *et al.*, 1999), Bensch *et al.,* (2009) assumed a conservative 80 km root mean square dispersal distance for the Swedish willow warblers. Time since secondary contact is equally difficult to assess, but given the willow warblers ability to occupy most habitats with trees and shrubs it is likely they recolonized Scandinavia soon after the last glacial ice sheets receded in Scandinavia (Bensch *et al.*, 1999, 2009). Bensch *et al.,* (2009) used an extremely conservative value of 150 years based on the first documentation of the ubiquitous distribution and high abundance of willow warblers across the entirety of Sweden by the early 19th Century (Nilsson, 1817). Using these two reference values the width of the trait clines for the willow warbler hybrid zone would be approximately 1200 km given neutral diffusion of the two migratypes across the migratory divide (Bensch *et al.*, 2009). In our case the width of the clines are significantly smaller than the estimated width suggesting strong selection resulting from assortative mating or reduced hybrid fitness (Bensch *et al.*, 2009).

Our previous work demonstrated that the migratory divide for willow warblers and the associated hybrid zone must be maintained by selection as clines for both phenotypic and genotypic traits are steepest at the exact location of the migratory divide (Bensch *et al.*, 1999, 2009). However, we do not know whether this selection is pre-zygotic (i.e. preventing hybridization) or post-zygotic (i.e. reduced hybrid fitness). In the current study we investigate whether any form of assortative mating (i.e. premating isolation) is occurring at a rate that would reflect the steepness of the previously described phenotypic trait clines. We address this question by studying a breeding site in the center of the hybrid zone where both willow warbler migratypes co-occur. We assessed the degree of assortative mating based on the comparison of the characteristic phenotypic traits of breeding pairs captured on their territories.

Materials and Methods

Background

Willow warblers are small 8 to 10 g insectivorous ground nesting long-distance migratory songbirds found in abundance across their Scandinavian breeding grounds from north to south (Ulfstrand and Högstedt, 1976; Cramp and Brooks, 1992; S. Bensch *et al.*, 1999). In central Sweden a migratory divide splits willow warblers into two recognized subspecies characterized by different migratory phenotypes (Hedenström and Pettersson, 1987; Bensch *et al.*, 1999, 2002, 2009; Chamberlain *et al.*, 2000). Despite an overall cline in both colouration and morphometrics from north-to-south, within site variance across their range precludes identification of either migratype in the hand (Salomonsen, 1945; Bensch *et al.*, 2009). (Salomonsen, 1928) first described the phenotypic differences between the two willow warbler migratypes in the early 20th Century. Later, ringing recoveries and δ^{15}N values derived from winter grown feathers shed light on the specific differences in migratory direction, routes, and wintering grounds (Hedenström and Pettersson, 1987; Chamberlain *et al.*, 2000; Bensch, Bengtsson, *et al.*, 2006). Although clear differences in migratory direction and distance to wintering grounds exist for the both migratypes, there is no apparent differences in the timing of autumn migration (Högstedt and Persson, 1982; Hedenström and Petterson, 1987; Lindström *et al.*, 1996). Spring arrival to the breeding grounds and nest initiation by *trochilus* in southern Sweden occurs on average 12 days earlier than the *acredula* in the mountains of northern Sweden (Hedenström and Pettersson, 1984). However, it is unknown if the two migratypes show similar differences in spring arrival and breeding dates within the migratory divide. Finally, in 2001–2002 Irwin and Irwin (unpublished data) made detailed song recordings of both migratypes across their breeding grounds in Sweden. A comparison of sonograms from both sides of the migratory divide did not reveal any phenotypic differences in song, making song an unlikely mode for subspecific identification and positive assortative mating.

Between 1996 and 2010 we sampled over 2000 individuals at 98 sites across Sweden to access trait differences between individuals and populations and to identify the location and structure of the migratory divide of the willow warbler. Here we measured three phenotypic traits, plumage colour, morphology (composed of six measurements), and δ^{15}N (proxy for wintering grounds) and one AFLP derived genetic marker (coincides with the migratory divide for migratory direction). Results from these efforts demonstrate coincident four steep trait clines (Bensch *et al.*, 1999, 2009). These analyses identified the location of the hybrid zone between 61.4° to 64.1° N (latitude) with the center of each cline close to 62.7° N (Bensch *et al.*, 2009).

Sample Methods

Between 10 May and 14 July, 2011 we conducted territory mapping and nest searching activities at the Flatruet breeding site (62.7° N, 12.7° E, 846 meters ASL) in the north-to-south center of the hybrid zone (Figure 1). In addition, we also conducted territory mapping at the Ljungdalen breeding site (62.8° N, 13.0° E, 593 meters ASL). We selected these study sites based on an assessment of our unpublished dataset collected between 2008 and 2010 to ensure we picked sites with a comparable frequency of both willow warbler migratypes. We based the migratype determination on the frequencies of winter grown feather δ^{15}N values and the WW2 AFLP marker. For δ^{15}N site mean values of ≤ 7 ‰ reflect sites dominated by the *trochilus* migratype and those > 9 ‰ the acredula migratype, south and north of the migratory divide, respectively (Chamberlain *et al.*, 2000; Bensch, Bengtsson, *et al.*, 2006). The terminal forms of each migratype in Sweden show significantly different allele frequencies for WW2 AFLP molecular marker on either side of the migratory divide (Bensch *et al.*, 2009).

Over the 2011 breeding season we identified all the territories (n = 33) held within our Flatruet study site, captured the males (n = 30), attempted to locate their nests and captured their social mates. In this study we captured males using mist-nets using the same conspecific song recording, while the females were captured at the nest. Each individual received a unique combination of metal and colour-rings to facilitate resighting throughout the breeding season. Social mates were all identified as females by the presence of a brood patch or observation during nest building (carried out solely by the females), during incubation or brooding nestlings at the nest attended by their respective male partners. We observed and confirmed both members of all pairs by colour-ring identification or direct capture at the nest. At the Ljungdalen study site we only mapped territories and captured males, but not females, as flooding limited our access to the study site after 11 June.

We followed previous protocols (Bensch *et al.,* 1999, 2009, Chamberlain *et al.,* 2000) to collect four morphometrics (i.e. wing chord, whole tail, tarsus, and bill-head), assess plumage colouration, sample blood for subsequent genetic analyses and collect the two first primaries to assess wintering ground $\delta 15$N for all individuals cap-

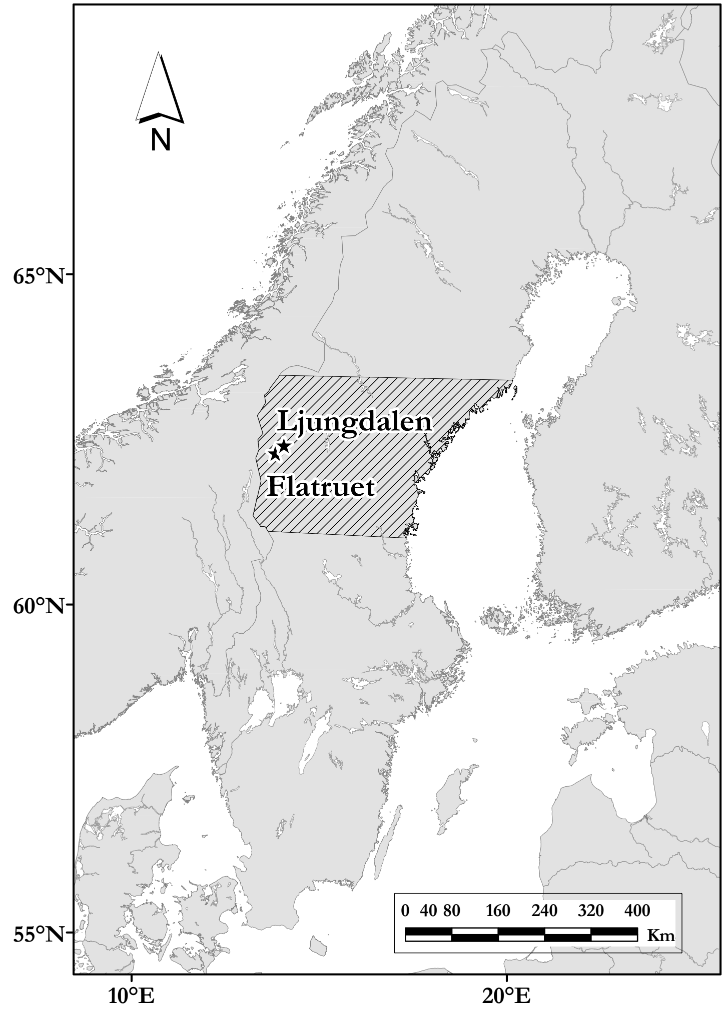


Figure 1. Faltruet (62.7° N, 12.7° E, 846 meters) and Ljungdalen (62.8° N, 13.0° E, 593 meters), Sweden, study site locations, cross-hatched area represents hybrid zone described in Bensch *et al.,* (2009) as between 61.4° to 64.1° N (latitude) in central Sweden, where both willow warbler migratypes meet in parapatry.

tured. Methods of analyzing δ15N from feathers are well described (Chamberlain *et al.,* 2000, Bensch *et al.,* 2006b) and for genotyping of the WW2 AFLP molecular markers (Bensch *et al.,* 2009). For this bi-allelic locus (S, southern allele; N northern allele) we scored the individuals as one of three genotypes (SS, SN, or NN). Plumage colour scores were assessed on a scale of 1 to 9 using three reference specimens representing the typical acredula (dull grayish-green plumage; score 2), intermediate (score 5) and the typical trochilus (yellow plumage; score 8) (Bensch et al. 2009). We summarized Morphometrics using a principal components analysis where PC1 explained the largest percentage of the variation in our data and was used for subsequent analyses. To test for trait correlations between each pair by trait (i.e. plumage colour score, PC1 for morphological measurements, WW2 AFLP genotypes, and δ15N values) we calculated Pearson's correlation. All statistical analyses were carried out using R (CRAN 2012).

Results

We identified 33 territories by capturing singing males in our Flatruet study population. Of these, we were able to locate 28 nests and captured the female partners of 20 territorial males. Several nests were lost to predation (n = 7), while 13 females and three males could not be captured. Thus, we were able to test for the occurrence of assortative mating for 20 confirmed social pairings based on comparisons of migratype specific phenotypic values using Pearson's correlations. Overall, within-pair comparisons of the four traits revealed a lack of evidence for assortative mating. For the δ^{15}N values previously shown to be a useful indicator of wintering grounds, there was a none-significant negative correlation (n = 20, R = –0.09, P = 0.65) (Figure 2A). Males had a higher mean δ^{15}N value (R = 8.07, SD = 1.52) than females (R = 7.70, SD = 1.68), suggesting a larger proportion of northern males or more southern females in our sample. For the AFLP WW2 locus, a marker associated with the intraspecific variation of the migratory program in willow warblers, there was a non-significant positive correlation (r = 0.18, P = 0.23) (Figure 2B). There were few homozygotes for the northern allele (1 male and 1 female) indicating that our sample was biased towards birds with a southern ancestry. This reduces our power for detecting a presence of assortative mating. For breast plumage colour there was a non-significant positive correlation (R = 0.26, P = 0.13) (Figure 2C) and we found a slightly negative non-significant correlation for body size as summarized by PC1 (R = -0.02, P = 0.53) (Figure 2D) and PC2 (R = 0.10, P = 0.34). Body size PCA results and trait values for all social pairs are summarized in the supplementary materials (Table S1 and S2, respectively). Finally, we compared capture dates (scored as the number of days since the first of May) with two phenotypic traits (δ^{15}N and AFLP WW2). For male δ^{15}N values we found a non-significant positive correlation with capture date (R = 0.15, P = 0.14) (Figure 3A) and for male AFLP WW2 a significant positive correlation (R = 0.37, P = 0.003) (Figure 3B), indicating approximately nine day difference in arrival dates between to two migratypes.

Discussion

We found no evidence for assortative mating between the two willow warbler migratypes co-occurring in the migratory divide, despite an apparent nine day difference in arrival dates for males of the two migratypes. A careful within-pair comparison of four traits previously shown to exhibit steep clines across the willow warbler migratory divide revealed no pattern of assortative mating. Our previous field work (1996 to 2010) at 98 field sites across Sweden including 53 sites within the migratory divide provide additional support for these results. Here we documented not only the previously described steep clines for four genotypic and phenotypic traits, but that both *trochilus* and *acredula* migratypes are indistinguishable by song with males and females responding to the same playback recording of a conspecific song (Bensch *et al.*, 1999, 2009). Further, both *trochilus* and *acredula* breed in all habitats with trees and shrubs across their range. These observations on territorial behaviour (i.e. antagonistic reaction to playback of conspecific song) and habitat choice are thus unlikely mechanisms for assortative mating.

Although we only sampled pairs at a single breeding site in the migratory divide, we carefully choose this site based on previous data, which indicated that our site is exactly in the middle of the divide and is a reliable breeding area for

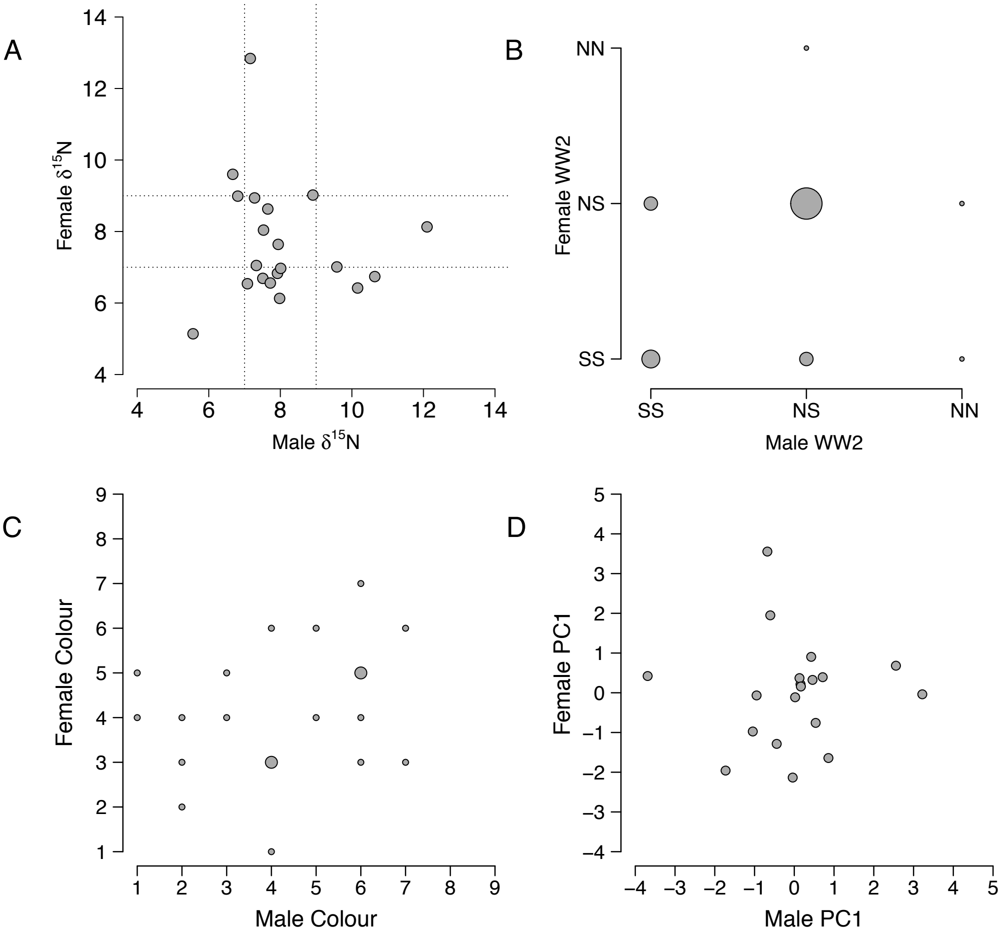


Figure 2. Phenotype and genotype comparisons for all pairings (n = 20). (A) δ^{15}N values (dashed lines at 7 ‰ and 9 ‰ represent cut-offs for birds likely wintering in West Africa and East to South Africa, respectively) (R = –0.09, P = 0.65), (B) pair counts for AFLP WW2 molecular marker (associated with migratory direction) are represented by the size of circle (R= 0.18, P = 0.23), (C) pair counts for colour scores (R = 0.26, P = 0.13), and (D) comparison of PC1 for body size (R = –0.02, P = 0.53).

both willow warbler migratypes. Our findings contrast with another well studied species, the European blackcap, where two migratypes breed in sympatry, but mate assortatively based on timing of arrival to the breeding grounds in central Europe (Bearhop *et al.*, 2005). In contrast, in our study we found a correlation between male arrival date and the migratype, but no assortative mating.

The lack of assortative mating shown in our study combined with our previous work gives further support for the suggestion that post-breeding selection against hybrids rather than pre-zygotic mate choice maintains this willow warbler migratory divide. Previous research with migratory divides suggest that hybrid migratypes have an intermediate endogenous migratory program to the parental migratypes that might result in reduced hybrid survival (Helbig, 1991a). Hybrid offspring with an intermediate migra-

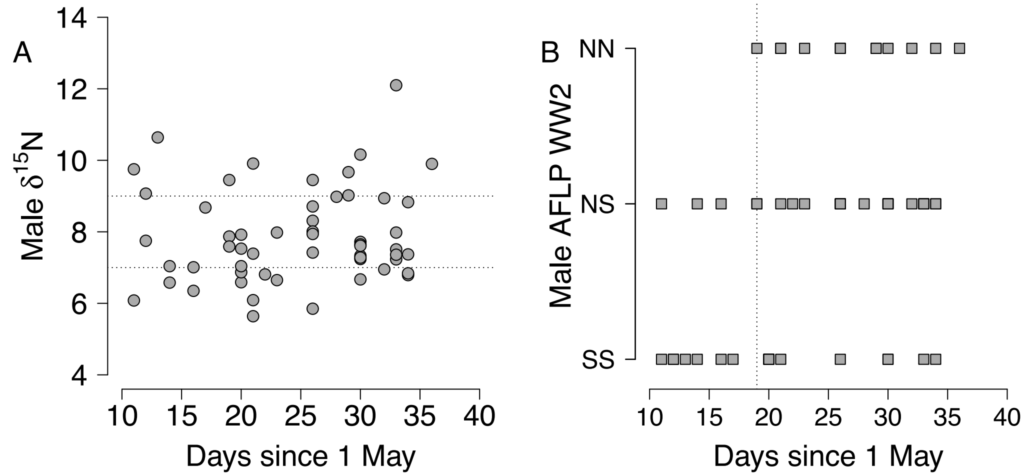


Figure 3. Capture date correspondences for all males captured at both Flatruet (n = 27) and Ljungdalen (n = 29) sites combined with (A) $\delta^{15}N$ values (dashed lines at 7‰ and 9‰ represent cut-offs for birds likely wintering in West Africa and East to South Africa, respectively) (n = 56, *R* = 0.15, *P* = 0.14, *LCL* = -0.08, *UCL* = 1.00), (B) pair counts for AFLP WW2 genotypes (SS homozygotes for the southern allele, NN homozygotes for the northern allele, SN heterozygotes) (n = 56, *R* = 0.37, *P* = 0.003, *LCL* =-0.16, *UCL* = 1.00). Capture dates are presented as the number of days after 1 May.

tory program would have to overcome significant ecological barriers that are avoided by the parental migratypes (Hedenström and Pettersson, 1987). During their first autumn migration, the intermediate willow warbler migratypes would have to undertake extensive flights across the Alps and over both the Mediterranean Sea and Sahara desert at their widest stretches with minimal stop-over opportunities, which are likely to result in increased hybrid mortality. Evidence for the possibility of an intermediate migratory phenotype comes from selection and cross-breeding experiments with European blackcaps taken from either-side of the central European migratory divide (Helbig, 1991a). Because our study demonstrates no clear assortative mating between the two willow warbler migratypes in central Sweden, hybridization should occur frequently. Without selection against the many hybrid forms, the observed steep trait clines would have dissolved in only a few hundred years (Bensch *et al.*, 1999, 2009). These results call for establishment of a long-term study with colour-marked populations to establish precise timing of arrival to the breeding grounds by parental migratypes, breeding behaviour, reproductive success, and survival at the center of the migratory divide.

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Supplementary Material

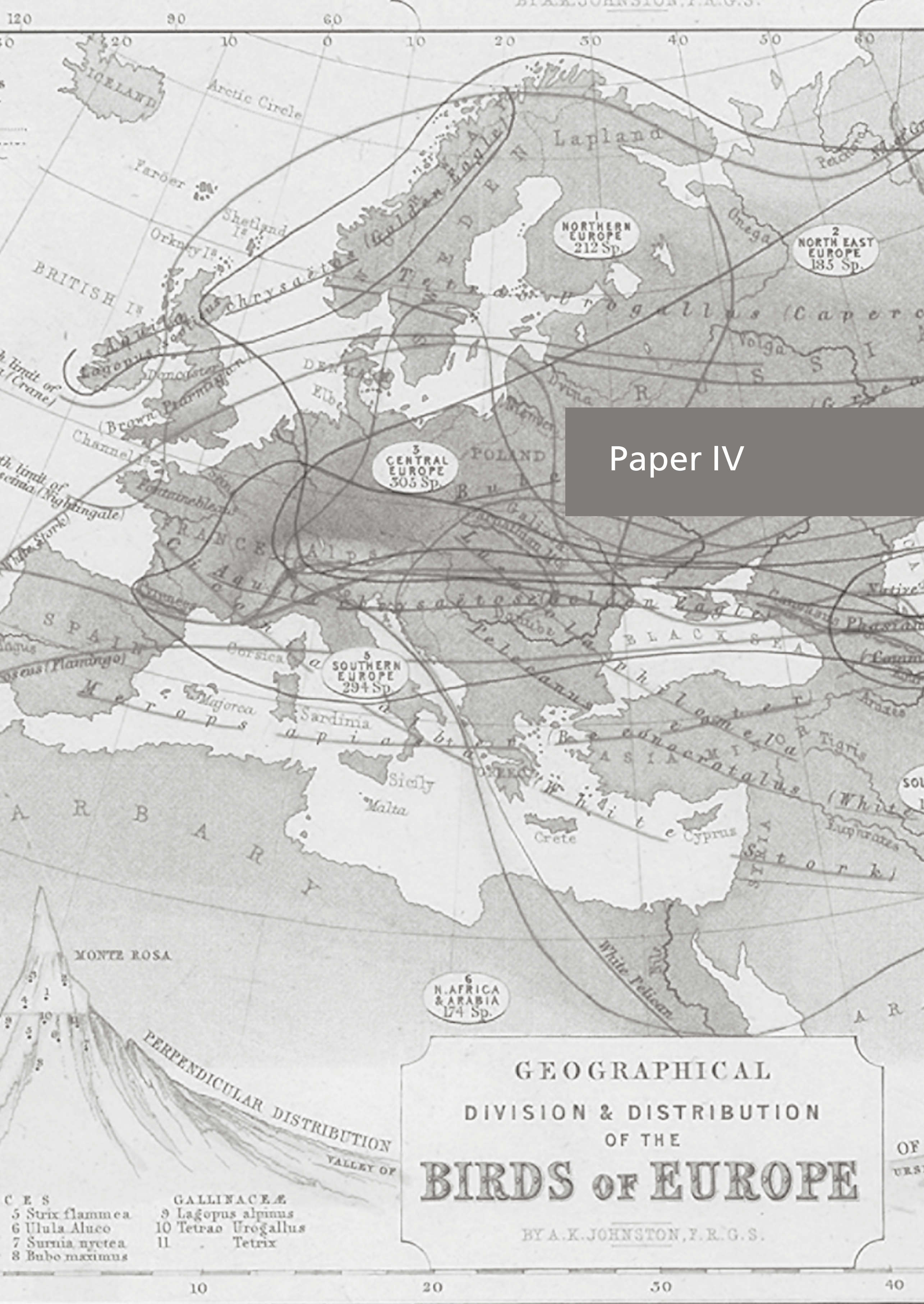
Table S1. Eigenvalues and proportion of variance explained by each principal component using four morphological measurements, i.e. wing chord, whole tail, tarsus, and bill-head length, separately for males and females.

	PC1	PC2	PC3	PC4
Males				
Wing	–0.60	0.23	0.42	–0.64
Tail	–0.59	0.47	–0.11	0.65
Tarsus	–0.41	–0.35	–0.80	–0.26
Bill + Head	–0.35	–0.78	0.41	0.32
Importance of components				
Standard deviation	1.42	0.99	0.91	0.40
Proportion of Variance	0.51	0.25	0.21	0.04
Cumulative Proportion	0.51	0.75	0.96	1.00
Females				
Wing	–0.49	0.48	0.65	–0.33
Tail	–0.55	0.39	–0.39	0.63
Tarsus	–0.38	–0.72	0.44	0.39
Bill + Head	–0.56	–0.32	–0.48	–0.59
Importance of components				
Standard deviation	1.35	1.03	0.79	0.71
Proportion of Variance	0.45	0.27	0.16	0.12
Cumulative Proportion	0.45	0.72	0.88	1.00

Table S2. Willow warbler capture date (day, month, year) trait data (δ^{15}N, 2 AFLP derived molecular markers) for all pairs (n = 21)

Male						Female					
ID	Captured	Colour	δ^{15}N (‰)	AFLP WW2	Body Size (PC1)	ID	Captured	Colour	δ^{15}N (‰)	AFLP WW2	Body Size (PC1)
11B/01	13-May	8	10.64	0	1.04	11B/35	2-Jul	6	6.74	0	–0.97
11B/04	20-May	5	7.92	0	0.08	11B/49	7-Jul	8	6.83	1	NA
11B/07	20-May	6	7.53	0	–0.54	11B/30	11-Jun	5	8.04	0	–0.76
11B/08	22-May	6	6.81	1	0.60	11B/36	3-Jul	8	8.99	1	1.95
11B/12	26-May	8	7.94	1	–0.71	11B/47	6-Jul	9	7.64	1	0.39
11B/14	26-May	6	8.01	1	0.44	11B/50	9-Jul	7	6.97	1	–1.29
11B/15	30-May	6	7.72	1	–0.02	11B/48	7-Jul	3	6.56	0	–0.11
11B/16	30-May	5	10.16	1	0.68	11B/44	5-Jul	6	6.42	0	3.55
11B/19	30-May	7	7.65	1	0.04	11B/40	3-Jul	5	8.63	2	–2.13
11B/21	30-May	9	7.28	1	–0.46	11B/43	5-Jul	8	8.94	1	0.32
11B/22	30-May	5	7.33	0	0.95	11B/38	3-Jul	9	7.05	0	–0.07
11B/23	30-May	8	6.67	0	–0.86	11B/34	16-Jun	7	9.60	2	–1.64
11B/24	2-Jun	7	7.98	1	1.73	11B/52	12-Jul	3	6.13	1	–1.96
11B/26	2-Jun	5	7.51	1	–2.56	11B/53	12-Jul	6	6.69	0	0.68
11B/27	2-Jun	5	12.10	1	3.69	11B/54	13-Jul	4	8.13	1	0.42
11B/32	16-Jun	4	7.08	0	–3.22	11B/33	16-Jun	4	6.54	1	–0.04
11B/42	3-Jul	6	8.91	2	–0.43	11B/41	3-Jul	4	9.02	1	0.90
11B/45	6-Jul	7	9.58	1	–0.13	11B/37	3-Jul	8	7.01	1	0.37
11B/46	6-Jul	7	5.56	0	–0.17	11B/39	3-Jul	8	5.14	0	0.16
11B/51	10-Jul	3	7.16	0	–0.16	11B/31	14-Jun	6	12.84	1	0.21

Paper IV


GEOGRAPHICAL
DIVISION & DISTRIBUTION
OF THE
BIRDS OF EUROPE
BY A.K.JOHNSTON, F.R.G.S.
1 NORTHERN EUROPE 212 Sp.
2 NORTH EAST EUROPE 185 Sp.
3 CENTRAL EUROPE 305 Sp.
5 SOUTHERN EUROPE 294 Sp.
6 N.AFRICA & ARABIA 174 Sp.
ICELAND
Arctic Circle
Faröer
Shetland Is.
Orkney Is.
BRITISH Is.
Lapland
Onega
Volga
Dwina
DENMARK
Elbe
Channel Is.
POLAND
FRANCE
Alps
SPAIN
Corsica
Sardinia
Majorca
Sicily
Malta
Crete
Cyprus
BLACK SEA
ASIA MINOR
Tigris
Euphrates
Danube
Nile
BARBARY
Aquila chrysaëtos (Golden Eagle)
Tetrao Urogallus (Capercaillie)
Lagopus
(Brown Ptarmigan)
White Stork
(Nightingale)
Flamingo
Merops apiaster
Pelecanus onocrotalus (White Pelican)
Phasianus
White Pelican
Stork
MONTE ROSA
PERPENDICULAR DISTRIBUTION
VALLEY OF
GALLINACEÆ
5 Strix flammea
6 Ulula Aluco
7 Surnia nyctea
8 Bube maximus
9 Lagopus alpinus
10 Tetrao Urogallus
11 Tetrix
120
90
60
10
20
30
40
50

Climate exerts direct selection on genomic region in a mountain living bird population

Keith W. Larson[a], Miriam Liedvogel[a], Max Lundberg[a], BriAnne Addison[b,a],
Oddmund Kleven[c, d], Terje Laskemoen[c], Jan T. Lifjeld[c], Susanne Åkesson[a], and Staffan Bensch[a]

[a]Department of Biology, Centre for Animal Movement Research, Ecology Building,
Lund University, SE-223 62 Lund, Sweden
[b] Current address: School of Science and Health, University of Western Sydney,
Locked Bag 1797, Penrith NSW 2751, Australia
[c] Natural History Museum, University of Oslo, PO Box 1172 Blindern, 0318 Oslo, Norway
[d] Current address: Norwegian Institute for Nature Research, PO Box 5685 Sluppen,
7485 Trondheim, Norway

Abstract

Local adaptation is an important process contributing to population differentiation which can occur in continuous or isolated populations connected by various amounts of gene flow. Two willow warbler subspecies, exhibiting distinct migratory phenotypes, meet in secondary contact in central Sweden. They are undifferentiated for neutral markers but show steep latitudinal clines for two genetic markers and stable nitrogen-isotopes. Two of these traits, stable nitrogen-isotopes and one genetic marker AFLP-WW2, show concordant cline patterns with steepest clines at the location of the secondary contact zone, suggesting an association with the different migratory programs. The selection processes governing AFLP-WW1, is less apparent. One of the alleles at AFLP-WW1 is associated with coastal lowland habitats and the other with mountains habitats. In this study, we sampled 2,285 individuals at 126 sites across Fennoscandia to study the geographic and climatic variables that explain allele frequency distributions for WW1. Our results demonstrate that 1) allele frequency patterns clearly differ between mountain and lowland populations, 2) these allele differences coincide with local adaptations to extreme temperature conditions and short growing season in the mountains, and milder conditions in coastal areas, and 3) the northern-allele or “altitude variant” of WW1 occurs in willow warblers occupying mountainous habitat regardless of subspecies. Finally our results suggest that climate exerts direct selection on the genomic region associated with this allele, making this marker suitable for monitoring climate change as a force of selection on bird populations.

Keywords: local adaption, *Phylloscopus trochilus*, altitude, ecological selection, hybrid zone, selection gradient.

Introduction

The early stages of speciation often begin with divergent selection for locally adapted traits. This can occur in either continuous or geographically separated populations connected by various amounts of gene flow. The degree of divergence reflects the balance between the selection for an adaptive trait, and gene flow from nearby populations (Via 2009, Nosil *et al.,* 2009). Thus in the early stages of population divergence most ancestral genetic variation is shared between both populations. Coding genes under directional selection diverge faster, whereas changes in neutral DNA occur through the much slower process of genetic drift (Ehrlich and Raven 1969, Slatkin 1987, Via 2009, Nosil *et al.,* 2009). Hence, neutral genetic markers often do not reveal population structure in recently diverged populations, because of insufficient time for divergence (Nichols 2001).

Local adaption can result from selection where adaptive traits separate contrasting phenotypes. For example, divergent selection in the spider, *Agelenopsis aperta*, has resulted in both desert and riparian habitat related phenotypes (Riechert 1993) and specialization on either alfalfa or red clover by pea aphids (*Acyrthosiphon pisum*) resulted in two divergent ecotypes (Via 1999, Via *et al.,* 2000). Local adaptations are not restricted to discrete habitats but can also arise along environmental gradients creating a cline (Bonin *et al.,* 2006). For example, the European common frog (*Rana temporaria*) shows clines in both morphological and reproductive traits in relation to altitude in mountain ranges across Europe (Bonin *et al.,* 2006), while some Caribbean island bananaquit (*Coereba flaveola*) populations have clines in colour polymorphisms associated with rainfall patterns and altitude (Wunderle 1981, Theron *et al.,* 2001).

In central Fennoscandia, two willow warbler (*Phylloscopus trochilus*) subspecies are represented by distinct migratory phenotypes, or “migratypes”, and meet in secondary contact. Contact occurred after the last glacial maximum, as habitats became suitable (Bensch *et al.,* 1999, 2009). The northern form, *P. t. acredula* migrates south-southeast through the Balkan Peninsula to winter on the eastern side Africa, while the southern form, *P. t. trochilus*, migrates southwest through the Iberian Peninsula to winter in West Africa (Chamberlain *et al.,* 2000). Each migratype likely colonized the formerly glaciated Scandinavia from two separate routes, *trochilus* from the southwest over Denmark into southern Scandinavia and *acredula* from the northeast along the eastern side of the Baltic Sea.

The two migratypes are nearly panmictic for mtDNA and 12 microsatellites, but show steep clines for three quantified trait markers. These include feather stable nitrogen-isotopes (δ^{15}N), which is a proxy for the wintering grounds in sub-Saharan Africa (Chamberlain *et al.,* 2000), and two bi-allelic AFLP derived genetic markers, WW1 and WW2 (Bensch *et al.,* 1999, 2002, 2009, Chamberlain *et al.,* 2000). While both allele frequency clines are steepest at the migratory divide in central Sweden and reached peak frequencies (> 0.9) in the northernmost part of Scandinavia, they displayed very different clines along the Baltic coasts of Sweden and Finland. At the WW2 locus, the “northern-allele” has a high frequency (> 0.9) along the entire Gulf of Bothnia followed by a northeast-to-southwest cline coincident to a parallel change in the migratory behaviour in central Scandinavia (Bensch *et al.,* 2009). In contrast, the frequency of the WW1 locus for the “northern-allele” drops in southern Finland, as it does in southern Sweden. These different cline patterns strongly suggest that the same selective processes cannot maintain variation at these loci.

The concordant cline patterns between WW2 and our proxy for migratory behaviour (feather $\delta^{15}N$) suggest that "northern" and "southern" alleles are associated with genes shaping the different migratory programs or represent adaptations to conditions on their different wintering grounds in Africa (Bensch *et al.,* 2009). The selection processes governing the clines for the WW1 locus are less apparent, although we can, based on the geographic distribution of the alleles, exclude its association with the two migratypes. From earlier data we know that the northern-allele of WW1 in *acredula* populations is predominantly found in high elevation populations of the western mountains of northern Sweden (Bensch *et al.,* 2002). As we follow the west-to-east or mountain-to-lowland altitude gradient the frequency of southern-allele WW1 increases in *acredula*. In lowland populations within Sweden the southern-allele is almost fixed in *trochilus* populations as it is for *acredula* populations in Finland where lowland habitats dominate. It is this contrast with the northern-allele found in the mountain populations of *acredula* and the substantial proportion of southern-alleles in the lowland *acredula* that suggests some form of local adaptation correlated with altitude and recognition that selection is acting on something not related to the migratory divide for the two subspecies. Because of the relatively few samplings sites, they were only able to statistically investigate longitude as a predictor of the northern-allele frequencies at this locus in (Bensch *et al.,* 2002).

Using the zebra finch (*Taeniopygia guttata*) genome (Warren *et al.,* 2010) as reference, WW1 could be mapped to a 2.5 Mb chromosomal region that is highly differentiated between southern *trochilus* and northern *acredula* willow warblers across Fennoscandia (Lundberg *et al.,* 2011). Several loci within this chromosome region showed a comparably high differentiation as with the WW1 marker, suggesting strong linkage disequilibrium (LD) to one or more genes under divergent selection, rather than being the selective target itself. Although we have yet to identify the gene under divergent selection and the high LD observed within the observed chromosome region, we expect the allelic distribution of WW1 to closely follow that of the focal gene.

In this study we focus on the geographic WW1 allele frequency distribution pattern using a substantially larger data set than (Bensch *et al.,* 2002) to investigate how the northern-allele is associated with latitude, longitude and altitude. Furthermore, if the northern-allele is under positive selection for local adaption to high latitude and altitude habitats, we would predict that environmental conditions related to breeding season temperature extremes and growing season length and productivity will best explain the observed pattern in allele frequency. High latitude and altitude habitats are associated with shorter and delayed growing seasons and larger and more extreme climate fluctuations, especially at the beginning and end of the breeding season, which provides a much shorter window for migratory birds to breed. Further, we predict the cline for WW1 allele frequencies, matching environmental parameters, to be smooth and gradual from south-to-north in Finland, where mountains are absent except in the far north on the border of Norway, reflecting gradual changes in habitats, with the extreme northern latitudes more similar to mountain habitats in Sweden and Norway. Due to the altitude gradient running perpendicular to the latitudinal gradient in the north of Sweden and Norway, we predict the clines to be more complex, narrower, and oriented east-to-west reflecting the lowland-to-mountain-to-lowland gradient across Scandinavia. Of particular interest from this expanded dataset is the addition of samples from across the entirety of Norway including mountainous sites south of the migratory divide (*trochilus* subspecies) and low elevation sites along the coast north of the migratory divide. This extensive dataset further allows us to examine whether the presumably beneficial northern-allele among *trochilus* populations breeding at high altitudes and whether selection favours the southern-allele in the habitats found at low altitudes and with milder climates along the coast in Norwegian *acredula*.

The prospect of identifying a genomic region under direct selection by climatic conditions or other factors significantly correlated with them make this marker potentially useful for monitoring changing climates. For example, cold adapted species often have restricted ranges during warm climate periods, while during colder periods they occur more broadly. This is especially important in high elevation or mountain regions where populations adapted to these habitats are limited in their ability to disperse upwards or northwards and face disproportionately greater risks of extinction in light of significant climate change (Thuiller *et al.,* 2005, Ohlemüller *et al.,* 2008).

Materials and Methods

Field and lab work

Between 1996 and 2011 we travelled to 126 sites across Fennoscandia, i.e., Sweden (n = 83), Norway (n = 35), and Finland (n = 8) to sample 2,285 willow warblers across their breeding grounds (Figure 1A). At each site we captured between 10 and 20 individual adult males and collected morphometric measures (e.g., wing chord, tarsus, body mass, and plumage colour), blood for DNA analysis, and the first primary flight feather for δ^{13}C and δ^{15}N analysis were collected (Bensch *et al.,* 1999, 2002, Chamberlain *et al.,* 2000). Some breeding sites were visited multiple years. We caught all males in mist-nets with an audio-lure playback using the same willow warbler song. All blood samples were stored in DNA buffer and brought back to the lab for analysis. Procedures for extracting DNA and subsequent bi-allelic genotyping of individuals for WW1 follow previously published methods (Bensch *et al.,* 2002).

Cline analysis

We used the full set of sampling sites (n = 127; Figure 1A) to determine the northern-allele frequency distribution for WW1 across Fennoscandia. To assess clinal variation in the northern-allele of WW1 we used a generalized additive model (GAM) to perform a logistic regression analysis. In this model the northern-allele frequency for WW1 was used as a response variable with a binomial error structure and the logit link function with the sample site geographic coordinates (i.e. latitude, longitude, and their interaction) and altitude as predictors. We tested the interaction between latitude and longitude as the Scandinavian Peninsula, and the mountains that dominate this region, is oriented northeast to southwest. Further, we predicted that the latitudinal effect will be stronger in Finland where lowlands dominate, while latitude in Scandinavia is highly correlated with altitude reflecting the northern position of the mountains, especially in Sweden. We used GAM, as implemented in the *mgcv* R package (Wood and Augustin 2002), which allowed us to incorporate both linear and non-linear predictors into the model. The significance of latitude, longitude, their interaction and/or altitude in this model would indicate geographic differentiation likely representing adaptions to mountain or high latitude habitats.

To visualize the cline we used the Spatial Analyst ordinary kriging function in ArcMap 10 (ESRI 2010) to create an interpolated continuous grid surface representing the observed northern-allele frequency distribution for the entire region. We then used the resulting interpolated surface to create allele frequency contours in 0.1 increments with the 0.5 isocline representing the center of the cline.

Environmental Analysis

Again, we used the northern-allele frequency for WW1 as a response variable in a generalized additive model (GAM) to perform stepwise logistic regression analysis with a binomial error structure, and the logit link function. Our initial model incorporated the long-term average maximum temperatures for April to August (Hijmans *et al.,* 2005a, 2005b), phenological or growing

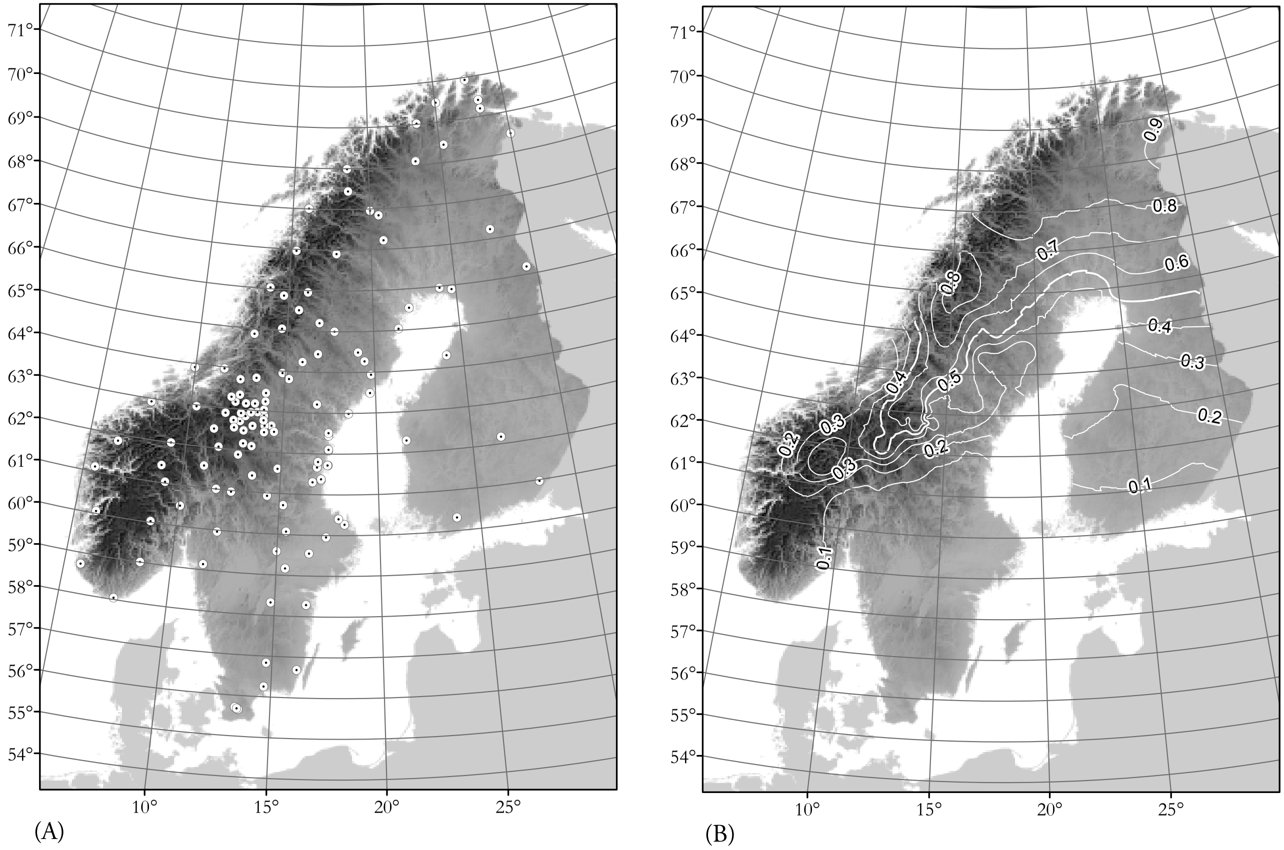


Figure 1. (A) Sample site locations where the background represents an altitude hill shading for study area. (B) Spatially interpolated observed 0.1 northern-allele frequency contours for the locus WW1 overlaid on altitude hill shade background. The 0.5 isocline represents the center of the cline. The background represents a digital elevation model (DEM) with hill shading for study area. Both maps have a 1 km or 30 arc-second resolutions and projected using the Swedish RT 90 0 gon Mercator projection.

season productivity predictor variables, net primary productivity, season begin date, and season length as calculated from Normalized Difference Vegetation Index (NDVI) (Ivits *et al.,* 2012), and potential evapotranspiration (Trabucco and Zomer 2009) to describe the conditions across our study area. Maximum temperatures in April through August were chosen as they represent temperature extremes during the months that willow warblers arrive, establish breeding territories, breed, and fledge their young in Fennoscandia. Potential evapotranspiration represents the atmospheres ability to remove water through evaporative transpiration and is affected by temperature, precipitation, winds, and solar radiation (which is highly correlated with latitude, *see* Table S1) (Trabucco *et al.,* 2008). Before running the model we assessed multicollinearity for all predictor variables and for variables with high collinearity (> 0.9) we only kept one of these variables in the model. We dropped the maximum temperature predictor variable for May as it was highly correlated with that in April ($R = 0.94$) as well as July and August due to their high collinearity with June ($R = 0.98$, $R = 0.96$, respectively). For each model we assessed the significance of each predictor variable by testing the reduction in deviance as measured by the χ^2 statistic and removed insignificant terms. Finally, to evaluate how well our final model described the conditions across our study area, we used a GAM with the residuals from the final environmental model as the response variable to determine if latitude, longitude, their interaction or altitude explained a significant amount of the residual variance.

To visualize the model predicted northern-allele frequency distribution for the entire region we used the Spatial Analyst ordinary kriging function in ArcMap 10 (ESRI 2010) to create an interpolated continuous grid surface. We then used the resulting interpolated surface to create allele frequency contours in 0.1 increments with the 0.5 isocline representing the center of the cline.

All analyses were performed in R version 2.14 (R Development Core Team 2012).

Results

Cline analysis

Geography (latitude, longitude, and their interaction) and altitude were strongly associated with the frequency of the northern-allele of WW1 ($R^2_{adj} = 0.93$, Deviance Explained = 91.9 %) across our 127 sites in Norway, Sweden, and Finland. The effects of latitude, the interaction between latitude and longitude, and altitude were all highly significant, while longitude was nearly significant (Table 1). The frequency for the northern-allele at locus ALFP-WW1 across Fennoscandia is virtually zero in southernmost Finland and Sweden, and reaches its highest levels (> 0.9) in the far north of Fennoscandia (Figure 1B). In Finland, on the east side of the Gulf of Bothnia, there is a gradual increase in the northern-allele frequency from 0.1 in the south to 0.9 in the north, thus the cline is shallow, with wide contour intervals, and oriented north-south. In Scandinavia, the cline is more hump- or saddle-shaped, having lower northern-allele frequency on both the coast of Norway and Sweden, and high allele-frequency in the mountains along the Norway-Sweden border. Here the cline is steep, with narrow contour intervals, and oriented both north-south and up-mountain (Figure 1B). Thus the distribution of the northern-allele is a north-to-south cline across the region with increasing cline width from west-to-east, as the landscape goes from mountainous to lowland.

Environmental analysis

The environmental model demonstrated the significance of climate and growing season variables in predicting the distribution for the northern-allele of WW1 across Fennoscandia ($R^2_{adj} = 0.92$, Deviance Explained = 90.6 %). The final model included the significant predictor variables April maximum temperature, net primary productivity (NPP), and potential evapotranspiration (PET)

Table 1. Parameter estimates for the GAM cline model examining geographical (i.e., latitude, longitude, and their interaction) and altitude variation in the northern-allele frequency for the AFLP marker WW1.

Parametric coefficients				
Parameter	Estimate	SE	Z	*P*
Intercept	–0.77	0.05	–14.68	< 0.001
Approximate significance of smooth terms	Est. *df*	Ref. *df*	χ^2	*P*
Altitude	1.95	9	59.47	< 0.001
Latitude	5.24	9	51.85	< 0.001
Longitude	2.51	9	2.72	0.08
Latitude*Longitude	9.32	22	25.77	< 0.001

Table 2. Parameter estimates for the GAM model examining variation in the northern-allele frequency for the AFLP marker WW1, where insignificant terms were removed using stepwise regression evaluating the reduction in deviance and significance. Final model variables include the mean monthly maximum temperature for April, mean annual potential evapotranspiration (PET), and mean annual net primary (growing season) productivity (NPP).

Parametric coefficients				
Parameter	Estimate	SE	Z	*P*
Intercept	–0.76	0.05	–15.55	< 0.001
Approximate significance of smooth terms	Est. *df*	Ref. *df*	χ^2	*P*
Max Temp. April	4.79	9	74.59	< 0.001
PET	3.92	9	112.70	< 0.001
NPP	8.42	9	47.06	< 0.001

(Table 2). June maximum temperature, first day of (growing) season and season length were not significant when evaluated using a χ^2 test for deviance reduction (Δ *Dev.* = –8.00, *P* = 0.19, Δ *Dev.* = 2.26, *P* = 0.42, Δ *Dev.* = –2.48, *P* = 0.42, respectively) and removed from the model. The final model demonstrates the significance of the maximum temperature in April, NPP, and PET (Δ *Dev.* = –94.59, *P* < 0.001, Δ *Dev.* = –52.89, *P* < 0.001, Δ *Dev.* = –125.78, *P* < 0.001, respectively,) in predicting the frequency distribution of the northern-allele across Fennoscandia (Figure S1). Although willow warbler do not arrive to the breeding grounds until May in the southern portion of our study area and early June in the north, April maximum temperatures were highly correlated with May and June temperatures (*R* = 0.94, *R* = 0.80, respectively) and likely reflect important local phenological conditions. Using a post hoc test of these results from the environmental model, we used regression to model the residuals against latitude, longitude, their interaction and altitude, and found no significance for any of these geographic variables (Table S2).

Discussion

Our results show that the northern-allele of the WW1 marker is strongly associated with low temperature conditions and short growing season on the breeding grounds, suggesting it is a marker of local adaptation for mountain and high latitude living in Fennoscandian willow warblers. The use of our expanded dataset also demonstrated that lowland *acredula* populations in the east of northern Sweden had a significantly higher frequency of southern-alleles, similar to those in coastal Norway where habitats tend to be milder and lower in altitude. Further, our results confirm the presence of both alleles in regions occupied by both *acredula* and *trochilus* populations inhabiting the mountainous regions of Norway and Sweden – thus clearly indicating a different selective force for WW1 that is independent from subspecies and migratory program. Finally, a test of the residuals from the final environmental model suggests that it was not geography or altitude *per se* that predicts the northern-allele frequency distribution, but the local environmental conditions that exist in these regions.

Although the environmental model found climate and growing season conditions to be highly significant at predicting the distribution of the northern-allele, it must be kept in mind that these variables are still highly correlated with latitude and altitude (Table S1). Therefore, these variables may simply reflect the conditions found in these habitats and serve as a useful proxy for other factors that represent the agent of selection. Birds breeding at high latitudes and altitudes often experience greater daily and seasonal stochasticity and extremes in climatic conditions, such as temperature, wind, precipitation, and snow cover than those in other habitats (Wingfield *et al.,* 1995, Pereyra and Wingfield 2003, Martin and Wiebe 2004). Between these regions the timing of summer snow melt can vary by as much as one month (Martin and Wiebe 2004). Further, years of exceptionally harsh conditions and frequent severe storms may make it difficult for potential breeders to acquire food, increase individual thermoregulatory costs, and limit breeding opportunities during the short summer season encountered at these latitudes and altitudes (Wingfield *et al.,* 1995, Pereyra and Wingfield 2003, Martin and Wiebe 2004).

Previous authors (Bensch *et al.,* 2002) have argued that the extensive sharing of genetic polymorphism between southern *trochilus* and northern *acredula* willow warblers is a signature from the time before the subspecies diverged (and secondary contact), rather than resulting from homogenizing gene flow following secondary contact. Further, given the previous estimates of dispersal of 80 km, and time since secondary contact as 150 years, the steep allelic north-south clines (< 300 km) at this locus makes it more likely that divergent selection is the mechanism to explain the present-day distribution rather than asymmetric gene flow and historical range expansion (Bensch *et al.,* 2009). Further, the north-south cline in the northern-allele in pure Finnish *acredula* populations argues against any link with current subspecific populations.

At present, we do not know what variables in these mountainous habitats drive the northern-allele to high frequencies or which gene or genes surrounding WW1 are the targets of selection. Many species of birds show adaptations to living at high altitudes. For example, the bar-headed goose (*Anser indicus*) breeds and migrates at high altitude on the Tibetan Plateau (Black and Tenney 1980) and the Andean goose (*Cheophaga melatoptera*) can reside year-round at over 6000 meters (Faraci 1991), where hypoxic conditions can limit effective transport of blood oxygen, requiring adaptations to respiratory and haemoglobin systems. Because of the relatively low altitudes of the Scandinavian mountains, altitude of our study sites ranges from sea level to 1093 meters, it seems unlikely that WW1 represents adaptions to hypoxic environments, but instead reflects phenological correlates, such as summer temperatures or food conditions in these high latitude alpine regions. We propose several alternative hypotheses for the adaptive value of WW1-linked genes relating to these selective constraints.

As previously discussed, climatic conditions can vary dramatically, daily and seasonally, at high altitudes and latitudes. Birds must be able to modulate their stress response to these conditions in order to successfully breed during the short breeding season (Silverin 1995, Wingfield *et al.,* 1995, Pereyra and Wingfield 2003). The adrenocortical stress response can suppress breeding behaviour in birds in order to buffer physiological needs essential for survival during harsh weather (Silverin 1995, Wingfield *et al.,* 1995). Experiments where high altitude and latitude birds were exposed to stress were better able modulate the adrenocortical response to stress than others at lower altitudes and latitudes, making high latitude and altitude birds resilient to the effects of stress during breeding efforts (Silverin 1995, Wingfield *et al.,* 1995). Thus, the northern-allele for WW1 may represent adaptations to these conditions that allow these phenotypes to buffer harsh conditions and maintain a constant reproductive output, increasing fitness where breeding opportunities are constrained by short summer season.

Alternatively, selection on genes linked to the northern-allele may confer adaptation to a particular diet found at high altitudes and latitudes. Stands of almost pure subalpine birch (*Betula pubescens czerepanovii*) characterize habitats occupied by the “mountain” phenotype willow warblers. During the breeding season willow warblers inhabiting subalpine birch communities forage extensively on autumnal moth larvae (*Epirrita autumnata*) which cyclically reach epidemic proportions (Selås *et al.,* 2001, Enemar *et al.,* 2004). In years where these moths reach peak densities, subalpine birch produce defensive secondary-chemical compounds known to be proteinase inhibitors (Selås *et al.,* 2001). It is therefore tempting to speculate that the northern-allele for WW1 represents an adaptation to dealing with accumulated secondary chemical compounds in larval autumnal moths.

Although our approach does not allow us to identify the mechanisms leading to positive selection for the WW1 northern-allele, we have identified environmental conditions that explain the distribution of WW1 alleles. Our results indicate that the genomic region associated with this marker is under direct environmental selection. These results open up an avenue for studies of functional genetics to identify the genes underlying the various adaptations to ecological/climatic conditions. Further, this marker provides a genetic tool to study how climate exerts direct selection on a gene or genomic region in a bird and hence would make an excellent candidate for monitoring bird population changes as a result of future expected changes in climate.

These results are especially significant given the predictions that cold-adapted and mountain populations of a diversity of taxa are particularly vulnerable to extinction due to the rapid warming of our climate (Thuiller *et al.,* 2005, Ohlemüller *et al.,* 2008, Pauli *et al.,* 2012). Studies of high elevation plants in Europe predict species loss maybe as great as 60 % due to their inability to disperse from these isolated habitats or adapt to warming conditions (Thuiller *et al.,* 2005). In particular, maps of regional plant species vulnerability show amazing concordance with the observed northern-allele frequency distribution in Fennoscandian willow warblers (Thuiller *et al.,* 2005 p. 8248).

Future work should focus on testing cold tolerance and food choice experiments contrasting mountain populations in Scandinavian with lowland populations along the Baltic Sea and Norwegian Coasts, to determine the physiological phenotype associated with the geographical and environmental distribution pattern revealed here. Experimental elucidation of physiological differences between genotypes will hopefully lead to the discovery of the direct selective mechanisms linked to these apparent adaptations and the genes associated with this AFLP marker.

Acknowledgements

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Supplementary Material

Table S1. Pearson's correlations for the predictor variables of the environmental model and altitude, latitude, and longitude.

Predictors	Altitude	Latitude	Longitude
PET	–0.37	–0.83	–0.08
NPP	–0.30	–0.21	–0.16
SBD	0.50	0.14	–0.02
SL	0.01	–0.17	–0.04
Temp Max April	–0.55	–0.84	–0.22
Temp Max May	–0.44	–0.85	–0.17
Temp Max June	–0.49	–0.71	0.04
Temp Max July	–0.59	–0.62	0.18
Temp Max August	–0.61	–0.75	0.02

Table S2. Parameter estimates for the GAM model examining the residuals from the final environmental model (Table 2) regressed against latitude, longitude, their interaction, and altitude.

Parametric coefficients				
Parameter	Estimate	SE	Z	*P*
Intercept	0	0	–0.88	0.38
Approximate significance of smooth terms	Est. df	Ref. df	$\chi 2$	*P*
Altitude	1.95	9	0	0.49
Latitude	5.24	9	0	0.38
Longitude	2.51	9	0	0.44
Latitude*Longitude	9.32	10	0.03	0.27

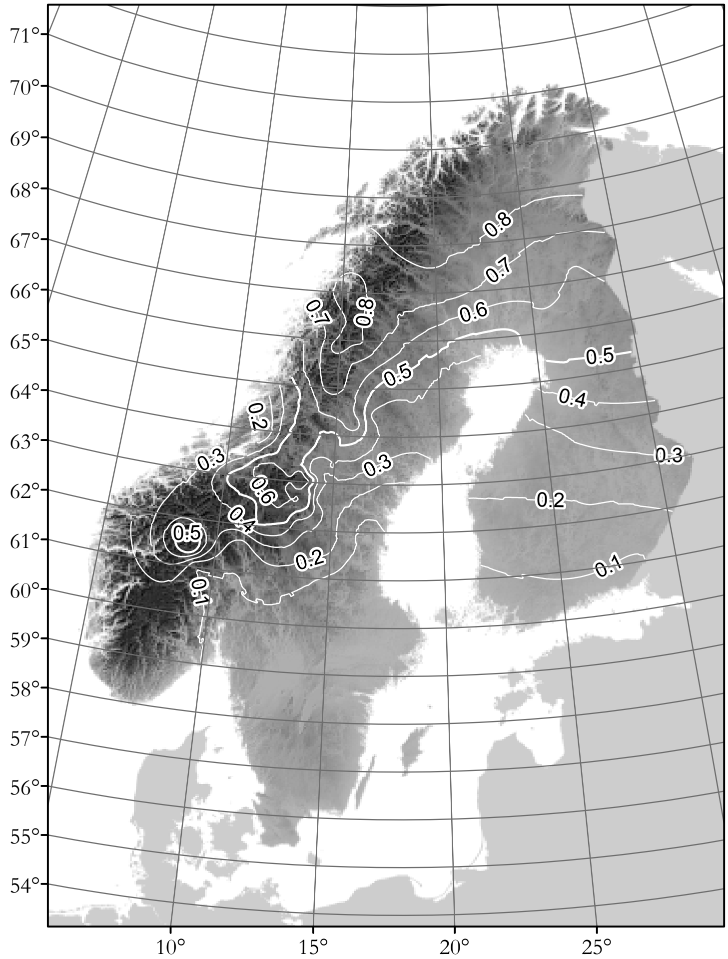

Figure S1. Spatially interpolated predicted 0.1 northern-allele frequency contours for the locus WW1 from the GAM logistic regression model overlaid on altitude hill shade background. The 0.5 isocline represents the center of the cline. The background represents a digital elevation model (DEM) with hill shading for study area. Map has a 1 km or 30 arc-second resolutions and projected using the Swedish RT 90 0 gon Mercator projection.

Paper V

Phenotypic turn-over and hybrid zone dynamics in the willow warbler migratory divide

Keith W. Larson, Miriam Liedvogel, Max Lundberg, Susanne Åkesson, Staffan Bensch

Department of Biology, Centre for Animal Movement Research, Ecology Building,
Lund University, SE-223 62 Lund, Sweden

Abstract

A migratory divide is a region where populations with distinct migratory phenotypes meet, potentially mate, and hybridize. In this study we characterize a willow warbler (*Phylloscopus trochilus*) migratory divide and hybrid zone spatially, both its geographic location and shape. Based on earlier findings of lower abundances in the west of the hybrid zone, we predict the zone to be wider in the west than in the east. Further, using data from repeated visits to sites across the zone, we predict low repeatabilities for migratory associated traits that would suggest that high annual turn-over in migratory phenotypes occupying the zone. The results of our analysis demonstrate that the hybrid zone is approximately 80 km wide in east along the Baltic Sea and 300 km wide in the mountainous west. These differences likely reflect contrasting in landscape heterogeneity. For example, weather in the east is milder and less stochastic and habitats relatively homogeneous, while in the mountainous west, habitats are heterogeneous, winters long, and temperatures more extreme. In the future it will be essential to develop genetic markers that allow for the identification of the two subspecies, hybrids, and backcrosses. Only then will we be able to elucidate differences in hybrid zone dynamics related to the region of low abundance in the west and areas of higher abundance in the east. Once markers allow for the identification of hybrid offspring, orientation experiments could be used to elucidate migratory directional preferences. These results would either support or refute our hypothesis that an intermediate migratory programme results in lower hybrid fitness.

Introduction

Many migratory bird species exhibit a migratory divide. A migratory divide is a region where neighboring populations with distinct migratory phenotypes meet, potentially mate, and hybridize. Thus migratory divides are often hybrid zones. These divides generally result from post-glacial secondary contact (Rohwer and Manning 1990, Bensch *et al.,* 1999, Ruegg 2008). Migratory divides thus make excellent ecological laboratories to investigate the effect of the migratory traits in pre- and post-zygotic reproductive isolation (Rohwer and Manning 1990, Helbig 1991, Ruegg 2008). To understand if and how the migratory program promotes reproductive isolation, it is important to assess the strength of selection on migratory associated traits and characterize gene flow across the divide (Ruegg 2008). Here we address these questions in the willow warbler (*Phylloscopus trochilus*), a good ecological model to study divergent selection on migratory traits.

Willow warblers are small long-distance migratory passerines that are represented by two subspecies in Sweden (*P. t. trochilus* in the South, and *P. t. acredula* in the North) with a latitudinal migratory divide across Sweden. The position of the hybrid zone is located between 61.4° and 64.1° north (latitude) and approximately 300 km wide (Bensch *et al.,* 2009). Both subspecies are differentiated by clines in wing length (northern birds have longer wings), migratory direction (*trochilus* has a southwesterly autumn migratory direction, *acredula* migrates south-southeast) and distance (*acredula* travels longer distances), and consequently their respective winter quarters (confirmed by stable nitrogen-isotope analyses) (Salomonsen 1928, Ticehurst 1938, Hedenström and Pettersson 1987, Bensch *et al.,* 1999, Chamberlain *et al.,* 2000). Thus subspecific differences largely reflect differences in their migratory phenotype, or "migratype".

Previous analysis of the willow warbler migratory divide have investigated (i) if the phenotypic pattern is best explained by neutral diffusion or the tension zone hypothesis in favour of the latter (Bensch *et al.,* 1999), and (ii) whether the two subspecies represented a ring subspecies around the Baltic Sea (Bensch *et al.,* 2009). Studies aiming to estimate the degree of genetic differentiation at neutral loci (mitochondrial DNA and microsatellites) across this migratory divide indicate that despite the phenotypic differences both subspecies are best described as one panmictic population (Bensch 1999, Bensch *et al.,* 2009). A genome wide marker-scan using amplified fragment lengths polymorphism (AFLP) lead to the identification of one bi-allelic marker (AFLP-WW2) with substantially different frequencies between the subspecies. Allele frequencies for AFLP-WW2 are highly coincident with the clinal values in morphological traits and isotope signature variation, which suggests that the AFLP locus WW2 is linked to a gene(s) encoding for intraspecific variation of migratype (Bensch *et al.,* 2009). This is further supported by the coincident clines for stable nitrogen-isotopes (δ^{15}N) and AFLP-WW2, geographically between Lithuania and Poland, which rules out that AFLP-WW2 is associated with size or colour (Bensch *et al.,* 2009).

The empirical analysis of hybrid zones is rooted on well-developed theory, typically on genotypic markers in one-dimension, e.g., single-locus cline analysis (Key 1968, Moore 1977, Barton and Hewitt 1985, 1989). In this study we extend this approach by analyzing a combination of both genotypic and phenotypic traits in two dimensions (Guillot *et al.,* 2009, Jombart *et al.,* 2010, Guedj and Guillot 2011). Here we use a high resolution dataset including both previously collected data (i.e. pre-2008) and a new dataset consisting of 50 densely sampled sites within the hybrid zone (including repeated visits to 36 sites, collected between 2008 and 2010).

Phenotypic traits may reflect local adaptations that could lead to the buildup of reproductive isolation through divergent selection, despite the presence of neutral gene flow (Via 2009). In our study system, the incorporation of phenotypic trait data into hybrid zone models is particularly important as willow warblers show no or little divergence in neutral markers even between the most distinct forms of the putatively hybridizing taxa (Bensch 1999, Bensch *et al.,* 2009). Thus inclusion of densely sampled phenotypic and genotypic sources of variation in hybrid zone models may provide key insights into evolutionary mechanisms that lead to reproductive isolation and maintain hybrid zone stability.

In this study we will first characterize the hybrid zone spatially, both with reference to geographic location (latitudinally) and variation in shape (longitudinally). A balance between dispersal (rate and distances) and selection against hybridization determines the width of a tension zone. Hybrid zone theory suggests that zone position and width often coincides with population sinks that trap zones as they generally move towards regions of low density, low fitness, and low dispersal (Barton 1979, Barton and Hewitt 1985). Earlier findings report lower abundances in the western mountains of Sweden versus the eastern part of the hybrid zone along the Baltic Sea (Larson *et al., submitted*). Given these findings, we predict the hybrid zone to be wider in the mountainous west than along the lowlands in the east. Further, using our data for sites with repeated visited, we predict low repeatabilities for various traits that would suggest that high annual turn-over resulting in a heterogeneous landscape of phenotypes. High-turnover would also suggest between year differences in opportunities for hybridization and thus the width of the zone is predicted to be wider in the west than the east where stable environmental conditions and high population abundance are typical. However, due to the lack of genetic markers for migratory traits we cannot determine if these heterogeneous populations are composed of pure parental forms, hybrids or backcrosses.

Materials and Methods

Sampling

The total dataset comprises material from 2,552 individual willow warblers sampled at 98 sites across Sweden (Figure 1). The total dataset can be separated into two temporal groups, with the data from 1996 to 2007 presented in earlier efforts (Bensch *et al.,* 1999, 2009, Chamberlain *et al.,* 2000), and those collected from 2008 to 2011 (Table S1). At each sampling location we trapped only males observed singing on their territories to ensure all individuals represented locally breeding birds. To capture the birds we used a single audio-lure playback at to attract territorial males into a mist-net. We sampled blood for genetic analysis, the first primary feather for stable isotope analysis, assessed plumage colour, and took four morphological measurements. For a detailed description of field and laboratory methods see previous publications (Bensch *et al.,* 1999, 2009, Chamberlain *et al.,* 2000).

Spatial analysis

We used both genotypic and phenotypic migratory trait data to characterize the position and shape of the hybrid zone using the spatial model found in the *Geneland* package for R software (Guillot and Santos 2010). We constructed two spatial models for Sweden. For both *Geneland* spatial models we ran the analysis for 500,000 steps with a thinning of 500 (i.e. saving every 1000th sample). For the post-process step we used 100 x 240 pixels for x, y and a burn-in of 200 steps. Both models include the traits AFLP-WW2, morphological data as summarized by the first two principal components from a principal components analysis (PCA), and feather stable carbon- and nitrogen-isotopes (δ^{13}C and δ^{15}N). This analysis excludes the AFLP marker WW1 used in previous cline analysis (Bensch *et al.,* 2009) as it has recently been shown to represent local adaptation to mountain environments, independent of subspecies (Larson *et al.,*

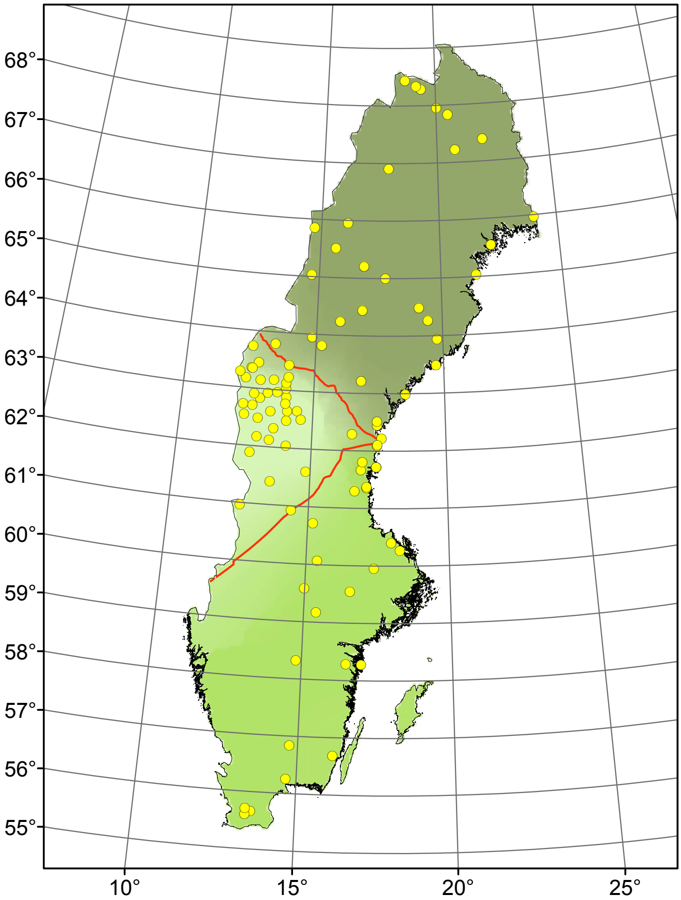

Figure 1. Spatially interpolated results derived from Geneland spatial models with two different a priori defined groups K = 3. The model allows incorporating both genotypic and phenotypic migratory traits. Specifically we here include five selected focal traits AFLP-WW2, $\delta^{13}C$, $\delta^{15}N$, body size PC1 and PC2 for all analyses investigating differences between the two migatpyes. The red lines mark the mid-point in the cline(s) separating the dark olive area representing *acredula* populations in the north and light green region of *trochilus* populations in the south. Sampling sites are indicated as red circles. For details of site coordinates, elevation, and site specific sampling effort see Table S1.

in prep.). The results of these models provide Bayesian prior probability assignments for each site based on the number of groups (K) defined by the model. The spatial model was run with K =2 based on the presence of two subspecies in our study area and K = 3 in order to define the "hybrid" populations most likely to include a mixture of parental forms, hybrid and backcross individuals. We assume the K = 3 model to best represent the spatial location and dimensions of the hybrid zone if it occurs within the previously identified hybrid zone (Bensch *et al.,* 2009). To

Table 1. Here we present repeatabilities measures (*R*) for each trait and by site and year (for details see Supplemental Table S1) across the hybrid zone that were visited more than once (alpha = 0.05). For description of the northern and southern-most latitudinal limits for comparison across the hybrid zone see Table S1. Indicative traits for characterization of both migratypes were selected as outlined in Table S1. This analysis was carried out for the period of 2008 to 2010, N is a reflection of the number of site and year combinations as only some sites where visited in 2009 and 2010. Due to limitations collecting DNA samples in those years, AFLP-WW2 is not presented.

						Variance	
Trait	**N**	***R***	**LowerCI**	**UpperCI**	***k***	**Within**	**Among**
Morphology PC1	52	0.08	0.04	0.14	17.47	1.59	0.13
Morphology PC2	52	0.10	0.06	0.17	17.47	1.14	0.13
δ^{15}N	59	0.02	0.00	0.06	16.53	3.38	0.07
δ^{13}C	52	0.00	–0.02	0.03	17.31	3.94	–0.02

visualize the results we used spatial interpolation or ordinary kriging in the Spatial Analysis toolkit for ArcView GIS 10 (ESRI 2010) to create continuous surfaces for our model results.

Statistical analysis

In order to summarize our individual trait data by site we first calculated a principal components analysis (PCA) on the four morphological measures, wing chord, tail, tarsus, and bill-head length (see Table S2 for results). Next we calculated the allele frequencies for AFLP-WW2 and site means for δ^{13}C, δ^{15}N, and the first two principal components (PC1 and PC2). For sites visited more than once within the hybrid zone between 2008 and 2010, we calculated repeatabilities or intra-class correlation coefficients (Lessells and Boag 1987) separately for each trait using the ICC package in R (Wolak 2011). We set the alpha level to 0.05 and used the "THD" method to calculate the confidence intervals for unbalanced data (Wolak 2011). All analyses where conducted in R (R Development Core Team 2012).

Results

Both of our *Geneland* spatial models for determining the location of phenotypic population clines conformed to previous studies that identified the willow warbler hybrid zone between 61.4° and 64.1° N latitude (Bensch *et al.,* 2009). The first model with two *a priori* defined groups showed a steep north-south cline between the two groups approximately at the previously described northern limit of the hybrid zone (Figure S1). The second model with three groups, identified three spatially contiguous regions (Figure 1) supported by previous cline analysis (Bensch *et al.,* 2009). In the second model (K = 3), the northern border is very similar to the cline described in the first model (K = 2) and the western border approximately the same width (approximately 300 km) as that previously described (Bensch *et al.,* 2009). Contrary to the results from previous one-dimensional models (Bensch *et al.,* 2009), the width of the hybrid zone in the east is narrower along the Baltic Sea coast (approximately 80 km). Finally, found no significant repeatabilities for both isotopes and significant but extremely low repeatabilities for body size traits in the hybrid zone (Table 1; Figure 2).

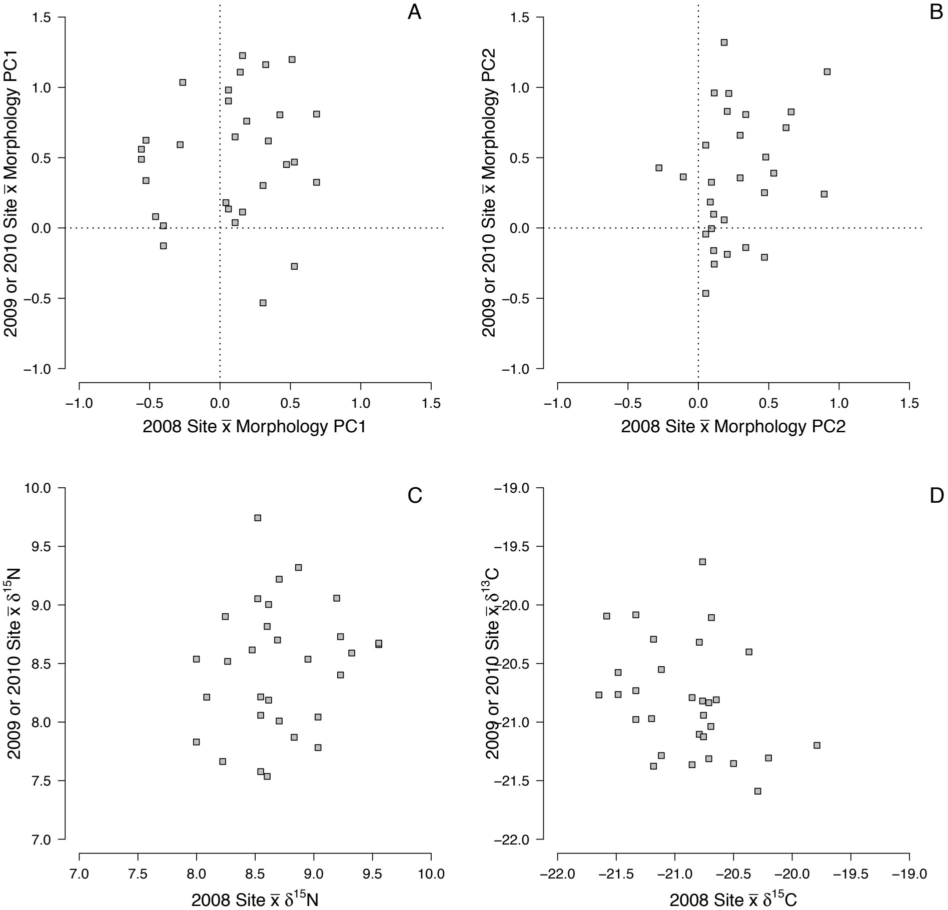

Figure 2. Mean site trait values for sites with repeated visits in the hybrid zone, with the mean site value for a given trait in 2008 on the *x*-axis, and the mean site values for 2009 or 2010 on the *y*-axis. Note some sites were visited three times. (A) morphology PC1, (B) morphology PC2, (C) δ^{15}N, and (D) δ^{13}C. Note that in 2008 and 2009 there were too few DNA samples collected for meaningful analysis.

Discussion

The coincidence of the *Geneland* results (K = 3) with the previously described location of the hybrid zone (Bensch *et al.,* 2009), suggests that the spatial model predicted the location of the hybrid zone in relation to *trochilus* and *acredula* populations well. In previous studies using one-dimensional model to fit both genetic and phenotypic clines separately for each trait, Bensch *et al.,* (2009) identified steep trait clines that were narrower than expected under neutral diffusion. These results gave support to the tension zone model, which predicts a balance between *acredula* and *trochilus* dispersal into the migratory divide and selection against hybrid offspring. Where our *Geneland* results differ is the shape of the hybrid zone, which uses a spatial approach to model the cline using both the genotypic and phenotypic traits simultaneously in two-dimensions. The resulting cline is much wider in the western mountains of Sweden on the Norwe-

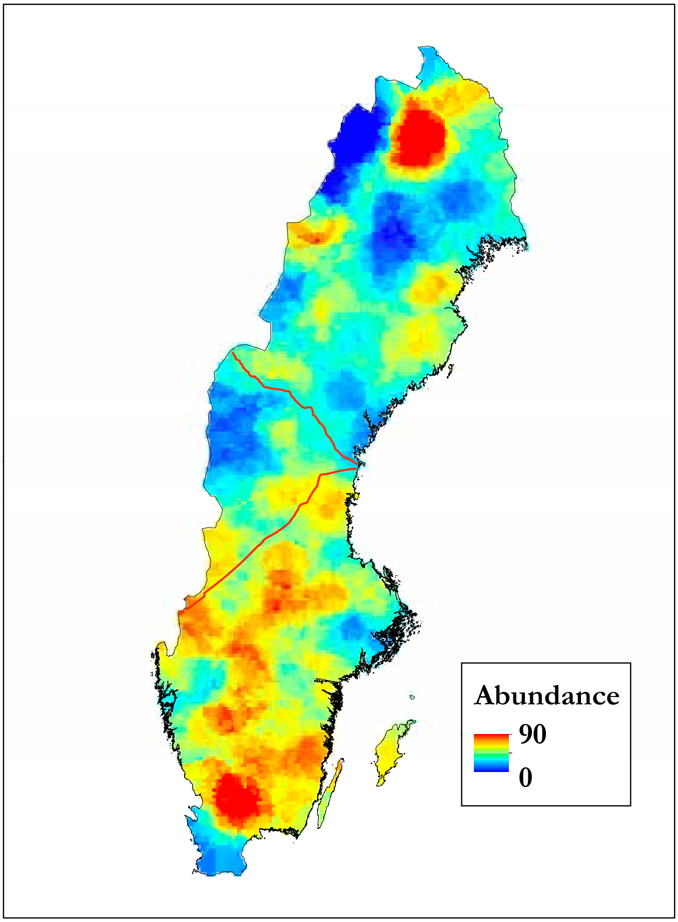

Figure 3. Spatially interpolated relative abundance of willow warblers across Sweden. Abundance data are derived from 706 sites collected between 1996 and 2011 as part of a breeding bird survey monitoring programme , see (Stjernman *et al.*, 2013) for survey methods.

gian border than on the Baltic Sea coast to the east. Although other studies have found hybrid zones associated with regions of low abundance (Rand and Harrison 1989, Ruegg 2008), we are unaware of studies that show such a dramatic difference in abundances and the shape of the hybrid zone across its width. It is possible that with improved molecular techniques to separate parental migratypes from hybrids and backcrosses, we would find population structure correlated with environmental conditions that characterize mosaic hybrids zones (Rand and Harrison 1989, Harrison and Bogdanowicz 1997).

This shape for the hybrid zone is intriguing because the general environmental gradients in this region are northwest to southeast following the watersheds out of the mountains southeast to the Baltic Sea. The northern boundary (cline) of the hybrid zone follows this general environmental gradient from the northwest to the southeast. When we compare the width of the zone in the west (Figure 1) to a map of willow warbler relative abundance generated from long-term monitoring data across Sweden (Figure 3), we can clearly see that the western portion of the hybrid zone appears to be trapped in a region of low abundance. Further comparison of the low abundance region with a map of the number of frost free days and winter temperatures reveals that this area has the lowest number of frost free days in the country (Sjörs 1965) and consistently has the lowest recorded winter temperatures (Sjörs 1965, Mellberg 2008). This region of low abundance appears to trap the hybrid zone in the west, keeping it wide (300 km from north-to-south), while it has narrowed in the east where the landscape reflects more mild conditions. The tension zone hypothesis predicts that hybrid zones are not directly associated with an environmental gradient and tend to float spatially while minimizing their width over time (Barton 1979, Barton and Hewitt 1985, 1989). Further, tension zones move and can become trapped on environmental gradients or regions of low abundance (Barton 1979, 1980, Barton and Hewitt 1985).

We found a lack of significant or extremely low repeatabilities for mean trait values at hybrid zone sites that suggests high turn-over in trait frequencies between years. As previously discussed, the hybrid zone in the west is wide and appears to be trapped in a region of low abundance. The sampling sites in this region vary in altitude from 300 to almost 900 meters above sea level and thus represent a wide spectrum of environmental conditions. Given the heterogeneous landscape in the west and the very short growing season characterized by the number of frost free days (Sjörs 1965) and very cold temperatures (Sjörs 1965, Mellberg 2008), it may explain why we see such high turn-over (as characterized by low repeatabilities for traits) at sites within the hybrid zone. Unfortunately, without markers that allow us to distinguish between parental migratypes, hybrids and backcrosses, we are unable assess the population structure across the hybrid zone.

A recent study on assortative mating in the hybrid zone demonstrated no clear assortative mating between the two willow warbler subspecies indicating that hybridization should occur frequently (Larson *et al., in prep.*). Without selection against the hybrid forms, the observed steep trait clines would have dissolved in only a few hundred years (Bensch *et al.,* 1999, 2009). The shape and position of the hybrid zone appears to be at least partially determined by a region of low population in the western portion of the hybrid zone. Our results also suggest that there is high turn-over in trait frequencies at sites across the hybrid zone. All of these factors give additional support for the hypothesis that some hybridization most likely takes place and hybrid offspring fledge and undertake their autumn migration south. If these offspring have an intermediate migratory program to their parental migratypes, they would likely suffer a cost to fitness as neither parental form possesses adaptations for long-distance non-stop flights. In the case of the willow warbler, an intermediate migratory direction would take hybrids over formidable barriers such as the Mediterranean Sea and Sahara Desert at their widest points.

Conclusions

The results of our spatial analysis support previous studies that identified the location of the hybrid zone and that the zone is narrower than expected under neutral diffusion (Bensch 1999, Bensch *et al.,* 2009). Our results demonstrate that the dynamics differ from east to west across the zone. In the east along the Baltic Sea the hybrid zone is approximately 80 km wide while in the west, approximately 300 km wide. These differences reflect the landscape heterogeneity across the zone. In the east, conditions along the Baltic Sea are mild and less stochastic and in the mountainous west winters are long and temperatures more extreme. To better understand the hybrid zone dynamics it will be essential to develop genetic markers that allow one to separate each parental migratype, hybrids, and backcrosses. Once markers allow the identification of hybrid offspring, orientation experiments should be conducted to elucidate migratory directional preferences, that would support our hypothesis that hybrids take an intermediate migratory direction to their parental migratypes (Helbig 1991, Bensch 1999, Bensch *et al.,* 2009).

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Table S1. Sample locations, geographic coordinates and elevation with number of visits and number of individuals sampled between 1996 and 2005, and 2008 and 2011.

				1996 to 2007		2008 to 2011	
Site	Latitude	Longitude	Elevation	Site Visits	N	Site Visits	N
Alnön	62.44	17.43	61	2	32	1	11
Altajärvi	67.83	20.53	381	1	10		
Ammarnäs	65.95	16.12	628	1	18	1	8
Anjan	63.73	12.54	439			3	40
Ånn	63.35	12.55	541	1	16	0	0
Åsarna	62.65	14.40	355	1	18	0	0
Aspberget	60.97	12.53	323	1	16	0	0
Bäcksand	62.08	17.44	26	2	23	1	11
Barkö	60.27	18.27	10	3	23		
Björka	55.66	13.63	27			1	10
Björkön	62.22	17.60	5	1	23		
Bollnäs	61.30	16.63	169	1	17		
Bunkris	61.40	13.55	512	1	16		
Ekorrsjö	64.49	19.07	211			1	11
Faxälven	63.21	16.81	208	1	20		
Fittjebodarna	62.88	13.97	662			2	33
Flatruet	62.71	12.70	846			4	91
Fotingen	62.64	14.02	510			2	31
Frankrike	63.79	13.41	500			1	14
Fullerö	59.55	16.55	27	1	11		
Gällivare	67.22	20.80	433	1	15		
Gardiken	65.51	15.64	440			1	13
Gestvallen	63.17	13.45	610			2	30
Gimsjön	62.28	16.50	342	1	6		
Glen	62.96	13.61	780			3	50
Gnarp	62.10	17.45	24	1	16		
Gräftåvallen	63.02	13.93	707			2	36
Granö	64.27	19.42	194			1	10
Gräsmarö	58.28	16.98	21	3	24		
Gunnarn	65.00	17.71	272			1	10
Haganäs	56.25	14.67	80	1	15		
Hällberget	63.44	13.99	325			1	15
Hallbodarna	63.12	13.92	501			2	33
Hotagen	63.95	14.83	528	1	15		

Table S1. *Continued*

				1996 to 2007		2008 to 2011	
Site	Latitude	Longitude	Elevation	Site Visits	N	Site Visits	N
Hudiksvall	61.72	17.42	48	1	17		
Idre	61.88	12.73	530	1	10	1	3
Insjön	60.72	15.18	280	1	18		
Jylkkyvaara	67.38	22.05	321	1	14		
Kaisepakte	68.28	19.32	377	1	22		
Kallax	65.53	22.13	9	1	9		
Kalmar	56.68	16.12	45	1	15		
Kaskeluokt	65.20	16.84	366			1	15
Klångstavallen	61.67	16.85	140	2	47		
Klimpfjäll	65.03	14.68	838	1	16		
Kramfors	62.98	18.50	29	1	15	1	11
Krokvik	67.95	20.02	487	1	15		
Kukkola	65.96	24.03	20	1	15		
Kullatorpet	55.60	13.46	122			1	12
Kvikkjokk	66.90	17.83	422	1	15		
Kvismaren	59.17	15.42	30	1	20		
Ljungdalen	62.84	12.97	593	1	22	3	75
Ljungris	62.90	12.73	816			3	49
Lofsdalen	62.12	13.42	626	1	15		
Ludvika	60.07	15.38	220	1	15		
Mårdsund	63.23	14.00	306			3	36
Medskogen	62.48	12.93	864			1	18
Meselefors	64.44	16.80	339			1	11
Midlanda	62.51	17.43	1	1	15		
Mora	60.93	14.38	312			1	10
Mörtsjön	61.72	17.41	47	3	87	1	10
Nordhallen	63.45	12.82	426	1	11		
Nordmaling	63.48	19.70	10	1	18	1	10
Orböle	60.40	17.97	17	1	14		
Öster Galåbodarna	62.77	13.94	593			2	30
Piteå	65.03	21.45	13	1	15		
Ramundberget	62.72	12.34	738			2	36
Rätan	62.50	14.56	362			3	47
Rönäs	65.85	14.70	499			1	15
Särvfjället	62.61	13.39	835			2	34

Table S1. *Continued*

				1996 to 2007		2008 to 2011	
Site	Latitude	Longitude	Elevation	Site Visits	N	Site Visits	N
Smedstorp	59.58	14.98	133	7	63		
Söderhamn	61.35	17.07	15	1	12	1	12
Sonfjället	62.32	13.55	725			1	15
Sörvattnet	62.17	12.95	736	1	10		
Stensoffa	55.70	13.46	17	9	155	3	70
Stordalen	68.33	19.10	515	1	22		
Storulvån	63.17	12.36	744			3	69
Storvallen	63.28	12.12	590			1	16
Sveg	62.03	14.05	453	1	16	1	11
Tåkern	58.32	14.82	104	1	15		
Tångböle	63.35	12.59	520	1	10	2	31
Tanna	61.80	16.90	94	2	49		
Tänndalen	62.54	12.41	831			2	35
Tåsjoberget	64.24	15.93	589			1	14
Tornehamn	68.43	18.58	445	1	19		
Tossåsen	62.94	13.25	776			2	34
Ulleråker	59.95	17.37	52	1	14		
Vålådalen	63.15	12.94	580			3	56
Valdemarsvik	58.28	16.47	44	1	15		
Vännäs	63.93	19.77	83			1	10
Varbergsviken	61.37	17.07	25	1	17		
Västbacka	61.60	14.83	410			1	15
Växjö	56.83	14.73	174	1	15		
Vemdalsskalet	62.48	14.01	333	1	10	2	36
Yxskaftkälen	63.81	15.22	375			1	13

Table S2. Results from principal components analysis on contributions of four morphological traits (in mm) measured in 2,552 individuals from 98 sites sampled across Sweden between 1996 and 2011.

Measurement	PC1	PC2	PC3	PC4
Wing	–0.55	0.43	–0.04	0.71
Whole Tail	–0.52	0.48	–0.05	–0.70
Tarsus	–0.43	–0.58	–0.69	–0.03
Bill + Head (length)	–0.48	–0.50	0.72	–0.04
Importance of components				
Standard deviation	1.34	1.12	0.72	0.66
Proportion of Variance	0.45	0.32	0.13	0.11
Cumulative Proportion	0.45	0.76	0.89	1.00

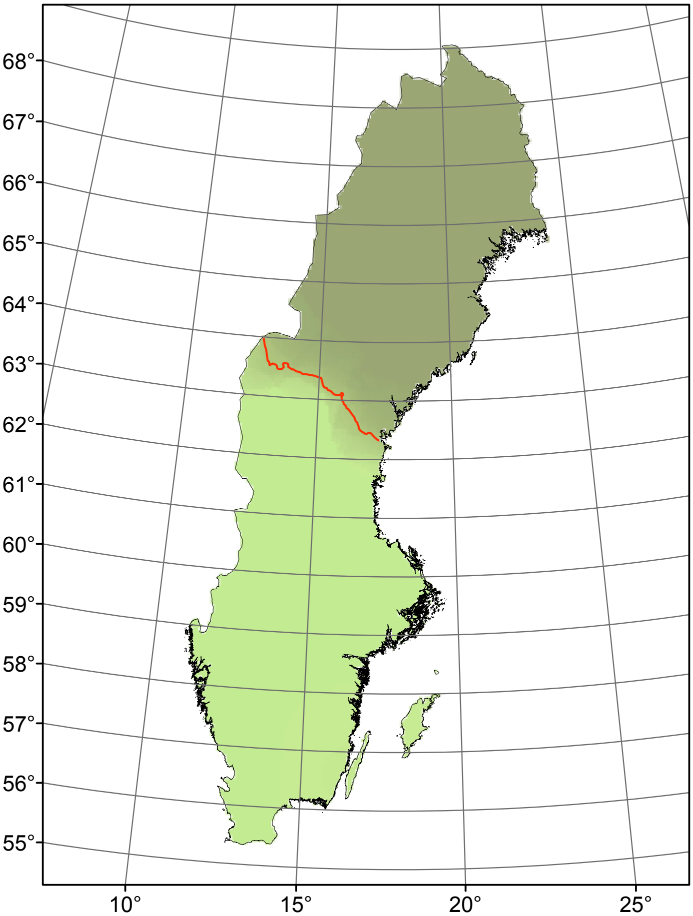
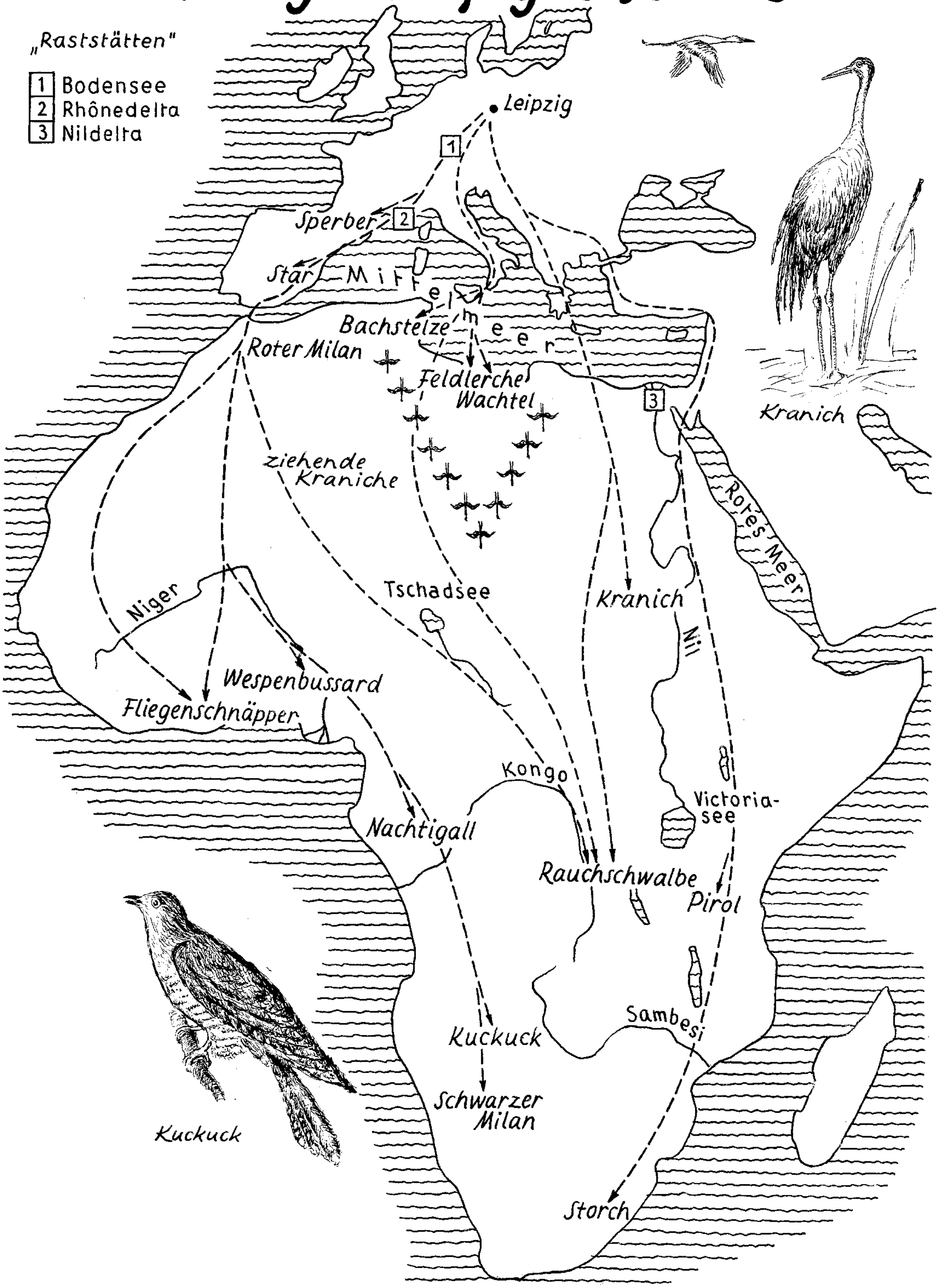


Figure S1. Spatially interpolated results derived from Geneland spatial models with two different *a priori* defined groups with K = 2. The model allows incorporating both genotypic and phenotypic migratory traits. Specifically we here include five selected focal traits AFLP-WW2, δ^{13}C, δ^{15}N, body size PC1 and PC2 for all analyses investigating differences between the two migratypes. The red lines mark the mid-point in the cline(s) separating the dark olive area representing *acredula* populations in the north and light green region of *trochilus* populations in the south.

Unsere Vögel auf großer Reise



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