MOLECULAR ECOLOGY

Molecular Ecology (2014) **23**, 3551–3565 doi: 10.1111/mec.12836

Differential high-altitude adaptation and restricted gene flow across a mid-elevation hybrid zone in Andean tit-tyrant flycatchers

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Abstract

The tropical Andes are a global hotspot of avian diversity that is characterized by dramatic elevational shifts in community composition and a preponderance of recently evolved species. Bird habitats in the Andes span a nearly twofold range of atmospheric pressure that poses challenges for respiration, thermoregulation, water balance and powered flight, but the extent to which physiological constraints limit species' elevational distributions is poorly understood. We report a previously unknown hybrid zone between recently diverged flycatchers (Aves, Tyrannidae) with partially overlapping elevational ranges. The southern *Anairetes reguloides* has a broad elevational range (0–4200 m), while the northern *Anairetes nigrocristatus* is restricted to high elevations (>2200 m). We found hybrids in central Peru at elevations between ~3100 and 3800 m, with *A. nigrocristatus* above this elevation and *A. reguloides* below. We analysed variation in haematology, heart mass, morphometrics, plumage and one mitochondrial and three nuclear loci across an elevational transect that encompasses the hybrid zone. Phenotypic traits and genetic markers all showed steep clines across the hybrid zone. Haemoglobin concentration, haematocrit, mean cellular haemoglobin concentration and relative heart mass each increased at altitude more strongly in *A. reguloides* than in *A. nigrocristatus*. These findings suggest that *A. nigrocristatus* is more resistant than *A. reguloides* to high-altitude hypoxic respiratory stress. Considering that the ancestor of the genus is suggested to have been restricted to high elevations, *A. reguloides* may be secondarily adapted to low altitude. We conclude that differential respiratory specialization on atmospheric pressure combined with competitive exclusion maintains replacement along an elevational contour, despite interbreeding.

Keywords: competitive exclusion, haemoglobin, local adaptation, low-altitude adaptation, respiratory physiology

Received 12 November 2012; revision received 10 June 2014; accepted 13 June 2014

Introduction

The Andes of South America exhibit dramatic environmental gradients over short geographic distances, providing a rich system to examine adaptation of organisms to their environments on a fine spatial scale (Monge & Leon Velard 1991; Rezende *et al.* 2005; Projecto-Garcia *et al.* 2013). Animal habitats in the tropical Andes extend from sea level to ~5000 m elevation, spanning atmospheric pressures of ~760–420 mmHg and a temperature range of ~33 °C (West 1996). At the top of this gradient, animals face hypodense air that increases both the energy requirements for powered flight and the rate of dehydration (Altshuler & Dudley 2006). Perhaps the most consequential effect of high altitude is that the partial pressure of oxygen (PO_2) in

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dry inspired air is reduced in proportion to the reduction in atmospheric pressure, with an additional diluting effect of water vapour that becomes more severe at altitude. At 3800 m elevation, above which reside at least 200 species of Andean birds (Parker *et al.* 1996), PO_2 in humidified air at the body temperature of a typical bird (40 °C) is reduced by 38.7% relative to sea level [calculated using model atmosphere equation of West (1996)]. Reductions on this scale cause arterial oxygen saturation to plummet, reducing the efficiency of O_2 uptake and transport (Mathieu-Costello 1990; Monge & Leon Velard 1991). Animals undergo dramatic compensatory changes in ventilation, perfusion and blood composition in response to acute high-altitude hypoxia exposure, and it is now clear that these respiratory responses extend to genetically based evolutionary adaptations in protein chemistry (Weber 2007; Storz *et al.* 2009; McCracken *et al.* 2010; Projecto-Garcia *et al.* 2013), morphology (Kiyamu *et al.* 2012) and gene regulation (Simonson *et al.* 2010; Cheviron *et al.* 2012).

A key question in the study of montane faunas is whether differential adaptation to altitude could play an important role in the origins or maintenance of species diversity. Strong diversifying selection along environmental gradients can promote intrapopulation divergence by favouring alleles that benefit individual fitness under local conditions, irrespective of their fitness consequences at other points along the gradient (Williams 1966; Kawecki & Ebert 2004). Gene flow along the gradient counters the effects of selection by homogenizing phenotypic and genetic variability. The selection-gene flow balance favours local adaptation when strong selection is coupled with low rates of migration (Kawecki & Ebert 2004; Cheviron & Brumfield 2009). Local adaptation plays a critical role in maintaining intraspecific variation (Felsenstein 1976; Hedrick *et al.* 1976; Hedrick 1986; Schneider *et al.* 1999), and it may be an important initial step towards speciation (Schluter 2001; Turelli *et al.* 2001; Via 2001). A further possibility is that differential local adaptation facilitates diversification at later stages of the speciation processes when reproductively compatible taxa come into contact and potentially interbreed along environmental gradients.

The classic model for speciation in Andean vertebrates is by geographic isolation along the latitudinal axis of the mountain chain (Graves 1985; Patton and Smith 1992; Krabbe & Schulenberg 1997; Graham *et al.* 2004; Cuervo *et al.* 2005; Gutiérrez-Pinto *et al.* 2012). Subsequent range expansion of latitudinal allotaxa often leads to secondary contact and parapatric elevational replacement (Dingle *et al.* 2006; Cadena 2007). Elevational replacement of closely related taxa is well documented in mountain regions throughout the world, but little is known about the mechanisms that promote and maintain this pattern (Terborgh 1971; Terborgh & Weske 1975; Graham 1983; Highton 1995, Sasaki *et al.* 2005; Poynton *et al.* 2007; Jankowski *et al.* 2013). Direct or diffuse competition has been shown to be the proximate cause of the upper or lower elevational distribution boundaries in many species of birds and other vertebrates (e.g. Diamond 1970; Jaeger 1971; Diamond 1973; Terborgh & Weske 1975; Mayr & Diamond 1976; Cadena & Loiselle 2007; Jankowski *et al.* 2010; Gifford and Kozak 2012). Hence, elevational replacement is a potential consequence of competitive exclusion due to differential performance of competing species in adjacent elevational zones (Terborgh 1971), although the mechanisms underlying differential competitive abilities remain uncertain and understudied. Physiological constraints related to atmospheric pressure or temperature comprise one potential mechanism, and these may be sufficient to promote elevational parapatry even if competition is weak or absent (Gifford and Kozak 2012).

In this study, we examine a case of elevational replacement in bird taxa that are sufficiently closely related as to have been considered conspecific in some classifications (Meyer De Schauensee 1970). *Anairetes reguloides* (Pied-crested Tit-tyrant) inhabits the dry, west slope of the Peruvian Andes from sea level to ~4200 m (ornisnet.org specimen data). The larger, montane-restricted taxon, *Anairetes nigrocristatus* (Black-crested Tit-tyrant), inhabits elevations from 2200 to 4200 m (ornisnet.org specimen data). The two species are arid-tolerant generalists that are nearly endemic to Peru. They have no apparent differences in habitat preference, inhabiting arid montane scrub, riparian thickets, *Polylepis* woodland and hedgerows (Parker *et al.* 1996; Schulenberg *et al.* 2007). Diversification within the clade appears to have occurred by allopatric isolation along the latitudinal axis of the Andean ridge, with *A. reguloides* in the south and *A. nigrocristatus* in the north (DuBay & Witt 2012). Subsequent range expansion led to latitudinal overlap of the two taxa in central Peru, with *A. reguloides* apparently restricted to lower elevations only where it overlaps *A. nigrocristatus* (Schulenberg *et al.* 2007).

In animals that are not adapted to high-elevation, high-altitude hypoxia triggers a systemic physiological shift that includes an increase in blood-oxygen-carrying capacity to compensate for reduced arterial oxygen saturation (Storz *et al.* 2010). This hypoxia response includes increased red blood cell production (erythropoiesis), leading to a higher ratio of red blood cells to total blood volume (haematocrit), which has the dual effect of increasing blood-oxygen-carrying capacity and causing potentially harmful increases in blood viscosity (polycythaemia; Winslow & Monge 1987). Another

aspect of the hypoxia response in nonadapted animals is the narrowing of pulmonary blood vessels (pulmonary vasoconstriction), which improves oxygen uptake but causes the right ventricle of the heart to work harder (Naeije & Dedobbeleer 2013), potentially leading to an increase in the relative size of the heart (Corno *et al.* 2004; Hoit *et al.* 2012). Lack of a haematological or pulmonary vasoconstrictive response in a mammal or bird at high elevation suggests 'cryptic' biochemical adaptation to high-altitude hypoxia (Storz *et al.* 2010). 'Cryptic' adaptation refers to the situation in which the phenotype of a population that is subject to stress evolves to resemble that of an unstressed population through modifications in underlying molecular architecture (Storz *et al.* 2010; Beall 2011). Consistent with the 'cryptic' adaptation hypothesis, the suppression of the erythropoietic response to hypoxia was recently found to be a key component of genetic adaptation to high altitude in Andean humans (Zhou *et al.* 2013). Thus, in high-altitude-adapted species, the physical characteristics of the blood and heart are expected to resemble those of native lowland species at sea level (Monge & Leon Velard 1991; Storz *et al.* 2010).

To the extent that differential haematological responses to altitude are found in *A. reguloides*/*nigrocristatus*, it would suggest a role for differential biochemical adaptation in promoting and maintaining the elevational replacement pattern. Direct comparison of the relationships between altitude and physiological characteristics is uniquely possible in these taxa because the lower altitude species, *A. reguloides*, extends its range to ~4200 m elevation in areas where *A. nigrocristatus* is absent. We predict that *A. nigrocristatus* will exhibit muted haematological and cardiac responses to high-altitude hypoxia because its restriction to upper montane zones may have facilitated genetic adaptation to low PO_2. On the other hand, *A. reguloides* is distributed contiguously across a >4000 m elevational gradient, potentially facilitating gene flow and inhibiting the spread of locally optimal alleles. We predict that *A. reguloides* will exhibit marked haematological and cardiac responses to high-altitude hypoxia because the homogenizing effect of gene flow with lowland conspecifics hinders local adaptation (see Feder *et al.* 2012). Alternatively, the physiological characteristics of high-elevation *A. reguloides* may resemble that of *A. nigrocristatus* if selection for high-altitude alleles in *A. reguloides* is sufficiently strong to overcome the homogenizing effects of gene flow, as has been demonstrated in some other montane vertebrate taxa (McCormack & Smith 2008; Cheviron & Brumfield 2009; Storz *et al.* 2012).

The question of whether *A. reguloides* and *A. nigrocristatus* are reproductively isolated biological species complicates the interpretation of differential adaptation and its potential role in the maintenance of diversity via elevational replacement. The elevational replacement pattern may be transient if introgressive hybridization is occurring, potentially causing the breakdown of locally co-adapted gene complexes and leading to fusion-extinction. Alternatively, unrestricted introgression could occur across selectively neutral regions of the genome, while regions of the genome involved in local adaptation or reproductive isolation remain distinct (Payseur 2010). We examined the distribution and characteristics of each taxon in the vicinity of a putative contact zone. We used cline analyses and population structure analyses to quantify and characterize phenotypic and genetic structure along an elevational transect spanning pure parental populations. A transient or unstable hybrid zone along an elevational contour would be fleeting because of the short physical distances involved. For example, in a study of an mtDNA contact zone in Rufous-collared Sparrows (*Zonotrichia capensis*) across the same Peruvian elevational gradient, Cheviron & Brumfield (2009) applied a simple model of cline width as a function of time in secondary contact to show that narrow elevational clines in Andean songbirds would have to be improbably recent in order to be consistent with neutral diffusion of genes across the cline. Therefore, we predict that the mid-elevation hybrid zone is stable and associated with reduced gene flow. The latter result would suggest favourable conditions for local adaptation and the stable persistence of elevational replacement.

Methods

Contact zone and transect

We discovered a contact zone between *Anairetes reguloides* and *Anairetes nigrocristatus* at middle elevations in Ancash, Peru where phenotypically and genetically intermediate birds occur. At present, this is the only location where these taxa have been found to overlap. We sampled a 212 km linear transect that spanned this contact zone between 310 and 4000 m elevation (Fig. 1). We analysed morphometrics, plumage characteristics, and genetic structure along this transect.

The transect was designed in Google Earth following Porter *et al.* (1997). The end points of the transect represent parental populations, determined by discrete differences in morphology and fixed variants of mtDNA. All localities were within 12 km of the transect line. Locality 1 is located ~65 km north along the coast from where the Rio Santa empties into the Pacific Ocean. Localities 2–6 lie within the Rio Santa valley. Locality 7 is isolated from the Rio Santa valley by the Cordillera Blanca, the highest range in the Peruvian Andes with a ridgeline that averages >5000 m elevation. The transect

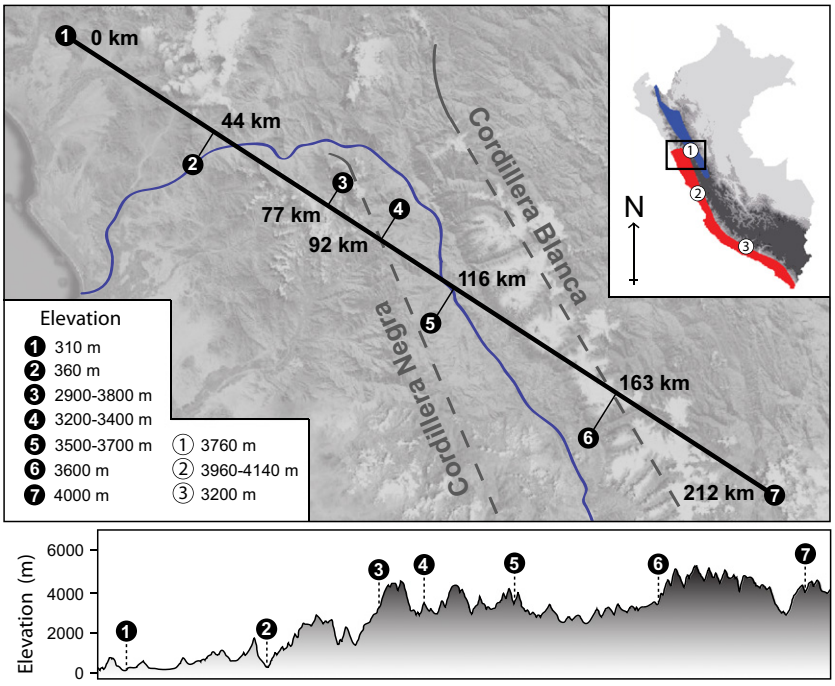

Fig. 1 Transect across contact zone between *Anairetes reguloides* and *Anairetes nigrocristatus*. Map of Peru in upper right corner. Red shading = range of *A. reguloides*. Blue shading = range of *A. nigrocristatus*. Black circles = transect sampling localities 1–7. Black numbers = distance in km from locality 1 for each locality. White circles = sampling localities away from the transect that are included in blood physiology analyses (the corresponding locality/Department in Table S1 (Supporting information) is: circle 1 = Ancash, 2 = Lima, and 3 = Arequipa). Grey-dashed lines = approximate ridge line of respective mountain range. The blue line represents the Santa River. Elevational contour at bottom was created in Google Earth by plotting straight lines between adjacent localities.

corresponds to an elevational gradient and a hypothesized dispersal corridor, the Rio Santa valley (Fig. 1). Elevation increases from locality 1 to locality 7 (Fig. 1).

Morphometric and plumage measurements

We measured two morphometric characters and two plumage characters from 58 individuals of *A. reguloides*/*nigrocristatus* across the transect. The two morphometric characters were body mass and wing chord. The two plumage characters were crest length (measured from the base of the culmen to the tip of the crest) and the length of the white tip on the outer rectrix (measured along the rachis of left R6 from the proximal point where the inner and outer veins are both white to the feather tip). A list of all vouchered specimens used for phenotypic and genetic analyses (with GenBank Accession nos and links to online specimen data) can be found in the supplementary materials (Table S1, Supporting information). Only adult birds collected between the months of May and August were used in phenotypic analyses. Individuals were considered adults if they lacked evidence of a bursa and had complete skull ossification.

Phenotypic cline analyses

We estimated clines for each morphometric and plumage character across the transect using CLINEFIT 2.0a (Porter *et al.* 1997; CLINEFIT 2.0a release date: August 2013). CLINEFIT uses a numerical maximum-likelihood algorithm to assess trait changes across geographic space (Porter *et al.* 1997). Unlike earlier versions, CLINEFIT 2.0a can handle quantitative traits that are continuously distributed. We tested a four-parameter and an eight-parameter model for each phenotypic character, comparing Akaike information criterion (AIC) and AIC with correction for finite sample sizes (AICc) to determine the best-fitting model (Burnham & Anderson 2002). We report AICc values in the manuscript, but it is important to note that AIC and AICc values resulted in the same ranking of models for all analyses. The four-parameter model estimates cline centre (c), cline width (w) and the phenotypic means at the left and right ends of the cline (P_L and P_R). The eight-parameter model estimates c, w, P_L, P_R and four parameters that determine the left and right tail shape (Z_L, Z_R, θ_L, θ_R) (Porter *et al.* 1997). Cline centre is defined by the location along the transect where the given character most rapidly shifts from one species to the other; cline width is defined by the distance over which this shift occurs (Szymura & Barton 1986; Cheviron & Brumfield 2009). Cline analyses were conducted with 1200 parameter tries per annealing step, 2000 replicates, and 50 replicates between saves. We report all cline parameter estimates in Table 1 with two-unit log-likelihood support limits that approximate 95% confidence intervals (Edwards 1972).

Genotypic structure analyses

Total DNA was extracted from frozen or ethanol preserved skeletal muscle using the DNeasy Tissue Kit

Table 1 Cline parameter estimates across the transect for phenotypic characters and genetic markers. Two-unit log-likelihood support limits are reported in parentheses for each parameter. Only parameter estimates from the model with the lowest AICc score for each locus are reported for clines (see Table 2 for model selection scores).

	Cline centre (km†)	Cline width (km)	P_L‡	P_R‡
Phenotypic character				
Mass (g)	97.5 (88.1–109.7)	71.4 (40.1–117.9)	6.5 (6.1–6.9)	9.6 (9.2–10.1)
Wing chord (mm)	103.7 (90.1–130.5)	80.0 (39.9–179.0)	47.7 (45.0–49.2)	57.7 (55.9–60.9)
Crest length (mm)	103.6 (90.4–119.1)	71.2 (38.5–131.2)	20.4 (17.7–22.2)	32.8 (30.6–35.4)
White tail tip (mm)	113.1 (105.5–117.6)	19.5 (2.4–49.0)	4.4 (3.5–4.9)	12.0 (10.8–13.1)
Molecular marker				
Cyt *b*	109.4 (101.2–120.8)	31.2 (18.8–56.6)	—	—
Myo2	118.2 (115.3–133.3)	7.9 (0.1–32.2)	0.12 (0.06–0.19)	0.91 (0.79–0.98)
IRF2	125.8 (112.1–137.1)	64.8 (28.9–81.3)	0.04 (0.00–0.11)	1.0 (0.87–1.0)
MUSK	115.5 (109.5–117.9)	5.0 (1.3–14.1)	0.00 (0.00–0.02)	0.93 (0.80–0.98)

†Kilometres from locality 1.
‡Phenotypic means (phenotypic characters) or asymptotic frequency (molecular markers) on the left and right side of the cline, respectively.

(Qiagen, Valencia, CA, USA). We sequenced four loci for each individual, including a mitochondrial gene [982 base pairs cytochrome *b* (Cyt *b*)], two autosomal nuclear intron loci [668 bp myoglobin intron2 (Myo2); 487 bp interferon regulatory factor 2 (IRF2)] and a sex-linked nuclear intron locus [498 bp muscle, skeletal, receptor tyrosine kinase (MUSK)]. We sequenced 82 individuals for Cyt *b*, 79 individuals for Myo2, 80 individuals for IRF2 and 79 individuals for MUSK across the transect (Table S1, Supporting information). We used primers and PCR amplification conditions as described by DuBay & Witt (2012), and used PCR and sequencing protocols described by Johnson *et al.* (2011). We detected no evidence of pseudogenes in the mtDNA. Unambiguous double-peaks of equal height in nuclear sequences were coded as heterozygous using the IUPAC ambiguity code. Sequences were aligned using MUSCLE 3.7 (Edgar 2004) and visually inspected in MACCLADE 4.08 OS X (Maddison & Maddison 2005).

We estimated the allelic phase for heterozygous nuclear sequences using the Bayesian probability algorithm of PHASE (Stephens *et al.* 2001) implemented in DNASP v.5 (Librado & Rozas 2009). We used PHASE protocols described by Sequeira *et al.* (2011). Individuals for which PHASE could not assign haplotypes with a probability >90% were excluded from further analyses (Table S1, Supporting information). Haplotype networks were constructed for each locus using the median-joining algorithm implemented in NETWORK 4.6 (Bandelt *et al.* 1999).

We used the program STRUCTURE 2.3.3 (Pritchard *et al.* 2000) to examine population differentiation across the transect in the four-locus data set, with haplotypes determined by PHASE. Structure computes the Bayesian probabilities of species assignment for individuals with admixed ancestry by maximizing Hardy–Weinberg equilibrium and minimizing linkage disequilibrium (Pritchard *et al.* 2000). We recoded haplotypes at each locus into different alleles, allowing the four loci to be treated as unlinked (similar to the thrush data in Pritchard *et al.* 2000). Structure was run with a two-population admixture model ($K = 2$, $\alpha = 1$), independent allele frequencies, and no a priori population information. Five independent runs were conducted with 200 000 generations after 100 000 generations of burn-in. We used the population assignment probability (*Q*-score) produced by Structure for each individual as an objective measure of species identification for further statistical analyses.

We characterized the cline shape and genotypic shift from *A. reguloides* to *A. nigrocristatus* along the transect for each locus using CLINEFIT 2.0a. We tested four-parameter and eight-parameter models as described previously for phenotypic characters, as well as two-parameter and six-parameter models. The two- and six-parameter models are appropriate for genetic data but not for continuous quantitative traits because the ends of the cline in these models are fixed at zero and one. The two-parameter model estimates c and w. The six-parameter model estimates c, w, Z_L, Z_R, θ_L and θ_R. For genetic data, P_L and P_R (in the four and eight parameter models) are the asymptotic frequencies on the ends of the cline. Cline analyses were conducted as described above for phenotypic characters.

Haplotypes were assigned to either species for cline analyses based on relative frequencies at the parental ends of the transect, outside of the contact zone. It is important to note that by doing this, all shared haplotypes are treated as introgressed, rather than resulting from ancestral variation. This conservative assumption provides the widest estimate of cline shape. If shared

haplotypes result from ancestral variation, then the true cline would be equal to or steeper than the cline estimated when all shared haplotypes are treated as introgressed. Two Myo2 haplotypes were unique to locality 4 (Table S1, Supporting information), where intermediate phenotypes occurred, and were thus excluded from cline analyses because their origin could not be confidently identified.

Haematological and cardiac measurements

We measured haematological parameters associated with blood-oxygen-carrying capacity in each species to test for differential physiological responses to high elevation. In allopatry, the two lineages both occur above 4000 m elevation, making it possible to compare their respective physiological responses to cold, hypoxic conditions at these elevations. We measured blood haemoglobin concentration ([Hb]) and haematocrit (Hct, the proportion of the total blood volume that is comprised of red blood cells) in individuals from the transect outside of the contact zone ($n = 36$ for [Hb] and 37 for Hct), as well as from 13 pure individuals sampled away from the transect, including *A. reguloides* individuals from the upper extent of its elevational distribution (Fig. 1; Table S1, Supporting information). For logistical reasons, we were unable to obtain blood measurements from localities 4 and 5. We calculated mean corpuscular haemoglobin concentration as the ratio of [Hb] to HCT (MCHC; Campbell & Ellis 2007). All three parameters are expected to increase in non-high-altitude adapted species at altitude but to remain relatively stable in genetically adapted high-altitude forms (Guyton & Richardson 1961; McGrath & Weil 1978; Black & Tenney 1980; Monge & Leon Velard 1991; Storz *et al.* 2010).

We obtained whole blood samples within 30 minutes of capture by venipuncture on the underside of the wing and collection with heparinized microcapillary tubes (Hct) and Hemocue HB201 + cuvettes ([Hb]). Hct (%) was measured with digital calipers after centrifuging the sealed microcapillary tube for 5 min at 13 000 r.p.m. to separate the red blood cells and plasma. Two Hct samples were taken for each bird, and the values were averaged. [Hb] (g/dL blood) was measured on ~5 μL of blood using a HemoCue HB201 + haemoglobin photometer. The HemoCue proprietary photometric method produces values for avian blood that are approximately 1.0 g/dL greater than those generated using cyanomethaemoglobin spectrophotometry (Simmons & Lill 2006); hence, we corrected [Hb] values by subtracting 1.0 g/dL.

After drawing blood, birds were euthanized by thoracic compression, and hearts were extracted, emptied of blood and weighed to the nearest milligram. Relative heart size (heart mass divided by body mass) was calculated for subsequent analyses. We were able to obtain heart mass from all birds along the transect (including birds not bled), allowing us to examined shifts in relative heart size across the contact zone ($n = 68$; Table S1, Supporting information). Relative heart mass is expected to increase in response to hypoxic respiratory stress, particularly in nonadapted species (Corno *et al.* 2004; Hoit *et al.* 2012). Finally, each bird was prepared as a study skin with partial skeleton and frozen tissues deposited at CORBIDI and/or the Museum of Southwestern Biology of the University of New Mexico (MSB; reference Table S1, Supporting information for catalogue numbers and links to online specimen data).

We tested for species differences in each of the four respiratory characteristics (relative heart mass, [Hb], Hct and MCHC) as evidence of differential physiological response to elevation. For each of these four dependent variables, we examined four explanatory variables (elevation, species identity, sex and body mass) and one interaction (elevation × species identity) and we used AICc to select the best model (Burnham & Anderson 2002; AIC and AICc proved to be consistent in all cases). Elevation and its interaction with species identity were central to our hypotheses and had generally stronger effects than other variables; accordingly, we tested all one- to five-parameter models that contained elevation or elevation × species identity, and we selected the model with the lowest AICc value. We eliminated models from consideration if they had equal or higher AICc scores compared to models that contained nested subsets of the same parameters (Arnold 2010). All analyses were restricted to samples from higher elevations (2970–4140 m) because there was no discernible effect of elevation on any of the four respiratory parameters below that range. For relative heart mass, we restricted the analysis to the birds from the transect, and we used the *Q*-score generated by Structure (Fig. 2) as an index of proportional species identity. All analyses were conducted using general linear models in SPSS (IBM).

Results

Contact zone analyses

Phenotypic structure. We observed phenotypic shifts from *Anairetes reguloides* to *Anairetes nigrocristatus* in both morphometric and plumage characters across the transect (Fig. 3). The estimated centres for the four phenotypic clines were all located within a ~16 km portion of the transect, between localities 4 and 5 (5.5 km from locality 4 and 2.9 km from locality 5; Table 1).

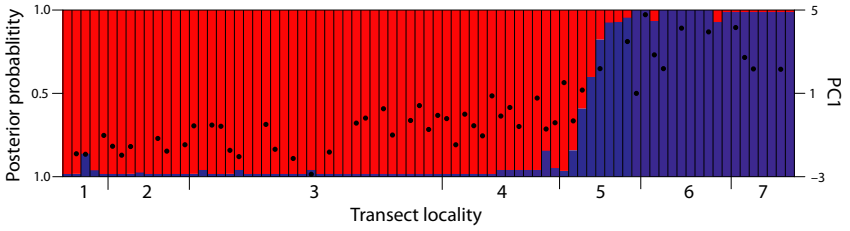

Fig. 2 Bayesian probability of species assignment in Structure across the transect for the four loci. Each coloured bar corresponds to the posterior probability of assignment to a parental population for a single individual. Red = *Anairetes reguloides*. Blue = *Anairetes nigrocristatus*. Numbers along the *x*-axis correspond to the localities along the transect. Black points represent principal component 1 scores based on four phenotypic characters (mass, wing chord, crest length, white tail tip). PC1 explained 83% of the variation. Individuals lacking any phenotypic measurements were excluded from principal component analysis.

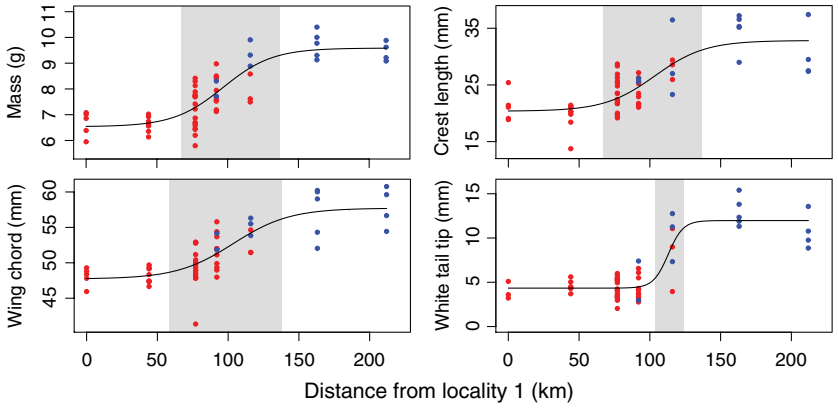

Fig. 3 Phenotypic clines across the transect between *Anairetes reguloides* and *Anairetes nigrocristatus*, determined using CLINEFIT 2.0a. Each point denotes an individual's measurement for the given character. Points are coloured based on mitochondrial haplogroup. Red = *A. reguloides*. Blue = *A. nigrocristatus*. Shading = cline width positioned around cline centre. Lines were plotted using equations described by Porter *et al.* (1997) and the models of best fit determined by AICc (Tables 1 and 2).

Estimated phenotypic cline widths ranged from 21 km (white tail tip) to 80 km (wing chord) (Table 1). Upper log-likelihood support limits for cline widths ranged from 49.0 km (white tail tip) to 179.0 km (wing chord) (see Table 1 for complete list of cline centres, cline widths and support limits).

Genetic structure. All loci contained informative single-nucleotide polymorphisms. Divergence was 20-fold greater in the mitochondrial DNA than the nuclear DNA. *A. reguloides* and *A. nigrocristatus* mitochondrial haplogroups differed by 36 base pairs, corresponding to ~3.7% divergence (Fig. 4). All individuals from localities 1–3 (n = 43) had *A. reguloides* mtDNA haplotypes, whereas all individuals from localities 6–7 (n = 18) had *A. nigrocristatus* mtDNA haplotypes (Fig. 4). Eighty-five percent and 33% of the individuals from localities 4 (n = 13) and 5 (n = 9), respectively, had *A. reguloides* mtDNA haplotypes (Fig. 4). Nuclear loci showed haplotype differentiation between *A. reguloides* and *A. nigrocristatus*, but neither species was monophyletic in the haplotype networks (Fig. 4). We observed two shared haplotypes in Myo2, two in IRF2, and one in MUSK (Fig. 4). One shared Myo2 haplotype was not contiguously distributed along the transect; it was found at localities 1–3 and 7. All other shared haplotypes occurred contiguously along the transect.

Structure analysis of the four genetic markers confidently assigned all, but one individual from localities 1–3 to *A. reguloides* (posterior probability of assignment >0.95; Fig. 2). The remaining individual could not be confidently assigned to either species. Twelve of the 13 individuals from locality 4 were confidently assigned to *A. reguloides*, while the remaining individual could not be confidently assigned to either species. One individual was confidently assigned to each species from locality 5, and the remaining seven individuals could not be confidently assigned to either species. Eight of the 10 individuals from locality 6 and all individuals from locality 7 were confidently assigned as *A. nigrocristatus*. The remaining two individuals from locality 6 could not be confidently assigned to either species.

All four loci exhibited clines from *A. reguloides* alleles to *A. nigrocristatus* alleles along the transect (Fig. 5). These genetic clines were best described by two-parameter (Cyt *b*) and four-parameter (Myo2, IRF2, MUSK) models using AICc (Table 2). The estimated cline centres were all located within a ~17 km portion of the transect, flanking locality 5 (Table 1). Estimated cline widths ranged from 5 km (Myo2) to 65 km (IRF2)

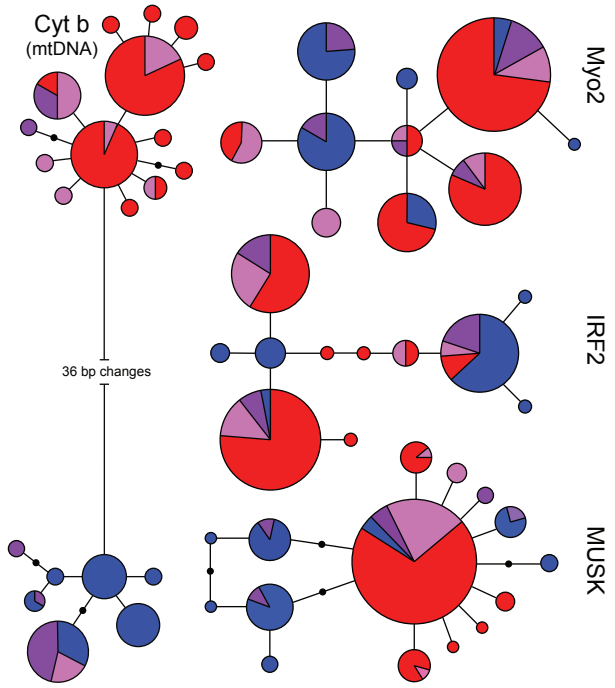

Fig. 4 Haplotype networks for the four loci. Red = individuals from localities 1–3 (*Anairetes reguloides*). Blue = individuals from localities 6 and 7 (*Anairetes nigrocristatus*). Pink and Purple = individuals from contact zone localities 4 and 5, respectively. Circles are scaled based on the Black points denote one mutational step.

(Table 1). Genetic clines, and associated support limits, were narrower than phenotypic clines overall.

Evidence of hybridization

We found eight individuals of mixed ancestry at localities 4 and 5 (Fig. S1, Supporting information). These intermediate individuals were diagnosed as follows: (i) individuals that phenotypically identify as one species but genotypically identify as the other, or (ii) individuals that possess diagnostic genetic markers for both *A. reguloides* and *A. nigrocristatus*. Parental phenotypes were determined by the range in a given character at localities 1–3 and 6–7 for *A. reguloides* and *A. nigrocristatus*, respectively. Individuals with intermediate characteristics were not observed away from localities 4 and 5.

Haematology and heart mass

At the highest elevations, *A. reguloides* showed steep increases in haematological parameters that were not evident in *A. nigrocristatus* (Fig. 6). The best model for haemoglobin concentration ([Hb]) included elevation × species identity ($P = 0.004$), species identity ($P = 0.121$), body mass ($P = 0.08$), and sex ($P = 0.033$), and elevation × species identity and/or species identity was included in all of the models that were within four AICc units of the best model (Tables 3 and S2, Supporting information). Haematocrit (Hct) was best predicted by elevation × species identity ($P = 0.025$), body mass ($P = 0.046$), and sex ($P = 0.009$), and elevation × species identity and/or species identity was included in five of the seven models that were within four AICc units of the best model (Tables 3 and S2, Supporting information). MCHC was best predicted by elevation × species identity ($P < 0.001$) and species identity ($P = 0.043$), and elevation × species identity and/or species identity was included in all of the models that were within four AICc units of the best model (Tables 3 and S2, Supporting information). In summary, each of the three measures of blood-O_2 carrying capacity increased with increasing elevation and did so more dramatically in *A. reguloides* (Table 3).

Relative heart mass increased with elevation across the transect but was lower in *A. nigrocristatus* than in *A. reguloides* (Fig. 7). The best model for relative heart mass included species genetic composition as measured by Structure (Q-score; $P = 0.001$), elevation × Q-score ($P = 0.004$) and body mass ($P = 0.003$; Table 3). Q-score

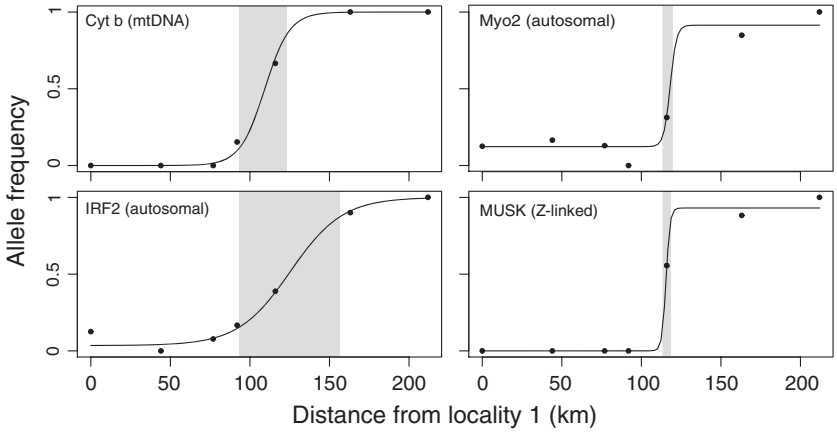

Fig. 5 Genotypic clines across the transect between *Anairetes reguloides* and *Anairetes nigrocristatus*, determined using CLINEFIT 2.0a. Black points denote the allele frequency at a given locality. An allele frequency of 0 denotes pure *A. reguloides* genotypes, and a frequency of 1 denotes pure *A. nigrocristatus* genotypes. Shading = cline width positioned around cline centre. Lines were plotted using equations described by Porter *et al.* (1997) and the models of best fit determined by AICc (Tables 1 and 2)

Table 2 Model selection for phenotypic and genotypic clines. The model with the lowest AICc score for each character or marker is bolded.

	Number of parameters†	lnL	AIC score	AICc score
Phenotypic character				
Mass	**4**	**−57.6944**	**125.3888**	**126.5427**
	8	−57.7755	133.5510	137.3010
Crest length	**4**	**−152.3688**	**314.7376**	**315.8914**
	8	−152.1772	322.3544	326.1044
Wing chord	**4**	**−134.8690**	**279.7381**	**280.8919**
	8	−134.8210	287.6599	291.4099
White tail tip	**4**	**−106.2196**	**222.4392**	**223.6892**
	8	−106.2074	230.4147	234.5056
Molecular marker				
Cyt *b*	**2**	**−11.9886**	**27.9772**	**28.1291**
	4	−12.0092	32.0183	32.5378
	6	−11.9886	35.9772	37.0972
	8	−12.0234	40.0469	42.0195
Myo2	2	−54.6514	113.3028	113.4874
	4	**−50.9965**	**109.9931**	**110.6280**
	6	−49.7044	111.4089	112.7859
	8	−49.8933	115.7867	118.2273
IRF2	2	−50.2011	104.4022	104.5666
	4	**−47.9316**	**103.8631**	**104.4265**
	6	−48.2538	108.5076	109.7249
	8	−47.9310	111.8991	114.0284
MUSK	2	−16.3005	36.6010	36.7610
	4	**−13.3912**	**34.7825**	**35.3305**
	6	−12.5957	37.1914	38.3745
	8	−13.3924	42.7848	44.8718

AIC, Akaike information criterion.
†The different parameters are defined within the text of the Methods section.

or its interaction with elevation was included in all of the models that were within four AICc units of the best model (Table S2, Supporting information), and in each model genetic similarity to *A. reguloides* was associated with a more striking increase in relative heart mass with increasing elevation (Table 3).

Discussion

Differential adaptation to altitude

Anairetes reguloides and *Anairetes nigrocristatus* showed differential respiratory response to low PO_2 at high elevation (Figs 6 and 7), suggesting a potential physiological basis for elevational segregation upon secondary contact. The observed increase in haematological parameters in *A. reguloides* at high elevation indicates that birds of this species accelerate erythropoiesis in response to ambient hypoxia, most likely to compensate for reduced arterial oxygen saturation. Acclimatization of native lowland vertebrates when ascending to high

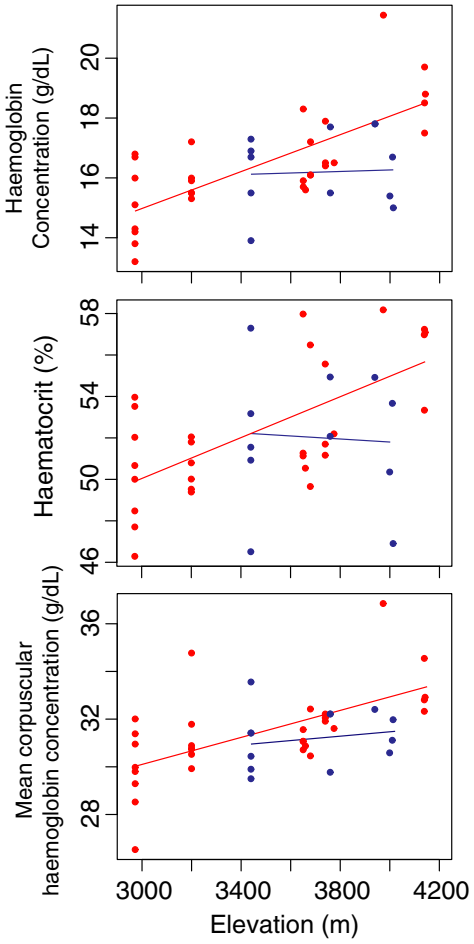

Fig. 6 Blood-oxygen-carrying capacity parameters plotted by elevation across the upper portion of the elevational range of *Anairetes reguloides* (red points) and *Anairetes nigrocristatus* (blue points). Each point denotes a sampled individual. Regression lines are plotted for each species. Haematological parameters for *A. reguloides* ranged from 13.2 to 21.4 g/dL for [Hb], 46.3–58.1% for Hct, and 26.5–36.9 g/dL for MCHC. Haematological parameters for *A. nigrocristatus* ranged from 13.9 to 17.8 g/dL for [Hb], 46.5–57.2% for Hct and 29.5–33.6 g/dL for MCHC.

elevations generally involves increases in [Hb] and Hct (Bullard 1972; Monge & Whittembury 1976; Winslow & Monge 1987; Monge & Leon Velard 1991; Storz *et al.* 2010). Moderate increases in these haematological parameters can increase blood-oxygen content, improving aerobic activity (Ekblom & Hermansen 1968; Kanstrup & Ekblom 1984; Ekblom & Berglund 1991). However, increases in [Hb] and Hct under severe hypoxia can be counterproductive because they are associated with increased blood viscosity that can cause reductions in cardiac output, aerobic capacity and venous return (Guyton & Richardson 1961; McGrath & Weil 1978; Black & Tenney 1980; Connes *et al.* 2006; Storz *et al.* 2010). Evidence from high-elevation humans suggests that the optimal [Hb] and Hct for O_2 diffusion

Table 3 ANOVA and ANCOVA results for haematological and cardiac measurements of *Anairetes reguloides* and *Anairetes nigrocristatus* sampled between 2970 and 4140 m elevation. Regression coefficients (*B*) for the explanatory variables 'species' and 'sex', apply to *A. nigrocristatus* and females, respectively; whereas coefficients for 'elevation × species' apply to *A. reguloides*. Relative heart mass data are from the transect only; blood parameters are from all localities including high and low portions of the transect but excluding individuals from localities 4 and 5, where hybrids occurred. A list of individuals used in these analyses can be found in Table S1 (Supporting information). For each dependent variable, the best model was selected from a range of one to five parameter models (Table S2, Supporting information). Significant predictor variables are bolded.

Dependent variable (R^2)	Source	Type III sum of squares	d.f.	Mean square	*F*	Sig. (*P*)	*B*(±SE)
Relative heart mass (0.234)	**Elevation × *Q*-score**	2.7×10^{-5}	1	2.7×10^{-5}	8.853	**0.004**	4.60×10^{-6} (1.55×10^{-6})
	Mass	2.9×10^{-5}	1	2.9×10^{-5}	9.656	**0.003**	0.001 (0.0003)
	***Q*-score**	3.4×10^{-5}	1	3.4×10^{-5}	11.133	**0.001**	−0.019 (0.006)
	Error	0	61	3.1×10^{-6}			
	Total	0.011	65				
[Hb] (0.388)	**Elevation × species**	24.954	2	12.477	6.257	**0.004**	0.002 (0.001)
	Sex	9.668	1	9.668	4.848	**0.033**	1.182 (0.537)
	Mass	6.408	1	6.408	3.213	0.08	0.695 (0.388)
	Species	5.002	1	5.002	2.508	0.121	8.811 (5.563)
	Error	83.758	42	1.994			
	Total	12601.41	48				
Hct (0.200)	**Elevation × species**	0.012	2	0.006	4.013	**0.025**	1.50×10^{-5} (1.67×10^{-5})
	Mass	0.006	1	0.006	4.23	**0.046**	0.021 (0.01)
	Sex	0.011	1	0.011	7.382	**0.009**	0.039 (0.015)
	Error	0.065	44	0.001			
	Total	13.31	49				
MCHC (0.378)	**Elevation × species**	65.175	2	32.588	12.72	**4.0×10^{-5}**	0.004 (0.001)
	Species	11.092	1	11.092	4.33	**0.043**	12.456 (5.986)
	Error	112.722	44	2.562			
	Total	46125.699	48				

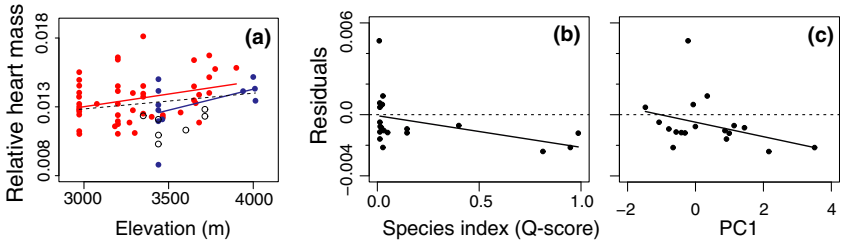

Fig. 7 (a) Relative heart mass as a function of elevation in individuals sampled along the transect. Red points = individuals assigned as *Anairetes reguloides* in Structure with a *Q*-score >95%. Blue points = individuals assigned as *Anairetes nigrocristatus* in Structure with a *Q*-score >95%. Open circles = Individuals that could not be assigned to either parental species with >95% confidence. Regression lines are plotted for each species. Dotted line represents the regression of all points. (b and c) Residual relative heart mass plotted against species identity of individuals from the contact zone (localities 4 and 5).Dotted line = zeroed regression line from plot (a). Black points = an individual's residual distance away from the dotted regression line in plot (a). (b) Species index represents the molecular identity (*Q*-score) of an individual. *Q*-score of 0.0 is *A. reguloides*. *Q*-score of 1.0 is *A. nigrocristatus*. (c) PC1 represents the phenotypic identity of an individual based on four characters (reference Fig. 2 caption). Smaller PC1 values are more *A. reguloides*-like and larger PC1 values are more *A. nigrocristatus*-like.

and transport efficiency is extremely close to that observed in sea level populations, and thus hypoxia-induced increase in the haematological parameters in *A. reguloides* might represent a maladaptive plastic response (Villafuerte *et al.* 2004; Storz *et al.* 2010; Zhou *et al.* 2013).

Relative heart mass across the transect supports our hypothesis of differential adaptation to altitude between the two taxa. If *A. reguloides* and *A. nigrocristatus* have the same respiratory biochemistry, then we would expect that relative heart mass as a function of elevation would be the same in both species, which is not the case. An observed positive relationship between relative heart mass and elevation in each species likely reflected a requirement for increased stroke volume or pulmonary blood pressure to meet metabolic demands in a

cold, hypoxic environment (Tucker 1968; Bishop 1997). The increase in cardiac size of *A. reguloides* compared with *A. nigrocristatus* (Fig. 7, Table 3) most likely reflects a more pronounced pulmonary vasoconstrictive response in the former species (Penaloza & Arias-Stella 2007; Hoit *et al.* 2012). The vasoconstrictive response to hypoxia increases pulmonary blood pressure, which is known to cause hypertrophy of the right ventricle and increase the relative size of the heart, at least in mammals (Corno *et al.* 2004; Hoit *et al.* 2012). An alternative explanation for the observed difference in heart size is that *A. reguloides* needs to pump blood at a higher rate to resist cold stress (Zheng *et al.* 2008; Liknes & Swanson 2011), as would be the case for a species with higher intrinsic thermal conductance or reduced thermogenic capacity under low PO_2 (Cheviron *et al.* 2012). If *A. nigrocristatus* has genetic adaptations that confer increased resistance to cold and/or hypoxia, it may be able to maintain homoeostasis at altitude with a relatively smaller heart than *A. reguloides*.

The muted haematological and cardiac responses to altitude in *A. nigrocristatus* are indicative of hypoxia resistance and 'cryptic' biochemical adaptation in that species (Storz *et al.* 2010). The biochemical mechanisms that provide hypoxia resistance in *A. nigrocristatus* are currently unknown, but studies of other high-altitude birds and mammals have documented genetic changes to haemoglobin structure (Weber 2007; Storz *et al.* 2009; Projecto-Garcia *et al.* 2013), the hypoxia-inducible transcription factor EPAS-1 (Simonson *et al.* 2010; Beall *et al.* 2010), or the erythropoiesis regulator SENP1 (Zhou *et al.* 2013), all of which have been shown to impact respiratory performance under hypoxia. It is important to note that genetic adaptations to high altitude can also reduce performance at low altitude. For example, a single base pair change is sufficient to increase the affinity of haemoglobin for O_2, a beneficial quality at high elevation. The same substitution can reduce the efficiency of O_2 unloading or reduce the plasticity in haemoglobin function that is associated with sensitivity to allosteric effectors, either of which could be maladaptive at low elevation (Perutz 1983; Weber 2007; Projecto-Garcia *et al.* 2013). It is plausible that segregating variants of haemoglobin or other respiratory genes provide more hypoxia resistance in *A. nigrocristatus* and more efficient respiration at low elevations in *A. reguloides*, but further study is needed to identify the molecular and functional basis of the observed phenotypic differences.

Previous research suggests that the ancestor of the genus *Anairetes* was restricted to high elevations and it therefore may have been genetically adapted to cold, hypoxic environments (DuBay & Witt 2012). If this is the case, the respiratory differences that we observed between *A. nigrocristatus* and *A. reguloides* most likely reflect 'low-altitude adaptation' in the latter species, implying the secondary loss of high-altitude adapted alleles. The low-altitude phenotype is characterized by pronounced hypoxic stress at elevations above ~3800 m. Contiguous gene flow with lowland conspecifics may limit the potential for high-altitude populations of *A. reguloides* to re-evolve hypoxia resistance.

Contact zone dynamics and restricted gene flow

Anairetes reguloides and *A. nigrocristatus* only come into contact at transect-localities 4 and 5, where hybrid or introgressed individuals were found to occur between the elevations of 3180–3720 m. We observed steep clinal shifts between parental populations (Figs 3 and 5), despite contiguous habitat corridors that provided the potential for introgressive hybridization. Restricted gene flow might be expected between elevationally segregating taxa if parental phenotypes and associated alleles are optimized to their respective elevations. For example, *A. nigrocristatus* appeared to experience reduced respiratory stress at high elevations; hence, birds possessing *A. nigrocristatus* genes may be expected to have higher fitness in these environments. However, genetic adaptations that confer cold and/or hypoxia resistance are expected to be maladaptive at low elevations; for this reason, haemoglobin adaptations in montane hummingbirds are predictably reversed upon secondary colonization of the lowlands (Projecto-Garcia *et al.* 2013). Restricted gene flow might occur at regions of the genome involved in local adaptation and that can occur while there is unrestricted gene flow at selectively neutral regions, particularly if local adaptation involves one or a few loci (Payseur 2010). Our modest sample of four loci, representing mtDNA, the Z-chromosome and two autosomes, suggests genomewide barriers to gene flow.

Two of the highest ranges in the Peruvian Andes about the transect: the Cordillera Blanca and the Cordillera Negra (Fig. 1). The contact zone is positioned in a northwards draining portion of the Rio Santa valley between the two ranges. Outside of this portion of the valley, the populations appear to remain pure (localities 1–3 and 7). The two taxa co-occur within the valley but segregate by elevation with *A. reguloides* predominating at lower elevations and *A. nigrocris*tatus predominating at higher elevations. Interestingly, the upper elevational limit of the observed contact zone (~3800 m elevation) corresponds to the elevation at which haematological parameters in *A. reguloides* markedly increased, suggesting that this elevation may be an important threshold for this species (Fig. 6). Conservatively, the cline estimates and physiological data jointly indicate that such a threshold occurs between 3100 and 3800 m for this species complex.

It is important to note that locality 3 spans high elevations (2900–3900 m) on the west slope of the Cordillera Negra, less than 20 km west of the contact zone (Fig. 1). Given the elevational position of the contact zone in the upper Rio Santa valley, we would expect to find *A. nigrocristatus* alleles and phenotypes at the higher elevations of locality 3, but this is not the case. All individuals from locality 3 appear to be pure *A. reguloides*, phenotypically and genetically. Locality 3 is isolated from the contact zone by the ridge of the Cordillera Negra (Fig. 1). The lowest pass in the Negra is ~4200 m, with no suitable vegetation for *Anairetes* above ~4000 m along the ridgeline or above ~1500 m around the northern tip of the range (personal observation and visual examination of high-resolution satellite imagery in Google Earth). *Anairetes* dispersing from one side of the Negra to the other (between localities 3 and 4) would be restricted to sparse shrubs that line the bottom of the Rio Santa valley as it hooks around the Negra (Fig. 1). The consequent restriction of the dispersal corridor to low elevations appears to have allowed *A. reguloides* to invade the upper Rio Santa valley from the coast but restricts *A. nigrocristatus* from invading upper elevation habitats on the west side of the Negra.

Implications for elevational distribution limits

If competition is important in determining the distributions of elevational replacement taxa, then we would expect to find *A. reguloides* extending to higher elevations and *A. nigrocristatus* extending to lower elevations in the absence of the other taxon. *A. reguloides* is found up to ~4200 m in the Department of Lima, Peru, where *A. nigrocristatus* is absent (ornisnet.org and arctos.org specimen data). Therefore, at the contact zone (3100–3800 m), competitive exclusion appears to be the proximate cause of the upper elevational limit of *A. reguloides*. Furthermore, in the absence of *A. reguloides*, *A. nigrocristatus* occurs locally down to ~2200 m in Cajamarca and Huánuco, Peru. However, *A. nigrocristatus* is absent from the lowlands and does not occur in places such as coastal Lambayeque and the Marañon River valley where no congeners are present. The failure of *A. nigrocristatus* to invade the lowlands is consistent with the predicted negative pleiotropic effects of high-altitude adapted alleles (Storz *et al.* 2010; Projecto-Garcia *et al.* 2013). These observations suggest that the lower elevational limit of *A. nigrocristatus* at the contact zone is influenced by competition with *A. reguloides*, but physiological limits likely set the lower elevational limit of *A. nigrocristatus* in other parts of its distribution, as predicted for cool-climate species (Gifford and Kozak 2012). Based on our comparisons of haematological and cardiac morphology, we hypothesize that the competitive displacement between these two species occurs deterministically as a result of their having different atmospheric pressures for optimal respiratory performance. One caveat is that parapatric elevational segregation and expanded elevational ranges in allopatry may simply reflect species occupying their preferred niches, rather than competition (Cadena & Loiselle 2007). This could plausibly occur when environmental parameters that affect the species' niche vary at least somewhat independently of elevation, but we are sceptical that this mechanism applies to *A. reguloides* and *A. nigrocristatus* because of the small geographical area across which their elevational limits vary.

Conclusions

Our study suggests that differential physiological adaptation to altitude is a mechanism that can promote elevational segregation upon secondary contact, even in the absence of complete reproductive isolation. *Anairetes reguloides* and *Anairetes nigrocristatus* co-occur in an intermediate elevational zone where they hybridize. Parental alleles segregate across elevations and do not appear to introgress across a geographically narrow contact zone that spans ~3100–3800 m elevation. Either there are genetic incompatibilities causing hybrid breakdown, or natural selection favouring the pure parental forms in their respective environments is sufficient to restrict gene flow across this elevational contour. The elevational generalist species, *A. reguloides*, showed evidence of elevated respiratory stress at high altitudes in comparison with the high-elevation restricted species, *A. nigrocristatus*. Although the specific genetic basis for this difference has not yet been identified, a growing body of literature on high-altitude animals supports the hypothesis that these differences reflect differential adaptation to altitude. In this case, phylogenetic evidence indicates that adaptation to low altitude occurred in *A. reguloides* after its divergence from a high-altitude restricted ancestor. These findings demonstrate the importance of differential adaptation to altitude in accelerating diversification of the fundamental niche, maintaining species diversity, and causing distributions limits, particularly at latter stages in the speciation process when discrete lineages come into secondary contact and potentially interbreed along elevational gradients. Relatively few advances in our understanding of the elevational replacement pattern of distribution have occurred since Terborgh's studies of Andean birds implicated interspecific competition as the key proximate mechanism (e.g. Terborgh & Weske 1975). The present study suggests that differential respiratory specialization on atmospheric pressure can lead to the

deterministic formation of stable elevational replacement. When superimposed on physiological differences, interspecific competition appears to further restrict the realized elevational distributions of both the high and low species.

Acknowledgements

This study would not have been possible without funding in part by NSF DEB-1146491, NCRR-NIH P20RR018754, the Centre for Evolutionary and Theoretical Immunology (CETI), The American Ornithologists' Union, and the Frank M. Chapman Memorial Fund of the American Museum of Natural History. Permits to conduct this research in Peru were provided by INRENA and DGFFS (087-2007-INRENA-IFFS-DCB, 135-2009-AG-DGFFS-DGEFFS, 0377-2010-AG-DGFFS-DGEFFS, 0199-2012-AG-DGFFS-DGEFFS). We thank Phred M. Benham, Natalie A. Wright, Matthew R. Jones, Emil Bautista Obispo, Homan Castillo, Andrew B. Johnson, Kevin G. McCracken, C. Jonathan Schmitt, Donna C. Schmitt, Glenn F. Seeholzer, and Centro de Ornitología y Biodiversidad (CORBIDI; Thomas Valqui, Willy Nanez) for fieldwork and logistical support; Adam Porter for advice in implementing CLINEFIT; Ida Moltke for advice in implementing Structure; and Staffan Bensch, Daniel Cadena, Joseph Cook, Steve Poe, Trevor Price, Jared Strasburg, and three anonymous reviewers for comments that improved this manuscript.

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S.G.D. and C.C.W. designed the research, analysed the data, and wrote the paper. S.G.D. collected the data.

Data accessibility

Table S1 (Supporting information) contains all the data analysed in this study and links to the online specimen records. Aligned sequences and input files can be found on Dryad: doi: 10.5061/dryad.f5t28.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Museum specimens used in study, with localities, GenBank Accession nos, morphological data, physiology data and links to online specimen records.

Table S2. Model selection for the statistical analysis of respiratory variables.

Fig. S1. Phenotypic and genetic characterization for all putative hybrid individuals from localities 4 and 5.